Matthias Giese *Editor*

Molecular Vaccines

From Prophylaxis to Therapy - Volume 1

Springer

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Mens agitat molem.

To my children Geraldine, Sebastian and Laura, who make everything worthwhile.

Preface

The idea of this book was born out of rage.

I am developing vaccines for more than 20 years – a troublesome and laborious business. It can be troublesome because pure theory and successful application of a theory in lab models, and later its translation into clinical practice, are poles apart. And it can be laborious because until now there is no complete scientific compendium covering all essential aspects of modern vaccine development in one book, what makes the busy normal lab day more difficult for a developer.

A developer is obliged to read several textbooks: You will need one book to understand all immunological key aspects; to learn the way of presentation of chemically different antigens by professional cells embedded in various tissues and organs, triggered by different cytokines; to understand the interactions between pathogen recognition receptors and their PAMP or DAMP ligands; to use bioinformatics for prediction of epitopes; to follow the pathways of gene activation and their regulations; and to understand how B- and T-cell memory work or what the consequences of immunosenescence are on vaccines for elderly or how malnutrition strongly influences the immune system in very young people with consequences on vaccine efficacy. Another book would be needed to understand all aspects of modern adjuvant developments, to get a feeling for the different classes and origins of immunostimulants, and to see the multiple immune reactions caused by various adjuvants. Other books would be required to understand the different vaccine types, the different delivery technologies, the right use of nanoparticles, or the helpful assistance of biomarkers.

The here presented two volumes of *Molecular Vaccines: From Prophylaxis to Therapy* cover most of all essential aspects of modern vaccine development in different fields such as infectious, non-infectious or cancer diseases. Moreover, patent claiming strategies will be discussed and also requirements for international licensing. These are two books that will satisfy a great need that up to now has been unfulfilled.

150 authors, from more than 20 nations, from five continents, Asia, Australia, Africa, America, and Europe, contributed to this magnificent book. I am deeply impressed by the enormous responses I got upon my invitation to join our international author team. So I trust that readers of this book, academic and industrial researchers, professors, physicians and graduate students in biochemistry, molecular biology, biotechnology, and (vet) medicine,

will benefit from the comprehensive expertise and will be enabled to provide successful innovative research and development in modern vaccines.

I would like to take this opportunity to thank all authors who generously contributed their knowledge and insights to this book. Special thanks go to Raphael Lekscha, Heidelberg, for his excellent technical preparations of my illustrations. I am grateful to Springer Publishing, particularly to Claudia Panuschka, Vienna, who made my book idea possible, and Wilma McHugh, Heidelberg, for her active support.

Heidelberg, Germany Matthias Giese

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Part I

Molecular Vaccines – From Prophylaxis to Therapy

From Pasteur to Personalized Vaccines

1

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1.1 1885: The First Therapeutical Vaccine

Anno Domini 1881. Louis Pasteur was on track to become the first leading vaccinologist at the time and to win the race against the development of the first artificial attenuated vaccine to his rival, the veterinarian Henry Toussaint. One year ago, he developed a vaccine against avian cholera (fowl cholera). Pasteur isolated the causative agent of this zoonotic disease, Pasteurella multocida (a gram-negative, nonmotile coccobacillus). The economic losses caused by avian cholera were enormously at this time and also today.

Cholera Vaccine. Emile Roux, Pasteur's assistant, came up with the idea to develop a series of immunization experiments with different aged cultures of Pasteurella: (a) immunization of 12 chicken with fresh cultivated Pasteurella, (b) immunization of 12 chicken with aged cultures of Pasteurella, and finally (c) immunization with old cultures of Pasteurella. 8 days later, 12/12 chicken of group A died, but 4/12 chicken of group B survived and surprisingly 11/12 chicken of group C survived. In the following challenge experiment, 8/11 immunized chickens survived. This was the confirmation that an old culture of Pasteurella could be used as vaccine to protect against avian cholera. Today, it is known that this vaccine developed serious side effects and was not able to reduce the shedding, and the duration of immunity was very short.

Pasteur and Oxygen. Pasteur concluded that the virulence of Pasteurella could be manipulated

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by the conditions of cultivation. The interruption or complete stopping of the process of cultivation resulted in a still living but weakened form of Pasteurella. Pasteur believed that only the exposure of the bacilli to oxygen was the reason for this attenuation.

The big difference between Jenner's smallpox vaccination and Pasteur's cholera vaccine was the source of the weakened form of the agent. It was known that a microorganism, e.g., smallpox, could exist in a virulent and in an avirulent natural form. And Jenner applicated the avirulent natural form. Pasteur however isolated the pathogen and attenuated this pathogen by special lab conditions. For the first time, he generated an artificially weakened vaccine against an infectious disease.

Also, Henry Toussaint was elaborating on vaccines but in contrast to Pasteur who believed that only a live vaccine would be the appropriate stimulus for the immune system. Toussaint used the antiseptic phenol to inactivate his anthrax vaccine and later potassium dichromate for the cholera vaccine.

Who would be the first scientist introducing the proof of concept of an artificially generated vaccine, demonstrating its feasibility and the potential of being used in the field?

Anthrax Vaccine in 1881. In the small village Pouilly-le-Fort in June 1881, Pasteur was invited to give the first public demonstration of a lab-generated vaccine for sheep against anthrax. Group A with 25 sheep was immunized, group B 25 sheep as control group. Both groups were subsequently challenged. The results were impressive: 24/25 vaccinated sheep have survived, but 23/25 unvaccinated animals have died. The real scandal followed immediately. Pasteur refused to open his lab protocols, to display all details of the development of this vaccine – with good reason.

Toussaint's Protocol. Pasteur used underhand Toussaint's inactivation protocol, heat combined with potassium dichromate treatment and not his own oxygen-based procedure; how the public and scientific community was convinced at this time? Pasteur's own studies were unsuccessful because oxygen was not able to kill the anthrax' spores. Pasteur won the competition with Toussaint. He never gave credit to Toussaint, the real inventor of this inactivation principle. Glory and publicity for Pasteur, and a deep fall into oblivion for Toussaint. *The winner takes it all*…. The medical historian Gerald L. Geison interpreted Pasteur's copy and paste behavior as fair scientific swindle (Gerald L. Geison: The Private Science of Louis Pasteur, Princeton University Press, 1997, USA).

Rabies Vaccine in 1885. Pasteur's worldwide breakthrough took place in 1885. Inspired again by Emile Roux's experiments with rabies, Pasteur developed a killed vaccine produced of emulsified and air-dried spinal cords of infected rabbits. The existence of any virus was unknown at this time. Well known was the zoonotic character of this disease and the neurological symptoms so that the doctors made the educated guess of affected sickened nerve tissue.

Joseph Meister was a 9-year-old boy when he was bitted by a rabid dog. The boy got 13 injections in which Pasteur used stepwise more fresh isolated nerve tissue with more virulent pathogens (Fig. 1.1). On October 26, 1885, Pasteur announced Joseph Meister's healing. Pasteur was hailed as a hero. Whether this series of immunizations was the reason for the healing is as yet not proven because the risk of contracting rabies after such an exposure is estimated at around 10 %. Marginal to notice, that Pasteur tested the rabies vaccine as prophylactic treatment in only 11 dogs, and only one therapeutical immunization of an infected rabbit was passed before Joseph Meister was vaccinated. Also, a first treatment of a rabies-infected girl, the 11-year-old Antoinette Poughon, shortly before Meister, failed. Girl and rabbit died.

Nonetheless, this was the first documented therapeutical vaccination in human medicine worldwide (Hervé Bazin: L'Histoire des vaccinations, John Libby Eurotext, 2008, France; Pasteur Vallery-Radot (ed): Œuvres de Pasteur. Volume 6: Maladies virulentes, virus-vaccins et prophylaxie de la rage – Méthode pour prévenir la rage après morsure, Masson, Paris 1933, France).

Fig. 1.1 Pasteur injected the first inactivated rabies vaccine to the 9-year-old Joseph Meister in 1885

1.2 Systems Vaccinology

Isolation of a pathogen, inactivation or attenuation, and injection are the basic rules for the development of any vaccine. Pasteur vaccines are with us since a century. Estimated 95 % of all global licensed vaccines for animal health are still Pasteur vaccines. The development is cheap and simple. The situation for human vaccines is quite different.

Without any doubt, these classical vaccines have dramatically improved our lives. And in almost the same manner but mostly unnoticed by the public, the huge progress in molecular sciences completely changed the vaccine research and development.

HBsAg Vaccine. The first registered human recombinant vaccine was against hepatitis B virus infection. In 1984, the cDNA of the surface antigen (HBsAg) was cloned into a vector for transfection of yeast cells as production system [1–3]. This recombinant product replaced the former hepatitis B vaccine, a so-called plasma vaccine: HBsAg particles were isolated from the blood of chronically infected patients, purified and inactivated. Preclinical and clinical studies demonstrated the safety, efficacy, and economy of the first recombinant vaccine. The stringent enhancements and consistent applications of molecular biology turned out to be the right way in biomedical research both in drug and in vaccine development.

Reverse Genetics. Established in the 1990s, reverse genetics start the functional analysis of a gene first by gene sequencing. Once decoded, this sequence is well directed (targeted) and altered by using various techniques and the effect on the organism is studied. Therefore, the causal relation between a targeted mutagenesis and the resulting new phenotype is stringent, precisely and reproducibly. The opposite direction is given by the classical so-called forward genetics.

Starting with a given phenotype, the genetic basis for this phenotype is investigated. The first pathogen altered by reverse genetics techniques was influenza virus [4, 5]. Sinsyne, site-specific mutations allow the engineering of viruses with defined biological properties.

More complex organisms as bacteria can also be studied for vaccine development by reverse genetics.

African Meningitis Belt. Neisseria meningitidis, a gram-negative diplococcus and often referred to as meningococcus, is an encapsulated bacterium that colonizes the nasopharynx and occasionally causes invasive disease. It infects only humans, and no animal reservoir is known. The classification of the 13 serogroups is according to the polysaccharide capsule surrounding the bacterium, but only six groups, namely, A, B, C, W-135, X, and Y, are associated with severe, invasive disease and can cause epidemics [6]. The WHO reports that the major disease burden is in the Third World, and every year, 400 million people living in the "African meningitis belt" (21 countries, from Senegal to Ethiopia) suffer on bacterial meningitis caused by Neisseria, with a case-fatality ratio (CFR) of 10.6 % in 2010 (GHO: Meningococcal meningitis, http://www.who.int/gho/epidemic_diseases/meningitis/en/index.html, WHO 2013).

Meningococcus. Meningococcal disease is a global health problem and sporadically occurs throughout the world. Mainly meningococcus serogroup A and recently also groups W-135 and Y are the causative agents in Africa, whereas serogroups B and C and Y (in the USA) are predominant in industrialized countries. Several vaccines are on the market. In 2010, the WHO prequalified the first serogroup A meningococcal conjugate vaccine (PsA-TT) developed solely for Africa (MenAfriVac, Serum Institute of India, Ltd.). Meanwhile, some quadrivalent meningococcal conjugate vaccines against serogroups A, C, W-135, and Y are licensed in Europe, North America, and Asia for persons 2–55 years. These conjugate vaccines replace the former polysaccharide vaccine based on capsule polysaccharides which are poorly immunogenic in infants. And because this type of vaccine is T-cell independent, no memory response is measurable and the induced immunity is short-lived (see also Chap. 2).

Reverse Vaccinology. The bottleneck is the meningococcus serogroup B (MenB). This pathogen causes 50 % of the meningococcal meningitis worldwide. A vaccine development based on the main capsule polysaccharide has been impeded. The antigenic structure of this bacterial carbohydrate molecule is identical to fetal brain-cell adhesion molecules [7]. Additionally, the sequences of the most surface proteins are highly variable. But the major concern about possible autoimmunity led to vaccine development with the use of noncapsular structures. Following the routes of reverse genetics demonstrated on a virus and the first publication of the entire genome of a bacterium [8], the genome of the virulent MenB strain MC58 was completely sequenced for identifying vaccine candidates [9]. Later on, this approach will be called *reverse vaccinology* including immunological and biochemical studies [10].

rMenB. Shortly, Neisseria meningococcus serogroup B, strain MC58: single singular chromosome, 2.2 megabase pairs in size, 2,272,360 nucleotides, 2,225 genes (2,063 for proteins). All genes were sequenced. 570 open reading frames (ORFs) for gene prediction were identified; from these, 350 ORFs could be successfully expressed in *E. coli*. From these potential vaccine candidates, among them lipoproteins, outer and inner membrane proteins, and transmembrane and unknown proteins, 28 novel proteins were found to generate neutralizing antibodies against MenB in mice and showed bactericidal activities. Only 7 recombinant proteins were positive in several tests and selected for further studies. Finally, five candidates were combined in a vaccine formulation named rMenB (Novartis) [11]. Clinical trials with this vaccine are ongoing.

The exclusive goal of reverse vaccinology is to discover novel antigens and to predict epitopes for B- and T-cell responses (Table 1.1). This approach focuses on isolated parts of microbial organisms and do not use systems biology. The entire process can be divided roughly in four major activities, with the first two steps in silico, in dry experiments.

Table 1.1 Key strategy of reverse vaccinology

1. Comparative genome sequencing and gene identification
2. Prediction of putative protein antigens and epitope mapping
3. Recombinant expression of candidate antigens
4. Immunization studies in vitro and in vivo

DNA Microarrays. Sequence analyses, finding of genes, comparative analysis with related pathogens, and predictions of thousands of proteins are to be done in real time by bioinformatics approaches performed on computer (in silico; see also Chap. 42). DNA chips (microarrays) are used to investigate the expression patterns of genes. Gene clusters can be recognized, up- and downregulated genes can be identified, and biological pathways and networks can be detected. Known and novel immunity-related genes and pathways can be described [12, 13].

In Silico. A first challenge is the prediction of proteins by in silico mapping of epitopes, also called dry experiments in contrast to wet experiments on the bench. Not every protein of the huge amount of proteins of a pathogen is suitable for vaccine development. Selection criteria and accurate prediction algorithms are essential, e.g., cross-reactivity and autoantigens, surface expression for immune recognition, conserved regions in contrast to sequence variability, membrane proteins, and number of helices. A serious protein prediction can save time and money. The exact localization of a protein helps to determine the potential antigenic character. For example, a signal peptide (leader sequence) on the N terminus serves an intracellular "postal code" and gives the way for exportation towards the cell surface. Such proteins are preferred because proteins of cell surface are exposed to antibodies, B and T cells. The physical and chemical complexity of a protein influences the expression in a heterologous system like *E. coli*, the most commonly used organism for industrial and pharmaceutical protein production. Not every heterologous protein can be cloned, expressed, and purified.

Prediction of T-Cell Epitopes. The prediction of epitopes (antigenic determinant), the binding portion of an antigen, helps to define possible vaccine targets. If the antigen is a protein, the epitope is a short peptide. MHC class I molecules present peptides 8–10 amino acids in length and are recognized by CD8+ T cells, resulting in a cellular response. In silico T-cell epitope prediction ranges from 90 to 95 % positive hits. Several bioinformatics databases are available [14–20].

Prediction of B-Cell Epitopes. Prediction of B-cell epitopes is much more complicated [21]. B-cell epitopes consist of a continuous and discontinuous structure. A continuous epitope is defined by the linear primary amino acid sequence of the antigen interacting with antibodies. A discontinuous epitope is defined by the conformational, characteristical 3-D shape of a protein. Discontinuous epitopes generate the majority of all antibody binding epitopes, but as yet, the epitope prediction is mainly for linear epitopes with a poor hit rate [22, 23]. Already the secondary structure of a protein almost resists for prediction much less a higher protein structure. Once produced in *E. coli*, each recombinant candidate antigen undergoes a series of biochemical and immunological experiments. Protein chips were developed for high-throughput screening to study activities, functions, and interactions of proteins [24]. Known and novel immunity-related proteins and pathways can be detected.

Proof of Concept. The combination of genomics and proteomics is a powerful approach to identify novel vaccine candidates and the computer helps to select putative antigens out of thousands of proteins [25]. But at the end of each in silico experiment, the proof of concept must be done in wet experiments, in cell cultures combined with immunization studies in animal models. This is the Lackmus test, not less. Figure 1.2 gives a schematic overview of reverse genetics embedded in systems vaccinology.

Bioinformatics. Meanwhile, reverse vaccinology has been applied to a large number of bacterial pathogens. Despite the rapid progress in bioinformatics and the development of better tools to manage the huge amount of information, we are facing enormous problems. Still the most important unmet needs are HIV, tuberculosis (TB), and malaria (WHO). The only currently licensed vaccine against TB, BCG (bacillus

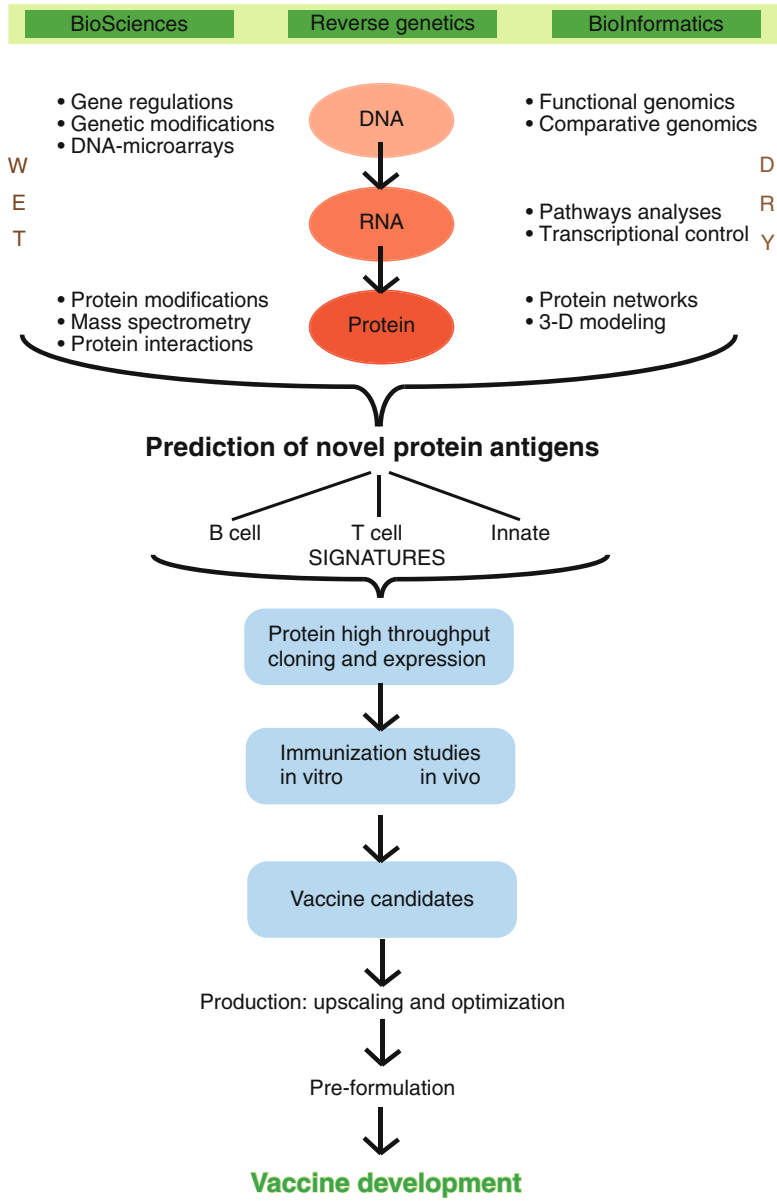

Fig. 1.2 Schematic overview of reverse genetics combined with systems vaccinology. The approach to define new antigens starts with dry experiments (in silico), bioinformatics studies to compare genomic data on DNA level. On RNA and protein level, transcriptional control, pathways, and networks are analyzed. Selection criteria and accurate prediction algorithms for B and T cells and the innate immune response are essential for prediction of novel protein antigens. The exact localization of a protein helps to determine the potential antigenic character. In wet experiments, predicted proteins are constructed, cross-checked (DNA microchips, mass spectrometry, protein networks), and tailored by molecular genetic tools. High-throughput cloning and expression systems deliver thousand of antigens. In vitro and in vivo immunization studies supported by bioinformatics data deliver candidate vaccines. The physical and chemical complexity of a protein influences the expression in a heterologous system like *E. coli*, the most commonly used organism for industrial and pharmaceutical protein production. Not every heterologous protein can be cloned, expressed, and purified. Upscaling and optimization of the production process for selected preformulated candidate vaccines is the last step before entering the clinical development process

Calmette-Guérin), is in use since 90 years and is not effective enough. A better, improved modern TB vaccine is strongly needed. Moreover, there are no vaccines available for infections with West Nile virus, dengue virus, hepatitis C, Ebola, etc. Even the development of a standard flu vaccine every year is a race against the time (see also Chap. 52). And what about highly variable pathogens causing persistent and latent infections, as HIV or HCV? Neither a prophylactic nor a therapeutical vaccine is available. The genes of these pathogens are identified but the bioinformatics approach, from genome to vaccine, does not work. Why?

Limitations. The identification of novel antigens is comparatively simple in contrast to understand the complex immune reactions in vivo, such as the antibody-dependent enhancement (ADE) in dengue vaccination (see Chap. 7) which leads to an increase of infectivity. Also, the establishment of a local immunosuppressive milieu by anti-inflammatory cytokines (see Chap. 2) diminishes the vaccination efficacy. Our knowledge of factors triggering the tissue microenvironment influencing B- and T-cell functions is absolutely poor (see Chap. 2). And how to design a vaccine against TB, a frequent coinfection for HIV in the third World, if on one hand the TB vaccine needs a strong T-cell epitope [26] and on the other hand the HI virus targets T cells (see Chap. 13)? Until now, there is no licensed vaccine against human respiratory syncytial virus (HRSV), an important respiratory pathogen especially for newborns, although the genome is sequenced and all proteins are detected since 1997. Obstacles are the immature immune system of newborns and the Th2 dominated immune response resulting in a vaccine-enhanced disease [27].

Holistic Vaccinology. We have to go deeper and understand biological systems as series of more or less dependent subsystems like the immune system, the nerve system, or the digestive system influencing each other by dynamic functions. And based on this insight, we must develop a holistic view on vaccinology. There is a need to learn more about the regulations of (immuno) genes. The human genome encodes for 22,000 genes.

MicroRNA. To date, more than 1,500 microRNAs (miRNA) are identified as regulator of gene activity (http://mirbase.org/index.shtml). miRNAs turn a gene on or off. Such small RNA molecules are important for B- and T-cell development and influence the immune response [28–31]. What are the specific signals for miRNAs and in which temporal sequence? Unsolved essential problems are also that some pathogens cannot be cultivated in cell cultures. Or for some pathogens, no animal model does exist (see Chap. 5). We are developing cheap vaccines for the Third World. But the severe poverty is the real cause of many infections. Chronic hunger may modulate the immune system through T-cell repertoire and still undiscovered other immune functions. Chronic hunger in early life dramatically impacts the programming of the young thymus, a very crucial phase, having long-term effects on T-cell development (see Chap. 2).

The Crux with '-omics. Maybe it is correct that we are living in the era of "-omics unlimited" [32]. Genomics and proteomics are well established and sufficiently describe these fields of main activities for vaccines. Meanwhile, there is an inflation of '-omics, such as allergenomics, bibliomics, cellomics, CHomics, chronomics, cross-omics, diagnomics, fragonomics, functomics, operomics, or recently vaccinomics. To understand this proliferating '-omics nonsense, you need a large glossary and the collaborative support of another database, the Vaccine Ontology (VO; http://www.violinet.org/vaccineontology/) with more than 5,000 terms (in 2012) with increasing tendency. What a crux with all these '-omics and terms. The same is for statistical analysis. The more statistics in a clinical trial, the more a critical mind is needed. A vaccine works or not.

> **We live in an era of '-omics were we no longer think we google.**
>
> (Matthias Giese)

Databases and statistics are not the holy grail; a traditional hypothesis-driven research of complex structures and experimental validation is still strongly needed. *"Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while*

imagination embraces the entire world, and all there ever will be to know and understand." (Albert Einstein)

1.3 Biomarkers: Correlates of Protection

Almost all licensed vaccines work through induction of antibodies, which neutralize or opsonize a pathogen making a bacterium or a cell susceptible for phagocytosis. For lots of classical vaccines, the serum antibody IgG titer correlates with vaccine-induced immunity [33].

Discrepancy. But there is a discrepancy observed in most diseases that the measured immune response does not correlate with the vaccine-induced protection. We measure a protection, but in many cases, the mechanism of immune protection is poorly known. In clinical descriptions, the terms "correlate" and "surrogate" are used for an immune response that is responsible for protection and an immune response of unknown players measured by a substitute [34]. In vaccine field trials, immune correlates or surrogates are evaluated to predict protection from infection or disease at both individual and population level, across populations, genders, and ages [35–37].

Correlate of Protection. The preclinical research phase is essential for the success of clinical development. Genomics and proteomics offer various tools to develop biomarkers to identify immune correlates of vaccine efficacy also for already licensed and novel vaccine developments especially for therapeutical vaccines for chronic infections or cancer diseases. The US Food and Drug Administration (FDA) defines a "correlate of protection" as laboratory parameter that has been shown to be associated with protection from clinical disease (FDA Guidance for Industry, 1997, report no.97N-0029, IV.C. Efficacy). Meanwhile, the huge hive of literature to "correlates of protection" is so confusing that focus in this chapter is given only on laboratory parameters.

Systems Biology. Antibodies provide the first line of defense which can prevent an early infection and correlate with protection. In case of an intracellular pathogen, antibodies can't prevent the replication and don't protect. This is where T cells come into play and the measurement of T-cell immunity is a more accurate correlate of efficacy.

To understand the immunological mechanisms of vaccination, the chronological steps of antigen uptake, processing, and presentation to B and T cells can now be studied in detail (see Chaps. 2 and 50). Using single genomic and single proteomic data, we are more and more able to understand the complex biological network of innate and acquired immunity. We are right in the middle of systems biology, investigating the (immuno) biology as a whole, with all immune cells, cytokines, and interactions with the environment [38]. Rightly, we speak of an immune system and should never forget the holistic complex.

Therapeutical EAV Vaccine. We are using systems biology in our laboratory to develop a therapeutical vaccine for persistent infections with equine arteritis virus (EAV) as a model system for humans. EAV, a small ssRNA virus of the family of Arteriviridae, causes respiratory diseases and abortions. The vaccine development has started backwards. First, we investigated the putative escape strategies in vitro by DNA chips (Fig. 1.3) and found that EAV upregulates 12 interferon (IFN)-related genes and downregulates 2 IFN-related genes. One of this downregulated gene was the IFN-γ inducible protein (IfI30, acc.no. NM-023065), an essential biomarker for the persistence of EAV in vitro.

In additional experiments, we could measure that the intracellular peptide transportation system TAP (transporter-associated peptides, also transporter associated with antigen processing) was downregulated following the infection with EAV and last but not least the MHC I molecules. All results taken together, we assumed that EAV triggers a cascade of intracellular and antigen processing-related events to escape the immune response (Fig. 1.4).

T-Cell Vaccine. With this knowledge that EAV reduces the cellular immunity, we developed a multivalent DNA T-cell epitope vaccine based on two envelope proteins together with the

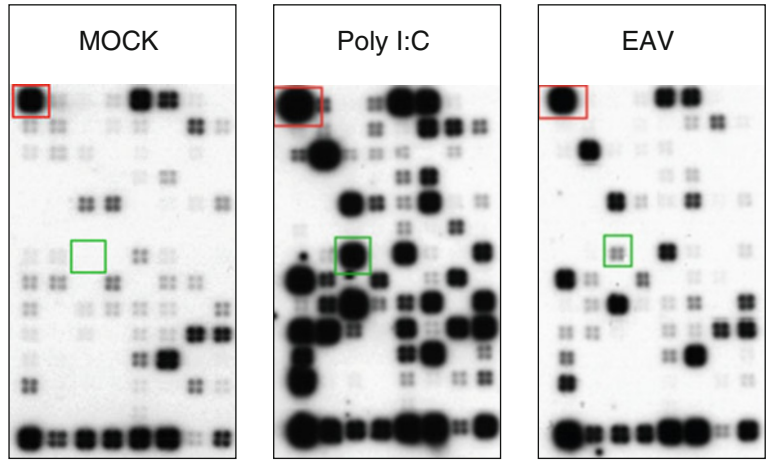

Fig. 1.3 Oligo GEArrays with RNA of C2C12 cells: Untreated MOCK control; polyI:C treatment; infection with EAV control genes, GAPDH (*red spot*); and IFN beta (*green spot*) EAV up- and downregulated genes can be detected in direct comparison with the MOCK and polyI:C control (Anett Heinrich-Schulz, PhD thesis 2010, Matthias Giese)

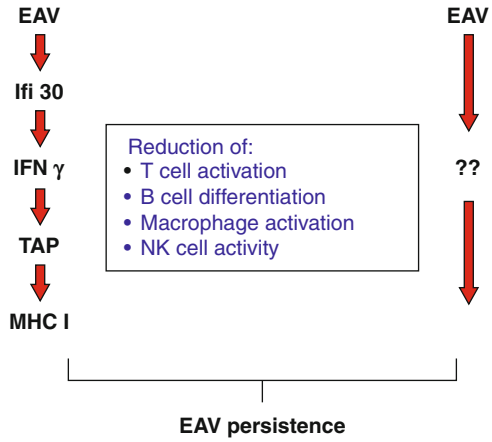

Fig. 1.4 Development of EAV persistence. After entering the target cell, EA virus suppresses among other genes the interferon inducible gene 30, Ifi 30. The protein encoded by Ifi 30 plays an important role in the MHC presentation. The lack of IFN-**γ** provokes a lower expression of TAP resulting in a diminished presentation of antigen via MHC I and the persistence of EAV. This downregulation seems to be part of the EAV evasion strategy. Further research is needed to uncover additional escape mechanisms of EAV

intracellular nucleocapsid protein [39]. In several therapeutical vaccination trials, we found that this vaccine is able to cure persistent EAV infected horses via a strong CD8+ T-cell activity (measured in an autologous cytotoxic T-cell assay) dependent on the virus load. Too much virus and the vaccine treatment fails. The measurement for the vaccine efficacy is the serum level of IFN-γ. Starting from backwards, from the biology of the virus, IFN-γ accurately correlates with the virus load in vitro. The correlation to the virus load in vivo needs further studies. However, IFN-γ could be confirmed as biomarker for the grade of EAV infection in horses and vv as hallmark for the efficacy of a therapeutical vaccination. The more virus, the less IFN-γ, the less therapeutical efficacy of the vaccine. The less virus, the more IFN-γ, the more vaccine efficacy.

IFN-γ, produced by T cells, NK cells, is an immune modulator influencing not only the expression of MHC I and II but also B- and T-cell differentiation.

The only licensed vaccine against tuberculosis (TB) is bacille Calmette-Guérin (BCG). The protective mechanisms of this vaccine are poorly understood. However, IFN-γ could be one biomarker for the BCG vaccine efficacy [40, 41].

Biomarker. But a biomarker is not per se a protein: "A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention…" [42] (Biomarkers Definitions Working Group, 2001).

With molecular tools, the traces of a vaccination can be followed up in real time and checked for molecular signatures on cell, protein, DNA, and RNA level. The type of DC carrying the antigen can be measured, the type of TLR and NF-kB-activated genes can be detected, and the direction of immune response with all effector arms can be predicted. With cDNA microarrays,

the response of host immune genes can be characterized, and by comparative analysis between vaccines and non vaccines, genes associated with protection can be isolated. Lots of putative biomarkers can be detected in blood samples as a snap shot of vaccination. To control a disease by vaccination may also include the study of chromosomal region that underlies genetic variation in response to the vaccination.

One biomarker is not sufficient, and a panel of various markers for innate and acquired immune factors, from genes to proteins to immune cells, is needed capable of characterizing the host response to vaccination. A robust panel will consist of real correlates combined with essential surrogates.

Yellow Fever Virus. Yellow fever virus (YFV) is a small RNA virus and causes mild infection with fever, headache, or in 15 % of all cases a life-threatening acute hemorrhagic disease. The WHO estimates 30,000 deaths every year in unvaccinated populations mainly in Africa. By using cDNA microarrays to investigate gene expression patterns and additional bioinformatics, the efficacy of the yellow fever vaccine YF-17B could be evaluated [43]. Until now, over 600 million people were immunized with this live attenuated vaccine. Lots of various laboratory parameters were measured before and over 60 days following the vaccination, e.g., B cells, CD4+, CD8+ T cells, TLRs, gene expressions, transcription factors, and cytokines. Aim of this study was to find molecular signatures correlating with the high magnitude of CD8+ T cells and the antibody titer. Surprisingly, the correlating biomarkers were some genes within the innate stress response system shaping the CD8+ T cell response [44]. Stress is known to be a potent immune modulator.

1.4 P-Vaccines: From Bench to Bedside

P (personalized)-vaccines use patient's antigens, tissues, or cells which are manipulated ex vivo and reinjected to the same patient as autologous vaccine, a procedure mainly used in oncology.

1964: The first scientific report on an experimental autologous cancer vaccine is published [45].

1994: Thirty years later, in December 1994, the first commercial approach of an anti-melanoma vaccine has started a multicenter clinical trial in Vienna [46].

Melanoma cells were isolated from a patient and sent to our laboratory. In a first step, the cancer cells were largely expanded in roller bottles and then inactivated by co-60 radiation. For the following gene transfer of interleukin 2 (IL-2), adenovirus 5 (Ad5) was chosen as vector. IL-2 was linked to Ad5 via a complex streptavidin-biotin bridge. Readout was the expression rate of IL-2 in vitro.

The whole laboratory process took at least 3 weeks and was going along with problems such as the successful isolation of a sufficient number of cancer cells for propagation, slow or no cell growth, and fluctuating transfection rates. The gene-modified irradiated cancer cells were s.c. reinjected to the patient. The theoretical concept behind this autologous cell vaccine is to present the immune system a panel of melanoma cancer antigens in combination with IL-2 as immune enhancer. In practice, this vaccine did not work and after PhII the study was finished.

At least two main reasons can be identified for this failure: (1) Already after the second application, anti-vector antibodies in all patients could be measured. The vaccine was neutralized. (2) But the basic mistake was done in the preclinical development, where a simple non-metastasizing (local encapsulated tumor) mouse model was used instead of the more realistic B-16 melanoma model. The correlation between IL-2 expression and the therapeutical efficacy in this non-metastasizing mouse model could not be confirmed in the clinical trial.

2010: Almost 50 years after the first experimental study with autologous cancer vaccines, FDA approved the first dendritic cell (DC) therapeutical cancer vaccine [47]. Since the first clinical trial in the 1990s with ex vivo manipulated DCs, about 50 more clinical trials were initiated, but DC-based vaccines have largely failed until today [48].

Medical Profiling. P-vaccines are mainly developed for cancer patients; however, they are also useful for patients suffering on chronic infections, metabolic disorders, and autoimmune diseases. The aim of individual profiling is to understand the patient-specific interplay between disease and immune system and to tailor a vaccine fitting with measured individual biological conditions. The prerequisite for a P-vaccine is the collection of individual data starting with the age, gender, and family history and the analysis of results from (epi)genomic epidemiology studies to define general genetic variants, and combined with the regular monitoring of the physiological state. Because generally the patient comes first to the doctor when he has fallen ill, the healthy baseline of gene and protein data are missing and also a longitudinal profile of healthy conditions is not available which limits the use of genomics and proteomics.

Fingerprints. The medical profiling starts with disease-associated fingerprints, a gene profiling technique using DNA microarrays. To simplify the complexity of up- or downregulated genes, analysis by grouping related genes based on their pathways will be done. Identification of active pathways is more predictive as the collection of different genes [49]. Loss or gain function of genes can be investigated by differential display and subtractive hybridization [50]. The vaccine-induced immune response is also monitored by functional gene and protein analysis using DNA/RNA and protein array technologies.

Peripheral blood mononuclear cell (PBMC) can be easily isolated, stimulated in vitro, and the gene expression profile can be routinely measured. In a clinical study with melanoma patients in which the patients got a therapy with IFN-α, it was found that the gene expression profile of in vitro IFN-α-stimulated PBMCs was similar to the gene expression of PBMCs following IFN-α treatment in vivo. This demonstrates the great benefit of gene analysis of individual PBMCs in vitro as predictor for immune response [51].

MicroRNAs. Increasing impact on medical profiling is given to microRNAs (miRNAs). As discussed before, gene expression is regulated on transcriptional and posttranscriptional level by short (~20nt) noncoding RNAs. Base pairing via complementary sequences within mRNA molecules results in gene silencing. Stable miRNAs in bodily fluids are used as diagnostic, prognostic, and predictive biomarkers. Moreover, targeted gene silencing via miRNAs is investigated for therapeutical treatments [52]. Numerous cancer-specific miRNAs have been identified [53]. Profiling of miRNAs in individual PBMCs, or more generally in blood and plasma samples, can be used for prediction of (immune) response of an individual anticancer treatment [54, 55]. Also in acute or chronic viral diseases, such as hepatitis A or B, a prognostic and predictive circulating miRNA biomarker can be used for the development and monitoring of therapeutical P-vaccines [56–58].

The molecular profiling of PBMCs raised to a key function in personalized medicine.

ABPP. The functional analysis of proteins instead of the mere recording of various proteins offers a platform for monitoring active proteins, referred to as activity-based protein profiling (ABPP) [59]. A chemical reagent, consisting of a reactive group (RG) and a tag, binds covalently to the active site of an enzyme. Inactive enzymes cannot bind an RG. A tag is a reporter such as a fluorophore or an affinity label such as biotin for measuring this chemoproteomic complex. ABPP can be used to study virus-host interactions by profiling serine hydrolases [60] and to develop individual P-vaccines against chronic viral infection. Serine hydrolases are involved in a variety of physiological and pathological processes, also in viral infection [61].

For translation of data from active pathways, or the interpretation of protein-protein interactions and networks, bioinformatics tools are indispensable to filter the huge amount of data [62, 63].

One Gene, Different mRNAs. In summary, P-vaccines use personal bioinformatics data from systems biology to design patient-specific individual vaccines for cancer diseases, chronic infections, and noncancer-noninfection (NINC) disorders (Fig. 1.5). The personal genetic profiling for prediction of diseases, the management of a therapy, or the development of individual

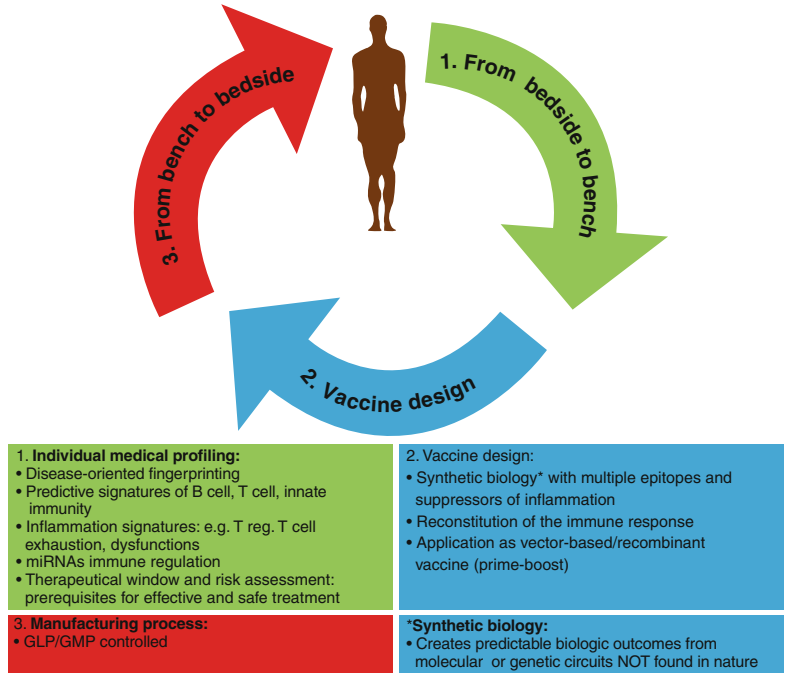

Fig. 1.5 P-vaccine development. (*1*) The medical profiling uses DNA microarrays for disease-oriented fingerprints. Loss or gain function of genes can be investigated by differential display and subtractive hybridization. The vaccine-induced immune response is also monitored by functional gene and protein analysis using DNA/RNA and protein array technologies. (*2*) After a risk analysis and the planning of a therapeutical window, an individual vaccine can be designed. The aim must be to reconstitute the immune response and to control the inflammation at the same time. Artificial carriers or synthetic adjuvants such as special nanoparticles can improve the efficacy of the vaccine. (*3*) Also, manufacturing and safety concerns of P-vaccines must be handled according to international standards in accredited laboratories

vaccines should belong into the hands of specialized accredited clinical genetic services and laboratories and not into the hands of private companies offering a direct-to-consumer DNA profiling. DNA fingerprints alone are not sufficient; they must be interpreted in the context with systems biology.

There is no one-to-one relationship between the analyzed DNA and the transcribed mRNA and the translated protein: Cells can use alternative splicing resulting in various mRNAs, and proteins undergo posttranslational modifications. Therefore, one gene can result in different mRNAs, finally in different proteins with different functions and different interactions with other proteins. Moreover, a serious genetic profiling is always linked with advice from clinical geneticists. The availability of a personalized medicine is of course no guarantee for recovery from a severe illness. Lots of tests have only limited informative value and can therefore not provide accurate individual predictions. Each test result is only a fractal-like use of a complex biological system, another piece in an unlimited puzzle.

1.5 Therapeutical Window

The efficiency of the immune system decelerates during life alone due to the immunosenescence (see Chap. 2). Age-associated immune changes take place in the innate and acquired immune systems and affect not only lymphocytes but also myeloid cells with a change in proinflammatory cytokines. Also, chronic infections caused by certain pathogens, such as CMV and HIV, remodel the immune system towards aged T cells. The loss of immune competence is also a

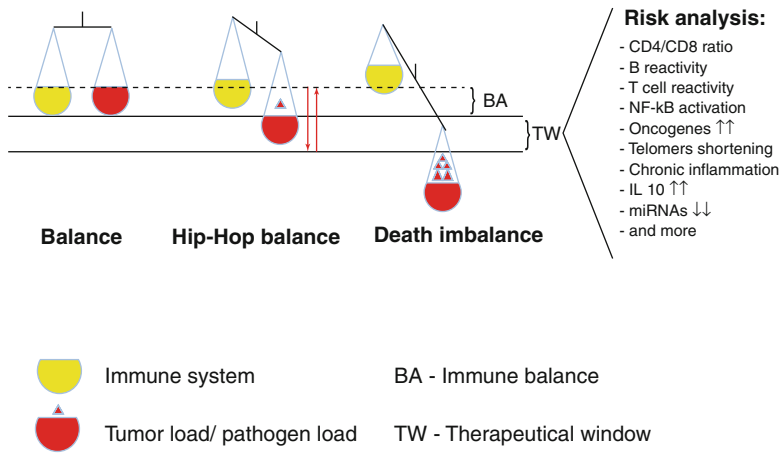

Fig. 1.6 Therapeutical window. A therapeutical window for each patient/each disease/each vaccination should be defined based on a risk analysis of various laboratory parameters, from proinflammatory cytokine production to B- and T-cell reactivity, overexpression ↑↑ of oncogenes, low expression ↓↓ of miRNAs, shortening of telomeres of lymphocytes, or search for specific miRNAs as part of gene regulations. This TW is the last chance to successfully reactivate most immune functions during the Hip-Hop balance before the imbalance of the immune system will get irreversible

major risk for many cancer diseases [64]. Immunosenescence and diseases reduce the efficacy of therapeutical vaccines.

A therapeutical window (TW) as part of the medical profiling should be based on a risk analysis of various parameters, e.g., cell samples, DNA and RNA arrays, and protein analysis (Fig. 1.6). This TW is the last chance to successfully reactivate most immune functions before the imbalance of the immune system will get irreversible. The TW is not comparable to the pharmacological window, the well-defined range of a drug, between the effective dosage and the toxic amount of dosage.

Vaccine-induced long-term effects require adequate innate and acquired immune functionality, especially a diverse repertoire of T cells that can be measured by genomic and proteomic approaches during medical profiling. New antigens are recognized mainly by naive T cells. A loss of T-cell repertoire diversity correlates with an impaired immune response. Therapeutical vaccines can also face functionally exhausted T cells and thus do not respond properly to therapeutical vaccination. Therefore, a therapeutical vaccination should combine the blocking of inhibitory pathways with stimulatory signals for the activation of CD8+ T cells, the major effector cells against cancer and chronic viral infections.

Balance. It seems easier to describe what an immunological disease-related imbalance does mean than to explain the immune homeostasis in healthy patients. The immune system is ambiguous, protective, and harmful; immunity on the one hand and calculated immunopathology on the other hand are well balanced by redundant regulatory mechanisms [65, 66]. The maintenance of the immune homeostasis with the ability to accurately regulate duration and intensity of humoral and cellular response is the basis for health, also against the early onset of many tumors.

The immune system is able to control at least in part growth of tumors. In 1863, Rudolf Virchow could find active leucocytes in tumor tissues. And it was Paul Ehrlich in 1909 who postulated a "body's own protection system" against tumor cells. "The immune surveillance hypothesis" was born and elaborated in the 1950s and 1960s: T-cell-mediated immunity evolved as a specific defense against cancer cells and that T cells constantly patrol the body, searching for abnormal body cells [67]. Until today, there are

many clinical data demonstrating the correlation between the immune system/surveillance and the development of tumors: spontaneous remission of colon carcinoma and acute myeloid leukemia or remission of lung and liver metastases of lung cancer (NSCLC). As long as the tumor load is controlled by the immune system and as long as this balance between disease and innate and acquired immunity works, the life is not threatened. The same is for infectious disease.

Hip-Hop Balance. The correlation of immune dysfunctions and the development of tumors are also described, such as modification of T cells, T-cell anergy, reduced expression of molecules of signal pathways, and reduced cytokine production. The immune-risk phenotype (IRP) is defined as CD4/CD8 ratio. In healthy conditions, the number of CD4+ T cells is higher as the number of CD8 + CD28- T_{reg} cells (formerly called T suppressor cells) and the CD4/CD8 ratio is rarely less than 1.0. The ratio may drop as low as 0.1 during a progressive disease. In the case of HIV infection, the decline of CD4+ T cells is proportional to the virus load; the higher the virus titer, the more rapid the decline. As described earlier with our own therapeutical vaccine against EA virus, the efficacy strongly depends on the virus load. Too much virus and the vaccine treatment fails. T_{reg} cells can damp the antitumor T-cell response; therefore, the number of T_{reg} cells can be used as prognostic factor [68, 69]. There are lots of host factors from cytokines to B- and T-cell reactivity which can be used as determinants of a therapeutical vaccine response.

The Hip-Hop balance is defined as an immunological condition in which the disease has partially destroyed some effector and regulatory functions but the immune system can recover and response to vaccination due its redundancy. This is the therapeutical window and must be defined for each disease and personalized vaccine.

Death Imbalance. Back to the clinical trial of 1994, and the anti-melanoma vaccine. The tumor load in all patients was so massive that some patients died during the treatment. The balance of T-cell subsets and their location within tumor tissues is a prognostic factor for the immunotherapeutic efficacy. The accumulation of T_{reg} cells at the tumor site combined with a decrease of functional cytotoxic T cells (CTLs) is of bad prognosis. The vaccination would be too little too late. Laboratory parameters for this death imbalance could be the permanent activation of transcription factors such as NF-kB resulting in an abnormal overexpression of oncogenes, lethal shortening of leucocyte telomeres, chronic proinflammatory cytokine production, absence of T-cell reactivity, and downregulation of miRNAs associated with advanced stage of cancer [70]. A risk analysis of these parameters has to be done.

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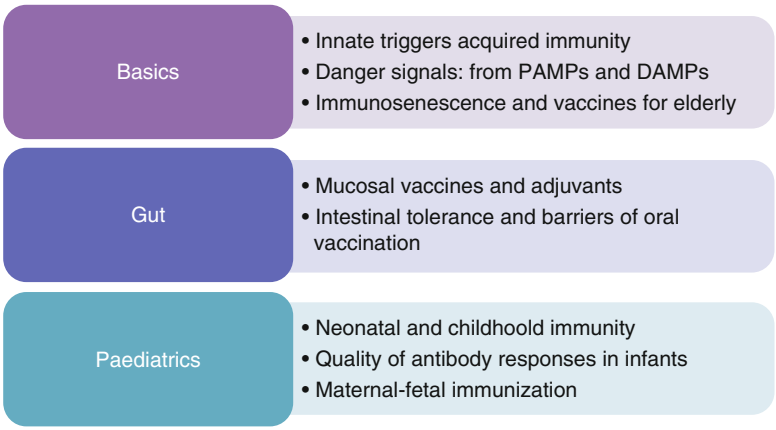
Part II

Vaccine Immunology

Overview of Part II



Long time the innate immunity was underestimated, was considered as relict from an ancient era, sufficient for insects but for mammals? This ignorance is reflected by classical vaccine development where the serum antibody IgG titer was the only critical read out of correlates of protection. Antibodies provide the first line of defense, can prevent an early infection, and correlate with protection. In case of an intracellular pathogen, antibodies cannot prevent the replication and do not protect. This is where T cells come into play, and the measurement of T cell immunity is a more accurate correlate of efficacy.

Vaccines for generating a robust CD8+ T cell response with a high effector capacity should address these fundamental key issues in an appropriate quality, magnitude, and duration of CD8+ T cell response. Live attenuated vector vaccines, also replicons and DNA vaccines, are able to stimulate CD8+ T cells, but not killed or conventional peptide/protein-based vaccines. Research on T cell vaccines is concentrated on accurate, conserved T cell epitopes. The development of therapeutical T cell vaccines for chronic infections or cancer diseases should address problems of adverse effects by persistent CD8+ T

cell activation. These T cell vaccines have the potential to generate immunopathology, such as exhaustion (loss of functions, beginning with a loss of IL-2 production) and inflammation. PD-1 high expression is one biomarker for exhausted T cells.

One of the goals of modern vaccinology is the development of vaccines as tools that can reduce the devastating morbidity and mortality caused by enteric diseases in children under the age of five living in developing countries. Understanding the processes involved in the induction of immune responses by orally delivered antigens, the delicate balance between immune activation and tolerance in the gut and the association between mucosal and systemic immunity is essential to assist the development and evaluation of vaccine candidates.

The main effector sites of adaptive immunity in the gut are the epithelium and the lamina propria. A large number of antigen-specific antibody-secreting cells (ASC) and activated T cells reside in these tissues and provide a first line of defense against intestinal pathogens. A hallmark of oral vaccination is the induction of long-lived antigen-specific mucosal IgA-secreting plasma cells.

There is an interface between malnutrition, premature T cell senescence, and susceptibility to infections. Chronic hunger may modulate the immune system through T cell repertoire. Hunger also influences the telomere length of CD8+ T cells resulting in a shortening. Chronic hunger in early life dramatically impacts the programming of the young thymus, a very crucial phase, having long-term effects on T cell development. Zinc is an essential mineral for the immune system, and a zinc deficiency results in thymic atrophy. It is suggested that the nutritional disturbance predisposes this population to infections. Malnutrition is one important reason for the loss of efficacy of oral vaccines in the third World.

More than half of the children that die under the age of five worldwide do so because of an infectious disease. Many of these diseases are vaccine preventable, and WHO estimates that around 1.5 million children below the age of five died in 2008 in such diseases. *S. pneumoniae* (pneumococcal) infections and rotavirus infections are leading causes, followed by infections caused by *Haemophilus influenzae B* (HIB), *Bordetella pertussis* (pertussis), measles virus, and *Clostridium tetani* infection in the neonatal period (neonatal tetanus).

A major disadvantage for the neonatal immune system is, independent of species, that most B and T cells are naïve. Small children are also impaired in both the maturation of the antigen-presenting cells and the capacity of such cells to respond to bacterial and viral antigens, with the important exception of TLR8. Using TLR8 ligands as vaccine adjuvants may represent a window of opportunity.

Whereas very young children cannot fully respond to an immunization, elderly patients have lost critical immune functions to fully respond to an antigen. The best vaccine approach for elderly is to start vaccination before aging is coming: development of novel (conjugate) vaccines with a broad antigenic, multivalent spectrum, e.g., influenza and pneumococcal infections,

inducing a strong complete long-lived memory B and memory T cell response with generating robust CTL activities; also a prime-boost regime with repeated booster vaccinations during adulthood. This expanded education of the immune system in young age is a solid basis for a recall and also a powerful immunological background against novel antigens in old age.

Basic Vaccine Immunology

2

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Contents

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Abstract

The most outstanding aspects of the immune responses following a vaccination are described here. But it is beyond the scope of this chapter to describe in detail all immunological functions. We focus on a more general description on what is known of the vaccine immunity.

2.1 Chemical Nature of Antigens

Viruses, bacteria, parasites, and fungi are permanent natural threats of our life. Also different types of allergies against pollen, mold fungus, animal hairs, or house dust mites are able to provoke an antigen-induced antibody response. Also environmental allergens, naturally or industrially produced ultrafine carbon particulate matter (PM), can cause allergies and promote asthma. PM is deposited in alveoli leading to a proinflammation response.

So manifold the world of antigens looks like, all known antigens (except for industrial PM) can be classified into five groups depending on their chemical nature (Fig. 2.1) – proteins, glycoproteins, carbohydrates, lipids (lipoproteins), and nucleic acids. An antigen is able to being bound by an antibody via surface epitopes. Epitopes or antigenic determinants are distinct molecular features of an antigen. Depending on the nature and the size of antigen, several different epitopes can decorate an antigen and can be bound by various antibodies. Special

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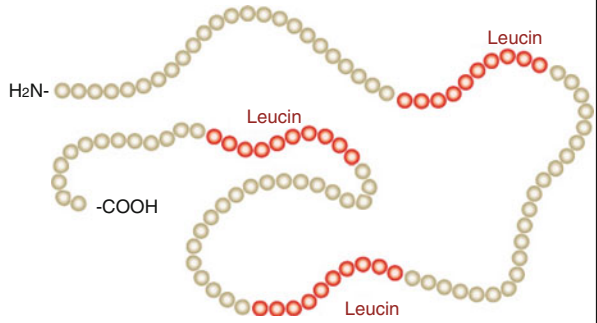
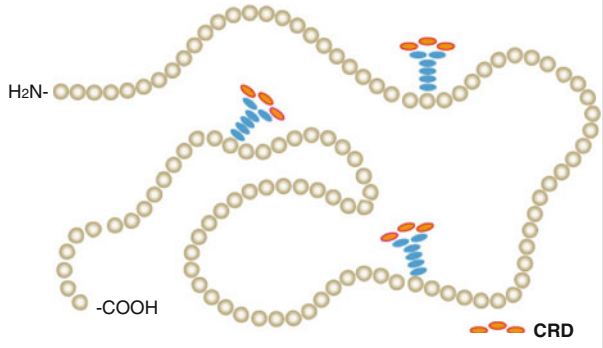
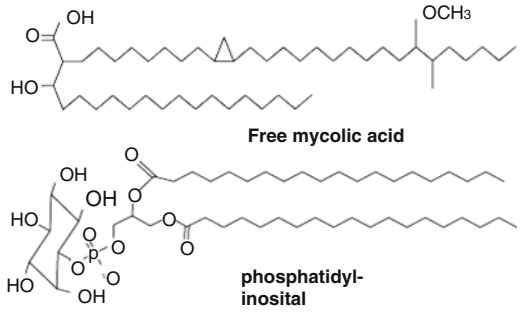
Antigen	Chemical structure	Antigenic recognition site
Pure protein		Leucine-rich repeats (LRR)-domain consisting of 2–45 LL repeats, with each repeat about 20–30 residues long.
Glyco-protein		Complex sugars, such as mannose, glucose or galactose, via their carbohydrate-recognition domain (CRD).
Lipids		Chain length is recognized of diverse sets of mostly bacterial lipids, glycolipids, and lipopeptides.

Fig. 2.1 Chemical nature of antigens. Each protein is encoded by genes and constructed of one or more chains of amino acids (primary sequence) linked by peptide bonds. The basic function of a protein is given by the tertiary structure, a stable three-dimensional structure. Many proteins are complexed with different sugars, like mannose, glucose, or modified by lipids (glycoproteins or lipoproteins). Glycoproteins are recognized as antigenic structure by their carbohydrate-recognition domain (CRD). Lipids can be recognized by their different chain lengths. Lipid antigens bind to CD1 molecules where their polar lipid head group is exposed to CD1-restricted T cells. After binding to TCR, these T cells interact with macrophages, dendritic cells, NK cells, and B and T cells, thereby contributing to innate and acquired immune responses. Various carbohydrates without protein binding are recognized via the C-lectin-like receptors. Compared to vertebrate DNA, microbial DNA (bacteria, viral, fungi) is hypomethylated and can be detected by Toll-like receptor 9. In contrast to DNA the antigenic character of foreign RNA is based especially on structural differences and the intracellular localization

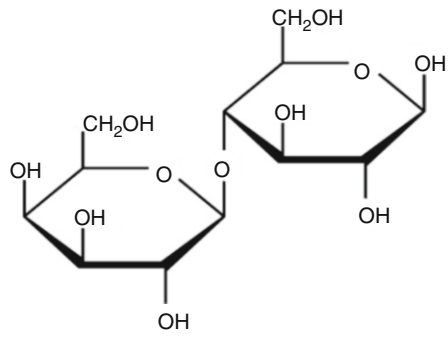
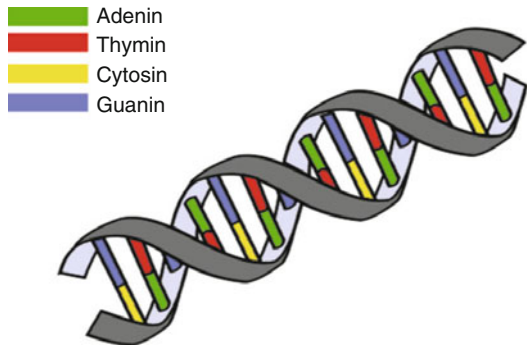
Carbo-hydrates		Recognition of mannose, fucose, galactose, glucan oligosaccharides via C-lectin like receptors.
Nucleic acids		**DNA**: Recognition of bacterial DNA by the hypomethylation (CpG) compared to vertebrate DNA. **RNA**: Recognition by primary nucleotide sequence, secondary and tertiary structure, or intracellular localization.

Fig. 2.1 (continued)

cell-surface, intracellular, and also secreted receptors, collectively called pattern recognition receptors (PRRs) and ubiquitously distributed in the body recognize various antigenic structures, thereby initiating an innate and acquired immune response.

PRRs identify invaders by their unique, highly specific, and highly conserved pathogen-associated molecular patterns, PAMPs. All sensors use PAMPs also to discriminate "self" from "nonself." This redundant PRR system guarantees to detect a pathogen in parallel by different sensors. If one sensor fails another PRR is active to trace this antigen. This high level of redundancy could be one reason why estimated 98 % of all infections in a healthy patient are detected and cured by the immune systems. PRRs and PAMPs are discussed later.

Self-antigens (cell debris, host-encoded proteins) are presented to T cells by dendritic cells (DCs) without any costimulatory molecules resulting in apoptosis of these T cells. Self-antigens, usually tolerated by the immune systems, become dangerous if the immune controlling fails.

The various antigens can be different in the quantitative and qualitative immune response, also in the duration and efficacy. The immunogenicity of an antigen is determined by several factors:

- Proteins are more immunogenic than lipids and carbohydrates.
- Chemical and structural differences of an antigen to a self-antigen.
- The size and complexity of an antigen: more epitopes for binding of antibodies.
- Particulate antigens are more immunogenic than soluble antigens.

A highly complex foreign protein, for which no similar structured self-antigen does exist, will be recognized as invader and induce a strong immune response.

2.2 Antigen-Presenting Cells

At best a vaccine will induce a complete immune response, comprising of components of innate and acquired immunity and humoral and cellular activities. Thereby B and T cells, monocytes, macrophages, natural killer cells (NK), granulocytes, or antigen-presenting cells (APCs) such as dendritic cells act in concert triggered by various cytokines, together with complement factors and antibodies.

APCs are in the early front for each vaccination and target cells for antigens. APCs bind different antigens and connect the innate and the acquired immunity. They use congenital, evolutionary, highly conserved patterns to recognize various antigens in order to activate B and T cells. Thus, the acquired immunity is regulated by innate immunological components.

The antigen recognition is part of the innate system and controlled by two corresponding elements. Element one is the pattern recognition receptor (PRR) [1]. Element two is the pathogen-associated molecular pattern (PAMP), exclusively expressed on microbes. Microbes, organized in one class, share the same PAMP [2].

The main drivers for the specific antigen (Ag) presentation are three types of professional APCs, monocytes [3]/macrophages [4], B cells [5], and dendritic cells belonging to the white blood cells (Table 2.1). All APCs are produced by hematopoietic stem cells in the bone marrow.

Dendritic cells. Monocytes/macrophages exhibit only a limited capacity to activate naive T cells, and the antigen presentation by B cells mainly serves as auto-presentation for antibody production. Especially dendritic cells (DCs) are the real professional cells for Ag presentation to T cells. DCs are mobile and can move as immature cells in the blood, and once activated, they migrate from blood to different tissues to secondary lymphoid organs. DCs are the main gatekeepers of the immune system. DCs possess complete molecular equipment to take up antigens, to process and present antigens to induce an immune response.

It is not surprising that DCs are in the focus of vaccine development since two decades. DCs are present in lots of tissues, lymphoid and non-lymphoid, including brain [6] and mainly abundant in tissues where external and internal environments run across: skin, lung, and gastrointestinal tract. Thereby, DCs are essential both for the immunotolerance and for induction of a protective response via MHC I and MHC II, in combination with costimulatory molecules such as B7, and proinflammatory cytokines. DCs are composed of a network of different subsets, which can be phenotypically and functionally differentiated.

Immature DCs. As immature cells DCs are always in motion to patrol through the body and to capture antigens at any time. They are sentinel cells of the immune system. At this stage the MHC molecules (HLA in humans) are only to a less extent prevalent. Also the costimulatory

Table 2.1 Professional antigen-presenting cells (APCs)

Cell type	Location	Function	Marker	Ref.
Monocytes:	Blood	Phagocytosis, Ag presentation		[3]
1. Classical			CD14+, CD16–	
2. Intermediate			CD14+, CD16+	
3. Nonclassical			CD14+, CD16++	
Macrophages	Tissue	Phagocytosis, Ag presentation	CD14+, CD40+, CD64+	[4]
B cells	Blood, lymphoid organs	Ag presentation, antibodies, regulation	CD19+, CD20+, CD21+	[5]
Dendritic cells	Tissue	Ag presentation	See Table 2.2	

Table 2.2 Functional subsets of dendritic cells (DCs) in peripheral human blood

Subsets of DCs	Localization	Hallmark and marker	Ref.
Myeloid (m)DCs:		T_{h2} cytokines,	
Dermal (d)DCs	Dermis	CD1a+, CD14+	[7]
Langerhans Cs	Epidermis	CD1a+, CD1b−, CD1c−	[8]
Brain (bDCs)	Brain	CD11b+, CD103+	[9]
Interstitial (iDCs)	Interstitium	CD11c+, CD11b+, CD1a+	[10]
Plasmacytoid (p)DCs	Blood, lymphoid organs	T_{h1} cytokines, interferon-α, CD11c−, CD11b−, CD1a+	[11]

signals are absent, necessary for the complete activation of T cells.

DCs cannot be detect and differentiated by any exclusive cell marker. Rather, a combination of markers, presence and absence, are used to identify DCs. Furthermore, the differentiation is complicated by the fact that monocytes, macrophages, and DCs share a common macrophage-DC progenitor and monocytes/macrophages can be developed into DCs after specific stimulation. Table 2.2 shows the functional subsets of DCs [7–11].

Depending on their ontogenetic origin, DCs are divided into a myeloid (also called conventional or classical) and a lymphoid line (nonconventional) [12]. Myeloid DCs (mDCs) are most similar to monocytes and can be divided in several subsets and are divided in migratory DCs and the lymphoid tissue-resident DCs. Surface marker for mDCs are CD11c+,CD11b+, CD1a+, and sometimes CD103+. These peripheral DCs are located in the epidermis as Langerhans cells, in the skin dermis, moreover in the mucosa, and interstitial spaces. All mDCs but LCs produce large amounts of different T_{h2} cytokines such as IL-4 and IL-10. Following the Ag capture and processing, mDCs migrate to regional lymph nodes in order to present the peptide antigens and to activate both CD4+ and CD8+ T cells.

Lymphoid DCs are referred as plasmacytoid DCs (pDCs) and look like plasma cells. They circulate in the blood stream and are characterized by their ability to produce huge amount of type I interferon [13] and other T_{h1} cytokines. To this, pDCs must be activated by viruses. Embedded in lymphoid and in peripheral tissues, pDCs form the group of lymphoid tissue-resident DCs. Surface markers are CD11c−, CD11b−, and CD1a+. DC subsets can also be differentiated by their life span and especially by their anatomical localization. Thus, the specific function of a DC is adjusted to its localization [14].

2.2.1 Capture of Topically Applied Antigens by Langerhans Cells

Langerhans cells (LCs) are a subset of skin DCs, radio-resistant, and also found in the mucosa of the noose, mouth, or in the genital tract and play a key role in the first line of defense. In particular, Langerhans cells in the basal and suprabasal layers of the epidermis are in a strategic advantageous position to capture vaccine antigens, process them, and migrate to draining lymph nodes. Almost 2 % of all epidermal cells are LCs. Following the antigen capture, LCs migrate through the dermis to the lymph vessels. Two different surface receptors of LCs capture glycoproteins via endocytosis (pinocytosis and phagocytosis), the C-type lectin receptor DEC-205 (CD205) and the langerin receptor (CD207). During maturation LCs express MHC I and II molecules.

Lectin-like receptors. Many cells of the immune systems, DCs, monocytes, and macrophages, are provided with lectin or lectin-like receptors (LLR). These LLRs are pattern recognition receptors and perform the same functions as the family of Toll-like receptors (TLR) which are discussed later. The lectin-like receptors DEC-205 (CD205) are part of the macrophage mannose receptor (MMR) family and recognize terminal monosaccharide residues of microbes and fungi via the carbohydrate-recognition domain (CRD). Also the langerin receptor (CD207), member of the type II receptor family,

recognizes and processes microbial glycoproteins. The difference between DEC-205 and CD207 is the different recognition of end-standing sugars. So diverse the carbohydrates look like in the body, so diverse are the carbohydrate-recognition domains of LLRs [15]. Even the lowest differences in the glycosylation pattern compared to the wild type activate a suitable LLR that binds with high affinity to its specific carbohydrate antigen. This implicates a very high degree of variability of the LLR-binding domain. Another trick of LLRs against foreign carbohydrate antigens is the multimerization of LLRs. As response to repetitive carbohydrate units of a given antigen, the LLR can form and consequently augments the binding capacity.

The C-type lectin and langerin receptors are generally used to identify Langerhans cells: LC (CD 207^{+}/$CD205^{+}$). Intradermally applied antigen is taken up by LCs within some minutes via these receptors. Even large molecules such as antibodies are able to cross the basal membrane which separates the epidermis from the dermis and are taken up by the dendrites of LCs [16].

At the same time these dendrites can expand to the opposite direction and, by passing the tight junctions, penetrate into the stratum corneum of epidermis without damage and take up antigens [8]. Hence, LCs control the space to the hard skin as well as the inner space to the dermis. LCs are mobile and literally sentinel cells of the immune system. However, LCs mainly capture bacterial and fungal antigens, also allergens, but no viral antigens [17].

Once taken up LCs present the processed antigens via cross priming to CD8+ T cells and induce a strong cellular immune response. During this activation step, LCs produce lots of IL-15 but also IL-6 and IL-8. These essential cytokines support the maturation of cytotoxic T lymphocytes (CTLs) [18].

Cross priming. Cross priming was first described in 1976 [19] and stands for the presentation of exogenously taken up antigens such as debris from apoptotic or necrotic cells by APCs such as LCs to CD8+ T cells via MHC I molecules. In contrast to direct (classical) priming, cross priming is the presentation of endogenously synthesized antigens from intracellular pathogens. Cross priming could be also another redundant mechanism of the immune system to make sure that a CTL response can be developed against such viruses which cannot infect APCs or infect APCs and deactivate the processing and presentation mechanism like MCMV [20]. The role of cross priming in natural viral infections remains controversial [21]. Cross priming is required for vaccination.

Effector mechanisms. Once primed and activated, CD8+ cytotoxic T lymphocytes (CTLs) have a large repertoire of effector mechanisms to destroy the target cells: (1) cytotoxic proteins, e.g., perforin and granzyme A and granzyme B [22]. In this process the direct cell-to-cell contact by the T cell receptor (TCR) and MHC I is essential. (2) CTLs directly bind to the Fas ligand (CD95L) by the Fas receptor on the target cell. This activates the caspase cascade leading to apoptosis [23]. (3) CTLs secrete large amount of TNF and IFN gamma. TNF binds to its receptor on the target cell, inducing apoptosis, whereas IFN gamma enhances the expression of MHC I and Fas ligand, resulting as well in apoptosis of the target cell [24]. Figure 2.2 shows the cross priming and direct priming of CD8+ CTLs and some effector mechanisms against a target cell.

2.2.2 Capture of Antigens by Dendritic Cells

The different layers of the skin are constructed like a phalanx. Could a pathogen successfully invade the epidermis, the next front line is the dermis with dermal dendritic cells (dDCs), member of the myeloid DCs family. These cells are also motile, express high levels of MCH II molecules, and can cross present viral antigens other than LCs. Two distinct populations can be described: dDCs CD1a+ and dDCs CD14+. Both populations are phenotypically and functionally quite different.

The first one exhibits a typical dendritic morphology with DC typical markers such as CD1, CD83, or CD208. Another feature is the uptake of antigens and processing and migration to the lymph node with activation of T cells. The other

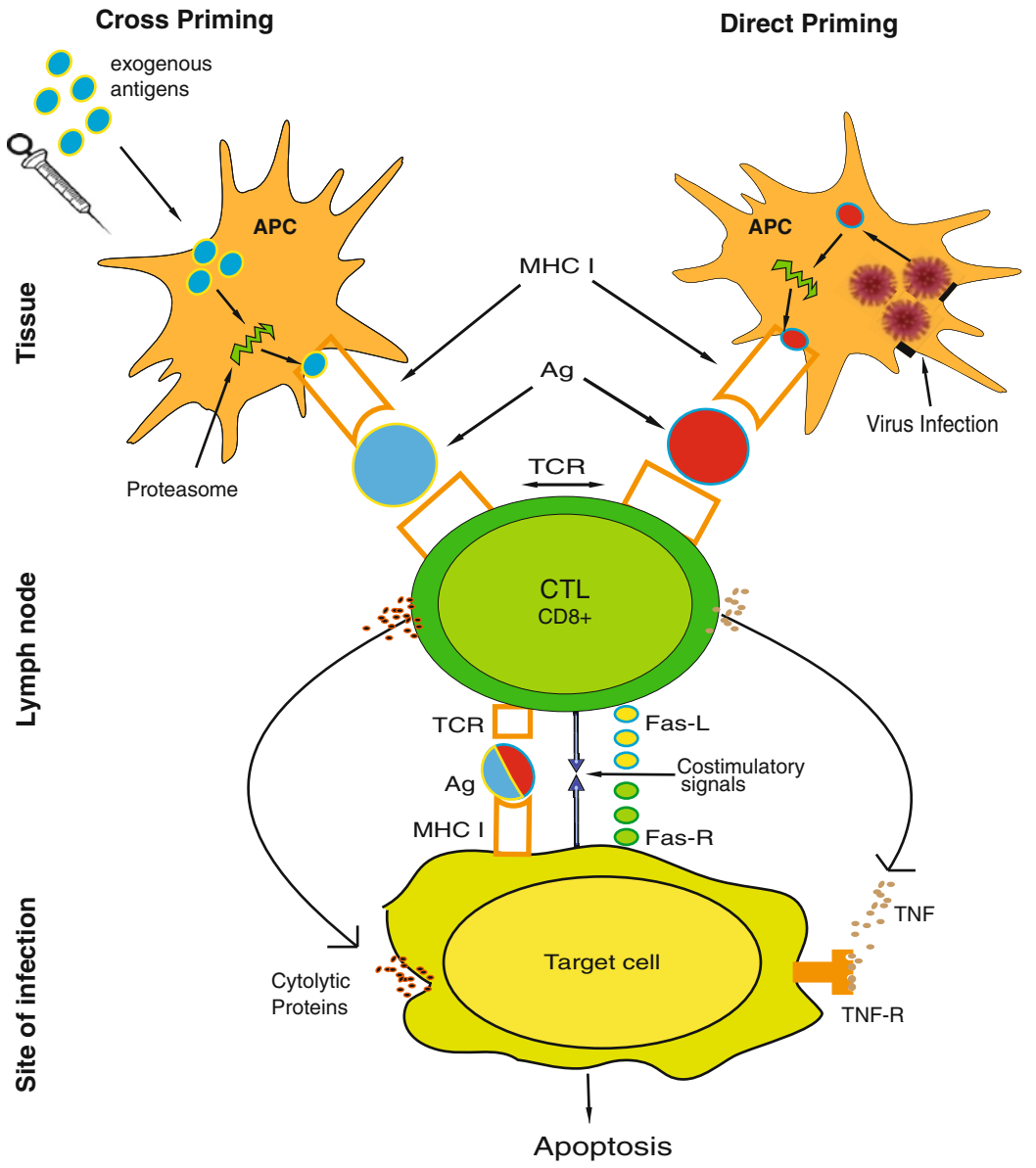


Fig. 2.2 Cross priming compared to direct priming with activated CTLs. Cross priming stands for the presentation of exogenous uptake of antigens such as debris from apoptotic or necrotic cells by APCs. Also DNA vaccination uses cross priming for activation of the cellular immune response. In contrast, direct (classical) priming stands for the presentation of endogenously synthesized antigens from intracellular pathogens. After intracellular processing, the antigens are presented via MHC I to CD8+ cytotoxic T lymphocytes. The activated CTL recognizes via its T cell receptor (*TCR*) the cognate antigen on the surface of a target cell. Several effector mechanisms can destroy the target as described before. *Ag* antigen, *Fas-L* Fas-Ligand, *Fas-R* Fas-Receptor, *TNF* Tumor necrosis factor, *TNF-R* Tumor necrosis factor-receptor

subpopulation exhibits a macrophage morphology and with typical macrophage marker, such as CD14, CD68, or CD209. Those dDCs also exert a typical macrophage function, the phagocytosis of bacteria and viruses. But among these two subpopulations, there are also hybrids, phagocytotic dDCs with a dendritic morphology [7]. The common ontogenesis helps to explain this mixture of dendritic cells and macrophages. DCs can develop from macrophages following a specific

stimulation. GM-CSF induces CD1, and LPS induces CD14 dendritic cells.

Cytokines. In contrast to LCs, which produce little cytokines except for IL-15, especially dDCs secrete large amount of different cytokines following stimulation of CD40: IL-1 alpha, IL-1 beta, IL-6, IL-7, IL-8, IL-10, IL-12, GM-CSF, TNF alpha, and TGF beta [25, 26]. Most of all these cytokines are produced by both dDCs subpopulations, but IL-10 is only secreted by CD14- DCs. The pattern of proinflammatory and anti-inflammatory cytokines indicates the direction of immune response.

Box 2.1
Impact of APCs for vaccine development

The largest human organ is the skin. Dependent from the body size, the skin decorates 1.52.0 m^2 (16.1–21.5 sq ft.). The skin is the anatomical barrier from tissue injury and pathogens and is the largest immunological organ.

A transcutaneous immunization (TCI) targets LC and other DCs. A topical application delivers a vaccine to LCs of the epidermis. Needle-free and noninvasive methods are hydrogel patches containing biodegradable nanoparticles such as chitosan as carrier, liposomes, or other polymers like polyethylene glycol-based gels (PEG). Hydrophilic macromolecules as peptides/proteins cannot penetrate across the skin and need a carrier. One limitation of this application method seems that only soluble and no particulate antigens are able to induce an immune response [27]. The particle size and the hydrophilicity/hydrophobicity of the carrier may influence the uptake by phagocytosis.

A low-invasive method is microneedle patches. Such patches are able to carry different kinds of antigens, from DNA to peptide/protein or inactivated or live virus. Lower vaccine doses are required compared to a classical intradermal injection. Needles are robust enough to penetrate the skin and to painlessly deliver the vaccine to LCs. This simple, safe, effective, and low-cost device is suitable for self-administration.

The application of antigens via electroporation targets DCs of the dermis. The electric pulses reversibly change the permeability of the cell membrane and therefore the antigen can penetrate the cell.

Besides this simple in vivo targeting of APCs by cutaneous immunization routes, a more complicated ex vivo method for direct targeting DCs was developed as whole cell vaccine. DCs were isolated from patient's blood, cultivated in vitro, and challenged with an antigen, e.g., a tumor-associated antigen (TAA). By the uptake of the TAAs, the maturation of the DCs is activated and the TAAs are processed. After a complex procedure in the laboratory of 3–5 days, the patient is injected with his autologous TAA-loaded DCs which in vivo alert T and B cells. In 2010 the US FDA has approved Sipuleucel-T, the first personalized cellular autologous immunotherapy for treatment of advanced prostate cancer [28].

For a deeper understanding of various delivery methods for vaccines, see Part 7 of this book.

2.3 Inflammation and Cytokines

Inflammation is following a microbial infection by bacteria, viruses, fungi, or other pathogens. Further inducers for inflammation are trauma or toxins, reactive T cells, and more. The function of an inflammatory reaction is to prevent an infection at a very early start by producing proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α. Other cytokines exert a pleiotropic nature acting both proinflammatory and anti-inflammatory [29].

Proinflammatory cytokines. IL-1 and TNF operate in a synergistic manner and upregulate a

cascade of genes, e.g., genes for type II phospholipase A2 (PLA2) and cylooxygenase-2 (COX-2), or the inducible NO synthetase (iNO) resulting in the production of chemotactic enzymes. These enzymes attract now immune cells like macrophages, NK cells, or neutrophils to the site of infection. At the same time blood vessels dilate and become more permeable. Other cytokines such as IL-4, TGF-β, and especially IL-10 control and suppress the inflammation process and hence they support the healing. These cytokines act as anti-inflammatory and account for the pivotal immune balance. Therefore, every therapeutic intervention of the immune homeostasis must be very carefully calculated.

Anti-inflammatory cytokines. IL-10 plays a key role in immune suppression. It is a master cytokine, powerfully monitoring the immunopathological milieu after infections. IL-10 is under normal conditions essential to regulate the inflammation. Different T cell subsets produce IL-10, such as T_{h1}, T_{h2}, and T_{h17}, but also eosinophils and neutrophils [30], NK cells, DCs, and B cells [31, 32]. This cell variability and redundancy in anti-inflammatory cytokine production gives rise to a multiple immune regulation on multiple levels with the only aim to suppress the inflammatory response triggered by IFN-γ, IL-2, or TNF and other proinflammatory cytokines.

Because IFN-γ and IL-2 are important for the establishment of a cellular response, the impact of IL-10 leads to a shift from a mainly T_{h1} response towards a mainly T_{h2} response. Not only the T cell-mediated immunity but also the maturation and function of DCs are influenced by this cytokine, and the overall cytokine pattern has changed following IL-10 secretion. IL-10 acts directly on APC cells to decrease accessory molecules such as B7, necessary for MHC I and II. Otherwise, IL-10 stimulates the proliferation of CD4+ T cells, thereby enhancing a humoral response. IL-10 is a key cytokine bridging the innate and acquired immune reactivity, on the one hand constitutively produced by macrophages and on the other hand produced by T cells after specific stimulation.

IL-10 as biomarker. IL-10 takes a special part in some intracellular infections by diverse pathogens. Some bacteria, fungi, viruses, or parasites induce IL-10 by binding to their specific pattern recognition receptor (PRR) and activating gene expression. For this reason IL-10 can be also used as biomarker, as surrogate marker of some infections. The level of IL-10 production seems to be dependent on the intensity of the PRR stimulation. TNF will be immediately downregulated; consequently, the complete inflammatory response falls down. Because of the lack of TNF, probably the main inducer, also the apoptosis gets down.

The significant contribution of IL-10 to chronic infections must be considered critically. In these special conditions, IL-10 acts as a devil molecule because it prevents a strong inflammation response and so avoiding the clearance of the pathogen [33]. Obviously, various microorganisms enact escape strategies of targeted induction of IL-10 for their persistence.

Box 2.2
Impact of IL-10 on vaccine development

Leishmaniasis is caused by an obligate intracellular and zoonotic parasite of the genus Leishmania, causative agent for a group of related diseases such as visceral (VL), cutaneous, or mucosal/mucocutaneous leishmaniasis as far as fatal systemic infection.

The estimated worldwide prevalence is 12 million cases, with an annual mortality of 60,000, occurring in 4 continents, endemic in 88 countries, 72 of which are developing countries. According to WHO, two million people will be newly infected annually.

A special situation is given to VL as an important opportunistic coinfection associated with HIV, an underestimated combination. Both pathogens reinforce each other. There is as yet no effective vaccine. One reason is given to the escape strategy of Leishmania spp., the evasion by IL-10. BALB/c mice were primed by a DNA vaccine, coding either for LACK (Leishmania

homologue of receptors for activated C-kinase) or for TRYP (tryparedoxin peroxidase), and boosted by the corresponding antigen in modified vaccinia virus Ankara (MVA) and challenged by L. major. LACK-vaccinated mice developed a high level of IL-10 due to T_{reg} cells. The T_{h1} response was at the same time downregulated, resulting in a lack of IFN-γ and TNF production. But the blocking of IL-10 receptors enhanced the IFN-γ production up to 3.5-fold. Conclusion: The DNA/MVA-LACK vaccine did not work. Other than the vaccination with TRYP did. The level of IL-10 was low, but the level of IFN-γ was high. TRYP-vaccinated mice were fully protected after challenge with L. major [34].

These studies demonstrate that Leishmania spp. are able to survive and persist by using the host cytokine IL-10. Furthermore, the level of IL-10 titer in correlation to the level of IFN-γ could be a predictive biomarker of the success of a vaccination. The choice of an appropriate antigen will influence the direction of response, towards a T_{h1} or T_{h2} and towards a cellular or humoral reactivity.

In order to monitor the clinical process of visceral leishmaniasis infection, the level of IL-10 titer is measured among others. The correlation to the parasite load is significant. Also the corresponding titer of IFN-γ is significant: A low IFN-γ titer goes along with a high titer of IL-10. So, the titer of IL-10 is an indirect biomarker for the severeness of this parasitic infection [35]. Other pathogens like Trypanosoma cruzi, Klebsiella pneumoniae, or Candida albicans also hijack IL-10 to prevent an immune attack.

The impact of IL-10 as immune modulator is also expressed in the context with some viruses. Such viruses code for their own IL-10 homologue (vIL-10) and can so directly influence the immune response against them. The first vIL-10 was described for Epstein-Barr virus (EBV) in 1990 [36]. The homology on protein level to the huIL-10 is 70 %.

There are also viruses not possessing own genes encoding such virokines. These viruses circumvent the lack of vIL-10 by expressing a special viral protein which upregulates the host production of IL-10 [37].

Meanwhile IL-10 is also used as prognostic factor for some cancer diseases. A high IL-10 expression is significantly associated with aggressive clinical manifestations in melanoma and squamous cell carcinoma [38, 39].

2.4 Therapeutic Vaccines and the Immunosuppressive Environment

Proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are responsible for the initiation of inflammation in response to infections, tissue damage, or cancer development [40]. This activates a cell-mediated immune response. Anti-inflammatory cytokines are also released to fine-tune, to balance this immune reaction, and to limit the sustained inflammatory reactions.

Immunosuppression. As discussed before, the central role of IL-10 to supervise the inflammation reaction, whether in microbial infections or in cancer diseases, is obvious. Other cytokines are also involved in controlling the inflammation, such as TGF-β, by blocking IL-1α/β of monocytes/macrophages by IL-1 receptor analogist (IL-1ra), or IL-4, by blocking the binding of proinflammatory cytokines to their specific receptors, or IL-6, by inhibition of TNF, or other cytokines such as IL-1 or IL-11 by promoting a T_{h2} response. It must be noted that with the exception of IL-1ra and further soluble cytokine receptors, all anti-inflammatory cytokines have a

dichotomous nature and can also perform proinflammatory activities [41].

In all immunosuppressive events, the aim of anti-inflammatory cytokines is to downregulate T effector functions resulting in a local immunosuppressive milieu. Only with this prior condition, pathogens can persist and tumor cells can proliferate. The immune regulatory network has changed from protection against pathogens to a severe protection of the nested pathogens. Some microbial pathogens encode for own IL-10 as part of their escape strategy, some tumors secrete IL-10 or TGF-β also as part of their escape strategy enhancing the local dominance of anti-inflammatory molecules and the establishment of an immunosuppressive environment to evade the protective immune response. Also tumor-associated macrophages secrete many cytokines which promote immunosuppression [42].

Any therapeutic vaccination has to overcome this strong bastion of anti-vaccine activities where the antigen presentation by DCs or the MHC I and II is reduced or abolished and the CTL activities reduced or stopped. The failure of such vaccines is to restore not effectively the T cell immunity against the persisting pathogens and then maintain this status of restored inflammatory response against the status of immunosuppression. The fact alone that bacteria, viruses, and parasites use pathogen-encoded or host-encoded proteins to survive within the host demonstrates the high priority of proinflammatory cytokines and the related essential cellular activities as defense strategy against invading microorganisms and cancer diseases.

Based on this knowledge a therapeutic vaccine must fulfil at least three fundamental functions as outlined in Table 2.3.

Table 2.3 Fundamental functions of therapeutic vaccines

1. Dam up the anti-inflammatory process
2. Reconstitution of the proinflammatory environment
3. Repeated exposure of multiple antigens to reconstitute the T cell immunity

2.5 Pathogen Recognition

The long cascade of an antigen-specific immune reaction is started upon the recognition of an antigen by APCs via their pattern recognition receptors (PRRs) to generate a B and T lymphocyte immune response.

These germ-line-encoded PRRs are part of the innate immune system and cross-link the innate with the acquired immunity which depends on clonal expansion of B and T cells. There is a permanent cross talk between innate and acquired immunity with a clear hierarchy. The PRRs form the base of a highly efficient and redundant receptor network for permanent monitoring of pathogens and dangerous self-antigens. PRRs are expressed on the cell surface or are located in endosomes or in cytoplasm. This redundant system guarantees that a pathogen can be detected in parallel by different sensors. If one sensor fails, another PRR is active to trace this antigen. This high level of redundancy could be one reason why estimated 98 % of all infections in a healthy patient are detected and cured by the immune systems.

Pattern recognition receptors (PRRs). The theoretical basis of the mechanisms of pathogen recognition was developed by Charles Janeway. In 1989, he postulated that each APC would express pattern recognition receptors (PRRs) recognizing pathogens on a specific conserved pattern of molecular structures called "pathogen-associated molecular patterns" (PAMPs) [43].

2.5.1 Non-Toll-Like Receptors

In addition to Toll-like receptors (TLRs), meanwhile other classes of innate PRRs, chemically and structurally different from TLRs, were described. As against TLRs little is known about the precise molecular mechanisms of most of all non-TLRs:

1. *NLRs* – nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). From the human NLR family to date 22 members are known; the most prominent are NOD1 and NOD2 which control bacterial infections and inflammation [44].

2. *RLRs* – the retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs). This family comprises three members, the retinoic acid-inducible gene I (RIG-I), the melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), and plays a key role in sensing viral RNA [45].
3. *CLRs* – C-type lectin receptors. Members of this diverse family of 17 subfamilies act as soluble PRR or also membrane bound. Dectin-1, and dectin-2 receptors, i.e., mainly detect mannose and glucan structures, but besides these dominant carbohydrates also proteins and lipids. CLRs have been implicated especially in antifungal immunity [46, 47].
4. *DNA sensors*, i.e., DAI, the DNA-dependent activator of IFN-regulatory factors, or AIM2 (absent in melanoma 2), or the indirect sensing of dsDNA by the RNA polymerase III, a polymerase which transcribes AT-rich dsDNA into dsRNA, which in turn is now able to activate (RIG-I)-like receptors [48]. These DNA sensors recognize cytosolic DNA [49].

Toll like. In 1996, the first Toll PRR was characterized in Drosophila [50], and only 1 year later the first "Toll-like" receptor (TLR) was described in humans [51]. The Toll gene of Drosophila was isolated in 1988, and the Toll protein was characterized as an integral membrane protein with a cytoplasmatic and a large extracytoplasmatic domain [52].

In contrast to most of TLRs which are mainly sensors for extracellular pathogens, non-TLRs are located within the cell [53]. Table 2.4 gives an overview on the different classes of PRRs.

The immense significance of the germ-line-encoded PRRs for the immune defense against pathogens is highlighted by their high quantity and distribution within the animal kingdom, from arthropods to vertebrates, from insects to mammals, the whole evolution preserved in this receptor family. A study from 2007 counts for around 500 PRRs, among them 177 TLRs, detected in 77 distinct organisms [54]. Insects do not possess a specific acquired immunity, but only an innate one, like plants. In addition, the discovery of the evolutionarily ancient PRRs emphasizes the dominant role of the innate immunity against the mammalian acquired immunity. The pacemaker of the immune response cascade is the innate system, even though innate and acquired immunity work hand in hand. A short view on the evolution of key steps towards the development of a complete immune system is given in Table 2.5 and shall illustrate the ancient role of the innate immunity.

Different PRRs work together to sense an individual pathogen and to adjust immune defenses to its evolving virulence structures displayed during infection.

Dependent on their functions, PRRs can be divided into two groups as shown in Table 2.6.

2.5.2 Toll-Like Receptors

TLRs are the best characterized sensors among the heterogeneous group of PRRs and recognize both exogenous and endogenous pathogen-associated molecular patterns (PAMPs), but also endogenous damage-associated molecular patterns (DAMPs). DAMPs arise from cancer cells, necrotic cells, tissue injury, or degradation processes.

Currently there are 10 TLRs discovered in humans, 13 in mouse. TLR-10 is restricted to humans only.

Distribution. TLRs can be found in DCs, macrophages and monocytes, T cells, B cells, and NK cells. Moreover, TLRs are also expressed on normal tissue cells, such as endothelial cells, fibroblasts, also on muscle cells, still on cartilage, and osteochondral tissues, namely, on chondrocytes, osteoblasts, and osteoclasts. Recently, TLR expression was also detected in fat tissue, on adipocytes [55]; in the liver, on hepatocytes [56]; and in the spleen [57]. This broad ubiquitous distribution of TLRs on various cells, in different tissues, and in different organs underlines the systemic significance for the innate immunity with its inflammation responses and the development of an acquired immunity [58].

TLRs and cell surface. The TLRs 1, 2, 4, 5, 6, and 10 are located on the cell surface. They are type 1 α-helical transmembrane glycoproteins: The leucine-rich ectodomain comprises the

Table 2.4 Classes of different PRRs, ligands, and cellular distribution

	Ligand	Cellular distribution
Toll-like receptors		
TLR2/1	Triacyl lipopeptides, soluble lipoproteins	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR2	Peptidoglycan, lipoteichoic acid, lipoarabinomannan, zymosan	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR4	LPS, flavolipin	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR5	Flagellin	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR6/2	Diacyl lipopeptides	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR3	dsRNA	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR7	ssRNAs rich in guanosine or uridine	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR8	ssRNAs rich in guanosine or uridine	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
TLR9	Unmethy. CpGs, hemozoin in parasites	B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells, and endothelial cells
NOD-like receptors		
NOD1	γ -D-glutamyl-*m*-diaminopimelic acid	e.g., DCs
NOD2	Muramyl dipeptide (MDP)	e.g., DCs
NLRP1	Activated by lethal toxin	e.g., DCs
NLRP3	Pathogen-derived toxins, e.g., M2 of influenza	e.g., DCs
NLRP7	Acylated lipopeptides	e.g., DCs
RIG-like receptors		
RIG-1	dsRNA with blunt ends, ssRNA with 5′ triphosphate, RNA panhandle structure	All cells except for pDCs
MDA5	Long synthetic dsRNA RNase L-cleaved self-RNAs with monophosphate ends	All cells except for pDCs
LGP2	Neg./pos. regulator for RIG-1/MDA5	All cells except for pDCs
C-type lectin receptors		
Dectin-1	β-1,3-glucan (fungal pathogens)	Phagocytic immune cells
Dectin-2	High-mannose structures	Predominantly expressed on tissue macrophages, DCs, and inflammatory monocytes
Macrophage-inducible C-type lectin	Variety of endogenous and exogenous ligands, e.g., SAP130, α-mannan, trehalose dimycolate, mycobacterial cord factor	Predominantly expressed by activated macrophages
DNA sensors		
AIM2	Cytosolic DNA	Macrophages
p202		
IFI16	Cytosolic DNA	
IFIX		
DDX41		

Table 2.5 Short timeline of some evolutionary major events in the development from innate to acquired immunity measured in millions of years ago (MYA)

Era	MYA	Evolution of life	Evolution of immunity
Cenozoic	1.8	Homo sapiens	Complete innate and acquired immune system
		Atmosphere: 0_2 = 20,95 %	
Mesozoic	100	Bees	Flowering plants (angiosperms)
	144	First mammals	
Paleozoic	350	Atmosphere: 0_2 = 26 %	Acquired immunity: MHC and antibodies
	450	Jawfish (lamprey)	
		Jawless fish (hagfish)	
Precambrian	600	Hydra (first animal?) Metazoa	Innate immunity: antibacterial activities and TLRs
	1.200	First multicell organisms (algae)	Sex and meiosis!
	3.500	Single-celled organisms	
	4.500		Origin of Earth

Table 2.6 Functional groups of PRRs

1. Scavenger receptors
   Responsible for phagocytosis of microorganisms without relaying intracellular signals, e.g., mannose receptors
2. Signaling receptors
   With relaying intracellular signals for triggering the innate and acquired immunity, e.g., TLRs

ligand-binding site, a single membrane-standing α-helix, and a cytoplasmatic Toll/interleukin-1 receptor (TIR) domain, responsible for the downstream signal transduction. These cell-surface TLRs are specialized to recognize and bind structures of gram-negative and gram-positive bacteria.

TLR1 recognizes soluble lipoproteins, e.g., Neisseria meningitides. TLR2 recognizes lipoproteins and also glycolipids of, e.g., Mycoplasma and Treponema maltophilum. TLR2 acts as heterodimer together with TLR1, TLR6, or TLR10. TLR2 is also specialized on fungal ligands like zymosan.

LPS. One of the best investigated pathogenic structures is lipopolysaccharide, LPS. TLR4 binds LPS via interacting with the myeloid differentiation protein, MD-2, forming a tandem TLR4/MD-2. This tandem is the molecular basis for the binding of lipid A, the biologically active component of LPS (for a schematic figure of LPS, see Chap. 33). In this process the co-receptor MD-2 is responsible for binding of the fatty acids by binding the acyl groups [59].

Additional nonbacterial ligands for TLR4 other than lipid A are, e.g., gp52 envelope protein of MMT virus, or fusion protein of RS virus, or endogenous ligands, such as the heat shock proteins HSP60 and HSP70.

TLR5 is the only TL receptor for binding pure proteins. TLR5 recognizes a highly conserved region of bacterial flagellins, and thus this receptor is able to recognize and bind a broad spectrum of microbes.

Little is known of TLR10 with the exception that TLR10 and TLR1 form a heterodimer. Until today the natural ligands of TLR10 are unknown.

In summary, TLRs 1, 2, 4, 5, 6, and 10 are specialized to bind bacterial and other pathogenic lipoproteins, glycolipids, and lipopolysaccharides, beyond that viral envelop proteins or endogenous structures described from tissue damage or other degradation processes, such as heat shock proteins.

TLRs and nucleic acids. TLRs 3, 7, 8, and 9 are located endosomally and recognize exclusively nucleic acids in terms of bacterial, viral, or host-derived DNA and RNA. TLR3 binds dsRNA and can also be activated by the synthetic dsRNA poly I:C. Furthermore, this receptor binds host-derived mRNA and tRNA.

The special character of TLR3 as antivirus receptor is demonstrated by the induction of type I interferons (IFNs) and the subsequent innate antiviral activities after binding to TLR3. Natural dsRNA normally arises during viral replication. However, naturally derived dsRNA is only a weak activator of TLR3 in vitro. TLR7

preferentially expressed on pDCs, like TLR8, and TLR9 bind ssRNA and are activated especially by viruses causing chronic infections. The activation of these receptors also induces IFNs. TLR9 recognizes unmethylated bacterial ssDNA, CpGs, but also viral DNA, e.g., HSV-1 and HSV-2 [60, 61], in contrast to other nucleic acid-binding receptors.

TLR7 and virus. Inactivated viral vaccines use TLR7. This could be very clearly demonstrated with inactivated whole influenza A virus. The viral RNA was bound by TLR7 on pDCs leading to a massive production of IFN-α. It is important to note that the process of virus inactivation does not influence the binding properties, respectively, the endosomal receptor capacities. This is in contrast to a recombinant flu vaccine which is only composed of proteins, or a split vaccine, where the viruses are destroyed by detergents, and the viral RNA is eliminated by purification procedures. An activation of TLR7 by recombinant or split vaccines does not happen [62, 63].

TLR9 and CpG. The natural bacterial backbone of a DNA vaccine itself is able to activate the TLR9. But this natural adjuvant effect is very weak probably due the fact that the vaccine DNA is mainly a dsDNA and not ssDNA. To improve this weak adjuvant effect, several synthetic species-specific CpGs can be incorporated into the DNA vector on the same plasmid or on an extra plasmid [60].

2.6 PAMPs: Pathogen-Associated Molecular Patterns

TLRs but also other PRRs identify bacteria, viruses, fungi, protozoa, and parasites by their unique, highly specific, and highly conserved pathogen-associated molecular patterns, PAMPs. All sensors use PAMPs also to discriminate "self" from "nonself." Not all ligand- recognition mechanisms are fully understood. Major PAMP ligands and some known structural features are:

1. *Lipids*: Chain lengths are recognized, e.g., triacyl (TLR1), diacyl (TLR2), and hexaacyl of LPS (TLR4). The conserved pattern of LPS is the lipid A. The immunostimulant monophosphoryl lipid A (MLP) used as adjuvant contains only five lipid chains. And, in contrast to the natural LPS, the inflammatory activity is about 100 less and a weak activator of the innate immune response [64].
2. *Proteins*: The leucine-rich repeat (LRR) domain consisting of 2–45 LL repeats, with each repeat about 20–30 residues long, is the target PAMP for PRRs. These PRRs contain themselves an LL domain and the resulting receptor-ligand complex consists of a protein-protein interaction [65].
3. *Carbohydrates*: Complex sugars form with proteins and glycoproteins or with lipids and glycolipids. Some TLRs and CLRs recognize complex sugars, such as mannose, glucose, or galactose, via their carbohydrate-recognition domain (CRD), a domain within the lectin multidomain protein [66].
4. *RNA*: Many PRRs sensing RNA are known for recognizing not only nonself but also self RNA under pathological conditions. Therefore, several RNA recognition features are essential for the identification of "nonself" RNA, e.g., primary nucleotide sequence, secondary and tertiary structure, or intracellular localization [67].
5. *DNA*: The molecular pattern for PRR recognition of bacterial DNA is the stringent hypomethylation compared to vertebrate DNA. Microbial DNA contains a high frequency of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides [68].

2.7 DAMPs: Damage-Associated Molecular Patterns

It is a common place to talk about immune system's ability to react against foreign pathogens. But perhaps the immune system does not react explicitly not a bit against nonself, but react only against any "danger," irrespectively of the source. This danger hypothesis was presented first by Matzinger in 1994 [69]: "*For many years immunologists have been well served by the viewpoint that the immune system's primary goal is to discriminate between self and non-self. I believe…*

that its primary driving force is the need to detect and protect against danger…."

DAMPs or alarmins. Today danger signals are defined analogous to PAMPs as endogenous host-encoded proteins which are released after cellular stress, tissue injury, and necrotic cell destruction, in the complete absence of any pathogen. Those molecules are collectively called damage-associated molecular patterns, DAMPs, sometimes also called alarmins. Many DAMPs are nuclear or cytosolic proteins, but also extracellular matrix proteins (ECM), which are either upregulated or released following tissue injury. These normally hidden proteins send a signal "danger" resulting in a sterile inflammation. Substances in this process released from damaged cells change now from the intracellular reducing to the extracellular oxidizing milieu, leading to a dramatical change of the extracellular redox milieu.

Oxidative stress. One consequence of this disordered redox milieu could be a chronic inflammation. Examples of such substances are different so-called leaderless secretory proteins (LSPs), like high-mobility group B1 molecule, HMGB1, or IL-18. These proteins are strong mediators of inflammation and are involved in the oxidative stress [70, 71]. DAMPs like PAMPs are bound by the same set of PRRs. DAMP ligands are described for TLRs 1, 2, 3, 4, 7, 8, and 9 [72]. There is also a synergy described between endogenously derived DAMPs and exogenously derived microbial PAMPs leading to a common innate immune response [73]. Besides intra- and extracellular proteins, lipoproteins, glycoproteins, or nonproteins like genomic DNA and RNA molecules, some major DAMP ligands which can activate the immune system are:

1. *Mitochondrial DAMPs, MTDs.* Peptides and DNA from mitochondria, the intracellular organelles responsible for providing the energy of a cell, and released after cell disruption after trauma [74].
2. *High-mobility group B1 molecule, HMGB1.* A ubiquitous nuclear chromatin-associated protein which acts as a transcription factor and is released, e.g., from necrotic but not apoptotic cancer cells or actively secreted by macrophages during the early proinflammation phase [75].
3. *Heat shock proteins, HSPs*. A family of highly conserved ubiquitous proteins acting as chaperons and responsible for the correct folding and transport of proteins. Hsp90, e.g., plays an important role in antigen presentation and maturation of DCs and is released by necrotic cells [76].
4. *S100 proteins.* A family of at least 21 members of calcium-binding proteins. Besides other functions in cell growth, differentiation, these proteins are also involved in inflammation. Some S100 proteins are useful as tumor markers, and the release of the intracellular S100A8 or S100A9 into the extracellular medium is a strong danger signal [77].
5. *Hyaluronic acid fragments, HA*. A carbohydrate polymer, non-sulfated glycoglycans, and part of the extracellular matrix (ECM). Degradation products of HA can be found rapidly after tissue injury at the site of inflammation [78].

2.8 TLR Intracellular Signal Cascade and Immune Response

The only function of antigen binding to TLR or another PRR, regardless of the origin, PAMP or DAMP, is to induce an immune response against this captured antigen. This response must be reasonable, a balance between T_{h1} and T_{h2} actions, a more T_{h2} or a more T_{h1} response, or a switch between T_{h1} and T_{h2}. This response should also be effective, and not directed against phantoms such as decoy epitopes. Based on these assumptions the immune reaction must be time limited, coordinated, controlled, well appropriate, and leading to a memory effect.

To meet all these assumptions a precisely adjusted signal cascade to the individual situation within the APC usually happens. The final outcome is the specific activation of selected immune-relevant genes. Thereby, about 150 genes can be activated and expressed. The cross talk within the signal cascade is essential in order that a protective and not pathological immune response will be induced. A set of (mitogen-activated protein) MAP kinases governs the activation of the signal cascade. A MAP feature is the three-tiered

classical pathway; three kinases are connected in series. In contrast to classical MAPKs, a two-tiered system is described for atypical MAPKs.

There are five main steps of TLR-dependent signal cascade:

1. Binding of Adaptor Molecules

The ligand binds to the N-terminal leucine-rich extracellular domain of TLRs and dimerizes the ectodomains, forming now a horseshoe structure. This results in a conformational change of the cytoplasmatic TIR intracellular domains, forming now a TIR-TIR homodimer. The TIR domain (Toll/interleukin 1 receptor) is the essential molecular key which connects the immune stimulus with a proper immune response and is highly conserved among all other TLRs. The TIR dimer recruits subsequently an adaptor molecule out of the cytoplasm. Five different cytoplasmatic adaptor molecules are available, each able to bind to TIR [79, 80]:

MyD88 Myeloid differentiation primary response gene 88
TRIF TIR-domain-containing adaptor inducing IFN-β
TIRAP Toll-interleukin 1 receptor domain-containing adaptor protein
TRAM TRIF-related adaptor molecule
SARM Sterile-alpha and armadillo motif-containing protein

Together with different cell types, also these different adaptor molecules account for a large variability in gene expression and hence for the different immune activities.

The main adaptor molecules are Myd88 and TRIF. Two pathways are distinguished, a MyD88-dependent and a MyD88-independent pathway.

Most of all TLRs use MyD88. TLR3 uses TRIF, and TLR4 uses MyD88 and TRIF. TLRs 2, 4, and 6 are located within the cell membrane and use the adaptors TIRAP instead of MyD88 and TRAM for TRIF.

2. Activation of IKK Complex

The N-terminus of MyD88, the so-called death domain (DD, a bundle of characteristic six alpha helices, found in all apoptotic proteins), is involved in the binding of IRAK (IL-1 receptor-associated kinase, a serine/threonine kinase family). IRAK is self-activated by autophosphorylation and binds to TRAF-6 after dissociation (TRAF-6, TNF receptor-associated factor 6). The activated TRAF-6 activates now the IKK complex, composed of IKK-γ also known as NEMO (inhibitor of kappa-B kinase) and the two catalytic subunits IKK-α (or IKK1) and IKK-β (or IKK2). The activation of IKK is the crucial step towards the release of transcription factors and a hallmark of the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway.

3. Release of Transcription Factors

TRAF-6 interacts with enzymes catalyzing the formation of polyubiquitin chains. These ubiquitins are required for IKK activation and end in two distinct pathways. Ubiquitin is attached to IkB protein, part of the cytoplasmatic NF-kB-IkB complex, and inhibitor of NF-kB. IkB is thereby labeled for proteolytic degradation. This inactivation of IkB starts the pathway for NF-kB which is now on the path into the nucleus. NF-kB is the most prominent transcription factor among a large group. The other pathway starts also with the ubiquitinated IkB protein and involves the c-Jun N-terminal kinase (JNK) for activation of AP-1 (activator protein 1). AP-1 translocates also into the nucleus, just as IFR3, another transcription factor.

4. Activation of Genes via NF-kB

NF-kB is often called "central mediator of the immune response." More than 150 genes are controlled by this transcription factor [81] (see also Table 2.7). Almost all of these genes are directly involved in the immune response. Based on the inducible gene regulation, the organism is able to react very quickly and very targeted on negative influences, such as infections, (oxidative) stress, or tissue injury. A dysregulation of NF-kB contributes to a pathoimmunity and is also discussed to play a major role in cancer diseases, because NF-kB also controls genes responsible for cell proliferation [82, 83].

NF-kB is no single protein, rather a complex of some proteins with a unique feature, the Rel homology domain (RHD) composed of 300 aa.

The RHD is also part of other transcription factors and responsible for the protein-DNA linkage. The NF-kB complex forms homo- and heterodimers, and by association with IkB,

Table 2.7 TLR signaling to target immune and nonimmune genes via NF-kB

Cytokines	Receptors	APPs	Reg P	GF	Others
IFN-β	MHC I	Tissue F1	TAP1	GM-CSF	p53
IFN-γ	B7.1	CRP	ICAM-1	G-CSF	IRF-1
IL-1α/β	CD48	CF C4	ELAM-1	M-CSF	IRF-2
IL-2	IgG heavy c.	CF B	FAS ligand	PDGF Bc	IkBα
IL-6	IgE heavy c.		IAPs		c-myc
IL-8	Ig light c.				c-myb
IL-9	TNFr				junB
IL-12	CD23				
IL-15	CD69				
TNF-α/β					

Abbreviations *APPs* acute-phase proteins, *Reg P* regulatory proteins, *GF* growth factors, *IFN* interferon, *IL* interleukin, *TNF* tumor necrosis factor, *MHC* major histocompatibility complex, *B7.1* costimulator of T cells, *CD48* lymphocyte antigen, *Ig* immunoglobulin heavy/light chain, *TNFr* tumor necrosis factor receptor, *CD23* cell-surface molecule, *CD69* C-type lectin protein, *Tissue F1* tissue factor 1, *CRP C-reactive protein*, *CF C4* complement factor C4, *CF B* complement factor B, *TAP1* transport protein for ER, *ICAM-1* intracellular adhesion molecule, *ELAM-1* endothelial cell leukocyte adhesion molecule, *FAS ligand* inducer of apoptosis, *IAPs* inhibitors of apoptosis, *CSF* colony stimulating factors/granulocyte/monocyte/macrophage, *PDGF Bc* platelet-derived growth factor B chain, *p53* tumor suppressor, *IRF* interferon regulatory factor, *IkB* inhibitor of Rel/NF-kB, *c-myc/c-myb/junB*, proto-oncogene

NF-kb quiets in cytoplasm. The dissociation of IkB activates NF-kB following translocating into the nucleus and binding to a specific 10 bp DNA site, called kB. The best known complex in mammalian cells is composed of the proteins p50 and RelA, also known as NF-kB.

NF-kB dimer has no intrinsic enzymatic activity and only mediates the transcriptional control of target genes, because the structure of the dimer does not allow a direct binding to nucleosomal DNA. The dimer binds to a co-regulator, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs). These co-regulators are enzymatically active and shift acetyl groups. DNA expression is regulated by acetylation and de-acetylation. After opening the chromatin structure through histone acetylation, the preinitiation complex (PIC) is assembled and helps the RNA polymerase II to activate the transcription via binding to the TATA box of the promoter, the transcription start site of any gene [84].

5. Release of Activated Immune Molecules

The transient change in the gene expression by NF-kB or another transcription factor is quick and enables the immune system to a prompt and robust response against danger, PAMP, or DAMP, arranged by any PRRs, such as TLRs.

TLRs are expressed on DCs, monocytes and macrophages, T cells, B cells, and NK cells, and furthermore on endothelial cells, fibroblasts, on muscle cells, also on chondrocytes, osteoblasts and osteoclasts, on adipocytes, on hepatocytes, and on splenocytes. This ubiquitous distribution assists a ubiquitous immune reaction.

Besides the differential activation of TLRs by PAMPS and DAMPs, also the cell type has great impact on the selection of immune genes and ultimately on the production and secretion of cytokines, receptors, acute-phase proteins (APPs), regulatory proteins (Reg P), or growth factors (GF).

Therefore, the diversity of the immune activities strongly depends on various stimuli, different PRRs/TLRs, and, respectively, the cell type-specific transcriptional profiles within the stimulated cell.

If viruses activate NF-kB as part of their escape strategy to regulate their replication, an immunopathological reaction will succeed [85, 86]. In this case a viral encoded motif binds to the kB site by bypassing the normal signaling pathway. A directed cytokine gene activation follows in support of an intact, not disturbed viral replication. And as discussed before, IL-10 again acts as immunosuppressive provirus cytokine.

The hepatitis B virus surface Ag (HBsAg), covering 90 % of the surface of hepatitis B virus (HBV), is part of a prophylactic vaccine and binds to NF-kB [87]. The findings from this binding studies and the observed viral manipulation of targeted immune genes could contribute to the development of therapeutic antivirus vaccines.

To this day there is no licensed drug which can directly block the activity of NF-kB, interesting not only for oncology. NF-kB can be easily experimentally manipulated and be blocked under controlled laboratory conditions. However, also after 25 years of intensive NF-kB research, the fears are massive that a therapeutic inhibition could induce nonpredictable and uncontrollable gene suppression with fatal outcomes.

Box 2.3
Impact of TLRs on vaccine development

TLRs identify pathogens and intracellular danger signals on distinct structures, PAMPS and DAMPs. In addition, lots of TLRs are involved in diseases, such as TLRs 2, 7, and 9 in systemic lupus erythematosus (SLE) and in the production of autoantibodies; TLR2 in bacterial and viral infections; TLR4 in allergy or rheumatoid arthritis and in various infection diseases, asthma, and cardiac and liver disease; and TLRs 3, 4, 7, and 9 in malignant melanoma.

TLRs bridge the innate and acquired immunity. Also the type of immune response, T_{h1} or T_{h2}, is triggered by them. What chance to develop ligands for some TLRs which dependent on the disease would promote a cellular activity, e.g., for viral infections, cancer, or more than the humoral response, e.g., in allergic asthma. TLRs are therapeutic targets and can be manipulated by agonists and antagonists [88].

Agonists binding to some TLRs are developed as vaccine adjuvants. Already licensed is Fendrix® as adjuvant for hepatitis B vaccine: HBsAg is adjuvanted by AS04C, containing monophosphoryl lipid A (MPL). MPL is structurally related to LPS (see also Chaps. 33 and 34) and a ligand for TLR4. It is reported that a full protection could be achieved after two immunizations in contrast to three immunization steps with a traditional alum-adjuvanted HBsAg vaccine.

In November 2012, the US Food and Drug Administration (FDA) Vaccines and Related Biological Products Advisory Committee (VRBPAC) granted Heplisav®, again an HBsAg-based vaccines but in this case combined with a synthetic CpG oligodeoxynucleotide (CpG-ODN), an immunostimulatory sequence (ISS) and ligand for TLR9. The VRBPAC also reviewed and evaluated data concerning a theoretical risk that vaccines with these CpG adjuvants might cause autoimmune diseases.

The immunostimulating capacity of CpG-ODN was first described by Krieg in 1995 [89]. He discovered that unmethylated CpG motifs, repeated cytosines followed by guanines and with a phosphodiester backbone, are widely distributed in microorganisms but absolutely rare in vertebrates. So unmethylated ssCpG-ODNs are considered PAMPs and are used to trigger the immune response. TLR9 is constitutively expressed only in B cells and plasmacytoid dendritic cells. It is not fully understood whether a specific sequence of CpGs, internal palindromes, or modifications of the phosphates are responsible for the immunostimulation or specific structures such as loops at both ends of the molecule. Accordingly the synthesized CpGs, 18–28 nucleotides in length, are very heterogeneous.

Besides CpG adjuvants which synergistically act, CpGs are meanwhile clinically tested as adjunctive immunotherapy after chemotreatment in some cancer diseases [90]. The efficacy of CpGs as monoimmunostimulant without a tumor-associated antigen is indeed disappointing. A significant prolongation of the survival time could not be achieved so far so that an approved immunotherapy for cancer patients based on CpGs does not exist as yet.

Antagonists of TLRs are in preclinical and clinical development. LPS binds to TLR4 and is involved in sepsis. Lipid A derivates and other compounds block the LPS-binding site of TLR4 and consequently inhibit the signal cascade. TLR2 and TLR4 are involved in cardiovascular diseases, in the progression of arteriosclerosis. A reduced expression of both receptors by an antagonist shows an antiatherogenic effect.

2.9 T Cells and Priming

While the innate immunity is based among others on highly conserved receptor structures, fixed in the germ line, the acquired immunity is characterized by clonal expansion of B and T cells. These cells carry antigen-specific receptors, whose diversity is dependent on gene rearrangements.

Following the capture of antigens by immature DCs, the most potent APC, via TLRs/PRRs, DCs migrate from non-lymphatic tissue via blood or lymph vessels to a lymph node, where they mature, process, and present the captured antigens via MHC to the T cell receptors (TCR) of naive T cells.

Priming. A T cell needs three distinct signals for a complete activation, called priming.

In addition to the presentation of antigen (signal 1), costimulatory molecules (signal 2) are essential for a complete T cell priming, and furthermore proinflammatory molecules (signal 3).

Signals. An incomplete priming results in anergy, a functional inactivation, but still alive, a status of tolerance. The major costimulatory signal of T cells is CD28 enabling T cell expansion and differentiation probably via enhancement of intracellular TCR signals [91]. CD28 binds to B7 molecules (B7.1 = CD80 and B7.2 = CD86) of DCs. At the same time additional signal molecules are upregulated both within T cells and in DCs (written in parentheses), such as 4-1BB (4-1BBL), CD27 (CD70), and CD40 (CD40L). A very intensive cross talk takes place between T cells and DCs during this process. DCs secrete specific proinflammatory cytokines (signal 3) which additionally support T cells in maturation, priming, and survival, such as IL-1α, IL-6, IL-7, IL-12, IL-15, IL-18, IL-27, IL-33, IFNs, IFN-γ, and TNF-α [25]. This is a quick immune response, a type 1 response.

T cell family. After complete priming, T cells differentiate to various effector cells, CD4+ T helper cells (T_H), with the traditional subsets T_{h1}, T_{h2}, a recently identified IL-17 producing subset T_{h17} [92], T follicular helper cells (T_{Fh} cells are specialized to regulate B cell response) [93] and regulatory T cells [94] (T_{reg}, formerly known as suppressor T cells), and CD8+ T cytotoxic T cells (CTLs). All these T cells mainly subsequently migrate into blood and to the site of infection or danger. Thereby T cells express migration receptors enabling infiltration into peripheral tissues. The common feature of the large T cell family is the unique T cell receptor (TCR).

CD4+ T_{h1} cells produce cytokines, such as IL-2, IL-12, and IFN-γ, and support a cellular immunity arranged, e.g., by macrophages and CD8+ cytotoxic T lymphocytes (CTLs). Those immune cells kill infected cells by granzyme B and perforin and secrete IFN-γ and TNF-α. CD4+ T_{h2} cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 to support B cell activation resulting in a humoral immunity. The development from an immature to a mature and primed T effector cell takes 3–5 days.

T cell-B cell connection. B cells, another type of APCs, bind, internalize, and process antigens in complex with MHC II molecules and present to CD4+ T Follicular helper cells (T_{Fh} cells), primed with the cognate antigen. T_{Fh} cells migrate into the B cell follicles of secondary lymph organs where they recognize via the TCRs this MHC II-antigen complex and activate the B cell to the production of antibodies.

The cycle is closed. Antigen-presenting cells broadly distributed through the body bind danger signals, whether pathogen or host derived, via ubiquitous TLRs/PRRs. Following the processing and presentation, up to 150 genes are expressed within the APC, ready to induce an immune response. The vaccine antigen mimics danger signals and occupies the same immune activation process like a pathogen. For a general overview, see Fig. 2.3.

2.10 Immune Memory

The anamnestic immune memory relies on cells of the acquired immune system, such as B and T cells. At this the duration of the immune memory differs dependently from the disease, the immune status and the age of the patient, and of the type of vaccine.

Basically, a natural infection can lead to a lifelong immunity, and a live vaccine results in a longer immunity than a recombinant vaccine. Responsible for the maintenance of specific memory immunity are special memory B and T cells. This is the conceptual basis of a vaccination. T cell memory in humans can be detected for up to 75 years [95].

2.10.1 Memory T Cells

Memory CD4+ T cells. At the end of the immune response, most CD4+ T cells undergo apoptosis. Only a small pool of effector memory CD4+ T_{EM} cells survives and continues to produce cytokines as a support of ongoing immune activities. There are roughly two groups of T_{EM} cells: memory CD4+ T cells which are less differentiated and highly differentiated multifunctional T cells. These highly differentiated cells can be distinguished by a diverse pattern of cytokines, like IL-2, IFN-γ, and TNF-α, resulting in a different level of protection [96]. Memory CD4+ T cells keep their ability to migrate through peripheral tissue, and they are predominant in peripheral tissue, such as localized dermal layer of the skin among all memory T cells. CD4+ T cells produce IL-2 and help B cell differentiation into antibody-producing plasma cells and account to secondary expansion of memory CD8+ T cells [97, 98].

Memory CD8+ T cells. Also the majority of T effector cells undergoes apoptotic cell death and immediately disappears from the circulation after clearance of the pathogen. Only a small population (up to 10 %) of memory T cells remains of which can very quickly respond to a reinfection with a cognate antigen or cognate vaccine antigen. The maintenance of these long-living memory T cells is antigen independent but dependent on cytokines mainly through IL-7, also known as T cell survival cytokine, and IL-15.

Another difference to effector T cells is the increased proliferation capability and longevity of memory T cells. This includes the increased expression of inhibitory molecules such as programmed cell death protein (PD-1) and the loss of effector functions, like the loss of production of granzymes, IFN-γ, or IL-12.

Additionally, the transcription of NF-kB-dependent (cytokine) genes will be reduced in memory T cells by an inhibitor of NF-kB.

Subsets. Three major subsets (in contrast to some literature speaking of only two) of memory T cells are identified based on their anatomical localization and functional status [99–102]: central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and tissue-resident memory T cells (T_{RM}).

T_{CM} cells express homing receptors for lymph nodes, CD62L and CCR7, residing in secondary lymphatic organs with a high proliferative but low effector potential [103]. T_{CM} cells are able to migrate and circulate in blood and therefore are believed to be the circulating and interconversion pool for other memory T cells.

T_{EM} cells produce cytotoxic proteins, reside in nonlymphoid tissues, are only low proliferative, circulate also in blood vessels, and provide efficient and rapid protection against systemic infections and only a limited efficiency against local infections. The pool of T_{EM} cells declines over time resulting in a progressive conversion to T_{CM} cells.

T_{RM} cells do not migrate and circulate. They prefer to reside in tissues that are either confronted with pathogens in first-line defense, like skin, intestinal (lung and gut), and vaginal mucosa tissue, or in such tissues which are highly sensitive to pathogens, like brain tissue. These cells exert effector functions and produce different cytotoxic molecules like granzyme B. Table 2.8 gives an overview on memory T cells.

Persisting antigen is not required for the maintenance of memory T cells [104], but the tissue-specific microenvironment influences the expression of migration receptors such as CD103+. Nonspecific local inflammatory cytokines are sufficient to arm T_{RM} cells [105]. This

protection by embedded TRM cells in peripheral sites can last over years.

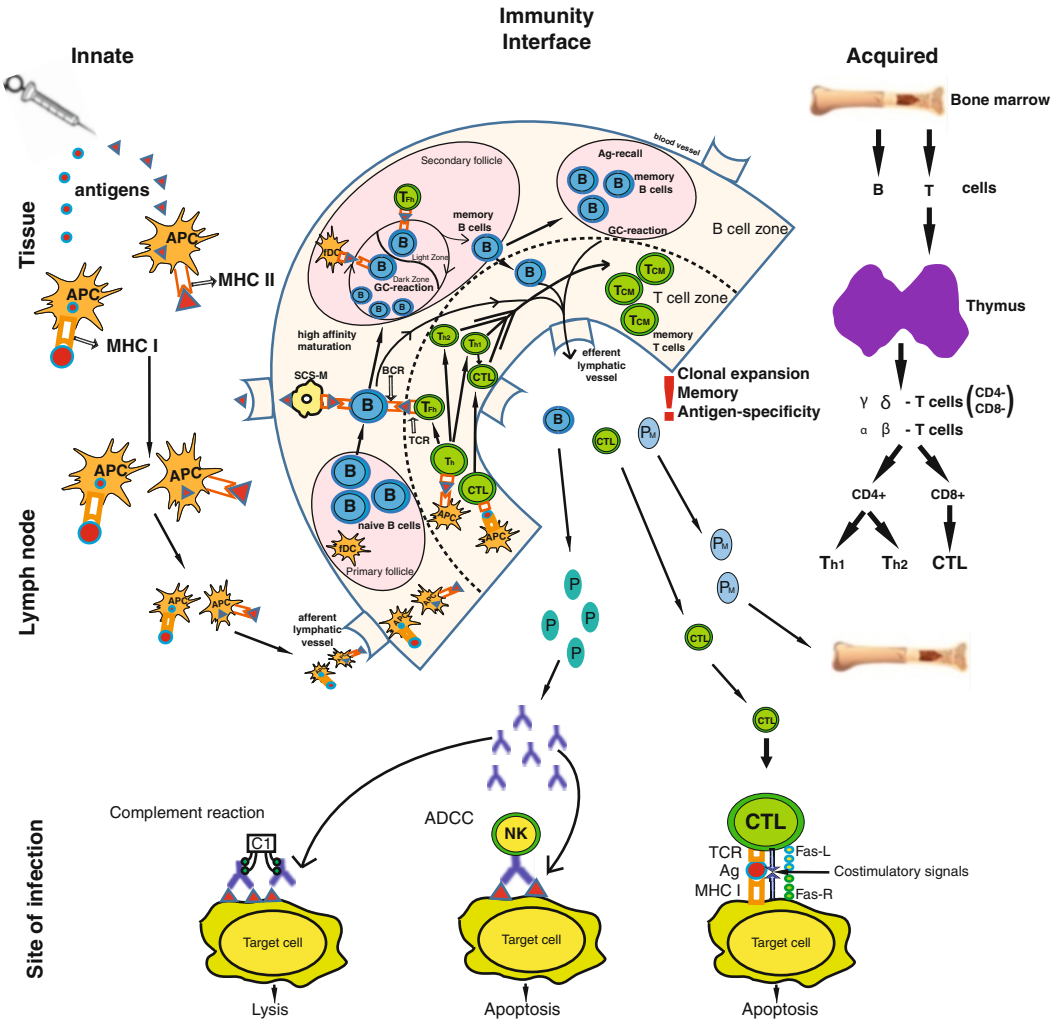
Pasta cells. The rapid change from resting naive T cells to highly differentiated, active, and migrating T effector cells, afterwards the rapid reduction of these effector cells and the generation of memory T cells, again provided with the ability to a rapid proliferation and rapid reduction on demand, postulates a precise regulation of lymphocyte homeostasis.

Any kind of dysregulation leads to a severe, sometimes life-threatening immune disease.

One key mechanism to control this homeostasis is the regulation of metabolism. In this process the production of energy in resting T cells switches from fatty acid oxidation and decarboxylation of glucose in citrate cycle and respiratory chain to an aerobic glycolysis (oxygen is present within the cell) in activated T cells [106]. Rapidly growing tumor cells produce energy as well from glycolysis in the presence of oxygen.

This discovery was described very early by Warburg and he was awarded the Nobel Prize in Physiology in 1931 [107]. Activated T cells and cancer cells have a high demand on energy which can only be covered via glycolysis.

While the oxidative decarboxylation is located in the mitochondria, the glycolysis takes place in the cytoplasm of the cell. The benefit of this location for activated T cell is the rapid offer of energy as ATP. This tremendous adjustment of metabolism enables T cells to effective activities against pathogens, against danger. Now T cells



can rapidly grow and proliferate, differentiate, migrate, and secrete cytokines.

Extrinsic factors control the increased metabolism. The T cell receptor (TCR) plays a key function. TCR regulates the expression of the glucose transporter, Glut1. Without TCR signals, the expression of Glut1 is decreased, resulting in a reduced absorption of glucose, subsequently leading in nutrient stress and apoptosis [108, 109]. IL-7 plays an additional key function in the metabolic switch. IL-7 promotes glucose uptake through activation of Akt kinases [110]. If this metabolic switch is absent, e.g., due to defect of enzymes or lack of glucose, T cells will develop anergy.

After completing the mission, effector T cells turn into resting T cells, and the metabolism change back to mitochondrial fatty oxidation and oxidative decarboxylation: *Pasta cells* – like a marathon runner who eats carbohydrates on the obligatory pasta party on the eve of competition in order to activate very quickly this biochemical simple energy depot later.

Table 2.8 Subsets of human memory CD8+ T cells

Cell type	Localization	Marker	Function
TCM	Blood, spleen, lymph nodes	CD62L+++, CCR7+ CD45RA–	Peripheral protection, circulating T cell pool, IL-2
TEM	Blood, spleen, liver, peripheral organs	CD62L±, CCR7–, CD45RA+	Fast and robust peripheral protection, cytotoxic proteins, IFN-γ
TRM	Skin, mucosa (lung, gut, vagina), salivary glands, brain	CD62L–,CCR7– CD103+,CD69+	Fast and robust local protection, cytotoxic proteins

Fig. 2.3 The interaction of innate and acquired immunity upon vaccination All lymphocytes (B cells, plasma cells, T cells or natural killer (*NK*) cells) originate from a common lymphoid progenitor cell which develops from a hematopoietic stem cell within the bone marrow. T cells. **T cells** migrate to the cortex of the thymus to undergo maturation in an antigen-free environment. Upon maturity, there are several forms of T cells (MHC restricted αβ-T cells, and T_{reg} cells – and non-MHC restricted γδ-T cells) ready to migrate to peripheral lymphoid tissues. **B cells**. B lymphopoiesis occurs exclusively in the bone marrow. Upon maturity, B cells leave the bone marrow and migrate to peripheral lymphoid tissues. **Lymph node**. Lymph nodes (*LN*) are the interface between innate and acquired immunity. Upon intramuscular injection, e.g., vaccine antigens are captured by antigen presenting cells (*APCs*/*DCs*) and transported to the local LNs where they are presented to B and T cells via MCH II/I molecules. Antigens can also enter the LN without APCs and are captured by residual subcapsular sinus (*SCS*) macrophages for presentation. B cell development in lymph nodes (*LN*) is regulated by T follicular helper (TFh) cells: (1) APC-primed TFh cells encounter in the primary B cell follicle of LN naive B cells that process cognate antigens relayed by SCS macrophages. This T-B cell interaction via CD27/CD70 provokes a rapid clonal expansion and differentiation of B cells into short-lived plasma cells and the production of IgM, the earliest immunoglobulin. (2) Following expansion and immunoglobulin class switch to IgA, IgE, and IgG, the germinal center (*GC*) reaction in the secondary follicle of LN begins which is the crucial basis for the generation of memory B cells, whereas in the extracellular follicle LN short-lived plasma cells secrete antibodies. (3) The GC reaction is carried out in a cycle of different steps. B cells scan follicular DCs for antigens in the dark zone of secondary follicle with the commitment to B cell memory. TFh cells support these B cells in the light zone and drive high affinity-maturation of antigen-specific B cells into non-secreting memory B cells and long lived secreting plasma cells to distally function at infectious tissues. One part of these antigen-specific memory B cells, however, do not leave the secondary follicle but re-enter the GC cycle and undergo another round of affinity-maturation. The GC cycle reaction can continuously produce stable long-lived memory B cells. Following cognate antigen recall, memory B cells are re-activated by memory TFh with a rapid proliferation of antibodies secreting memory plasma cells and induction of another GC reaction. Memory plasma cells (PM) survive in the bone marrow and provide continuously serum antibodies to the body. T cells develop to effector cells and central memory cells (TCM). TCM cells are able to migrate and circulate in blood and therefore are believed to be the circulating and interconversion pool for other memory T cells. The unique hallmark of the acquired immunity is the clonal expansion, the memory effect and the antigen specificity. After education B and T cells leave the LN and migrate to the site of infection. Cytotoxic T lymphocytes (*CTLs*) kill target cells following direct cell-to-cell contact and release, e.g. of cytolytic proteins. Antibodies bind to cell surface antigens leading to an anti-body dependent cell-mediated cytotoxicity (*ADCC*), mediated, e.g., by NK cells. The classical complement pathway is activated to lyse a target cell by binding of the C 1 complex to antigen-bound antibody molecules

Box 2.4

Impact of T cells on vaccine development

An ideal vaccine should induce a complete, humoral, but also cellular immune response. The natural T cell response CD4+ and CD8+, is influenced by several factors, such as nature of the pathogen and its target tissue in the host, in an acute or chronic infection.

Vaccines for generating a robust CD8+ T cell response with a high effector capacity should address these fundamental key issues in an appropriate quality, magnitude and duration of CD8+ T cell response. Live attenuated, or vector vaccines, also replicons and DNA vaccines are able to stimulate CD8+ T cells, but not killed or conventional peptide-/protein-based vaccines. Research on T cell vaccines is concentrated on accurate, conserved T cell epitopes.

Epitopes are immunodominant peptides and their binding to MHC alleles is the requirement for epitope presentation to specific T cells. The length of peptides differs for MHC I and MHC II, depending on the allele between 8 and 11 for MHC I, respectively, between 13 and 25 residues for MHC II. By comprehensive series of epitope mapping using a panel of synthesized overlapping peptide sequences (peptide library), TCR binding sites can be identified and characterized. The prediction of novel T cell epitopes and thus the screening of vaccine candidates are also done by in silico methods [111] (see Chap. 42). However, wet experiments must follow and confirm or drop the identified epitopes.

Since 2007 the Immune Epitope Database and Analysis Resource (IEDB) provides a catalog of T and B cell epitopes and tools to predict novel epitopes [112, 113]. Currently, data from 197,005 T cell, 221,898 MHC binding, and 7,621 MHC ligand elution assays are listed in this catalog. The access to IEDB's website is free: http://www.iedb.org.

The most gallant and definitely most difficult approach to activate CD8+ T cell is the isolation of antigens which directly bind to MHC I molecules [114]. Despite lots of efforts, there is no successful clinical trial with a protein-based T cell vaccine. As demonstrated in a recent study, peptide pulsing of fresh blood cells ex vivo could help to control an HIV infection via induction of CD8+ T cells [115]. A broader approach aims to directly target DCs via a defined TLR selected antigen. However, there are many subsets of DCs. It is therefore important to understand which type of DCs is targeted, where these DCs are located, what the TLR repertoire is, and which cytokines are involved (adjuvant development).

The maintenance of memory T cells was shown to be independent of cognate antigens. Whereas TEM provide effective protection for systemic infections, TRM are responsible for protection for local infections. Nonspecific local inflammatory cytokines seem to be sufficient to arm TRM cells [105]. A successful T cell development should include a highly specific adjuvant to force these necessary local inflammatory cytokines combined with an appropriate delivery system, such as skin delivery for activation of skin resident TRM cells in HSV-1 infection [116].

The development of therapeutic T cell vaccines for chronic infections or cancer diseases should address problems of adverse effects by persistent CD8+ T cell activation. These T cell vaccines have the potential to generate immunopathology, such as exhaustion (loss of functions, beginning with a loss of IL-2 production) and inflammation. PD-1 high expression is one biomarker for exhausted T cells. A mathematical model helps to predict potential immune adverse effects of T cell vaccines for chronic viral infections [117].

Table 2.9 Subsets of human B cell memory cells

Cell type	Localization	Marker	Function
Memory B cell	Secondary lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches)	CD19+, CD20+, CD27+, CD38–	Cell pool for rapid production of memory plasma cells; secondary GC reactions
Memory plasma cell	Bone marrow	CD19+, CD20– CD27+, CD38+++	High-affinity antibodies after recall

2.10.2 Memory B Cells

The measurement of antibodies in response to a vaccine is presumed to be level of protection and duration. This vaccine immunogenicity is only an indication for a possible protection but definitely not equal to the real factual protection. There are also some reports of patients who developed antibodies after vaccination but indeed are not protected. Last but not the least, some pathogens induce a primary immune response, but after their clearance, a memory response is missing and the body is not protected against a reinfection with the same pathogen. This is the case, e.g., for respiratory syncytial virus (RSV), causative agent of respiratory disease in infants, and morbidity in children and adults [118, 119].

Like T cells also B cells generate long-lived memory cells following the primary antigen contact. And also like memory T cells, memory B cells are able to persist without a new antigen contact [120, 121].

Memory subtypes. Two B cell subtypes are described [122]: memory B cells and memory plasma cells. Memory B cells reside in secondary lymphoid organs and memory plasma cells survive in the bone marrow and provide continuously serum antibodies to the body (Table 2.9).

The ever measured longest survival of memory plasma cells in man, about ten decades, was found in people born in or before 1915. Survivors to the 1918 H1N1 influenza virus pandemic were enrolled in a study and tested for antibody response to the recombinant 1918 hemagglutinin (HA) protein. All survivors possess neutralizing antibodies to this H1N1 pandemic virus which also cross-reacts with a genetically similar HA of 1930 influenza infection [123].

Affinity maturation. Immature B cells develop into mature B cells in secondary lymphoid organs. After protein vaccination B cell development in lymph nodes (LN) is regulated by T_{Fh} cells and following in three phases [124]: (1) DC-primed T_{Fh} cells encounter in the primary B cell follicle of LN naive B cells that process cognate antigens relayed by subcapsular sinus (SCS) macrophages [125]. This T cell-B cell interaction via CD27/CD70 provokes a rapid clonal expansion and differentiation of B cells into short-lived plasma cells and the production of IgM, the earliest immunoglobulin. (2) Following expansion and immunoglobulin class switch to IgA, IgE, and IgG, the germinal center (GC) reaction in the secondary follicle of LN begins which is the crucial basis for the generation of memory B cells, whereas in the extracellular follicle, LN short-lived plasma cells secrete antibodies. (3) The GC reaction is carried out in a cycle of different steps. B cells scan follicular DCs for antigens in the dark zone of secondary follicle. T_{Fh} cells support these B cells in the light zone and drive affinity maturation of antigen-specific B cells into non-secreting memory B cells and long-lived secreting plasma cells to distally function at infectious tissues. One part of these antigen-specific memory B cells, however, does not leave the secondary follicle but reenters the GC cycle and undergoes another round of affinity maturation. The GC cycle reactions can last up to 8 months after protein vaccination [126] and continuously produce stable long-lived memory B cells. Following cognate antigen recall, memory B cells are reactivated by memory T_{Fh} with a rapid proliferation of antibodies secreting memory plasma cells and induction of another GC reaction. But a survival cytokine, like IL-7 described for T cells, does not exist for B cells.

2.11 Immunosenescence and Vaccination

The reduced efficacy of vaccines in the elderly (>65 years) is generally attributed to immunosenescence [127–129]. Consequently, older adults are more susceptible to infectious diseases. Age-associated immune changes take place in the innate and acquired immune systems and affect not only lymphocytes but also myeloid cells with a change in proinflammatory cytokines.

It is of note to underline that immunosenescence is not only restricted to aging. During chronic infections, certain pathogens such as CMV and HIV remodel the immune system towards aged T cells [130, 131].

Malnutrition. There is also an interface between malnutrition, premature T cell senescence, and susceptibility to infections [132]. Chronic hunger may modulate the immune system through T cell repertoire. Hunger also influences the telomere length of CD8+ T cells resulting in a shortening [132]. Chronic hunger in early life dramatically impacts the programming of the young thymus, a very crucial phase, having long-term effects on T cell development. Zinc is an essential mineral for the immune system and a zinc deficiency results in thymic atrophy [133]. It's suggested that the nutritional disturbance predisposes this population to infections. Malnutrition is one important reason for the loss of efficacy of oral vaccines in the Third World (see also Chap. 3).

T cells. Beginning with the puberty by the early teens and directed by circulating sex hormones, the thymus undergoes involution: Thymic epithelial cells (TEC) (niche for lymphocyte development) are primarily replaced with fat cells, 3 % per year during adulthood [134]. Size and function dramatically decline. The generation and the export of naive T cells decrease [135]. The thymic epithelial tissue shrinks to 10 % by the age of 70 years. A loss of crucial cytokines, essential for thymopoiesis and also produced by these epithelial cells, such as IL-1, IL-3, IL-6, IL-7, and transforming growth factor (TGF-β). IL-7 is known as survival cytokine for the development and homeostasis of T cells [136]. The naive T cell immunity is waning with the age and diminishes responsiveness to novel antigens during elderly life. The decreased production of naive T cells is followed by a diminished response of TCR signaling (NF-kB, MAPK) leading to a blunted T cell proliferation to antigen stimulation [137] and reduced T cell differentiation [138]. This is in contrast to the memory pool of CD4+ and CD8+ cells, induced at an early age, that can persist for a life-time of an individual [139]. More than this, the loss of naive T cells leads to a memory T cell clonal expansion so that the overall number of T cells in the young and elderly are comparable [140]. The increase of memory T cells could be explained by a long-lasting antigen contact of the elderly. However, this expansion is associated with an age-dependent dramatic loss of TCR repertoire [141]. Another drawback is the telomere shortening of senescent CD8+ T cells, called replicative senescence. It does not affect only the replicative potential but also significantly antiviral effector functions, such as IFN-γ production or antigen-specific cytotoxicity [142, 143]. Old T cells lack important costimulatory molecules such as CD28 and CD27 but express senescence markers: PD-1, CD57, and KLRG-1. This count to a lesser extends for CD4+ T cells but especially for CD8+ T cells which develop the most functional defects.

Thus, the age-related changes in the T cell life comprise quantitative and qualitative alterations and lead to a reduced immune responsiveness.

B cells. Most results on age-related changes in the B lymphopoiesis come from mouse models. But recently studies in humans share some common mechanistic features and similarities. Mouse hematopoietic aging is a reasonable model of human hematopoietic aging [144].

The decline of high-affinity antibodies results partially from defects in T cells. Besides extrinsic factors/microenvironmental factors influencing B cell functions, important intrinsic changes in senescent B cells occur [145].

The human B cell number in the periphery (blood, spleen, lymph nodes) declines moderately with aging or not [146, 147]. But the peripheral B cell repertoire in the elderly is less diverse correlating with poor health status [148]. The B lymphopoiesis is limited by a loss of naive follicular B cells. All blood cell lineages, lymphoid (T and B cells) and myeloid (polymorphonuclear cells, monocytes/macrophages), are continuously generated from hematopoietic stem cells (HSCs) in

bone marrow that can self-renew and are critical for maintaining cellular homeostasis throughout life. One dramatic change in the HSC compartment is the shift towards production of myeloid progenitors at the expense of lymphoid progenitors [149–151]. Changes on gene transcription level but also epigenetic changes are responsible for these alterations [152]. Collectively, elderly human hematopoietic stem cells undergo quantitative as well as qualitative changes with functional modifications.

Intrinsic changes are shown in murine and human B cells in vitro. Old B cells show an impaired ability to undergo immunoglobulin class switch recombination (CSR) measured by a reduced antibody production. Activation-induced cytidine deaminase (AID) is essential for immunoglobulin (Ig) gene CSR and somatic hypermutation (SHM). The AID expression in elderly B cells is significantly reduced compared to the young due to the impaired transcription factor E47 [145].

DCs and TLRs. DCs are the most potent APCs and target for cell-based vaccines. There is an age-related reduction of Langerhans cells (LCs) in the skin resulting in a reduced immunosurveillance [153]. Murine aging LCs have the same ability to migrate to LNs as young LCs; however step by step they lose their capacity to stimulate T cells [154] what should be considered for the application of vaccine patches in the elderly.

The number and the functions of plasmacytoid DCs (pDCs), present in the peripheral blood and in secondary lymphoid organs, are changed in the elderly, in contrast to unchanged number of myeloid DCs [155]. Impaired pDCs show a reduced ability for antigen presentation to CD4+ and CD8+ T cells with lack of induction of cytotoxicity in CD8+ cells. In addition, aged pDCs secrete lesser IFN as young pDCs resulting in a loss of antiviral activities [156]. The reactivity of DCs to self-antigens is increased and can cause autoimmunity [157, 158]. Recently it was shown that not only the expression of some TLRs on DCs is reduced but especially the TLR signaling pathways in elderly individuals what correlates with a diminished vaccine responsiveness [159, 160]. In contrast, no significant differences in TLR expression were observed between young and aged macrophages in mice and in humans [161]. But an age-related reduction in the levels of major signaling adaptors for TLRs, such as MAPK, NF-kB, or MyD88, was observed [162, 163].

Inflammaging. The upregulation of certain cytokines, such as IFN-α, TNF-α, IL-6, and CRP, in elderly individuals is collectively called inflammaging probably provoked by a continuous overload of antigens and stressors [164]. Other studies explain that inflammaging is partially independent of such immunostimulants and rather dependent on the microenvironment, such as a diminished cross talk between tissue-specific cytokine-producing cells [165]. Various cell types are involved in the continuous overproduction of proinflammatory cytokines, such as T cells, DCs, macrophages, and endothelial cells, and raise the risk to come down with age-associated disorders [166]. Table 2.10 summarizes some important key features of immunosenescence.

Box 2.5
Impact of immune aging on vaccines for the elderly

The proportion of the world's population over 60 years will increase to 22 % by 2050, or in absolute number, 2 billion people aged 60 years and older (according to WHO estimates, March 2012). Vaccination of the elderly is recommended especially against infections with influenza virus or pneumococcal infections. Travelers' vaccines against (sub)tropical pathogens, such as typhoid or yellow fever, are recommended due to the mobility of elderly travelers.

Immunosenescence is a multicell and multifactorial process in the innate and acquired system. A multiple decline of major immune functions. Several intrinsic defects could be identified. Some apparent extrinsic factors are described. But little is known on the deep influence of the tissue-specific microenvironments on immune cells. Every in vitro experimental design fails to imitate this specific natural niche in vivo.

Is there an early key event synchronizing all other alterations? Are aging HSCs the door opener to understand the process

of immunosenescence? Or is the thymic atrophy the pacemaker?

Several vaccine developments for the elderly focus on increased antigen dosages [167], novel adjuvant strategies [168], shortened intervals, vector-driven vaccines, change of application routes [169], or the rejuvenation of the thymus by growth hormones and cytokines [170, 171]. Can such approaches circumvent the decreased ability of HSCs to replenish the follicular B cell compartment, the impaired immunoglobulin class switch, loss of high-affinity antibodies, reduced capacity for novel antigens, loss of naive T cells, replicative senescence, loss of TCR repertoire, impaired TLR signaling, decline of transcription factors, or shortening of telomere lengths of leukocytes? It would be squaring the circle!

The best vaccines for the elderly start before aging is coming: Development of novel (conjugate) vaccines with a broad antigenic, multivalent spectrum, e.g., influenza, pneumococcal infections, induce a strong, complete, long-lived memory B and memory T cell response with generating robust CTL activities. A prime-boost regime with repeated booster vaccinations during adulthood. This expanded education of the immune system in young age is a solid basis for a recall and also powerful immunological background against novel antigens in old age.

Table 2.10 Some key features of immunosenescence

Aged cell type	Dysfunction	Consequence
CD4+ T cell	Reduced IL-2 production Reduced cognate helper activity	Reduced support for B cells
CD8+ T cell	Suboptimal signaling pathways Replicative senescence Reduced differentiation Loss of TCR repertoire	Reduced CTL activity
B cell	Loss of naive follicular B cells Reduced number of GCs Reduced B cell differentiation Reduced Ig class switch	Decreased diversity of antibody response
Plasma cell	Accumulation of antigen-experienced old cells in the bone marrow	Reduced antibody production and loss of high affinity
DCs	Impaired TLR signaling Increased reactivity to self Overproduction of cytokines	Decreased T cell immunity Loss of self-tolerance Autoimmunity Chronic inflammation
Inflammaging	Increased secretion of proinflammatory cytokines by various cell types (IFN-α, TNF-α, IL-6, CRP)	Atherogenic profile related to age-associated disorders (e.g., atherosclerosis)

2.12 Booster and Vaccination Strategies

Antigens. At the beginning of this chapter, we introduced the palette of different antigens: glycoproteins, lipoproteins, carbohydrates, glycolipids, and nucleic acids.

So different the chemical nature of antigens is, so different the induced immune response will be. The different vaccine types correspond to the pathogen's nature, like attenuated live virus/bacterial vaccines, inactivated virus/bacterial vaccines, conjugate and polysaccharide vaccines, recombinant protein, and DNA/RNA vaccines.

The nature of an antigen, like the chemical structure (the more complex, the more immunogenic), the size (the larger the molecule, the more immunogenic), and the physical form (particulate antigens are more immunogenic than soluble ones), determines the kind of immune response.

The exposure of proteins and nucleic acids to their corresponding PPRs/TLRs on T cells leads to an acquired, T cell-dependent immune response. A key specificity of this is the development of a memory response. The establishment of a long-lived immunological memory is critical for a revaccination, a booster. Once primed by a vaccination (or natural infection), the second response upon a booster mounts generally the antibody and CTL response to the cognate antigen.

Lipid antigens. Bacterial polysaccharides (S. pneumoniae, S. typhi, H. influenzae) and lipids activate the innate immune system without involvement of CD4+ T cells. Lipid antigens are recognized by CD1 molecules on, e.g., Langerhans cells, following the presentation of the lipid-CD1 complex in an MHC-independent way to invariant NKT cells (iNKT) cells [172, 173]. iNKT cells are a subset of natural killer (NK) cells which are a subset of T cells.

Polysaccharide antigens. Two classes of polysaccharide antigens have been described: Thymic independent type 1 antigens (TI-1) activate directly B cells by binding to the B cell receptor (BCR), first signal, and binding to a TLR on B cells, second signal. TI-2 antigens bind to BCRs, first signal, resulting in clustering, second signal. This CD4+ T cell-independent activation leads to antibody production. Lipopolysaccharides provoke a TI-1 response via binding to TLR4. However, B cell response is short-lived, memory B cells are not produced (only generated upon T cell-dependent activation), and therefore a booster cannot not enhance the antibody response. Moreover, as shown recently, a booster with a pure polysaccharide vaccine can lead to a state of immune tolerance or hyporesponsiveness caused by apoptosis of memory B cells [174, 175].

Conjugate vaccines, the carboyhydrate is coupled to a protein carrier, first developed for newborns and infants, because they are unable to make antibodies to repeating polysaccharide antigens due the immaturity of their B cell receptors, (see also Chap. 4) induce a complete T and B cell response, are now also used in adults and elderly with significant better results than immunization with pure polysaccharide [176].

Table 2.11 Prime-boost strategies

Priming with rec virus A – boosting with rec virus B [177]
Priming with DNA vaccine – boosting with rec virus [178]
Priming with DNA vaccine – boosting with rec protein [179]
Priming with virus-like particle (VLP) – boosting with DNA [180]
Priming with rec protein – boosting with rec virus [181]

Prime-Boost. Traditionally revaccinations are given by homologous booster. Heterogeneous prime-boost strategies involve two different vector platforms for the same antigen and are in (pre) clinical studies especially for chronic infections, such as HIV, malaria, and TB. The combination of recombinant (rec) vectors used for prime-boost can be very different (Table 2.11). But the basic principle in all prime-boost regimes is the application of different vaccine constructs targeting the same antigen.

Also 20 years after the first prime-boost vaccination, the mechanisms behind this remain unclear. More clear are the results, mostly increase of CD8+ T cell numbers with increased lytic capacity, broaden antibody specificities with improved antibody quality. These results reflect that antigens presented by different platforms could bind to different TLRs so broadening the B and T cell response. Also the use of plasmid DNA as prime offers naive and correct folded proteins to the immune system. These DNA-encoded proteins share dominant and subdominant epitopes leading to a broader response. In contrast, recombinant proteins share mostly immunodominant epitopes resulting in a narrow response only.

Drawbacks. However, the bottleneck of a DNA vaccine is the low immunogenicity. Pure naked DNA does not work and must be supplemented with a strong adjuvant (Giese, unpublished results).

The drawback of any viral vector as prime or boost is the antibody response against the vector.

Repeated administrations of a viral vector vaccine automatically induce neutralizing antibodies that dramatically reduce the efficacy of this vaccine. One of the mostly used viral vectors is adenovirus serotype 5 (Ad5). The seroprevalence is very high and preexisting immunity also reduces the efficacy of this vector [182]. To circumvent this handicap, other human but also simian adenovirus serotypes [183], such as Ad26 and Ad35, are under investigation. They differ inter alia in their innate profile and immunologic potential. Not every tested adenovirus is able to strongly prime CD4+ and CD8+ cells. The same is for pox viral vectors; they are limited to prime CD8+ T cells but are a potent boost for CD8+ T cells. The lessons learned from these experiments are the right temporal order.

To summarize this vaccine strategy, prime-boost is superior in eliciting a robust complete immune response compared to DNA, protein or vector vaccines alone. Some single vaccines fail on their own but work in combination. Nevertheless, we are far from a miracle linked to prime-boost. The clinical phase III HIV vaccine RV-144 trial in Thailand, a prime-boost strategy, resulted in 30 % protection only [184]. Further research is needed.

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Gut Immunology and Oral Vaccination

3

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Contents

Abstract

Oral immunization is sought as a practical way to reduce the devastating morbidity and mortality caused by enteric diseases in children under the age of five living in the developing world. Licensed oral vaccines against polio, rotavirus, cholera and typhoid fever have had a major impact reducing global disease burden and mortality. A quandary that remains to be solved is the diminished immunogenicity and efficacy of these vaccines when given to subjects living in underdeveloped areas of the world as compared to people living in industrialized nations. Low socioeconomic status, poor living conditions, malnutrition and natural barriers that affect people living in less privileged countries are major determinants of vaccine performance. Novel

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protective antigens, adjuvants and immunization approaches to overcome these barriers are being explored. Understanding the processes involved in the induction of mucosal and systemic immunity by orally delivered antigens, the influence of multiple competing elements and the delicate balance between immune activation and tolerance in the gut is essential to assist the development and evaluation of vaccine candidates. Modern technology (e.g. genomics, proteomics, high throughput immunological assays using non-invasive specimens and mathematical modeling to dissect correlates of protection) will be essential to this task. Ultimately, well conducted clinical trials in high and low income countries will be needed to determine safety and effectiveness of vaccine candidates that appear to be promising in animal models. This chapter reviews the basis for immunological priming by orally delivered antigens in the gastrointestinal tract and the ensuing effector responses in humans. It also provides a summary of oral vaccines available, factors that affect efficacy of oral vaccines in developing countries as well as the ongoing efforts to develop more effective vaccine candidates.

3.1 Introduction

Oral vaccination is sought as a practical and effective way to prevent diseases caused by enteric pathogens. It has the potential to induce local immune responses providing a front line of defense at mucosal surfaces as well as systemic immunity. Compliance is also higher for oral vaccination in all ages compared with parenteral vaccination and can be easily implemented to benefit large populations. One of the goals of modern vaccinology is the development of vaccines as tools that can reduce the devastating morbidity and mortality caused by enteric diseases in children under the age of five living in developing countries. Licensed oral vaccines against polio, rotavirus, cholera, and typhoid fever have had a major impact on reducing global disease burden and mortality. A quandary that remains to be solved is the diminished immunogenicity and efficacy of these vaccines when given to subjects living in underdeveloped areas of the world as compared to people living in industrialized nations.

Low socioeconomic status, poor living conditions, malnutrition, and natural barriers that affect people living in less privileged countries are now recognized as major determinants of vaccine performance. Novel protective antigens, adjuvants, and immunization approaches to overcome these barriers are being explored. Understanding the processes involved in the induction of immune responses by orally delivered antigens, the delicate balance between immune activation and tolerance in the gut, and the association between mucosal and systemic immunity is essential to assist the development and evaluation of vaccine candidates. This chapter reviews the basis for immunological priming by orally delivered antigens in the gastrointestinal tract and the ensuing effector responses in humans.

It also provides a summary of the licensed oral vaccines available as well as the success, hurdles, and ongoing efforts to develop more effective vaccine candidates and to achieve successful immunization using oral vaccines.

3.2 Vaccine Uptake and Immunological Priming in the Intestinal Mucosa

Understanding the molecular and cellular mechanisms by which orally delivered antigens and adjuvants are sampled, processed, and stimulate immune cells to develop protective immunity is critical to assist in the design of more effective oral vaccines and immunization strategies. Dissecting the host factors that influence these processes is also important when targeting specific groups (e.g., infants and young children). Antigens that reach inductive sites of the intestinal mucosa are captured by the microfold (M) cells within the follicle associated epithelium (FAE) and actively transported to reach dendritic cells (DC) and other antigen presenting cells (APC, e.g., macrophages) underlying the FAE. Chemokines produced by the epithelial cells

recruit and activate DC, B, and T cells in mucosal inductive sites [1]. Alternatively, antigens can be taken up directly by DC in the lamina propria that extend their dendrites throughout the intestinal epithelium.

Lamina propria DC have been associated with the induction of systemic IgG to orally delivered antigens, whereas DC in the Peyer's patches (PP) have been associated (mainly) with the production of intestinal T cell-dependent IgA [2]. Antigen-loaded DC migrate to interfollicular T-cell areas of the lymphoid follicles or to local/regional lymph nodes where they present antigens and stimulate naïve T cells. These cells will expand and differentiate into $CD4^+$ and $CD8^+$ effector and memory T cells, Th17 or T-regulatory (Treg) cells, and will migrate to the lamina propria of mucosal effector sites via efferent lymphatics and blood [3]. Gut mucosal DC (i.e., conventional $CD11c^{hi}$, plasmacytoid DC, and CD103 DC) play a key role in the outcome of mucosal responses [4] by determining the nature of the T-cell (i.e., effector, memory, regulatory) and B-cell (i.e., IgA, IgG) responses induced and imprinting specific homing receptors (i.e., α_4/β_7, CCR9, and CCR10) on antigen-committed lymphocytes [4]. B cells activated by vaccine antigens will cluster in the Peyer's patches or mesenteric lymph nodes (MLN) to form germinal centers (GC), a special microenvironment that promotes B cell growth and differentiation. In the GC, B cells will undergo IgA class switch and affinity maturation, becoming IgA^+ plasmablasts.

The GC reaction depends on cognate B cell-T cell, CD40-CD40L interaction and IgA-inducing signals including TGF-β, IL-4, IL-10, and retinoic acid (RA) [1]. In the GC, B cells also interact with stromal cells called follicular dendritic cells (FDC) and receive signals and costimulation from follicular helper T (T_{FH}) cells, which further promote GC formation and IgA class switching (e.g., TGF-β1) [5]. The IgA^+ plasmablasts generated in the GC will migrate to the lamina propria where they will terminally differentiate into polymeric IgA-secreting plasma cells in a process that involves cytokines produced by $CD4^+$ T-helper cells (e.g., IL-2, IL-5, TGF-β, and IL-10), DC (e.g., RA, IL-10, TGF-β, IL-6, B-cell activating factor [BAFF], and proliferation-inducing ligand [APRIL]), and intestinal epithelial cells (e.g., TGF-β, IL-6) [4, 6, 7]. Activation of B cells and the production of IgA through T cell-independent mechanisms have been described in mice and humans [8, 9].

This process appears to occur in the Peyer's patches but also in isolated lymphoid follicles and in the lamina propria, which, unlike the PP, lack segregated T-cell zones. It involves recruitment and activation of B cells that respond to antigens (or microbial products) recognized by their own receptors (including "innate receptors") or antigens presented by DC, as well as molecules such as TGF-β, BAFF, APRIL, IL-6, IL-10, and RA produced by various subsets of DC, local stromal cells, and epithelial cells [5]. T-independent, low affinity, "natural" IgA responses are produced against commensal organisms, whereas "classical" high avidity T cell-dependent IgA appears to be produced by pathogenic bacteria, toxins, and viruses [10]. It should be noted, however, that while a wealth of mechanistic information on gut immunology has been derived from mouse studies, their relevance and applicability to humans remains to be determined. The processes involved in vaccine uptake and immunological priming in the gut are depicted in Fig. 3.1.

3.3 Immunological Effectors Induced by Oral Vaccination

The main effector sites of adaptive immunity in the gut are the epithelium and the lamina propria. A large number of antigen-specific antibody-secreting cells (ASC) and activated T cells reside in these tissues and provide a first line of defense against intestinal pathogens. A hallmark of oral vaccination is the induction of long-lived antigen-specific mucosal IgA-secreting plasma cells; this process occurs mainly in the PP and isolated lymphoid follicles. In a seemingly robust and self-sufficient manner, these cells produce dimeric IgA, which is transported through the epithelial barrier into the intestinal lumen via the polymeric Ig receptor (pIgR) [5]. In the lumen, secretory IgA (sIgA) binds to microbial surface antigens and prevents their attachment to the mucosal

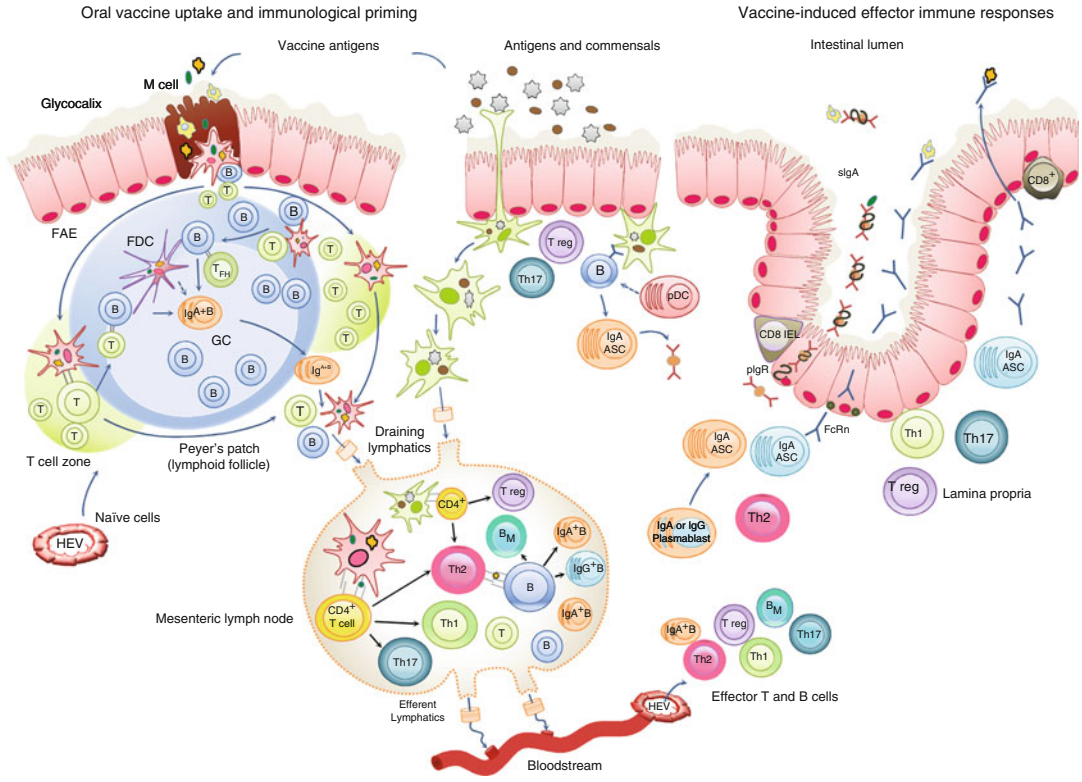

Fig. 3.1 The follicle associated epithelium (*FAE*) contains microfold (*M*) cells that specialize in endocytosis and rapid transport of intact antigens. Vaccine antigens are transported by the M cells across the epithelial barrier and taken up by subepithelial immature dendritic cells (*DC*). DC within the epithelial layer of the intestinal lamina propria may also capture antigens from the lumen through their extended dendrites; some of these DC ($CD103^+$, $CD11b^+$) are involved in the induction of tolerance to commensals. Immature DC as well as naïve and memory lymphocytes enter the mucosa through high endothelial venules (*HEV*); these cells are attracted by chemokines (CCL20, CCL23, and CXCL16) produced by the FAE enterocytes and other mediators. DC carrying vaccine antigens migrate to the interfollicular T-cell areas or to the draining lymph nodes to present antigens to naïve $CD4^+$T cells. B cells are antigenically primed outside of the lymphoid follicle by interaction with DC and T cells; these activated B lymphocytes reenter the follicle, and after interaction with follicular dendritic cells (*FDC*), which capture migrating antigens and Treg-derived T-follicular B-helper (*TFH*) cells, they become germinal center (*GC*) cells. In the GC, B cells undergo IgA class switch and somatic hypermutation differentiating into IgA^+ plasmablasts (or plasma cells) and potentially memory B (B_M) cells. Effector $CD4^+$Th cells primed in the interfollicular T-cell areas may also enter the B-cell follicle where they activate B cells (through CD40-CD40L interaction) and release cytokines (i.e., IL-4, IL-5, IL-10, TGF-β) that induce GC reaction, leading to initial maturation of B cells and IgA isotype switching. IgA plasma cells can also be induced through a T cell-independent mechanism that involves B-cell activation by direct contact with DC and the production of B-cell activation and growth factors (e.g., BAFF, APRIL, TGF-β, and nitric oxide) by distinct DC subsets (e.g., FDC and plasmacytoid DC) and epithelial cells. T-independent (low affinity, "natural") IgA responses are induced in the PP but could also be generated in isolated lymphoid follicles and the lamina propria. Simultaneous with the activation of T and B cells, retinoic acid (*RA*) produced by mucosal DC imprints gut-homing receptors (i.e., $\alpha_4\beta_7$, CCR9, CCR10) on antigen-specific Th cells and IgA-committed B cells. B and T cells activated in the gut mucosa by vaccine antigens migrate to the regional (mesenteric) lymph nodes, where they further differentiate into distinct effector populations (e.g., Th1, Th2, Treg, Th17, and B_M cells). Vaccine-specific T and B cells exit the lymph nodes through the thoracic duct and access the bloodstream; they disseminate through circulation and reenter the mucosal effector sites. In the lamina propria, mucosal B cells terminally differentiate into plasma cells, most of which produce IgA that is exported through epithelial cells as secretory (*sIgA*). Mucosal IgG is produced by local plasma cells or may exudate from blood. The processes depicted are simplified; it should also be noted that the mechanisms involved in the induction of adaptive (activating or regulatory) immune responses following oral immunization are only partially understood

interface. Through this mechanism of "immune exclusion," IgA blocks invasion of potential pathogens [11, 12].

Mucosal sIgA can also neutralize toxins, viruses, and microbial antigens within epithelial cells or in the lamina propria and escort them out to the lumen. In addition to stopping pathogens from entry, IgA helps maintain harmony in the interplay between commensals and the host immune system [5]. Two IgA subclasses are present in human mucosal secretions: IgA1, found primarily in saliva and the proximal small intestine (this is also the subclass found in serum), and IgA2, present mainly in the distal small intestine and colon [3, 13]. IgA1 appears to recognize mainly proteins, whereas IgA2 recognizes polysaccharides and lipopolysaccharides (LPS) [9]. IgG is also produced by mucosal plasma cells and transported across the epithelial barrier through the neonatal Fc receptor (FcRn) [14]. It is also believed that serum IgG diffuses across the epithelial barrier by paracellular leakage [15], and this could explain the protection induced by parenteral vaccines to some enteric pathogens (e.g., *Shigella* spp.) [16]. Both IgA and IgG can transport antigens through the epithelial cells from the intestinal lumen to the lamina propria or from the basolateral cell surface back to the lumen [17].

In mice, antibody-mediated transport of microbes through M cells has been associated with reduced inflammation and mucosal destruction [18]. Conceivably, similar approaches that could shuttle vaccine antigens across the intestinal epithelium might provide a means to improve oral vaccine take. Alternatively, vaccine uptake could be enhanced by targeting antigens to the M cells, enterocytes, and DC using vectors and specific ligands [2, 19].

B and T lymphocytes primed by vaccine antigens in the intestinal mucosa migrate to the mesenteric lymph nodes, where they further differentiate. They exit the lymph nodes through the thoracic duct, access the bloodstream, and disseminate systemically to reach mucosal effector sites. The presence of circulating IgA ASC is a typical indicator of intestinal immunological priming following enteric infection or oral vaccination [20]. These cells are detected transiently in circulation (7–10 days after antigen exposure) while migrating to effector sites or other lymphoid tissues. Virtually all IgA and some IgG ASC induced by oral immunization express integrin α_4/β_7, which binds the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) expressed by lamina propria high endothelial venules. Many of the intestinal and all colon IgA ASC also express CCR10 which enables them to respond to the mucosal epithelial chemokine CCL28 [21].

A smaller proportion of these orally primed ASC express peripheral lymph node-homing receptor CD62L (L-selectin), which is mainly induced after systemic immunization [22]. It is now well accepted that the local lymphoid environment, rather than the nature of the antigen, determines the homing properties of the activated lymphocytes. DC from the gut and the MLN (such as $CD103^+$ DC) stimulate IgA production and imprint gut-homing molecules on mucosally primed B cells in a process that requires RA, IL-6, or IL-5 [21]. The presence of IgA and IgG-specific ASC expressing gut-homing receptors has been demonstrated in humans following infection or oral vaccination against *V. cholerae* [23], *S.* Typhi [24, 25], *Shigella,* and rotavirus [26].

Ideally, oral vaccination will also result in the production of strong cell-mediated immunity that includes antigen-specific intraepithelial and lamina propria T lymphocytes. Most of the T lymphocytes present in the lamina propria (in both mice and humans) are effector memory $CD4^+$ T cells ready for rapid defense [27] and include the classical subsets (Th1, Th2, and Th17), Treg cells, and natural killer T (NKT) cells [8]. Through interaction with $CD103^+$ DC in the lamina propria and MLN, T cells are induced to express CCR9 and $\alpha4\beta7$ acquiring the capacity to migrate to mucosal effector sites. CCL25, which is expressed by crypt epithelium cells in the jejunum and ileum, mediates chemotaxis of CCR9-bearing memory $\alpha4\beta7^{hi}$ $CD4^+$ and $CD8^+$ T lymphocytes into the lamina propria [28].

Cytotoxic $CD8^+$ T lymphocytes in mucosal tissues can mediate lysis of infected cells and

promote an inflammatory milieu through the secretion of IFN-γ and TNF-α that activate phagocytic killing. $CD8^+$ T cells also participate in immune regulation; $CD8^+$ T cells with suppressor activity have been shown to expand upon interaction with epithelial intestinal cells and to mediate immune suppression via cell-to-cell contact [29]. $Foxp3^+$ $CD4^+$ Treg cells also play a key role in preserving immune homeostasis and tolerance against commensal organisms and food antigens in the gut [30, 31]. $CD4^+$Th17 cells, a subset defined by the production of Th17, are present in the lamina propria in the small and large intestine [32]. They have been found to increase upon signals from intestinal commensals [33] and have also been implicated in protection against *H. pylori* and other bacterial infections [34]. Th17-related cytokines such as IL-22, IL-17A, IL-17F, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are important for protection against extracellular pathogens. These cytokines, however, have also been associated with the development of pathologic disorders such as inflammatory bowel diseases (IBD), which can be counteracted by $FoxP3^+$ Treg cells [35]. It is also believed that many of the functions in gut inflammation typically attributed to IL-12 and Th1 cells might be due instead to Th17 cells and IL-23 [36]. Adult volunteers who received the oral typhoid vaccine Ty21a developed $CD8^+$ T cells secreting IL17A and other cytokines, in response to vaccination [37].

Another desirable attribute of oral vaccination is the capacity to induce immunologic memory. Mucosally primed antigen-specific B cells in the gut mucosa can differentiate into long-lived plasma cells or memory B (B_M) cells. B_M cells are detected in circulation while in transit to niche organs; these cells do not secrete antibody but rapidly differentiate into ASC upon antigen exposure [38]. In humans, the presence of B_M cells has been demonstrated in response to infection or oral vaccination with rotavirus, [39] *V. cholerae* [40], *Shigella* [41], and *Salmonella* [42, 43]. A summary of the main immunological effectors that can be induced by oral vaccination is shown in Fig. 3.1.

Depending on the mechanism by which each pathogen causes disease, different vaccine approaches would be needed to generate specific immunological effectors that could target distinct virulence factors and interrupt pathogenesis. A mucosal vaccine might be sufficient to prevent illness by noninvasive organisms such as *V. cholerae* and enterotoxigenic *E. coli* (ETEC), whereas a combined mucosal-parenteral approach may be more suitable to protect against pathogens that cause gut inflammation and cell destruction such as *Shigella spp.* and rotavirus and those inducing a systemic disease, such as *S.* Typhi and poliovirus. The antigen of choice, the method of delivery, and the inclusion (or not) of adjuvants, in addition to host-related factors, will determine the characteristics and the magnitude of the responses induced. A careful evaluation of such responses is important to ascertain the likelihood of achieving protection against a particular pathogen in light of known immune correlates (or surrogates) of protection [44].

Correlates of protection are defined, immunological end points that can predict vaccine efficacy. They can be derived from animal models that recapitulate disease; randomized, controlled efficacy studies in humans; experimental challenge models in human volunteers; and observational epidemiological field studies (including case–control studies) comparing immune status in naturally infected versus exposed individuals who remain asymptomatic [45]. This knowledge is extremely valuable to move vaccine candidates through the regulatory pathway for clinical testing. It also allows for post-licensure vaccine evaluation and optimization, comparing formulations, immunization schedules, or other modifications to the final product. A correlate allows one to predict the performance of a vaccine in different populations and settings and to compare vaccine candidates. National regulatory agencies typically accept well-defined immunological endpoints as the basis for licensure in cases where efficacy studies cannot be conducted or are not warranted.

3.4 Intestinal Tolerance and Immune Regulation Related to Vaccination

There is a delicate balance between promoting the activation of the gut mucosal immune system to mount an adaptive immune response to a potential pathogen while maintaining tolerance to prevent immune disorders such as food allergy, inflammatory bowel disease (IBD), and celiac disease. It could be argued that considering the prevalent tolerogenic environment of the gut, it is counterintuitive to expect orally delivered antigens to engender a strong immune response without disturbing immune balance and physiological homeostasis. The nature of the antigen and the mode of delivery (e.g., dose) can determine the end result (immune activation or tolerance). Oral vaccines that have been the most successful involve active replication (e.g., rotavirus) or strong immunogenic stimuli (e.g., live attenuated strains, immunomodulatory molecules).

Large particles are typically captured by M cells and likely to undergo the normal process of antigen presentation and immunological priming, whereas soluble antigens (administered orally in a single high dose or in multiple small doses) are likely to be taken up and presented preferentially by APC in the gut to induce tolerance by clonal anergy/deletion or suppression of effector cells [2, 46]. Several T-cell subsets involved in maintaining oral tolerance and homeostasis in the gut have been described as follows: $Foxp3^+$ $CD25^+$ $CD4^+$ nTreg cells, $CD25^-$ $Foxp3^-$ IL-10-secreting $CD4^+$Tr1 cells, and TGF-β-secreting Th3 T cells [47]. nTreg cells develop in the thymus and are believed to mediate systemic homeostasis and prevent autoimmune disease. Both antigen-specific and antigen-nonspecific regulatory mechanisms of intestinal immunity have been described, the first involving regulatory cytokines that maintain an immunosuppressive environment and the second the generation of antigen-specific anergy or Treg cells that can maintain systemic immunologic quiescence [47].

Soluble antigens that have diffused into the lamina propria through tight junctions or translocated through transcellular transport may be taken up by special lamina propria $CD103^+$ DC, which have a unique tolerogenic phenotype [48]. These cells migrate to the MLN where they promote the differentiation of $CD4^+$ T cells into $FoxP3^+$ $CD25^+$ $CD4^+$ Treg cells through the production of RA, which acts as a cofactor for TGF-β-mediated conversion of T cells into Treg cells [49, 50]. An essential component that drives and maintains mucosal tolerance is IL-10, which is produced mainly by Tr1 and nTreg cells but also by myeloid cells in the gut. Both IL-10 and TGF-β (produced by Tr1, Th3, and nTreg) have suppressive functions inhibiting the production of cytokines by activated T cells, the expression of costimulatory molecules on APC, and the production of antibodies [47]. The microbiota is also known to influence the induction of $Foxp3^+$ Treg cells and as a consequence, the state of tolerance. Some of the $Foxp3^+$ Treg cells in the PP differentiate into T_{FH} cells which are involved in GC formation and support IgA responses; [1] this agrees with IgA supporting (unperturbed) housekeeping functions at the mucosal interface.

3.5 Licensed Oral Vaccines and Their Performance in Different Populations

A handful of oral vaccines have been licensed and are commercially available for prevention of polio, rotavirus, *S.* Typhi, and *V. cholerae* O1 (Table 3.1). They consist of live attenuated organisms obtained by tissue culture passage (Rotarix® and poliovirus) [57] or reassortant technology (RotaTeq®) [58], deletion of genes encoding virulence factors (cholera) [59], or chemical mutagenesis (typhoid) [55] as well as inactivated organisms combined with immunomodulatory recombinant toxin subunits (cholera) [60]. These vaccines have been successful in preventing disease in specific target populations: infants living in industrialized countries

Table 3.1 Licensed oral vaccines

Target pathogen	Licensed vaccine	Details of vaccine	References
Poliovirus	Sabin's tOPV (trivalent oral poliovirus vaccine)	Live attenuated poliovirus strains of serotypes 1, 2, and 3	[51]
Rotavirus	RotaTeq® (Merck & Co, Inc)	Pentavalent attenuated reassortant rotavirus vaccine	[52]
	Rotarix® (GlaxoSmithKline Biologicals)	Live attenuated monovalent rotavirus vaccine	[53, 54]
Salmonella Typhi	Vivotif® (Berna Biotech, Ltd)	Live attenuated *S.* Typhi Ty21a	[55]
Vibrio cholerae	Dukoral® (Crucell)	Whole killed *V. cholerae* O1 Inaba and Ogawa (El Tor and classical biotypes) admixed with recombinant cholera toxin B subunit (CTB)	[56]
	Shanchol® (Shantha Biotechnics-Sanofi Pasteur)/mORCVAX® (VaBiotech)	Whole killed *V. cholerae* O1 strains and a O139 strain (without CTB)	[56]

(rotavirus vaccines), infants in developing countries (polio vaccine), and school-age children in developing countries (typhoid and cholera vaccines). Despite their success in industrialized nations, however, there is mounting evidence of their reduced immunogenic capacity and efficacy in certain subpopulations in less developed areas of the world [61–64].

3.5.1 Oral Polio Vaccine

There are three serotypes of poliovirus: 1, 2, and 3. This single-stranded RNA virus replicates in the lymphoid tissue underlying the gastrointestinal epithelium and generally produces either mild (fever and sore throat) or no symptoms. In <1 % of cases, however, the virus disseminates to motor neurons and causes flaccid paralysis. Albert Sabin developed a trivalent oral poliovirus vaccine (tOPV) containing live strains from serotypes 1, 2, and 3 attenuated by repeated passage through nonhuman cells [51]. As part of the Global Polio Eradication Initiative, this vaccine has eliminated the transmission of wild-type polioviruses throughout the Americas, the Western Pacific, and Europe [65]. Type 2 poliovirus has been globally eradicated as of 1999. However, poliovirus still remains endemic in Pakistan, Afghanistan, and Nigeria [66].

Sabin's tOPV has been the vaccine of choice for use in poliovirus-endemic countries due to the ease of delivery, its capacity to induce lifelong immunity in the intestine (the primary site of virus entry) [67], and the fact that it can be shed in the stool and indirectly immunize other individuals in the community [68]. Despite its overall success, however, tOPV has proven less effective in the remaining reservoirs [69, 70]. In the states of Uttar Pradesh and Bihar in India, some children under 5 years of age have received ~15 doses of tOPV compared to 10 doses given to children elsewhere in India. Despite this high coverage, the former group developed lower immune responses to the vaccine, which has been attributed to diarrhea and other enteric infections caused by poor sanitation and high population density [62, 71, 72].

The composition of the vaccine may also be responsible for the reduced immunogenicity. The type 2 strain efficiently colonizes the intestine and elicits stronger immune responses than the type 1 and 3 strains. Since it is possible that the

type 2 virus can outcompete the other virus strains, tOPV contains $\geq 10^{6.0}$ infectious units of type 1 virus, $10^{5.0}$ infectious units of type 2, and $10^{5.8}$ infectious units of type 3 virus per dose [65]. In the states of Uttar Pradesh and Bihar in India and in other developing countries, monovalent type 1 (mOPV1) and type 3 (mOPV3) vaccines and the bivalent type 1 + 3 (bOPV) vaccine have been used to interrupt transmission of the type 1 and 3 polioviruses [73]. Due to these efforts, India has been wild-type poliovirus free since 13 January 2011 [74]. One of the disadvantages of tOPV is the occasional reversion to virulence that causes vaccine-associated paralytic poliomyelitis (VAPP) in 1 out of 2.5 million doses [75]. As such, in some countries where transmission of wild-type poliovirus has been successfully interrupted, tOPV has been replaced with inactivated poliovirus vaccine (IPV) [76].

3.5.2 Rotavirus Vaccines

Rotavirus is a double-stranded RNA virus and the leading cause of severe gastroenteritis in children less than 5 years of age. It affects both developing and industrialized countries and it is estimated to cause 527,000 deaths annually, the majority of which occur among children in the developing world [77]. Effective vaccines are considered the best prophylactic intervention to reduce the burden of disease [78], and the WHO Strategic Advisory Group recommends their inclusion in national immunization programs [79].

Serotyping is based on the VP7 glycoprotein (G serotypes) and the VP4 protease-sensitive protein (P serotypes). Five strains (P[8], G1; P[4], G2; P[8], G3; P[8], G4; and P[8], G9) are commonly detected and constitute the main vaccine targets [80].

Two oral vaccines, RotaTeq® and Rotarix®, are currently licensed in most countries. An oral vaccine previously licensed in the USA, RotaShield®, was withdrawn from the market due to its association with intussusception, an unexpected complication that appeared shortly after the first dose in which a part of the intestine invaginates into another part of the intestine; the vaccine is no longer available [81]. The induction of serum IgA has been associated with protection against rotavirus infection in children [82]. The levels of serum IgA but in particular the frequency of circulating rotavirus-specific IgA ASC were found to correlate with intestinal ASC measured in biopsies [83].

3.5.3 Rotarix®

Rotarix® (also known as RV1) is a live attenuated monovalent rotavirus vaccine developed by GlaxoSmithKline Biologicals. It is administered orally at 6–14 weeks of age and again ~4 weeks later. This vaccine contains the RIX4414 strain which is a G1P[8] serotype and is derived from the parent vaccine strain 89–12 [53, 54]. The vaccine was attenuated by passaging 43 times. A large multinational, double-blind Phase 3 trial performed in 11 Latin American countries and Finland showed that Rotarix® is highly effective in protecting infants against severe rotavirus gastroenteritis (vaccine efficacy of 85 %, $P < 0.001$ versus placebo), significantly reduces the rate of severe gastroenteritis from any cause (rate reduction of 40 %, $P < 0.001$), and is not associated with an increased risk of intussusception [57]. A post-licensure study performed in Mexico and Brazil showed that Rotarix® was associated with a short-term risk of intussusception in approximately 1 of every 51,000–68,000 vaccinated infants [84]. However, the benefits of the vaccine far outweighs the risks as it prevented approximately 80,000 hospitalizations and 1,300 deaths from diarrhea each year in these two countries.

In contrast to the excellent efficacy seen in high- and middle-income countries of Europe and Latin America, a randomized, placebo-controlled, multicenter trial in Africa (South Africa and Malawi) showed a vaccine efficacy of 61.2 % [61]. Efficacy against severe gastroenteritis from any cause was only 30.2 %. Possible reasons for the reduced efficacy in African infants include malnutrition, coinfection with other enteric pathogens, anti-rotavirus antibodies in

breast milk, and interference by the oral poliovirus vaccine. These factors will be discussed later in this chapter.

3.5.4 RotaTeq®

RotaTeq® (also known as RV5) is a pentavalent attenuated rotavirus vaccine developed by Merck through reassortment of ten genes from the bovine rotavirus strain WC3 and an individual capsid gene from five of the most common human serotypes (G1, G2, G3, G4, and PIA) [52]. Three doses of the vaccine are given orally, starting at 6–12 weeks of age with the subsequent doses given at 4- to 10-week intervals. A final formulation of 12×10^7 infectious units per dose was evaluated in a large field trial performed in 70,000 infants predominantly from the USA and Finland but also from Central and South America, Europe, and Asia [58]. The vaccine had a 98 % efficacy in protection against severe rotavirus gastroenteritis, and there was no difference in the risk of intussusception between vaccine and placebo recipients. In a post-licensure study in the USA, 786,725 total doses were administered to 4- to 34-week-old infants and showed no increased risk of intussusception due to vaccination [85]. A combined efficacy of 33.9 % against severe rotavirus gastroenteritis and 51.2 % against very severe gastroenteritis was reported in two recent multicenter, double-blind, placebo-controlled vaccine trials performed in 4- to 12-week-old infants in Africa and Asia through the entire follow-up period [86]. These studies also showed cross-protection against non-vaccine serotypes. Although the vaccine efficacy in these trials was substantial, the values obtained were lower than in trials performed in developed countries or in developing countries in Latin America [58, 64, 87–89]. The African trial involved 5,468 infants in rural sites in Ghana and Kenya and an urban area in Mali. The vaccine efficacy against severe rotavirus gastroenteritis declined from 64.2 % in the first year of life to only 19.6 % in the second year of life (resulting in an overall efficacy of 39.3 %). This reduction in efficacy was most likely due to waning immunogenicity. Serum IgA geometric mean titers (GMT) measured 14 days after the 3rd dose were five to ten times lower than for subjects in trials performed in developed countries [64]. Similarly, postimmunization serum neutralization antibody responses were fourfold lower for G1 and threefold lower for serotype P1A[8] in the African subjects. The Asian trial involved 2,036 infants in Matlab, Bangladesh, and urban and peri-urban Nha Trang, Vietnam [90]. Three doses of RotaTeq® resulted in a vaccine efficacy of 48.3 % against severe rotavirus disease. However, unlike the African study, the vaccine efficacy was similar in the first and second years of life (51 and 45.5 %, respectively).

Possible explanations for the lower efficacy of rotavirus vaccine in African and Asian infants are the same as for Rotarix® as well as the younger age of the infants enrolled in this study compared to other studies. Additionally, many of the infants in these trials received the oral poliovirus vaccine and RotaTeq® at the same time. It has previously been shown that coadministration of the oral poliovirus vaccine results in reduced immunogenicity of oral rotavirus vaccines [63, 91, 92].

3.5.5 Cholera Vaccines

Cholera is still a major global public health problem, occurring mainly in developing countries. In 2010, 317,534 cases of cholera and 7,543 deaths were reported to the World Health Organization (WHO) worldwide, although the true global burden is estimated at 3–5 million cases and 100,000–130,000 deaths annually [93]. Vaccination is recommended in endemic areas in high-risk groups and during outbreaks, using available oral killed vaccines [93]. *Vibrio cholerae* serogroups O1 and O139 are responsible for most of the cholera epidemics seen worldwide [94]. Serogroup O1 is further divided into biotypes, El Tor and classical, and serotypes, Inaba and Ogawa. An effective cholera vaccine needs to protect against both biotypes and serotypes.

V. cholerae has a high-infectious dose and is acquired through fecal-oral contamination, usually via food and waterborne transmission. The disease is caused by the cholera toxin (CT) which consists of five B (binding) subunits and one A (active) subunit. The B subunits bind to GM1 ganglioside receptors and serve as a conduit for the A subunit to access the epithelial cells, causing profuse secretion of water and electrolytes. Unlike other enteric infections, prior exposure to wild-type *V. cholerae* infection induces a serogroup-specific protection against clinical disease that is robust and durable [95, 96]. The precise immunological effectors responsible for this protection are not fully known. Individuals from endemic areas infected with *V. cholerae* develop LPS-specific IgA ASC, which peak 7 days after the onset of disease [97, 98] and express the gut-homing integrin receptor α4β7 [23]. Serum vibriocidal antibodies have been inversely correlated with susceptibility to infection but are unlikely the protective effector mechanism [99–101]. Intestinal IgA, on the other hand, is believed to play a critical role blocking the pathogen at mucosal surfaces. Mucosal LPS-specific IgA ASC were detected in duodenal biopsies of cholera-infected patients, even in the absence of detectable anti-LPS IgA in secretions. These cells might resume antibody production when appropriately stimulated [102]. IgG and IgA B_M cells specific for *V. cholerae* LPS, CTB, and TcpA were found in cholera-infected patients for up to 1 year following disease [40, 103], and the frequency of LPS-specific IgG B_M cells has been associated with protection against infection in household contacts of cholera patients [104]. A mucosal anamnestic response mediated by B_M cells (directed primarily to the LPS) appears to be necessary for long-term protection against cholera [105].

Two types of oral cholera vaccines are commercially available, both consisting of whole killed organisms. Dukoral™ (Crucell, Sweden) contains heat or formalin-inactivated *V. cholerae* O1 Inaba and Ogawa strains of both El Tor and classical biotypes admixed with recombinant cholera toxin B subunit (CTB). Although it is internationally licensed, it is mainly used in travelers because of its high cost [56]. Two doses are recommended, 10–14 days apart. In a randomized double-blind trial, protective efficacy was ascertained in children aged 2–15 years and women older than 15 years in rural Bangladesh. Among 62,285 subjects, vaccine efficacy after 3 years was 50 % (26 % for 2- to 5-year-olds and 63 % for older subjects) [60]. After 5 years of follow-up, protective efficacy was still 49 % [106]. Shanchol™ (Shantha Biotechnics-Sanofi Pasteur, India) or mORCVAX (VaBiotech, Vietnam) contains several *V. cholerae* O1 strains and an O139 strain, without CTB, and represents a less expensive alternative. Shanchol™ was tested in 101 adults and 100 children (1–17 years old) in a cholera-endemic region, Kolkata, India. Following two doses of vaccine, 53 % of adults and 80 % of children showed seroconversion (defined as a fourfold rise in serum vibriocidal antibody titers following vaccination as compared with baseline) against *V. cholerae* O1, and only 10 % of adults and 27 % of children seroconverted for *V. cholerae* O139 [107].

In another study performed in a cholera-endemic region, 2- to 5-year-old toddlers and 12- to 23-month-old infants living in the Mirpur area in Dhaka, Bangladesh, were given two doses of the vaccine which was safe and immunogenic in all age groups [108]. However, in both of these studies performed in cholera-endemic regions, the geometric mean fold rise in vibriocidal antibodies in adults was lower than that seen in a non-cholera-endemic area (Sơn La, Vietnam) [109]. This is most likely due to the high baseline titers observed in Kolkata and Dhaka, suggesting that preexisting immunity due to natural infection may negatively affect the immune response of live oral attenuated vaccines. Toddlers and infants had lower baseline levels presumably due to lack of exposure to *V. cholerae* O1. In a large double-blind trial in Kolkata, India, Shanchol® showed 67 % efficacy ($P<0.0001$) against *V. cholerae* O1 diarrhea in recipients aged 1 year and older. Recent studies suggest that a single dose of Dukoral® or Shanchol® may be sufficient to elicit protection [110, 111].

Compared with wild-type *V. cholerae* infection, the inactivated whole-cell cholera vaccines induce a more limited and short-lived protection in adults and particularly in children [95, 112, 113]. Hence, live vaccine strains that can mimic natural infection are sought to harness the immunological effectors of naturally derived immunity, ideally after a single immunization.

CVD 103-HgR is a live attenuated vaccine derived from a *V. cholerae* O1 classical biotype, Inaba serotype that contains a deletion in the gene that encodes the A subunit of cholera toxin and also carries a gene that encodes mercury resistance (inserted into the hemolysin A [hlyA] locus). Clinical trials performed in the USA [114] and Europe [115] showed that a single oral dose of 5×10^8 colony-forming units (CFU) of CVD 103-HgR resulted in seroconversion (fourfold or greater rise in serum vibriocidal antibodies) in >90 and 88 % of vaccinees, respectively. The vaccine was also able to protect volunteers against cholera due to both biotypes (El Tor and classical) and serotypes (Inaba and Ogawa) of *V. cholerae* O1 in challenge studies [59, 114, 116].

However, clinical trials performed in several developing countries showed diminished immunogenicity. In Indonesia, 5×10^8 CFU of CVD 103-HgR elicited seroconversion in only 16 % of 5- to 9-year-old children [117]. Similar results were observed in 2- to 4-year-old children [118]. Clinical trials performed in Peru [119] and Thailand [120] showed similar results. Nonetheless, 5×10^9 CFU of CVD 103-HgR was able to elicit high seroconversion rates in Indonesian children and Peruvian and Thai adults [117, 119, 120]. CVD 103-HgR was also tested in a randomized large-scale controlled field trial in a highly endemic area of North Jakarta [121]. Contrary to the results in US volunteers, the efficacy was low due to a decrease in the incidence of disease at the end of the study [121]. One possible explanation is that the extensive vaccination led to indirect protection and when the disease disappeared, the estimates of efficacy became insignificant [122].

In a randomized, placebo-controlled, double-blind, crossover clinical trial in Mali, lower vibriocidal seroconversion rates following immunization with CVD 103-HgR were observed among HIV seropositive versus HIV seronegative subjects [123]. CVD 103-HgR was previously manufactured by the Swiss Serum and Vaccine Institute (Berna, Switzerland) under the trade names Orochol® and Mutacol® and licensed as a single-dose cholera vaccine [124–126]. Production is being resumed by PaxVax; clinical trials are currently being performed to make it available in the USA (NCT01585181; ClinicalTrials.gov). Other live vaccines in the front line include Peru 15 (Haikou VTI Biological Institute, China), also a genetically engineered toxin-deficient strain found to be safe and immunogenic in adults and children in Bangladesh, although not yet tested in Phase 3 studies, and live attenuated *V. cholerae* 638 [127] and CTB-expressing strain VA 1.3 [128], both attenuated El Tor derivatives.

3.5.6 Oral Typhoid Vaccine

Salmonella enterica serovar Typhi (*S.* Typhi) causes an acute systemic infection characterized by persistent high fever. It is a food and waterborne infection uncommon in modern industrialized countries but endemic in underdeveloped areas lacking adequate sanitary conditions. The peak incidence of typhoid fever is seen among school-age children [129, 130]. In urban slum environments in South Asia, systematic household and health center-based active surveillance demonstrated a high incidence of bacteremic typhoid infection among febrile toddlers and preschool children [130–132]. Travelers from industrialized countries to developing countries are also at increased risk of developing typhoid fever [133].

Natural *S.* Typhi infection induces serum antibody responses to bacterial LPS, flagella, outer membrane proteins and other bacterial antigens, gut-derived ASC, and sIgA [134–136]. High titers of serum IgG against the capsular Vi antigen are found in ~80–90 % of chronic biliary *S.* Typhi carriers, in 12–38 % of acute typhoid fever patients who do not become chronic carriers, but only

rarely in healthy individuals from endemic areas [137–139]. Proliferative responses to *S*. Typhi antigens were observed in healthy adults living in typhoid-endemic areas who have no known history of clinically overt typhoid fever [140]. Elevated serum levels of Th1-type cytokines (e.g., IFN-γ, TNF-α, IL-1, and IL-6) were seen in patients with culture-confirmed typhoid fever [141].

A live attenuated oral typhoid vaccine, strain Ty21a, developed in the early 1970s by chemical mutagenesis, is licensed for use in the USA for travelers to endemic areas and in other countries worldwide [55]. Ty21a provides significant protection without causing adverse reactions. In large-scale efficacy field trials with Ty21a, involving approximately 514,000 schoolchildren in Chile [142–144], 32,388 in Egypt [145], and 20,543 subjects from 3 years of age to adults in Indonesia [146], no vaccine-attributable adverse reactions were found through passive surveillance [142–144, 147]. Two formulations are licensed: enteric-coated capsules and a lyophilized vaccine to be reconstituted with buffer. In recent years, however, only the enteric-coated capsules have been commercially available. A field trial in Santiago, Chile, showed that three doses of Ty21a in enteric-coated capsules given every other day conferred 67 % protection over 3 years of follow-up [147, 148]. Based on these results, this formulation and dose regimen are used throughout the world, except for the USA and Canada where a four-dose regimen is used. The four-dose North American regimen is based on results of another large-scale, randomized comparative trial carried out in Chile where four doses of Ty21a in enteric-coated capsules were significantly more protective than two or three doses [149].

Ty21a confers a long-lived protection [148]. Individuals who received Ty21a in two Chilean field trials were followed up for a total of 7 years and over this period, the enteric-coated capsule formulation of Ty21a conferred 62 % vaccine efficacy [148], while the liquid formulation had a point estimate of vaccine efficacy of 78 % over 5 years of follow-up. Immune responses in Ty21a recipients include serum IgG against the O polysaccharide [142, 150] and mucosally primed O-specific ASC that are detected 7–10 days after vaccination [20]. Most of these ASC carry the intestinal-homing integrin $\alpha_4\beta_7$ [20, 24]. O-specific serum IgG [142] and gut-derived ASC were found to correlate with the protection conferred by different formulations and immunization regimens of Ty21a in field trials. Intestinal sIgA antibody responses [151] and antibody-dependent cellular cytotoxicity have also been described following oral Ty21a immunization [152, 153]. Additionally, Ty21a stimulates strong T-cell proliferative responses, Th1-type cytokine secretion (e.g., IFN-γ, TNF-α), and multifunctional $CD8^+$ cytotoxic T lymphocytes [154–157]. T-cell clones with T_{EM} phenotype (i.e., $CCR7^-$, $CD27^-$ $CD45RO^+$ $CD62L^-$) co-expressing gut-homing molecules (i.e., $\alpha_4\beta_7$ and CCR9) were derived from peripheral mononuclear cells of Ty21a vaccinees [158].

3.6 Barriers Associated with Oral Vaccination

There is growing recognition that oral vaccines meet a very different host in underdeveloped populations compared to industrialized nations. At a global level, one of the greatest challenges of modern vaccinology is to identify and overcome the barriers that lead to the diminished immunogenicity and reduced protection of oral vaccines in impoverished populations [159–162]. Some of the factors that may explain this phenomenon include small bowel bacterial overgrowth, which is indicative of environmental enteropathy in individuals living in poverty, heavy intestinal helminth infestation [163], malnutrition, maternal antibodies, interaction with the microbiota, and interaction with other vaccines. These factors are summarized in Table 3.2 and are herein described separately but they are interrelated [170].

3.6.1 Small Bowel Bacterial Overgrowth (SBBO) and Environmental Enteropathy

Many children living in poverty have small bowel bacterial overgrowth, which manifests itself as

Table 3.2 Barriers to oral vaccination

Barrier	Example of reduced immunogenicity due to the barrier or increased immunogenicity when the barrier was overcome	References
Small bowel bacterial overgrowth (SBBO) and environmental enteropathy	In Chile, children with SBBO showed reduced vibriocidal seroconversion following immunization with the live oral cholera vaccine CVD 103-HgR	[164]
Malnutrition	In Bangladesh, supplementation with zinc-improved vibriocidal seroconversion induced by Dukoral®	[165, 166]
Maternal antibodies and immune components of maternal milk	In Bangladesh, temporary withholding of breast-feeding for 3 h before immunization with Dukoral® resulted in increased magnitude of vibriocidal antibodies	[166]
Microbiota	In Finland, IgA seroconversion to the live oral rotavirus vaccine RotaShield® was improved when coadministered with the probiotic *Lactobacillus casei* GG	[167]
Oral immunization of newborns	Increased seroconversion has been observed in infants given a monovalent oral poliovirus vaccine in contrast to trivalent oral poliovirus vaccines	[168]
Coadministration of vaccines	tOPV can affect the immunogenicity of oral rotavirus vaccines when coadministered	[169]

excessive colonization of the proximal intestine and environmental (or tropical) enteropathy characterized by histopathological changes in the small intestine, including inflammation and blunting of villi. It is believed that children (and adults) living in poverty in developing countries are continually exposed to fecal contaminants, which lead to SBBO and environmental enteropathy. Interestingly, environmental enteropathy can be reversed and eventually disappears then the individual is transferred to a clean environment [171].

SBBO may be one reason why CVD 103-HgR showed reduced efficacy in clinical trials performed in developing countries. In a clinical study performed in Chile, SBBO (measured by lactulose breath H_2 test) was detected in 10 out of 178 5- to 9-year-old children [164]. These children had lower vibriocidal geometric mean titers than other children, though this was not significant (160 versus 368, $P=0.25$). Logistic regression showed that peak H_2 (cleaved by bacterial enzymes following ingestion of lactulose) was associated with diminished vibriocidal seroconversion ($P=0.04$).

SBBO and environmental enteropathy may have a direct inhibitory effect on live oral vaccines through the production of short chain fatty acids or other small molecules, or an indirect effect, through the activation of innate immune cells (as shown by increased lymphocytes in the mucosa) and a proinflammatory milieu that destroys the vaccine. Alternatively, fecal contaminants may compete with the live oral vaccines for colonization sites in the gut and prevent the vaccine from accessing key receptors or cells [172].

Evidence to suggest that coinfections of the intestine affect the immune response generated by live vaccines is shown in a clinical trial performed with CVD 103-HgR in a high helminth prevalence region in Ecuador [163]. One hundred and thirty-nine children were given two doses of albendazole (a broad-spectrum anti-helminth) or placebo followed by CVD 103-HgR after the second dose. Seroconversion (serum vibriocidal responses) was higher in the albendazole group compared to the placebo group (29.3 % versus 15.5 %), though this difference was not significant ($P=0.06$).

3.6.2 Malnutrition

Maternal and child malnutrition is highly prevalent in low- and middle-income countries [173]. For example, 33 % of children in South Central Asia and 28 % of children in Eastern Africa are underweight. Many of these children have vitamin A and zinc deficiencies. Zinc is required for many cellular functions and for effective innate and acquired immunity [174]. Deficiency of vitamin A is well known for causing immune defects in the gut, affecting distinct populations of intestinal DC. In mice, the vitamin A metabolite, retinoic acid, is critical for trafficking of vaccine-induced T cells to the gastrointestinal mucosa and for protective vaccine efficacy [175]. Indeed, children with vitamin A deficiency have depressed immune responses compared to children who have been supplemented to normal vitamin A levels [176].

Some studies have shown that supplementation of zinc or vitamin A improved immunogenicity induced by oral vaccines. In a study performed in Bangladesh, 2- to 5-year-old children were given zinc, vitamin A, or both 1 week before the oral cholera vaccine Cholerix/Dukoral and vibriocidal titers were measured 2 weeks after vaccination [165]. The study showed that supplementation with zinc, but not vitamin A, improved the rates of vibriocidal seroconversion. Conversely, when sera from these children were tested for antibody to cholera toxin (CT), children who received zinc had significantly lower levels of CT antibodies than children who did not receive micronutrient supplementation [177]. Therefore, zinc enhanced vibriocidal but suppressed CT-antibody responses to cholera vaccination. A second study in Bangladesh confirmed that zinc supplementation could increase the magnitude of vibriocidal responses induced by Dukoral [166].

The effect of vitamin A supplementation on the immune response to oral polio vaccine was ascertained in a study performed in 1,085 infants in Ghana [178]. Vitamin A or placebo was given at 6, 10, and 14 weeks of age at the time the infants received their diphtheria/pertussis/tetanus (DPT) vaccine. Neutralization titers against poliovirus types 1, 2, and 3 were determined, and no significant differences were observed between the group that received vitamin A supplementation and the placebo group. In a similar study performed in New Delhi, India, infants that were given vitamin A at 6, 10, and 14 weeks with OPV showed increased neutralizing antibody titers against poliovirus type 1 (but not type 2 or 3) compared to the placebo group (who did not receive vitamin A supplementation) [179]. These results are consistent with a previous study performed in Indonesia that found that vitamin A did not affect immune responses to any of the polioviruses [180].

Overall, the data suggests that zinc and, to a lesser extent, vitamin A supplementation may improve immune responses to oral vaccines, but more studies need to be performed to determine the effect on specific vaccines.

3.6.3 Maternal Antibodies and Immune Components of Maternal Milk

Some live oral vaccines may be inhibited by elevated levels of antibodies in breast milk. In addition, human breast milk contains immunomodulatory molecules and cytokines that promote the development of the gut immune system, some of which have tolerogenic functions such as TGFβ, IL-10, and vitamin A [181]. The most well-known example of maternal milk interfering with vaccine take is that of rotavirus vaccines [161]. Breast milk contains IgA antibodies that neutralize rotavirus and also lactadherin (a receptor analog) that can bind to the virus and prevent its attachment [182]. Various studies have been performed to examine the effect of breast milk on the immunogenicity of rotavirus vaccine with mixed results. Goveia et al. [183]. examined the effect of breast-feeding frequency (never breast-fed versus sometimes breast-fed versus exclusively breast-fed) on the ability of RotaTeq® to protect against rotavirus gastroenteritis in 5,098 infants from 11 countries from three regions: Europe, USA, and Latin America / the Caribbean [88]. Breast-feeding did not affect the efficacy of the vaccine. On the other hand, analysis of breast milk from Indian women has shown that it possesses high titers of IgA and neutralizing activity which could theoretically reduce vaccine potency [184].

Breast milk from Korean and Vietnamese women had slightly lower IgA and neutralizing titers and American women had the lowest titers. A clinical trial performed in Bangladesh has shown that temporary withholding of breast-feeding for 3 h before immunization with Dukoral® resulted in an increased magnitude of vibriocidal antibodies and frequency of responders compared to subjects who just received the vaccine [166]. This difference was seen in 10- to 18-month-old infants but not 6- to 9-month-old infants.

3.6.4 Microbiota

It is unclear how the gut microbiome interacts with orally delivered vaccines and affects immunization. It is known, however, that socioeconomic, geographic, and cultural settings imprint differences in gut microbiome profiles [185]. Turnbaugh et al. [186]. studying the gut microbiome in obese and lean twins in the USA found that there is no single abundant bacterial phylotype in the human gut. Instead, it appears that a core gut microbiome exists at the level of metabolic functions. This suggests that not only is the gut microbiome different between different populations but that it is also different within a population (in terms of the bacterial phylotypes). Evidence to suggest that the microbiota can alter immunogenicity induced by oral vaccines is shown in a study from Finland [167]. Investigators orally administered *Lactobacillus casei* GG in conjunction with RotaShield® vaccine to 2- to 5-month-old infants and observed rotavirus IgA seroconversion in 93 % cases versus 74 % of the placebo controls who did not receive *L. casei* ($P=0.05$). The potential to improve vaccine responses by modifying the gut microbiota in infants and the possibility of using probiotics as adjuvants and/or delivery vehicles are currently being explored [187].

3.6.5 Oral Immunization of Newborns and Coadministration of Vaccines

The immune system of newborns and older infants differs in their composition and functional capacity. It is generally accepted, however, that the neonatal immune system is not underdeveloped but rather "inexperienced," and conventional vaccines fail to activate key cells involved in antigen presentation and immune stimulation. Newborns have been shown to respond to oral polio vaccine, and protective immunity can be achieved after a single immunization at birth. Interestingly seroconversion rates are higher when newborns are given the monovalent, as opposed to the tOPV vaccine [168]. A recent study reports a reduction in Th1 and Th2 immune responses to intradermal BCG vaccination in newborns who were simultaneously given OPV [188], but cautions about potential confounding factors.

As mentioned above, the efficacy of live oral vaccine efficacy can be affected by coadministered vaccines. The tOPV vaccine is known to affect immunogenicity to oral rotavirus vaccines when both are given to infants [169]. As more oral vaccines are licensed and added to the Expanded Program on Immunization (EPI), well-designed clinical trials will be needed to measure the effect of different vaccine combinations.

3.7 New Oral Vaccines and Approaches for Immunization Against Enteric Pathogens: Typhoid and Paratyphoid Vaccines

Live vaccines with the capacity to induce both antibodies and cell-mediated immunity embody one of the most efficient approaches to prevent disease caused by typhoid, non-typhoid, and paratyphoid *Salmonella*. The challenge remains to generate strains that are safe and yet sufficiently immunogenic. A live attenuated, single dose, *S.* Typhi vaccine, Typhella™ (also known as M01ZH09, recently acquired by Prokarium Ltd., UK, from Emergent BioSolutions™, USA), proved to be safe and immunogenic in US adults and Vietnamese children [189–192]. This vaccine has mutations in *aroC* (aromatic biosynthesis pathway) and *ssaV* (SPI-2 type III secretion system) [193]. Larger field trials would be needed to determine its protective efficacy. A series of

attenuated *S*. Typhi vaccines, CVD 908-*htrA* (with deletions in *aroC, aroD,* and *htrA*) and CVD 909 (CVD 908-*htrA* that constitutively expresses Vi), have been shown to be safe and immunogenic after a single dose [194].

While enteric fever due to *S.* Typhi has been substantially reduced, paratyphoid fever still remains. A live attenuated *S*. Paratyphi A vaccine, CVD 1902, harboring deletions in the *guaBA* and *clpX* loci and developed at the Center for Vaccine Development, University of Maryland Baltimore, is currently being tested in a Phase 1 clinical trial (NCT01129452; ClinicalTrials.gov). The data obtained to date suggest that the vaccine is well tolerated and immunogenic (Kotloff, personal communication).

3.7.1 *Shigella* and Enterotoxigenic *E. coli* (ETEC) Vaccines

Diarrhea caused by *Shigella* spp. and ETEC remains a notable disease burden associated with substantial mortality globally, but particularly in children in much of the developing world. The genus *Shigella* contains four species (or groups), *S. dysenteriae, S. flexneri, S. boydii,* and *S. sonnei,* which in turn include one or multiple serotypes [195]. These invasive pathogens often cause profuse dysentery which is often refractory to oral rehydration. A vaccine against these pathogens is greatly needed and such a vaccine should induce broad protection against *S. dysenteriae* I (causes epidemics of severe dysentery), all 14 serotypes of *S. flexneri* (cause endemic pediatric shigellosis), and *S. sonnei*. A number of *Shigella* vaccine candidates based on killed or live attenuated organisms have been developed and evaluated in human studies. An inactivated *S. sonnei* whole-cell vaccine was shown to be safe and immunogenic in volunteers ingesting three to five doses of 10^{10} killed organisms [196]. A series of live attenuated vaccines developed at Walter Reed Army Institute have been shown to protect vaccinated adult volunteers in experimental challenge studies and adult and children living in endemic areas in controlled field trials [197, 198]. The live attenuated *S. flexneri* 2a SC602 strain harbors mutations in the plasmid-encoded gene *icsA* and the chromosomal gene *iuc* [199]. Five clinical trials performed in the United States showed that SC602 is well tolerated and elicits mucosal immune responses [200]. However, when the vaccine was tested in Bangladeshi adults and children, although it showed minimal vaccine shedding and reactogenicity, it also produced very minimal immune responses [201]. The dietary deficiency of iron in Bangladeshi volunteers has been suggested as one reason for the lower immunogenicity of this vaccine which has a mutation in iron uptake. A pentavalent *Shigella* vaccine consisting of multiple attenuated strains (*S. sonnei*, *S. dysenteriae* 1, *S. flexneri* 2a, 3a, and 6) is being pursued by investigators at the CVD to provide broad-spectrum protection [195], and several candidate strains containing mutations in *guaBA* and *sen/set* loci (e.g., CVD 1204, CVD 1208) have been evaluated in Phase 2 studies in adult human volunteers. This approach has not yet been tested in endemic populations.

A live oral vaccine, ACE527, comprising three attenuated strains of ETEC was safe and well tolerated in humans at doses up to 10^{11} CFU in adult volunteers [202]. In a Phase 2b efficacy study whereby vaccinated and unvaccinated volunteers were challenged with ETEC 10407, this vaccine reduced the incidence and severity of diarrhea but did not meet the primary end points of the study in reduction of moderate to severe diarrhea [203].

3.7.2 Norovirus Virus-like Particles

Noroviruses (NoV) are single-stranded, positive-sense RNA viruses that cause gastroenteritis in humans. The NoV capsid can be expressed in insect or plant cells, and it has been shown that the VP1 capsid protein can self-assemble virus-like particles (VLPs) that resemble the NoV capsid. Phase 1 clinical trials using VLPs of the prototype NoV, Norwalk virus, showed that Norwalk virus-like particles are safe and immunogenic in humans following oral [204] and intranasal [205] administration and protective against NoV experimental challenge [206]. Appealing

features of this system include the possibility of expressing VLPs in plants, which can be ingested [207], and the potential to display vaccine epitopes on their surface for heterologous vaccination [208].

3.7.3 Novel Adjuvants for Mucosal Immunization

The choice of adjuvants to accompany a mucosally delivered antigen is critical as it may have a dramatic impact in the magnitude, quality, and duration of the immune responses induced. Toll-like receptor (TLR) agonists, detoxified bacterial enterotoxins, mucoadhesives, saponins, and particles are among the most extensively tested [34]. A genetically detoxified double mutant of the *Escherichia coli* heat-labile enterotoxin (LT) harboring deletions on 192G and L211A, also known as dmLT, is presently one of the most promising oral adjuvant candidates [209]. In mice, oral administration of dmLT was able to enhance responses to coadministered tetanus toxoid [209]. Clinical trials are ongoing to evaluate the safety and immunogenicity of dmLT fed to adult volunteers (NCT01147445; ClinicalTrials.gov). While oral adjuvants could be powerful tools to improve the immunogenicity of orally delivered antigens, they could also disturb the delicate balance between immune stimulation and tolerance, particularly during the first year of life when the gut immune system is not yet fully developed [210]. Intranasal delivery of a detoxified single deletion mutant of *E. coli* LT was associated with Bell's palsy (transient facial nerve paralysis), emphasizing the need for a careful assessment of safety of novel candidates [211].

3.7.4 Improved Immunization Strategies

The administration of vaccine antigens in the same or different formulations by different routes (e.g., mucosal and parenteral), also known as the "prime-boost" strategy [212], might provide a means to overcome the limited efficacy associated with oral vaccination and to improve responses to antigens that are poorly immunogenic. This approach could be useful to enhance the breadth and magnitude of immune responses to pathogens that breach the mucosal barrier and become systemic. Mucosal prime-parenteral boost regimens have been successful at inducing protective immunity in various animal models [213, 214]. In humans, oral priming with *S.* Typhi vaccine strain, CVD 909, followed by parenteral boost with *S.* Typhi Vi capsular antigen induced Vi serum IgG responses and classical ($CD19^{+}IgD^{-}$ $CD27^{+}$) IgA and IgG B_M cells specific for bacterial antigens [43]. The combination of OPV followed by parenteral IPV (or in reverse order) has been implemented in several countries to elicit both gut and systemic immunity, minimizing the risk of VAPP [215]. In fact, IPV was found to induce mucosal IgA only if preceded by OPV vaccination [26]. Israeli soldiers who received a parenteral O polysaccharide-based *Shigella* vaccine were protected against infection and this may have been facilitated by prior natural (oral) exposure to the organism [216]. Interest for the prime-boost strategy is growing and more studies in humans are expected in the near future. Alternative and potentially more efficient routes for mucosal immunization include the sublingual, aerosol, ocular, and vaginal; [217] their effectiveness and practicality will ultimately determine their use in humans.

3.8 Conclusions and Future Directions

Despite the obstacles, the global success achieved so far with the existing licensed vaccines exemplified by the eradication of type 2 poliomyelitis and the remarkable reduction of diarrheal disease burden shows that enteric infections can indeed be prevented by oral immunization. Further research is needed to uncover novel broad-spectrum protective antigens and effective delivery systems and adjuvants to improve oral vaccine take. Efforts should also be focused on

elucidating the events associated with immunological priming in the gut that lead to the elicitation of robust mucosal and systemic immunity and immunologic memory. Understanding the barriers that interfere with oral immunization is also paramount to meet the need of less privileged nations and to help reduce the burden of disease where it is most needed. Modern technology (e.g., genomics, proteomics for antigen discovery, high throughput immunological assays using noninvasive specimens, and mathematical modeling to dissect correlates of protection) can assist in the development of vaccine candidates and in the characterization of immunological outcomes. Over the years, scientists have evaluated a number of approaches to efficiently shuttle vaccine antigens via the gut such as recombinant live vectors, nonliving particles, replication-deficient viruses, and antigen-expressing plants. Well-conducted clinical trials will be needed to determine safety and effectiveness of many candidates that appear to be promising in animal models. Future success may lie in the implementation of novel immunization strategies such as prime-boost regimens that combine live attenuated organisms that mimic natural infection with protective subunit vaccine antigens. A major hurdle in creating successful oral vaccines for global use remains the need to achieve adequate safety for industrialized country populations, while retaining immunogenicity and efficacy in developing country populations.

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Pediatric Immunology and Vaccinology

4

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Abstract

Induction of protective immunity in infants has the potential to reduce morbidity and mortality in childhood infections but it has long been known that long-lasting protective immunity is difficult to induce in the neonate. This chapter describe the basis of the neonatal adaptive immune system. It also gives an update on current childhood immunisation programmes on a global level as well as the known side effects to vaccines in children and young adults. Finally, it also provides data on how to improve childhood vaccinations and future much needed vaccines.

4.1 Introduction

The first months of life are a time of high risk for infections in the newborn infant, and it would therefore be useful to induce adaptive immunity by immunizations early in life. However, it has long been known that long-lasting protective immunity is difficult to induce in neonates both upon immunization and also after infection. Some of these problems could in theory be solved by vaccination of the pregnant women to enhance the passage of protective maternal antibodies to the child, but this may later affect the adaptive immune response of the infant. The difficulties to induce proper vaccine responses will be described and put into a context of the neonatal immune system that in many other aspects is capable of protecting the infant against disease [1, 2].

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4.2 Cellular Components of Innate and Adaptive Immunity

A major disadvantage for the neonatal immune system is, independent of species, that most B and T cells are naïve. Although the majority of cells and soluble factors appear early in fetal life, their numbers and relative ratio and activation status differ from adults [3]. There is a lack of knowledge in human infants since the majority of studies in neonatal immunology have been generated in rodent models. It is difficult to translate these findings to humans since newborn mice are underdeveloped compared to human infants [4]. The studies of cells isolated from umbilical cord blood have provided new insight, but these data may not be representative of the circulating cells in the neonatal immune system. Data obtained from cord blood should therefore be combined with data acquired from infants [5].

4.3 Neonatal and Childhood Antigen-Presenting Cells

Monocytes and dendritic cells (DCs) function as antigen-presenting cells and are key players in innate immunity but also responsible for initiation of adaptive immune responses. Two subtypes of DCs are identified in peripheral blood: the myeloid DC (mDCs) and the plasmacytoid DC (pDCs). The mDC is the main antigen-presenting cell and plays a crucial role in B cell differentiation by the release of cytokines such as IL-12, IL-6, BAFF, and April, which drive the formation of antibody-producing B cells. On the other hand, pDCs produce interferons and thereby play a vital role in antiviral immunity [6]. There are age-associated differences in the proportion of DCs in peripheral blood in children, where pDC numbers are much higher in infants than in older children, probably reflecting their importance for protection against viral illness in early life before protective adaptive immune responses have been initiated [7]. However, that comes at the cost of less efficient B cell activation early in life.

Several studies on cord blood DCs have confirmed an immature phenotype with low, or no basal expression of CD40, co-stimulatory molecules CD80/CD86 or MHC class II molecules [8–10]. Functionally, this translates into suboptimal human neonatal DC responses to most stimuli [11]. In addition, the importance of the toll-like receptors (TLR) pathway on DCs for induction of adaptive immune responses is apparent and well established. Despite comparable expression of TLRs on cord blood DCs and adult cells, the capacity of cord blood DCs to respond to TLR agonists is also significantly reduced and characterized by low production of the pro-inflammatory Th1 cytokine TNFα and IFN [12, 13]. However, recent data suggests that neonatal TLR-mediated impairments are selective since the TLR8 agonist R848 is able to induce a robust immune response in cord blood DCs comparable to adult cells. This finding has also been confirmed in infant cells and may have important implications for the choice of adjuvants in neonatal vaccine research [14, 15].

The innate responses of monocytes and antigen-presenting cells develop within the first year of life [15, 16]. Phenotypic analysis of peripheral blood monocytes and DCs has shown that circulating DCs acquire an adult-like phenotype around 6 months of age. Cytokine production after TLR stimulation at birth is skewed towards a Th2 response with production of IL-6, IL-8, and IL-10 and low levels of the Th1-polarizing IL-12p70 cytokine [17]. However, the IL-6 levels are comparable to adults already at 3 month of age. For IL-10, the production remains significantly higher also at 12 month of age, and the same trend is shown for IL-8 [16].

Thus, small children are impaired in both the maturation of the antigen-presenting cells and the capacity of such cells to respond to bacterial and viral antigens, with the important exception of TLR8. Using TLR8 ligands as vaccine adjuvants may represent a window of opportunity.

4.4 Neonatal and Childhood T Cells

The peripheral blood T lymphocyte subsets in infants differ from that of adults. At birth, there is a gradual increase in the absolute number of CD3+ T lymphocytes and, from the age of

2 years, a decrease to levels similar to adults [18]. Helper CD4+ T cells and cytotoxic CD8+ T cells also increase in the first months of life and decline after 9–15 months of age [19]. Analysis by flow cytometry has revealed several CD4+ memory cell populations in blood, where only the central memory T cell population is present at birth. As a result of antigenic stimulation, effector memory helper T cells increase during the first year to levels comparable to adults and remain stable during childhood. The recently described population of CXCR5+ memory T cells, also defined as follicular helper T cells, is absent at birth but increase in number during the first year of life in parallel to the increase in serum IgA and IgG [19]. Follicular helper T cells were first described as cells able to efficiently support the differentiation of switched B cells in secondary lymphoid organs with the subsequent production of IgA and IgG [20]. The absolute numbers of regulatory T cells (Treg) increase the first month of life similar to CD4+ memory T cells and remain stable thereafter. Neonatal Treg exert potent immunosuppressive activities and suppress antigen-specific T cell proliferation and IFNγ production [21] which may modulate the development of a memory CD4+ T cell pool later in life [22].

Intrinsic defects in T cell immunity has been described for neonatal T cells. A key feature of signalling via T-cell receptor (TCR)-CD3 on naïve CD4+ T cells is the upregulation of CD40 ligand on the cell surface. Neonatal CD4+ T cells have reduced capacity to express CD40 ligand after TCR-CD3 activation, which in turn negatively affect antibody production, Ig switch, and memory B cell generation [23]. Helper T cell responses after immunizations in newborns have been investigated in many contexts [1], and several factors (antigen dose, adjuvant, routes of immunization) influence whether a predominantly Th1 or Th2 response will be elicited. A majority of current childhood vaccine elicit a predominantly Th2-biased response with the exception of BCG and whole-cell pertussis vaccines [24].

So, the T cell responses early in life are skewed towards a Th2 response, and neonatal helper T cells have reduced capacity to support B cell differentiation and antibody production.

4.5 Neonatal and Childhood B Cells

Several studies show age-dependant developmental changes in peripheral blood B cell subsets during the first 5 years of life with a significant decrease in total B cells with age. Most striking is the shift from a predominantly naïve and transitional blood B cell pool during infancy to an increase of the memory B cell fraction in the older child and adult. The transitional B cells are increased in infants compared to adults, which may bridge the gap between innate and adaptive immunity early in life. Transitional B cells produce IgM upon TLR 9 stimulation and thus may be an important mechanism for a first line defense against bacteria at birth [25]. The expansion of the memory B cell pool is most evident during the first year of life, where after the absolute number is stable over time. Taken together, these findings suggest that the decrease in total B cells with age is mainly related to a reduction in the output from the bone marrow (BM) of transitional and naïve cells [26, 27].

Using CD27 as a surrogate marker of human memory B cells together with the surface expression of IgD, several memory B cell populations have been characterized. Classical switched memory B cells increase during infancy and reaches a peak between 5 and 10 years of age [27, 28]. Differentiation of classical switched memory B cells occurs in the germinal center (GC) of secondary lymphoid organs; immunohistochemistry studies show that GCs are absent at birth and gradually develop to adult size between 12 and 24 months of life [29]. Interestingly, gut colonization of *Escherichia coli* promotes the early development (0–4 months) of the CD27+ memory pool in infants [30]. The IgM memory subset appears gradually in circulation from around birth and reaches adult levels at 2 years of age [31]. Several studies have shown that IgM memory B cells confer protection against *Streptococcus pneumoniae,* both after infection and immunization [32].

Less is known on the terminally differentiated plasma cell pool in infants and children. In preschool children, the plasma cell compartment is similar in size as reported for adults [33]. However, in the mouse model of KLH-NP immunization, it has been shown that survival of plasma cells is

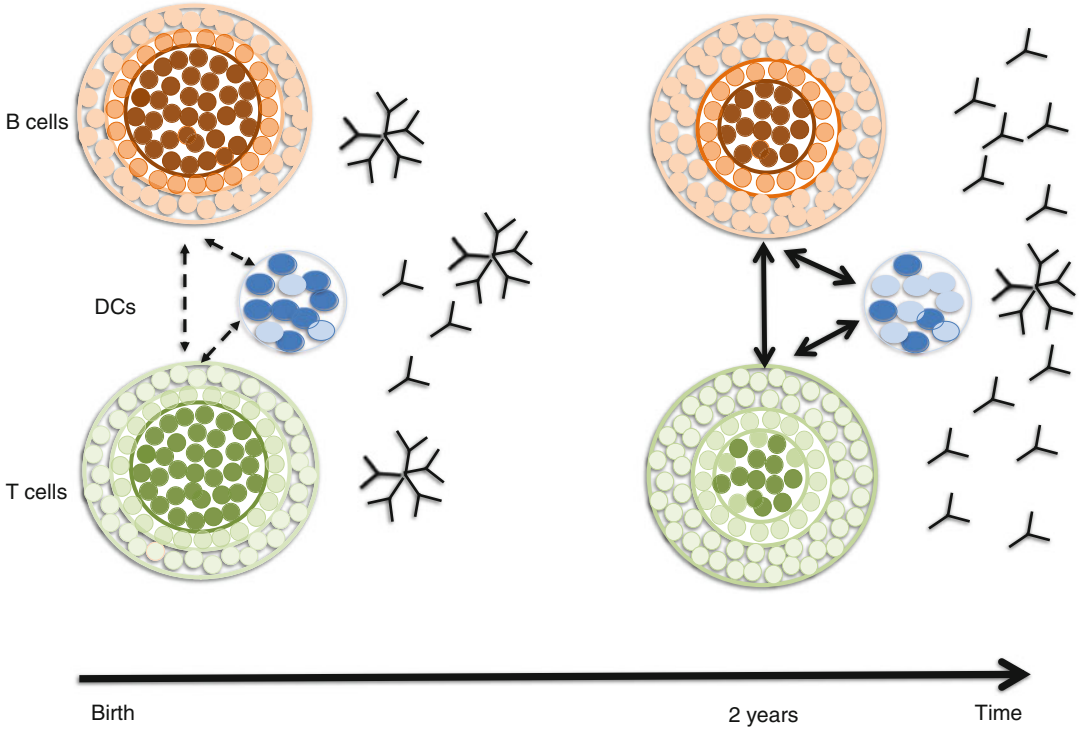

Fig. 4.1 Maturation of peripheral blood cell populations involved in adaptive immune responses occurs during the first 2 years in children. At birth, translational (*orange*) and naive B cells (*dark orange*) are most abundant, and the T cell compartment is dominated by naive T cells (*dark green*). After stimulation by environmental antigens, the memory B cell (*pale orange*), memory CD4 T cell (*pale green*), and follicular T cell (*green*) pool increases. Accordingly, the interactions between DCs, B cells, and T cells increase, and the antibody response matures with the production of IgG and IgA. Dendritic cells (DCs) at birth are predominantly plasmacytoid DCs (*blue*), but the myeloid DCs (*pale blue*) increase after 1 year

impaired in neonatal mice compared to adults [34]. The supportive network of BM stromal cells was less capable of supporting plasma cell survival factors in neonatal mice compared to adults.

In summary, postnatal maturation of the B cell compartment occurs in the presence of antigenic stimulation by microbes and also requires maturation of lymphoid organs and the bone marrow (Fig. 4.1).

4.6 Quality of Antibody Responses in Infants

During fetal and early neonatal ontogeny, the peripheral B cell population is much less diverse that that of adults. Early studies showed that the B cell repertoire expressed early in life is skewed towards specific V_H genes and that early neonatal cells lack molecular mechanisms utilized by adult cells for diversification [35]. It is also known that in vivo antibody (Ab) responses are of lower affinity and restricted heterogeneity compared to adults.

One important difference in outcome of B cell activation is that neonatal B cells produce less amounts of Abs than adults after antigen-specific activation [36]. The differences in Ab secretion could be due to impaired antigen presentation by DCs or macrophages as well as suboptimal secretion of cytokines by T cells. However, there are also intrinsic B cell differences in that neonatal B cells show little or no proliferation after B cell receptor (BCR) cross-linking [37] even though signal transduction occurs upon Ig ligation [38]. It has been demonstrated that neonatal B cells are more prone to tolerance induction and/or apoptosis after BCR ligation. Neonatal B cells also express less MHC class II and the co-stimulatory molecules CD80/CD86 are not upregulated after BCR triggering

[39]. While these impairments may render neonatal B cells hyporesponsive, CD40 ligation and IL-4 stimulation leads to B cell activation and proliferation allowing B cell differentiation. Thus, in the presence of T helper mechanisms, the neonatal B cell response is adequate, although more stimulatory signals may be needed to achieve similar outcomes as for adult B cells [35].

Somatic hypermutation (SHM) occurs predominantly in germinal centers of spleen or lymph node and is essential to diversify and improve the antibody formation as it leads to selection of antibodies with high affinity [40]. This process is dependent on the enzyme activation-induced deaminase (AID), which inserts point mutations in to the Ig heavy and light chain genes and thus, play an essential role in repertoire diversification and affinity maturation [41]. Data on SHM in human infants are rare, but one early study reported SHM in IgG and IgA heavy chain transcripts in cord blood [42]. In peripheral blood of newborns, few or no mutations could be detected when sequencing the V_H6 gene, but in older infants (10–60 days), more mutations were found in the same locus [43]. By 8 months of age, the range of mutations reached adults levels, and there were signs of repertoire selection [44].

So, neonatal B cells are less responsive to BCR ligation and more prone to apoptosis or tolerance induction. Antibody maturation is limited. The responses to the majority of antigens will be less efficient due to both T and B cell inabilities (Fig. 4.2). For type 1 T cell-independent antigens, which themselves can activate immature B

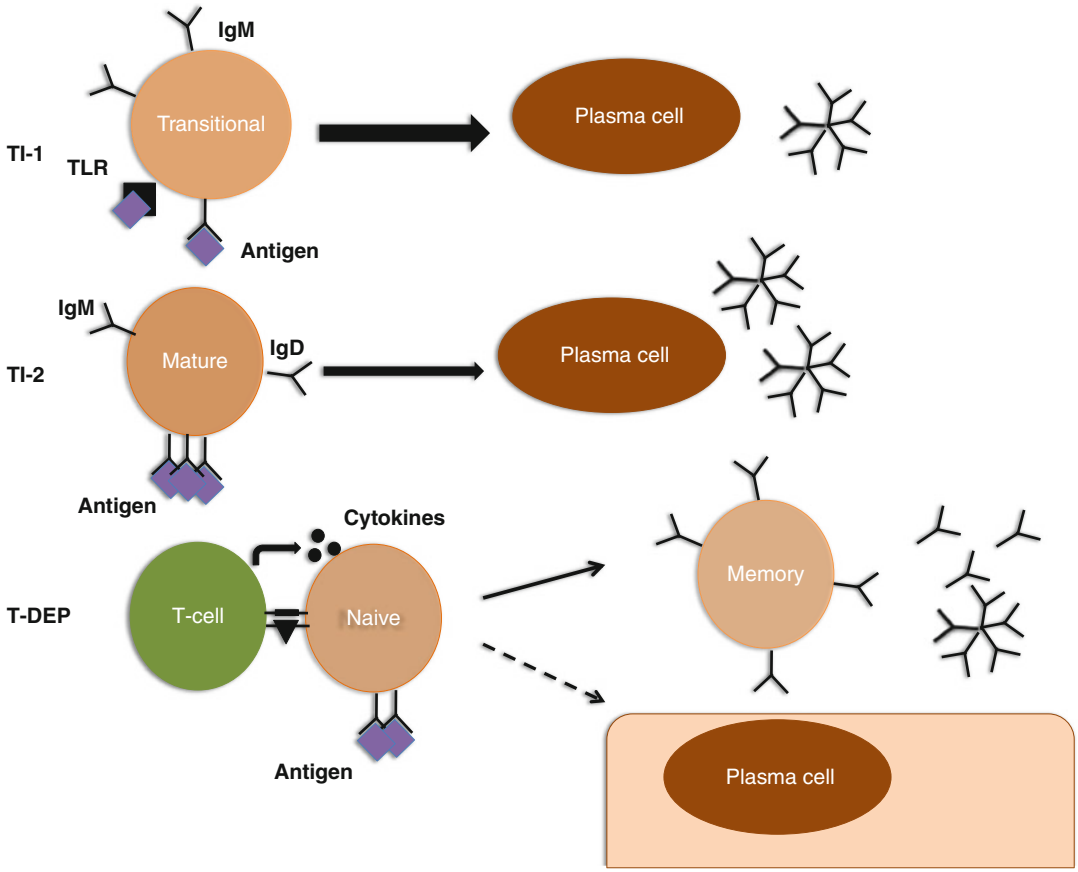

Fig. 4.2 A schematic figure on adaptive immune responses in young children. At birth, translational B cells respond to certain T-independent antigens (*TI-1*) with the formation of short-lived plasma cells that mainly produce IgM as a first line defense. IgM+ mature B cells are also able to respond to T cell-independent antigens (*TI-2*) which cross-link several BCR through binding of repetitive antigenic structures. The response to T-dependent antigens is not present at birth but mature during the first 2 years. The production of switched memory B cells and homing of plasma cells to the bone marrow are therefore impaired in the infant

cells, these are partially hampered by lack of TLR and BCR signalling (Fig. 4.2). For type 2 T cell-independent antigens, which are repetitive structures that can cross-link BCR by multiple binding on mature B cell responses are very limited in young children, both due to few mature B cells and also poor BCR function. Children hence respond poorly to vaccines consisting of such antigens (polysaccharide vaccines).

4.7 Soluble Factors in Neonatal Blood Affecting Adaptive Immune Responses

Although most components of the immune system appear during fetal development, the concentrations of soluble components can differ markedly from those of adults. In particular, the plasma complement proteins and their activity are low in infants. The complement system is an important part of innate immunity, but it may also impact on adaptive immune responses, it enhances the effects of specific immunoglobulins, it primes antigen-presenting cells and aid their maturation, and finally, it enhances the antigen-driven maturation of antibody responses by B lymphocytes. The level of classical complement components in newborns is decreased compared to adult levels, which probably contributes to the deficit in early adaptive immune responses [45]. During the first 6 months of life, there is an evolution towards adult levels for several of the complement proteins [46].

In recent years, neonatal plasma has been shown to contain other molecules with immune modulatory functions mainly affecting the outcome of TLR activation on antigen-presenting cells. Adenosine, an endogenous purine metabolite, selectively inhibits TNF production from TLR2-activated monocytes while IL-6 production is preserved. Thus, adenosine contributes to the Th2-polarizing properties of neonatal plasma [47]. In addition, yet unidentified factors in neonatal plasma have the capacity to polarize TLR4-mediated cytokine responses with low IL-12p70 production and high IL-10 production, thus mediating immunosuppression during the first month of life [48].

The influence of maternal antibodies on adaptive immune responses is debated. Potential mechanisms by which maternal antibodies could affect infant vaccine responses include specific masking of infant B cell epitopes by maternal antibodies and the uptake of maternal antibodies: antigen complexes by APC [49]. Abundant data in the literature favors these models, and it also fits well with the observation that maternal antibodies lack the capacity to interfere with infant T cell priming in vivo. This issue will be discussed more below.

4.8 Current Pediatric Vaccines, Worldwide

More than half of the children that die under the age of five worldwide do so because of an infectious disease. Many of these diseases are vaccine preventable, and WHO estimates that around 1.5 million children below the age of 5 died in 2008 in such diseases. *S. pneumoniae* (pneumococcal) infections and rotavirus infections are the leading causes, followed by infections caused by *Haemophilus influenzae B* (HIB), *Bordetella pertussis* (pertussis), measles virus, and *Clostridium tetani* infection in the neonatal period (neonatal tetanus).

The vaccine schedules used in the world differ due to economic issues and the endemic infection situation of the region. The goals from WHO are focused on reaching a 90 % vaccine coverage rate of each country's national policy. In almost all parts of the world, these include vaccinations against diphtheria, tetanus, pertussis, and polio, which have lead to a drastic reduction in the incidence the past 30 years. Neonatal tetanus is still quite prevalent, mostly due to low vaccine coverage rates in mothers, hence less antibodies are transferred in utero. The MMR vaccine (measles, mumps, rubella) is used in Europe, America, Australia, and some Eastern Mediterranean countries, whereas the plain measles vaccine is used in most African countries and South East Asia. The measles vaccination coverage is 85 % worldwide; however, the coverage is poor in mid- and southern Africa. Furthermore, the introduction of the pneumococcus vaccine is recently established in Europe, North America, Australia, and many

African and South American countries now follow. Vaccines against HIB are also becoming very prevalent worldwide, but only recently, thus less than half of the world population were protected in 2010, though this number is likely to rise. The introduction of the varicella vaccine has been less successful, and it is primarily used in America but also in other countries for risk groups.

In the coming years the rotavirus vaccines will be introduced with a focus on African countries, but also in America, Europe, and Eastern Mediterranean areas. Vaccines against local endemic infections including *Neisseria meningitidis* (meningococcus), Japanese encephalitis, *Mycobacterium tuberculosis*, hepatitis, rabies, *Salmonella typhi* (typhoid fever), and yellow fever may also be included in childhood vaccination schedules. In the older children, utilization of the human papillomavirus (HPV) vaccine, reducing cervical cancer, is increasing in all parts of the world, with the exception of the East Mediterranean countries.

4.9 Vaccine Side Effects

Side effects of vaccinations are a debated field. Immediate reactions, such as allergy [50] and local reactions, with swelling, sourness, and pain at the site of injection, are easily measured and described. In addition, common early systemic side effects including irritability and fever can be measured on a population basis. Less common side effects, occurring in close proximity to vaccination, are also described. For example, febrile seizures 7–10 days post MMR vaccination have long been recognized occurring in 1 of 600 doses [51]. Similarly, MMR vaccination associates with a 1/50,000 doses risk of immune thrombocytopenic purpura [52].

When it comes to rare and long-term side effects, the burden of proof is more challenging. Furthermore, such reactions/diseases are often multifactorial. There are basically two approaches to long-term side effect investigations: either a purely logical hypothesis based on immunological data from the vaccine or a suspicion from epidemiological data. One example of the first is the effects of childhood vaccination on allergy and atopy. It is known that children who retain the neonatal Th2 profile longer may have an increased likelihood of allergies and that an early Th1 tilted response will decrease incidence of allergy and asthma [17]. Pediatric vaccines that give a predominant Th1 or Th2 response may hence affect development of allergy and atopy. Circumstantial clinical reports suggest such an association for the pertussis vaccine, and controlled clinical trials point away from allergy promoting or preventing effects [50].

One example of the second type, where epidemiological data has prompted further investigation, comes from the increased frequency of childhood narcolepsy in Scandinavia post H1N1 influenza vaccinations in 2009. For the group under 11 years, the frequency rose from around 0 cases/100,000 inhabitants to around 3.4/100,000 in 2010. For 11–16 year olds, it rose from around 1/100,000 to around 8.7/100,000 in 2010 [53]. This occurred almost exclusively in Finland and Sweden and has been attributed to the genetic background and possibly other unknown factors. It has been suggested based on genetic data and the lowered onset age that vaccination brought forward the onset of a disease that normally would have occurred later [53]. In much the same way, the pertussis vaccine has been described as a trigger of severe myoclonic epilepsy (Dravet syndrome) in genetically susceptible individuals. Time for vaccination coincides, however, with characteristic onset time for this disease, and fever (which may be associated with vaccination) is known to trigger first events. Pertussis vaccination does not associate with any altered outcome of disease as compared to unvaccinated genetically susceptible individuals [54]. The proposed link between autism spectrum diseases and vaccination (in particular MMR) is hereto not proven [55, 56].

In parallel, data also suggest that you are more likely to have side effects if you are vaccinated early in a vaccine campaign. One interpretation of this is that people with underlying disease and/or risk factors are vaccinated early, and such individuals appear more prone to side effects. This

was shown for the H1N1 vaccine, where the early cohorts had increased frequencies of Bell's palsy, paresthesia, and inflammatory bowel disease [57].

Finally, it is not only the antigen as such that is important for the side effects but also the formulation. This has been demonstrated by comparing the seizure frequency when giving a combined MMR-varicella (MMRV) vaccination or MMR and varicella (MMR+V) vaccine separately on the same day. Using MMRV the seizure frequency rises to 1/2,300 doses, as compared to MMR+V where the seizure risk is comparable to MMR alone [58].

So when it comes to side effects causing long-term morbidity, these are linked to the onset of a disease that most likely would have occurred in an unvaccinated child as well. Children with underlying disease may be at higher risk of side effects. For the short-term side effects, formulation appears to play a role.

4.10 Vaccination During Pregnancy

Since vaccinations are less efficient in very young children, one way to protect neonates against severe infection is to vaccinate women during pregnancy, utilizing the transmission of antibodies from the mother to the child in utero. This strategy is already in use in some countries, and US health authorities recommend the seasonal trivalent inactivated influenza vaccine and the tetanus/diphtheria/acellular pertussis vaccine to be used during second and third trimester of pregnancy. Furthermore, a number of other killed vaccines are recommended to pregnant risk groups including vaccines against hepatitis A, hepatitis B, meningococcus, and the 23-valent pneumococcus polysaccharide vaccine. Live viral vaccines are contraindicated in pregnant women.

General concerns include the safety and efficacy of maternal-fetal immunization. Safety has been shown using killed vaccines against seasonal as well as H1N1 influenza [59]. Furthermore, similar data are available for the adult-type tetanus, reduced diphtheria toxoids, and acellular pertussis vaccine [60].

For the discussion of efficacy, several aspects have to be taken into consideration. First, whether fetal immunization is efficient in preventing fetal illness, either from the community or from the mother during fetal life or not. In addition, whether there will be negative effects of the passive antibodies transferred on later immunizations of the child or not. For the efficacy two types of vaccines will be discussed: the vaccines containing T-dependent antigens and the vaccines containing T-independent antigens. One can assume that T-dependent antigens will be more efficient, as they give rise to antibodies that are more efficiently enriched over the placenta. For T-dependent antigens, such as the influenza vaccine, epidemiological data show that children born to vaccinated mothers have milder influenza-like symptoms and a reduced incidence of verified influenza [61].

For T-independent antigens, the efficacy is less clear. It has been shown that pneumococcal antibodies are transferred in utero, at sufficient levels, post maternal immunization with the pneumococcal polysaccharide vaccine [62]. These are results for serotypes 1 and 5, where only 5 can be really seen as T-independent due to the zwitterionic nature of the serotype 1 polysaccharide [63]. Maternal immunization does in this case interfere with early childhood vaccination (7–17 weeks postpartum), in that vaccination does not increase the amount of specific antibodies, if already present at high concentration in the infant. No effect of maternal vaccination was seen on vaccination efficacy at 3 years of age [64]. There is little evidence for a role of these antibodies in preventing disease, and neonatal pneumococcal colonization is not affected [65].

One argument against fetal immunization is the possible interference with later childhood immunizations. This can be mediated both by the ability of neutralizing antibodies to interfere with specific T cell responses and the ability of neutralizing antibodies to interfere with humoral responses upon immunization. As for T cell responses, these are clearly less prominent, as discussed above, in small children as compared to in adults. The presence of maternal neutralizing antibodies does not affect efficacy of T cell responses, measured as IFN-γ production [49, 66].

When it comes to the humoral response, the situation is more complex. It is clear that lower levels of neutralizing antibodies are produced upon vaccination in children that are still retaining maternal antibodies at the age of 9 month [66] and that at 6 months, the ability to produce antibodies upon vaccination is poor in general irrespective of maternal antibodies. The potential interference with childhood immunization at 9 months will then depend of the persistence of maternal antibodies. Antibodies against different antigens show different half-life in vivo, and antibodies against measles virus and rubella virus persist longer than, for example, antibodies against mumps virus. Mumps antibodies also persist even shorter if the mother has been vaccinated as compared to infected naturally [67]. Furthermore, transport across the placenta will not depend only on subclass, as both IgG1 and IgG2 antibodies against pneumococcus are transferred less well than antibodies of similar subclasses against tetanus [68]. Clearly, the potential interference will have to be judged for each vaccine. Most children that were vaccinated while still immune from the mother, however, have a good response upon second dose vaccination [66]. Some studies have found a slightly higher vaccine failure rate upon second vaccination, where the first early vaccination has failed [69]. If this is a result of the child's intrinsic ability to respond to vaccination or a result of interference by maternal antibodies is not known.

Vaccination with killed vaccines, preferentially in third trimester, appears safe and at least for some vaccines efficient. There are conflicting data on interference with childhood immunizations, but most data point towards little interference. Recommendations will have to be specific for each vaccine, and more studies are required.

4.11 Immunization Responses in Children with Primary or Acquired Immunodeficiency

A growing number of children survive infancy and early childhood despite severe immunodeficiency including transplantation and chemotherapy, primary inherited immunodeficiency disorders, or congenital HIV infection. These children are vulnerable to infections and thus would benefit from effective immunizations. It is also possible that a better understanding of the molecular deficits behind impaired vaccine responses in these patients could contribute to the development of better vaccines.

Common variable immunodeficiency disorder (CVID) affects antibody production and is characterized by low serum concentrations of IgG and IgA and/or IgM and increased susceptibility to respiratory infections with encapsulated bacteria (*H. influenzae*, *S. pneumoniae*). CVID is a heterogeneous disease with several genetic defects involving important molecules for B cell signalling and/or T-B cell interactions. In adult CVID patients, who had switched memory B cells (CD27+) in peripheral blood before immunization, a protective antibody response could be detected against several antigens [70]. Similarly, in a pediatric study, 11/16 children were found to respond to the meningococcal group C polysaccharide vaccine [71]. In addition, vaccine responses against polysaccharide vaccines were associated with the presence of IgM memory B cells in these patients. A similar study of CVID patients indicates that there is a block in the formation of plasmablasts after immunization against both *Clostridium tetani* and *S. pneumoniae* [72].

Immunization responses in HIV-1-infected patients are severely impaired, both in adults and children [73] for the majority of antigens. The introduction of highly active antiretroviral therapy (HAART) has improved vaccination outcome for the majority of patients [74]. Immunization guidelines for this vulnerable pediatric group have recently been published [75]. Vaccination is safe with few side effects, and the only vaccine that is contraindicated as of today is BCG for HIV-1-infected children. However, there are still unresolved questions regarding immunizations in pediatric HIV-1 infection and, in particular, how durable the antibody response in HAART-treated children will be compared to healthy individuals.

Re-immunization of children posttransplantation and after chemotherapy is required since the

different treatment modalities eradicate protective antibody-mediated memory [76]. As for HIV-1-infected children, most vaccines are safe although revaccination with live vaccines should be postponed 12–24 months after completion of therapy. There is no consensus on the optimal time to start revaccination or how many doses that should be administered to achieve long-lasting protection [77, 78].

4.12 Development of New Pediatric Vaccines

Traditional vaccines often consist of whole-killed viruses, administered by intra muscular injection (i.m.). For some important childhood pathogens, for example, respiratory syncytial virus (RSV), such attempts have failed. The development of a RSV vaccine will be discussed as an example of a novel vaccine strategy in infants. Initial clinical studies using formalin-inactivated RSV for administration to small children resulted in disease aggravation upon infection, hospitalization, and in some cases death [79]. This has later been attributed to a devastating Th2 response, resulting in lung pathology. RSV causes a localized respiratory disease, without general viremia, resulting in significant hospitalization, morbidity, and mortality rates. From an immunological perspective, RSV is challenging because of the failure of adaptive immunity to prevent reinfection.

This has been ascribed to the poor quality of the T cell response and the short durability of the antibody response. One hypothesis is that the mucosal immunity is to slow for this rapid virus and that the serum antibodies are present at low levels in the tissue. Finding alternative strategies for RSV vaccination is key. One such way is to deliver the vaccine at mucosal linings such as intranasally (i.n.), instead of i.m. Then immune activation will take place within the mucosa-associated lymphoid tissue (MALT) with activation of microfold epithelial cells (M cells) and subsequently underlying antigen-presenting cells such as DCs. This will induce both local IgA and systemic IgG.

Depending on which route that is used, different local immunity will result. For example, using i.n. administration, immunity in the upper respiratory tract and the cervicovaginal tract will prevail, whereas oral administration will induce IgA production mostly in the small intestine and in the mammary glands. Experiments using live-killed RSV nanoemulsions for i.n. administration in mice have shown an IgA response in the lung as well as protective capacity [80]. Current most promising results come from clinical phase II trials where live attenuated RSV strains have been given i.n [79]. Still, RSV vaccine development suffers from the initial failures, and no good vaccine is yet available. Results are to be awaited from clinical studies of naked DNA and vector-expressed DNA vaccines in the pediatric population.

Development of new vaccines also includes the introduction of adjuvants with enhanced immune stimulatory capacity to compensate for low intrinsic immunogenicity of antigens [81]. Novel adjuvants aim at optimizing B cell responses and generating appropriate T cell responses, which could be of particular importance in childhood vaccines. One of new adjuvants is the TLR4 agonist monophosphoryl lipid A (MPL) which of to date is licensed or in phase III clinical trials [82]. MPL, when combined with alum, acts on DCs and promotes IFN-γ production and thus overcomes the Th2-bias response associated with alum. It is now licensed in a human papillomavirus (HPV)-16/18 vaccine and has been shown to induce long-lasting B cell memory and persistent antibodies [83]. In children immunized at 1–4 years of age against *Plasmodium falciparum*, an MPL-containing adjuvant-induced high antiparasite antibodies with long-term protection against clinical disease as a result [84]. An additional potential new adjuvant, the non-toxic mutant of heat-labile enterotoxin (LT) of *E. coli (*LTK63*),* was shown to overcome delayed maturation of follicular DCs and thus induce germinal centers when given parentally in mice together with a polysaccharide conjugate vaccine [85]. In addition to an improved B cell response, LTK63 upon binding to macrophages, induces a balanced Th1/Th2 cytokine production as well as cytotoxic T cell responses [86].

So, the development of new efficient vaccines to be administered early in life may take advantage

Fig. 4.3 The key points of this chapter are summarized in a text box

- During the first 2 years in life, the immune system develops in a step-wise manner. These age-related steps are important to consider when designing new vaccines or when vaccination of new age groups is warranted.
- The risk of long-term side-effects of childhood vaccination is very low.
- Vaccination guidelines for children with underlying disease is needed.
- Vaccination during pregnancy, with subsequent passive protection by transferred antibodies, is one way to protect the neonate. The potential risk for interference with future childhood vaccinations will have to be judged for each vaccine, but in general appears to be low.
- Future childhood vaccines will be based on new adjuvants, new immunisation routes and involve important diseases such as RSV-infection and Malaria.

of novel adjuvants to circumvent the hyporesponsiveness of neonatal adaptive immunity. Finally, a summary of the key points outlined in this chapter is shown in Fig. 4.3.

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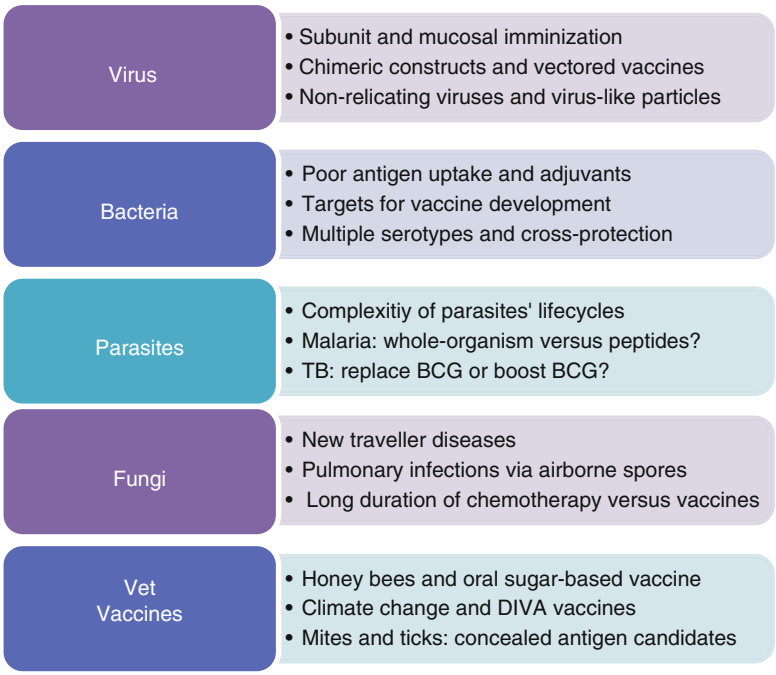
Part III

Vaccines for Infectious Diseases

Overview of Part III



In the Western world, roughly 26 % of all human emerging diseases are caused by infectious agents: viruses, bacteria, parasites, and fungi. About 60 % of these infections are of zoonotic origin. About two-thirds of all known 1,400 pathogens are transmitted by animals and are zoonotic pathogens. But only 2 % of all global products for human health are vaccines (20 % for veterinary vaccines). The oldest licensed vaccine, BCG, against tuberculosis (see also Chap. 13) was introduced 90 years ago and is still in practice. Without any doubt, current vaccines are successful against invariant

pathogens. But there are no effective vaccines against variable pathogens or latent infections.

RSV: More than half of the century after the RSV discovery, no vaccine is still available. Recent advances in immunology and vaccinology have contributed to more progress in understanding how RSV escaped immune responses and how it caused pathology in humans.

Ebola: Studies with a recombinant adenovirus-based vaccine, a vaccine based on recombinant vesicular stomatitis virus (VSV), a vaccine using virus-like particles, and a vaccine base on a recombinant human parainfluenza virus have been shown to be protective.

Dengue: Live attenuated viral vaccines are in advanced phase of clinical development. The need to protect against all four serotypes in order to avoid immune enhancement of disease has made dengue vaccine development a challenging task.

Norovirus: In experimental studies NoV VLPs produced in plants have been shown to induce potent cellular and humoral immune responses. Since NoV naturally causes infection in the gastrointestinal tract, the VLPs are stable at low pH and resistant to digestive enzymes.

Measles: Measles remains one of the leading causes of death in children under 5 years of age. Measles is one of the most contagious human diseases. To overcome the limitations of the live vaccine, several alternatives are under investigation such as vector-driven vaccines.

Shigellosis-Streptococcus-Salmonellosis: A promising approach is the use of proteins conserved across multiple serotypes as part of vaccine formulations. The inclusion of conserved proteins in vaccine compounds potentially solves the issue of serotype specificity. Multivalent subunit vaccines induce cross-protection.

Malaria and Tuberculosis: Recent work has improved our understanding of host-*Plasmodium* parasite interactions and has provided critical insight into new strategies for enhancing antimalarial immunity via prophylactic vaccination. BCG against TB is the only vaccine recommended by the WHO, with more than three billion doses administered since its introduction in 1921.

Paracoccidioidomycosis: Antifungal chemotherapy can last up to 2 or more years, with a significant frequency of relapsing disease. Peptide vaccines are a powerful agent in severe cases and in cases of poor response to chemotherapy.

Vet Vaccines: Paving the Way to Products for Human Health?

Mites and Ticks: With the uptake of the blood meal from a vaccinated host, damage to the gut wall occurs which may result in death or decreased reproductive capacity.

Borreliosis and Bluetongue: Reducing vector competence for an organism by vaccination, the host may be an effective strategy for preventing Lyme

disease in humans. Current commercial vaccines that have been used to control BT virus spread in Europe are mainly based on inactivated viruses; these are not DIVA compliant. A serological discrimination between vaccinated and non-vaccinated animals is not possible.

5 Subunit Vaccine Candidates Engineered from the Central Conserved Region of the RSV G Protein Aimed for Parenteral or Mucosal Delivery

Thien N. Nguyen, Christine Libon, and Stefan Ståhl

Contents

Abstract

Respiratory syncytial virus (RSV) is a major pathogen causing severe upper and lower respiratory disease in infants and in elderly worldwide. According to WHO, an RSV vaccine is urgently needed. Here, we describe the design of various types of subunit vaccine concepts based on molecular engineering aimed to deliver RSV antigens. Gene segment encoding parts of the conserved central region of the RSV G protein (G2Na) were prepared for various expression and delivery formats: (1) prokaryotically expressed and purified G2Na alone or fused to different carrier proteins, one of them, namely, BBG2Na (Alum), has reached clinical trials in the elderly; (2) G protein-derived antigens surface displayed on lived vectors (non pathogenic bacteria) and (3) nucleic acid vectors.

These subunit vaccines were administered with or without adjuvant in rodents and in non-human primates by different routes (parenteral or mucosal). We summarise and compare immunogenicity, protective efficacy and safety conferred by each immunisation format in RSV experimental animal models. Among these, G2Na proved to be the most promising component for an RSV subunit vaccine.

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Respiratory syncytial virus (RSV) is a highly infectious pathogen causing severe bronchiolitis and pneumonia in newborns, young infants, immunocompromised individuals and the elderly.

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Despite 60 years of RSV research, no human vaccine is available. RSV vaccine development history has been "marked" by a dramatic clinical trial of the formalin-inactivated RSV (FI-RSV): two children died among the vaccinees due to unexpected exacerbation of pulmonary disease. The enhanced immunopathology is still not completely elucidated [11]. Non neutralising antibodies (Abs) induced by denatured FI-RSV antigens (Ags), TLR missed priming of innate immunity and aberrant Th2-induced adaptive immunity were among the plausible hypotheses [16]. Prophylactic treatment with humanized monoclonal antibody (hMAb) (Synagis®) is available for high-risk infants but the cost benefits are still controversial.

A safe and efficient hRSV vaccine is still highly needed to save hundreds of thousands children's lives yearly in the world. Ideally, for children, the RSV vaccine should be combined with the existing paediatric vaccine. Elderly is another target population in the winter season where RSV symptoms of illness are very similar to influenza infections [19]. An RSV subunit vaccine should hopefully be combined with the current influenza vaccine. At the Institut de Recherche Pierre Fabre (France), we have dedicated our R & D in the development of hRSV vaccine for more than 12 years in collaboration with the Royal Institute of Technology (Sweden) and the Institut Armand Frappier (Canada). Here we summarise our different approaches in designing subunit vaccines based on the central domain of the RSV G protein in various forms (non-replicating and live vaccines) aimed for parenteral or mucosal delivery. Among these, one subunit vaccine candidate, namely, BBG2Na adjuvanted with Alum, reached clinical trials in the elderly [34, 58, 61].

5.1 Disease

RSV is characterised by the ability to repeatedly infect the upper respiratory tracts (URTs) of human throughout their life. As well, a primary RSV infection in infants which results in serious lower respiratory tract (LRT) pathology does not necessarily prevent a second serious infection [11]. After an incubation period of 4–7 days, the first symptom of RSV is a runny nose which is usually associated with fever. One to three days later, the infant develops a worsening cough, and as the disease progresses, tachypnoea and dyspnoea. RSV is one of the most common causes of bronchiolitis, and RSV infection is also associated with development of childhood asthma and recurrent wheezing up to 7 years. In adults, mild to moderate URT illness is a characteristic. However, severe pneumonia can occur, particularly in the elderly with co-morbidities or compromised immune status [73]. Elderly have a higher risk than adults for involvement of the LRT upon RSV infection, with rales and wheezing as the most common symptoms, but fever, cough and nasal discharge also occur.

5.2 Pathogen: RSV Classification and Structure

hRSV is a member of the genus *Pneumovirus* and the family *Paramyxoviridae*. Its genome is a 15,222-nucleotide-long, negative-sense RNA molecule which encodes 11 viral proteins, among which the nucleoprotein (N), the fusion protein (F), the surface glycoprotein (G), the small hydrophobic protein (SH), the matrix protein (M) and several nonstructural proteins including the L protein (replicase) and virulence factors NS1 and NS2 that mediate resistance to interferon-alpha. RSV strains are divided into two different subtypes, RSV-A and RSV-B, based on their reaction patterns with mAbs panels and amino acid (aa) sequence divergence between essentially G proteins [11].

The fusion protein F is involved in viral penetration into the host cell during entry, and subsequently in formation of syncytia. The G protein binds to cell-surface proteoglycans with high affinity but is not required for infection. Overall, the roles of F and G in mediating receptor binding are not elucidated, although both have been shown to interact with heparin [11, 20]. Recently, nucleolin was identified as a cellular receptor for RSV [67]. The G protein was shown to be a

structural and functional mimetic of fractalkine, a proinflammatory CX3C chemokine that mediates leucocyte migration and adhesion, which explains its role in pathogenesis [69]. F and G are the only two components known to induce RSV-neutralising antibodies [11, 12].

Cytotoxic T lymphocyte (CTL) response is important both for the eradication of RSV-infected cells as well as for the downregulation of Th2 cytokines. On the other hand, RSV-specific $CD8^+$ T cells can also increase pulmonary pathology in RSV-infected mice [6]. The poor CTL response found in young children [27] and in the elderly RSV-infected patients [14] might be one of the possible causes of more severe disease. In humans, a number of CTL epitopes have been found in the N, F, M and SH proteins. To date, no human CTL epitope has been found in the G protein. Immunisation of mice with vaccinia virus expressing H-2^d-restricted epitope of $M2_{82–90}$ protein conferred protection against RSV infection. However, this protective effect mediated by CTL waned within several weeks, and it did not seem to contribute to long-term protection [12]. Although neutralising antibodies is a major goal of RSV vaccination, an interesting strategy might be to combine long-lived antibody and T cell immunity to provide robust protection against reinfection and/or enhanced disease.

5.3 Transmission

RSV is a highly infectious pathogen. The infected child is contagious for 2 weeks from the beginning of the illness. Usually the illness, thought to be noncontagious when the wheezing disappears, is spread when droplets containing the virus are sneezed or coughed into the air by an infected person. If someone inhales the particles or the particles contact their nose, mouth or eye, they might become infected. RSV is often introduced into the home by school-aged children who are infected with RSV and have a mild upper respiratory tract infection, such as a cold. RSV can be rapidly transmitted to other members of the family.

5.4 Diagnostic and Classical Therapy

The bronchiolitis diagnosis is often made on the basis of history and physical examination: clinical signs and symptoms may include increased respiratory effort or shortness of breath, wheezing, rhinorrhoea, tachypnoea and nasal flaring. Rapid RSV diagnosis could rely either on immunofluorescence (IF) or ELISA. IF techniques are fast and easy to perform but the interpretation of results is subjective and the specimen must contain adequate nasopharyngeal cells. ELISA assays offer the advantages of more objective interpretation, speed and the possibility of screening a large number of specimens.

Nucleic acid techniques such as reverse transcription polymerase chain reaction (RT-PCR) assays are now available for RSV detection. There are multiplex kits including also other respiratory pathogen detections. The sensitivity of these assays often exceeds the sensitivity of virus isolation and Ag detections methods [57]. Use of highly sensitive RT-PCR assays should be considered, particularly for low viral loads in the respiratory specimens.

In young infants with respiratory distress, treatment of the LRT requires considerable supportive care: mechanical aspiration of secretions, supply of humidified oxygen and respiratory assistance in most cases. Ribavirin is the only antiviral compound which showed effectiveness for treatment of RSV disease. However, its toxicity limits its use in paediatric patients. Passive immunisation in the form of RSV-neutralising immunoglobulins (RespiGam®) or hMAbs (Synagis®) given prophylactically has been shown to prevent RSV infection in newborns with underlying cardiopulmonary disease and in small premature infants. However, these expensive treatments are not accessible to all children worldwide. Vaccination would be the best alternative in reducing of RSV transmission and infection.

5.5 Vaccination Strategies

Since the 1960s, RSV vaccine development has been hampered by the “enhanced disease” of the infamous FI-RSV vaccine. Therefore, in research,

not only RSV vaccine should fulfil the protective efficacy, but it should be first totally safe. The long-standing paradigm stating that a Th2-type cytokine was the principal cause of FI-RSV-induced enhanced disease has been recently invalidated. A more complex and generalised dysregulation of the immune response revealed by a significant enhancement of not only Th2 cytokines but also Th1-type cytokines and chemokines [3, 56]. There were several approaches investigated when designing RSV vaccines: (1) live vaccine given by intranasal (*in*) route which could mimic natural infection without the pathogenicity of the native RSV strain. (2) Non-replicating vaccine, consisted of F and/or G proteins, when formulated with an appropriated adjuvant, could achieve both a good humoral (neutralising RSV-Ab) and balanced cellular immune responses.

5.5.1 Live Vaccine

The development of live attenuated and temperature-sensitive (*ts*) strains constitutes approaches to generate RSV strain capable of replicating in the URT but not in the LRT. Intranasal administration of live attenuated RSV could confer both URT and LRT protection in seronegative infants. Obtaining an effective (immunogenic) and genetically stable (no revertance) *ts* strain is still to overcome. Since natural infection does not assure a sustained protective immunity in infants, the question is still: can live attenuated RSV do better than wild-type RSV?

There are also other approaches to express RSV F/G proteins using heterologous live vectors hosts such as Sendaï virus, parainfluenzae (hPIV 3) or bovine RSV to express human F/G proteins [9, 11]. As an alternative to viral vectors, we explored the possibility of using commensal or food-grade gram-positive bacteria as carrier for G protein derived. Here we started with a subunit Ag based on central domain of G protein, characterised by its capacity to induce high IgG antibody responses after parenteral immunisations or both IgA and IgG antibody responses after mucosal immunisations.

For simplicity and clarity, we present our data as follows: (1) rationale for choosing central domain of G protein (Fig. 5.1), (2) different G constructs generated in different formats (Fig. 5.2), (3) subunit G protein vaccine candidates aimed for both parenteral and mucosal administrations (Fig. 5.3) and (4) the surface-displayed G protein derivatives on gram-positive bacteria aimed for mucosal administrations (Fig. 5.4). We summarised the experimental data in table formats.

5.5.2 Subunit Vaccine

Rationale Native F and G glycoproteins are recognised as the most potent antigens in inducing antibody-mediated protection against RSV. In our hands, beside the native F extract of virion origin, recombinant fragments or synthetic peptides did not reproduce the authentic and conformational structure of the F protein.

After extensive screening of numerous peptide sequences from both proteins, our studies were focused on the central conserved domain of RSV-A $G_{130–230}$ protein, named G2Na (Fig. 5.1). The extracellular domain of G is divided into three regions: the N terminal $G_{66–130}$, the central domain $G_{130–230}$ and the C terminal $G_{230–298}$. In the G2Na segment (yellow), glycosylation is significantly reduced and the sequence is relatively conserved between the A and B RSV subtypes. In contrast, the N and C terminal sequences (orange) are highly variable and heavily glycosylated. G2Na has several specific features in particular:

- A hydrophobic domain $G_{164–176}$ that is strictly identical between RSV subgroups A and B and which could induce cross-protective immune responses.
- A cysteine-rich region:
  - With four conserved cysteine residues which form a conformational cysteine loop structure (disulphide bridges occur between Cys_{173} and Cys_{186} and between Cys_{176} and Cys_{182}): this is critical for the induction of protective antibody responses [2, 70]. A potent protective peptide $G1a_{174–187}$ peptide was first identified by Trudel et al [70].

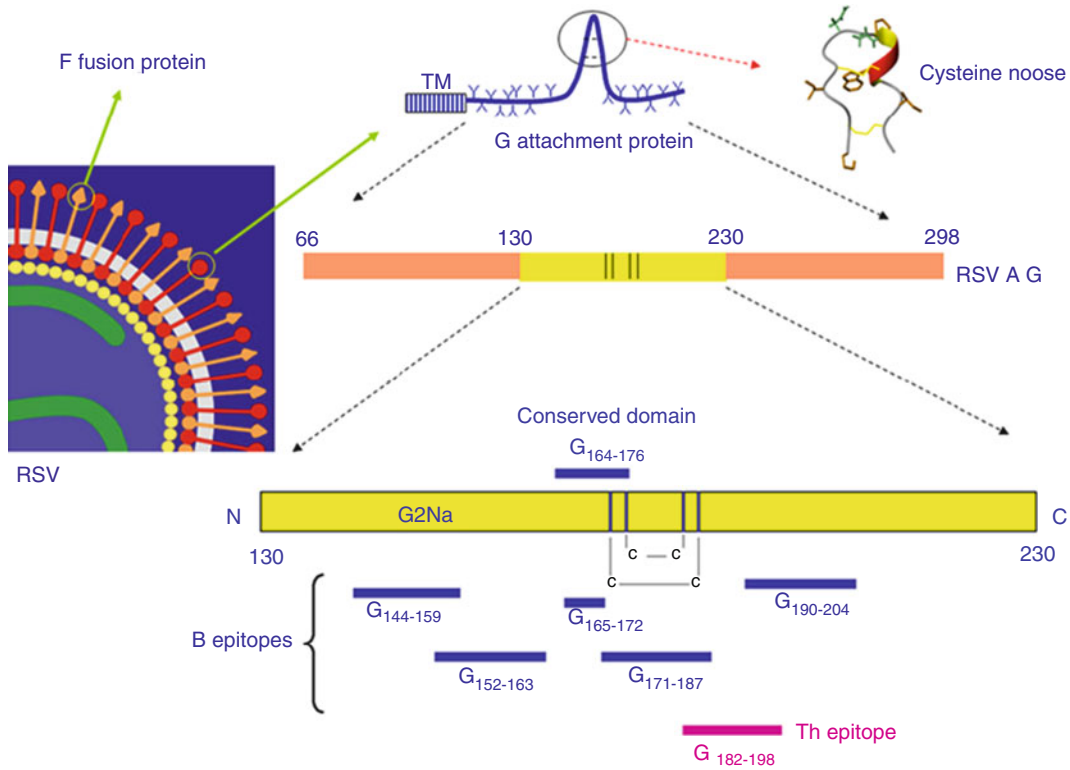


Fig. 5.1 RSV with surface glycoproteins F and G. Attachment glycoprotein G with a transmembrane (*TM*) region at the N terminal. Both the N and C termini of the extracellular domain are highly glycosylated (Y), while the central portion is non-glycosylated. Note the cysteine-bridge loop structure enlarged on the right. Linear representation of $G2Na_{130–230}$ of RSV-A with the B cell protective epitopes and the Th cell epitope. Note the 4 conserved Cys residues (*vertical blue stripes*) and disulphide bridges between them

  - With Cys_{182} and Cys_{186} located in the CX3C chemokine motif [25, 69]. Peptides containing this motif generate Abs that inhibit RSV G protein binding, reduce lung virus titres [76] and neutralise both the A and B subtypes of RSV [10].
  - Which acts as immunomodulator and confers as adjuvant effect [16, 30, 41].
  - Which, if substituting the cysteines to serines, affects immunogenicity, affinity maturation and protective efficacy [16, 53].
  - A Th1/Th2 epitope ($CD4^+$ Th epitope). $G_{182–198}$ responsible for URT protection in mice [26, 53, 54, 68, 72].
- A proline-rich conserved segment $G_{184–198}$ which confers conserved secondary structure.
- A lysine-rich heparin-binding domain important for attachment of RSV G to the cell [20].
- Five epitopes recognised by and inducing protective Abs (protectopes) in the G2Na domain: $G_{150–157}$, $G_{163–174}$, $G_{178–185}$, $G_{171–187}$ and $G_{190–204}$ [52, 59].

This rationale encouraged us to use G2Na as the principal antigen for an RSV vaccine. To ensure a better immunogenicity in humans, different carrier proteins were tested: an albumin-binding domain BB [35], diphtheria toxin derivatives DT [48] and outer membrane protein P40 [24, 47]. They were evaluated in the form of fusion proteins or as conjugate vaccines (Fig. 5.2, Tables 5.1 and 5.2). BB was finally selected as a carrier: firstly, due to its high affinity to human serum albumin, it extended the half-life of the fusion protein in vivo [50], and secondly, BB was shown to enhance the immunogenicity of G2Na mostly by inducing an earlier antibody response [35]. BBG2Na was extensively documented in animal models in terms

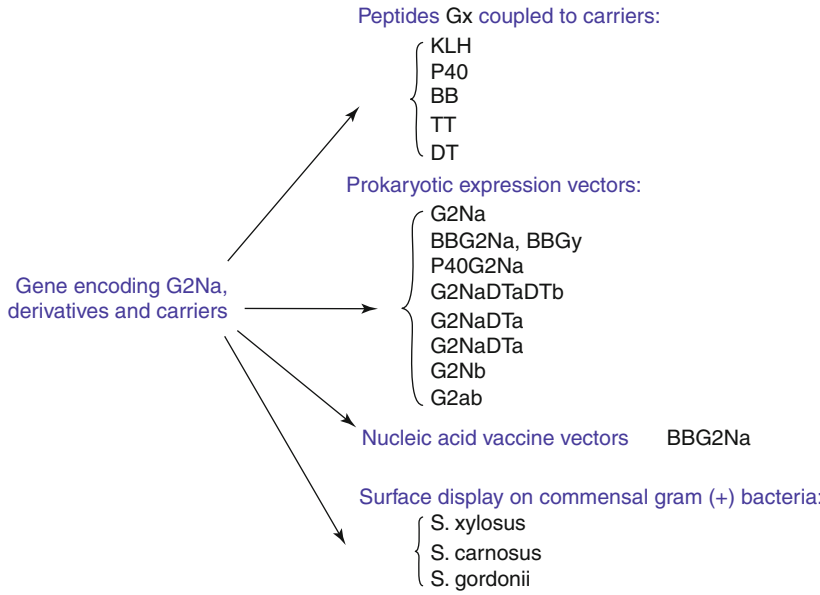


Fig. 5.2 Different G2Na derivatives and RSV peptide antigens: (1) Peptides Gx were chemically coupled to various carrier proteins – G2Na and G2Na fused to BB, to P40 or to DT derivative carrier proteins; (2) $G2Nb_{130–230}$ of RSV-B and fusion protein G2ab of RSV-A and RSV-B $G_{130–230}$ proteins; (3) BBG2Na in nucleic acid vaccine vectors; (4) G peptides or G2Na expressed on the surface of gram-positive bacteria

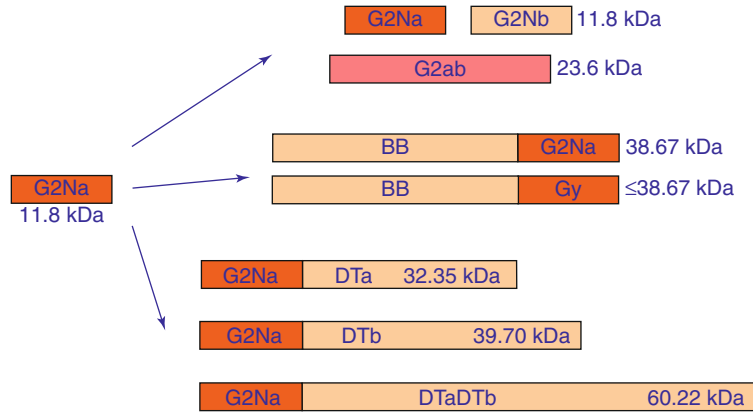


Fig. 5.3 Different constructs of recombinant proteins: G2Na, G2Nb, G2ab, BBG2Na, BBGy (Gy: G2Sera, G2DCa, $G_{140–190}$, $G_{140–192}$, $G_{140–194}$, $G_{140–196}$, $G_{140–198}$, $G_{140–200}$) – G2Na fused to DT derivatives

of immunogenicity, protective efficacy and safety [13, 56, 58] before the clinical trials.

5.5.3 Animal Models

5.5.3.1 BALB/c Mice

BALB/c mice is an albino inbred strain, semi-permissive to RSV infection, and is generally used for primary screening of vaccine antigens. We used neonatal BALB/c mice to document BBG2Na immunogenicity in the presence or absence of maternal RSV-Abs [4, 64]. In contrast, to mimic the elderly situation, RSV-primed mice were used to document Ab responses in the seropositive population [23, 48]. This convenient model allowed us to study of RSV infection (protection and immunopathology) in SPF inbred animals, for which a multitude of immunological reagents were available.

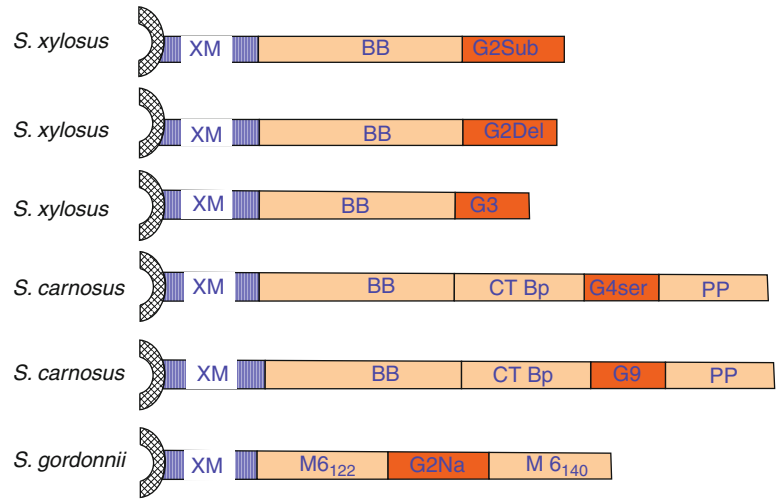
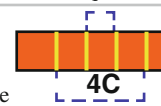
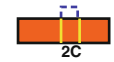
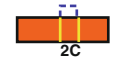
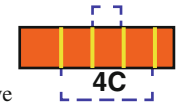


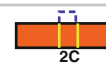
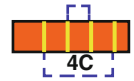
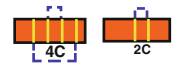
Fig. 5.4 Surface display of different G fragment antigens constructs on various gram-positive bacteria

Table 5.1 Different G2Na derivatives and peptides

	Name	G (aa–aa)	Nature of aa sequence	References
RSV A	G2Na	130–230	 Native	[48, 58]
	G200a	140–200		[53]
	G198a	140–198		[53]
	G196a	140–196		[53]
	G194a	140–194		[53]
	G192a	140–192		[53]
	G190a	140–190		[53]
	Gcf	131–230		[29, 30]
	G2DCa	130–230	C_{173} and C_{186} substituted by S 	[53]
	G2Sera	130–230	all 4 C substituted by S	[53]
	G1a	174–187	C_{186} substituted by S 	[70]
	G3a	3 X G1a	trimerized G1a peptide	[45]
	G4a	172–187	 Native	[52, 59]
	VG4a	171–187		[52, 59]
	G5a	144–159		[52, 59]
	G7a	158–190		[32]
	G8a	158–200		[32]
	G9a	190–204		[6, 52, 59]
	G11a	164–176		[52, 59]
	G2Dela	G130–161 G171–230	Chimera	[46]
	G20a	G190a G5a	Chimera	[32]
	G4S	171–187	all 4 C substituted by S	[8, 65]

(continued)

Table 5.1 (continued)

	Name	G (aa–aa)	Nature of aa sequence		References
RSV B	G2DCb	130–230	C_{173} and C_{186} substituted by S		[48]
	G1b	174–187	C_{186} substituted by S		[65]
	G2Nb	130–230	Native		[48]
	G4b	172–187			[2]
	VG4b	171–187			[2, 65]
AB	G2ab	G2NaG2DCb	 Chimera		[48]

Column from left to right: (i) Antigens are divided into subgroup A (in *yellow bar*), subgroup B (in *green bar*) and both A and B subgroup (in *blue*). (ii) G2Na and the names of the derived antigens. (iii) Amino acids included. (iv) Nature of aa sequence: with the four native Cys_{173}, Cys_{176}, Cys_{182} and Cys_{186} (*red bar* with four *yellow stripes*) or with only two central Cys_{176} and Cys_{182} (or all four Cys substituted by Ser). (v) Reference of the study

Table 5.2 Carrier proteins

	Name	Origin	References
Carrier protein	BB	Albumin binding domain of Streptococcal protein G	[35, 49]
	DTa	Diphtheria toxin subunit A	[48]
	DTb	Diphtheria toxin subunit B	[48]
	DTaDTb	Fusion of DTa to DTb	[48]
	KLH	Keyhole limpet haemocyanin	[70]
	P40	Outer membrane protein A of *Klebsiella pneumoniae*	[24, 47, 59]

Column from left to right: (i) Different names of the carrier proteins. (ii) Origin of the carrier. (iii) Reference of the study

5.5.3.2 Cotton Rats

Sigmodon hispidus are more permissive to RSV infection. To compare immunogenicity between our Ag formulations, they were more discriminant in terms of RSV-Ab responses compared to the BALB/c mice. They are also very helpful to document enhanced immunopathology [63]. The only disadvantage is the limited number of available immunological reagents.

5.5.3.3 Non-Human Primates

African green monkeys and macaques were used for the evaluation of our vaccine candidate BBG2Na [15, 71]. Macaques seemed to be less susceptible to hRSV infection making this model not yet reliable. A disadvantage of these models is that ethical and economical constraints often limit the number of animals per group, which can result in statistically insignificant data.

5.5.3.4 Human Primates

Chimpanzee is the best model, highly permissive to RSV infection, respiratory symptoms mostly closed to human disease, but they belong to protected species: no sacrifice [11].

5.5.4 Parenteral Immunisation

The most important feature for an RSV vaccine is to protect the LRT where most fatal cases in children occurred. Serum IgG inhibiting RSV replication is a sufficient component to prevent lung disease as demonstrated by the licensed Synagis®. Majority of the licensed human vaccines are administered via the parenteral route, mostly intramuscularly (*im*). In rodent models, *im*, intraperitoneal (*ip*) and subcutaneous (*sc*) were tested [22, 59].

Antigen preparation G2 protein derivatives (>60 aa) were expressed alone or fused to carrier

proteins, *i.e.* BB or DT subunits. These recombinant proteins were expressed with high yield in *E. coli* and purified by affinity chromatography after denaturation and refolding from inclusion bodies [48]. Clinical grade of G2Na and BBG2Na were obtained from pilot scale production at the Centre d'Immunologie Pierre Fabre. Peptides (<20 aa) were chemically coupled to carrier proteins: BB or P40 or KLH [52, 59, 70].

We show in Table 5.3 the different combinations of peptides/proteins used for parenteral immunisations. For conjugated peptides Gx, $G1a_{174–187}$, $G5a_{144–159}$, $VG4a_{171–187}$ and $G9a_{190–204}$ protectopes were coupled to KLH or to P40 or to BB carrier proteins; most of them induced moderate to highly protective antibody responses against RSV-A in mice. G2Na alone or fused to BB or DT derivatives adjuvanted with Alum; administered *ip*, *im* or *sc* induced high RSV-A antibody responses.

Consistently, the elicited antibody responses protected lungs of all immunised animals challenged by RSV-A. Recombinant fragments of G2Na (Gy) having two amino acid residues deleted from the C-terminus were generated, enabling us to define $G194a_{140–194}$ as the smallest protective amino acid sequence [53]. Another chimeric protein G20a ($G_{140–190}$ fused to $G5a_{144–159}$) showed to be protective in mice [32] but not in cotton rats where it required to be fused to DT carrier to induce protective immune responses [44]. The cysteine residues were shown to be critical for the activity. When all four cysteines were substituted for serines (BBG2Sera), immunogenicity dropped significantly [53]. When Cys_{173} and Cys_{186} were substituted, immunogenicity was decreased but not enough to compromise lung protection.

In contrast, URT protection was affected, most likely due to alteration of the Th epitope $G_{184–198}$ which contains the Cys_{186}. BBG2Na showed to be immunogenic in neonatal BALB/c mice even in the presence of maternal RSV antibodies without any enhanced FI-RSV-like disease symptoms [4, 54, 56, 64]. BBG2Na and G2Na induced persistent (≥24 weeks) and high level of RSV-A antibody responses in RSV-primed mice [23, 48]. It should be noted that elicited antibodies also bind to a recent clinical isolate BT2a [48]. Similarly, Jang et al. showed that trimerised G2Na antigen induced antibodies that recognised other recent hRSV-A and hRSV-B clinical isolates [28, 74]. BBG2Na was also evaluated in a nucleic acid vaccine format.

When injected *im* in mice, the vector induced partial immune protective responses to RSV challenge. For better efficiency, the nucleic acid vectors needed to be optimised in terms of in vivo expression of G2Na [1].

Table 5.4: Heterologous protective efficacy. As expected, $G1a_{174–187}$ and $VG4a_{171–187}$ subgroup A peptides did not induce cross-reactive antibodies against RSV-B. In contrast, the subgroup B $G1b_{174–187}$ peptide induced moderate RSV-B IgG level which protected animal lungs against RSV-B challenge [65]. Surprisingly, G2Nb (RSV-B) the equivalent of G2Na induced weak and did not boost RSV-B antibody response, neither alone nor admixed or fused to G2Na [48]. This might be due to the conformational problem of the G2Nb structure. In stark contrast, G2Na and BBG2Na both induced moderately cross-reactive antibodies against RSV-B: the viral titres in immunised animal lungs were highly reduced upon RSV-B challenge [48, 58, 62]. This was confirmed by other groups who showed that the central conserved cysteine-rich domain of G2Na was important in inducing cross-reactivity antibody response to an RSV-B strain [10, 28, 43, 76]. BBG2Na was selected as a human vaccine candidate for several reasons: BB, primarily used as an affinity tag for purification purpose [48], showed to act as a carrier protein [35] and prolonged the half-life time of the fused molecule in vivo [50].

BBG2Na was immunogenic in the naïve neonatal mice model even in the presence of maternal antibodies [4, 64]. Strategies to vaccinate pregnant mothers were investigated and found feasible as illustrated by a clinical trial with subunit F protein [42]. In contrast to FI-RSV-vaccinated animals, BBG2Na did not evoke enhanced immunopathology after challenge [51]. Although BBG2Na/Alum elicited a Th2 type of response, RSV challenge triggered a mixed Th1/Th2 response [13, 56, 60]. Furthermore,

Table 5.3 Parenteral immunisations

	Antigens	Adjuvants	Route	Animal models	RSV A ELISA	LRT protection vs RSV A	References
Peptides Gx	KLH-G1a, KLH-G5a	Freund	*ip*	Mice	+++	+++	[70]
	P40-G1a, P40-VG4a, P40-G5a, P40-G9a	Alum	*ip, im*	Mice	++	+++	[52]
	BB-G1a. BB-G5a,	Alum	*ip*	Mice		+++	[59]
	G20a	Alum	*im*	Mice	+++	+++	[32]
	G20aDTa	Alum	*im*	Rats	+++	+++	[44]
	G125-203	Alum	*ip*	Mice	+++	+++	[65]
G2Na	G2Na	Alum	*ip, im*	Mice and rats	+++	+++	[48]
	G2Na	PLGA	*im*	Mice and RSV^+ Mice	+++	+++	[48]
	G2Na	PLGA and/or Alum	*im*	Mice and rats	+++	+++	[44, 48]
	G2Na+F	Alum	*im*	Mice	+++	+++	[44]
	G2Na+G2Nb	Alum	*im*	Mice	+++	+++	[48]
	G2ab	Alum	*im*	Mice	+++	+++	[48]
	G2NaDTa, G2NaDTb, G2NaDTaDTb	Alum	*im*	Mice	+++	+++	[48]
BBGy	BBG200a, BBG198a, BBG196a and BBG194a	Alum	*ip*	Mice	+++	+++	[53]
	BBG192a and BBG190a	Alum	*ip*	Mice	++	++	[53]
BBG2Na	BBG2Sera	Alum	*ip*	Mice	+	++	[53]
	BBG2DCa	Alum	*ip*	Mice	++	+++	[53]
	BBG2Na	Alum	*ip, im, sc*	Mice and RSV^+ Mice	+++	+++	[23, 58]
	BBG2Na	Alum or Freund	*im*	Monkeys	+++	+++	[71]
	BBG2Na	Alum	*im*	Macaques	+	–	[15]
	BBG2Na	DDA	*im*	Mice and rats	+++	+++	[31]
	BBG2Na	DDA+Alum	*im*	Mice and rats	+++	+++	[31]
	BBG2Na	CpG+Alum	*im*	Mice	+++	+++	[44]
	BBG2Na	MPL+Alum	*im*	Mice	+++	+++	[44]
	BBG2Na	Titer Max	*ip, im*	Neonatal mice	+++	+++	[4, 64]
	BBG2Na (Nucleic acid vaccine)	No adj.	*im*	Mice	–	+	[1]

Column from left to right: (i) Antigens (G1a, G4a, G5a and G9a peptides coupled to KLH, to P40 or to BB carrier proteins). G2Na alone or fused to diphtheria derivatives DTa, DTb or DTaDTb; Gy fragments fused to BB and BBG2Sera, BBG2DCa, BBG2Na. (ii) Adjuvants: *Alum* Alhydrogel or Adju-Phos, *PLGA* poly(D,L-lactide-co-glycolide, *MPL* monophosphoryl lipid A, *DDA* dimethyldioctadecylammonium bromide, *CpG* CpG oligodeoxynucleotide. (iii) Route of administration: *ip* intraperitoneal, *im* intramuscular, *sc* subcutaneous. (iv) Animal models: mice (BALB/c naïve animals unless mentioned, RSV^+mice: RSV-primed mice), rats (cotton rats), monkeys (African green monkeys). (v) RSV-A IgG ELISA titre: +++ high, ++ medium, + low, – undetectable. (vi) LRT protection against RSV-A challenge: +++ complete, ++ good, + partial, – no protection. (vii) References of the study

Table 5.4 Parenteral immunizations – cross reactivity RSV A and RSV B

Antigens	Adjuvants	Route	Animal models	RSV A ELISA	LRT protection vs RSV A	RSV B ELISA	LRT protection vs RSV B	References
KLH-G1a	Freund	*ip*	Mice	+++	+++	–	–	[65, 70]
KLH-VG4a	Freund	*ip*	Mice	+++	+++	–	–	[65]
KLH-G1b	Freund	*ip*	Mice	–	–	++	++	[65]
G2Na	Alum	*im*	Mice and rats	+++	+++	++	++	[48]
G2Nb	Alum	*im*	Mice	+	NT	+	NT	[48]
G2aba	Alum	*im*	Mice	+++	NT	++	NT	[48]
G2Na+G2Nb	Alum	*ip, im*	Mice	+++	NT	++	NT	[48]
G2Na+F	Alum	*im*	Mice and rats	+++	+++	+++	NT	[44]
BBG2Na	Alum	*ip, im, sc*	Mice and rats	+++	+++	++	++	[58, 62]
BBG2Na	Alum or IFA	*im*	Monkeys	+++	+++	++	NT	[71]
G2 (aa148-198)	TiterMax	*im*	Mice	+++	+++	++	NT	[10, 76]

Column from left to right: (i) Names of antigens. (ii) Adjuvants. (iii) Route of administration: *ip* intraperitoneal, *im* intramuscular, *sc* subcutaneous. (iv) Animal models: BALB/c mice or cotton rats. (v) RSV IgG ELISA titre: +++ high, ++ medium, + low. (vi) LRT protection (lungs) against RSV challenge: + complete, ++ good, + partial, – no protection. (vii) *NT* not tested. (viii) Reference of the study

when BBG2Na was adjuvanted with DDA, CpG, MPL, Freund's or TiterMax, a mixed IgG1/IgG2a antibody response was raised, in contrast to the only IgG1 antibody response induced by BBG2Na/Alum, indicating that a Th response could be modulated by several human adjuvants. The protective efficacy was not altered whatever adjuvant used [4, 31, 44].

In African green monkeys, by *im* injection, BBG2Na was highly immunogenic when adjuvanted with incomplete Freund's adjuvant (IFA), while moderately high RSV-A antibody titres were induced with Alum. Antibodies induced also cross-reacted with the RSV-B. In a subsequent RSV-A challenge study, it was found that BBG2Na in either Alum or IFA demonstrated protective efficacy [71]. In macaques, BBG2Na showed to be weakly immunogenic [15]. RSV susceptibility in this model and limited number of animals rendered interpretation of the data not reliable. To mimic the elderly situation, we showed that BBG2Na was immunogenic in RSV-primed mice and no inhibition of the antibody response was observed [23, 48]. BBG2Na successfully went through phase I and phase II clinical trials in terms of safety and immunogenicity [34, 55, 61]. Unfortunately, clinical phase III was halted due to rare adverse events in a very small number of vaccinees.

The chronology of the events, the delayed to onset and the symptoms were suggestive of a vaccine-related type III hypersensitivity-like reaction. When tested in a rabbit model of type III hypersensitivity, we found that BBG2Na induced an Arthus reaction and that the BB component, rather than G2Na, was responsible for this effect [36]. This provided the impetus for further studies on the immunogenicity and protective efficacy of G2Na.

Although in our previous work we showed that BBG2Na induced an earlier and higher G2Na Abs response than G2Na alone [35], we've recently showed that with or without carrier proteins (DT derivatives or BB), G2Na induced a similar level of RSV-A and RSV-B IgG responses in mice and cotton rats [48]. In cotton rats, G2Na/Alum induced high and persistent antibody responses to RSV-A and RSV-B up to 148 days. These antibodies protected animal lungs from RSV-A infection equivalent to animal treated with Synagis® control. Importantly, we provided robust evidence that immunisation with different G2Na formulations in the presence of anti-RSV-Abs did not compromise avidity maturation of the antibodies over time [48]. To get a better cross-reactivity and long-term CTL response, ideally, G2Na should be associated with the F and/or M proteins. Indeed, G Cys-rich domain has demonstrated to enhance CTL response either when it was coadministered with RSV matrix M protein Ag [5] or when it was fused to

F and M2 proteins: $G_{125–225}F/M_{81–95}$ [75]. The disadvantage with this strategy would be that purification of F protein from RSV virions is still poor in yield and up to date, no recombinant F has been successfully produced at industrial scale. We are currently investigating whether RSV-B G peptides to be combined with G2Na would be possible (Fig. 5.3).

5.5.5 Mucosal Immunisation

In addition to the parenteral routes, we also explored the alternative mucosal immunisations (Table 5.5). Mucosal immunisation does not readily prime for enhanced immunopathology in animal models and might further promote vaccine efficacy by induction of mucosal IgA as a first-line immune defence against the virus. Although their role in protection in humans remained unclear, RSV-specific IgA antibodies are secreted rapidly in the upper airways of mice following a primary RSV infection and confer protection. Transcutaneous immunisation (*tsc*) of G2Na adjuvanted with cholera toxin (CT) induced a low RSV-A IgG titre which conferred partial protection [21]. However, intranasal administration is the common way to achieve protection. The main advantage of this route is its easy administration (needle-free) and thus better acceptance by parents. But it requires the use of mucosal adjuvants such as the cholera toxin B subunit (CTB) or dimethyldioctadecylammonium bromide (DDA) [33]. CTB is of significant interest: its immunopotentiating capacity as a carrier molecule is considered to be related to its ability to bind to the monosialoganglioside GM1, present on mucosal epithelial cells [7, 37]. When administered in mice, BBG2Na adjuvanted with CTB or DDA induced both local IgA and systemic IgG which protected lungs against RSV-A challenge. Another group showed that either by sublingual (*sl*) or by *in* administration, with or without CT adjuvant, $G2_{131–230}$ induced both protective IgA and IgG in mice [30]. These data confirm and reinforce that

Table 5.5 Mucosal immunisations

	Antigens	Adjuvants	Route	Animal models	RSV A ELISA IgG	RSV A ELISA IgA	LRT protection vs. RSV A	References
Protein	G2Na	CT	*tsc*	Mice	+	*ND*	++	[21]
	G2cf (131–230)	± CT	*sl and in*	Mice	++	++	+++	[30]
	BBG2Na	CTB	*in*	Mice and rats	+++	++	+++	[33]
	BBG2Na	DDA	*in*	Mice and rats	+++	++	+++	[33]
S. xylosus	XM | BB | G2Del		*in*	Mice	–	*ND*	NT	[46]
	XM | BB | G2Sub		*in*	Mice	–	*ND*	NT	[46]
	XM |BB|G3		*oral*	Mice	+	*ND*	NT	[45]
S. carnosus	XM | BB | CTBp | G4ser | PP		*in*	Mice	+	*ND*	+	[8]
	XM | BB| CTBp | G9 | PP		*in*	Mice	+	*ND*	++	[8]
S. gordonii	S. gordonii XM | M6122 | G2Na | M6141		*in*	Mice	+/–	+	++	[18]
BAC	Baculovirus BAC-G2Na		*in*	Mice	++	++	+++	[29]
AD	rAdenovirus rAD-3xG2Na		*in*	Mice	+++	*ND*	+++	[28]

Column from left to right: (i) Name of the antigens used with a mucosal adjuvant – surface display of G protein derivatives on gram-positive bacteria *S. xylosus*, *S. carnosus* and *S. gordonii*; baculovirus and adenovirus vectors expressing G2Na. (ii) Adjuvants for proteins Ags: *CT* cholera toxin, *CTBp* cholera toxin subunit B, *DDA* dimethyldioctadecylammonium bromide. (iii) Route of administration: *in* intranasal, *sl* sublingual. (iv) Animal models: BALB/c mice and/or cotton rats. (v) RSV-A IgG ELISA titre or RSV-A IgA ELISA titre: *ND* not determined, +++ high, ++ medium, + low, +/– very low, – undetectable. (vi) LRT protection against RSV-A challenge: +++ complete, ++ good, + partial, *NT* not tested. (vii) Reference of the study

G2Na is a potential subunit RSV candidate for mucosal immunisation.

Live vaccines constitute an alternative vaccination principle and these could be administered by *in* or by the oral route (per os). Live attenuated RSV is currently being developed [9, 11]. We chose a very different approach of employing food-grade recombinant staphylococci, namely, *Staphylococcus xylosus* and *Staphylococcus carnosus*, as vaccine-delivery vehicles (Fig. 5.4 and Table 5.5). Both nonpathogenic strains were demonstrated to be safe for mucosal or subcutaneous routes up to very high doses [66]. In mice, the oral administration of *S. xylosus* expressing G3, a trimerised $G1a_{174–187}$ epitope, at the surface was able to trigger serum G3-specific IgG that could be detected until day 143 post-immunisation [45]. Hydrophobicity engineering on the conserved domain $G_{164–176}$ of G2Na was employed to enable secretion and surface display of G2Sub (substitution of phenylalanine to serine) or G2Del (deletion of the hydrophobic stretch) on the surface of *S. xylosus* [46]. However, RSV antibody levels induced by *sc* immunisations of these recombinant bacteria were rather weak.

Although systemic antibody responses were reported to surface-exposed antigens after oral delivery with the *S. carnosus* system [38, 40, 66], attempts were made to improve the system in terms of Ab responses elicited to the surface-exposed antigenic determinants upon immunisation via the mucosal routes.

To increase the Ab response, a bacterial adhesion factor, the fibronectin-binding domain from *Streptococcus dysgalactiae*, was co-expressed on the surface of the bacteria to target the vaccine to the mucosa [7, 37–39]. This resulted in a 1.5 $\log_{10}$ increase in the serum IgG responses to the co-displayed antigen upon intranasal immunisation of mice [39]. In another approach, $CTB_{50–75}$ (CTBp), shown to have an immunopotentiating effect [17], was co-expressed on the *S. carnosus* surface. It significantly increased (close to 2 $\log_{10}$) serum antigen-specific IgG responses upon intranasal administration [7]. Further investigations demonstrated that live staphylococci were required to obtain this effect.

In a subsequent vaccination study, the *S. carnosus* system, improved by co-display of CTBp, was used for delivery of G peptides [8]. Three peptides, $G_{144–159}$ (G5), $G_{190–203}$ (G9) and $G_{171–188}$ (G4S), were expressed on the *S. carnosus* system, improved by co-display of CTBp by recombinant means as surface exposed on three different staphylococci. Intranasal immunisation of mice with the live recombinant staphylococci elicited significant anti-peptide as well as anti-RSV-A IgG2a/IgG1 responses. Upon RSV-A challenge, lung protection was demonstrated for approximately half of the mice in the G9 and G4S immunisation groups. To our knowledge, this represented the first study in which protective immunity to a viral pathogen has been evoked using food-grade bacteria as vaccine-delivery vehicles.

Later, in a similar approach, a commensal bacterium *Streptococcus gordonii* was used successfully for expression of the entire G2Na at the cell surface. Intranasal immunisation with live bacteria induced IgA against RSV-A and G2Na. Upon RSV-A challenge, immunised animals had significantly lower virus titres in the lungs than the controls [18].

Another group has recently demonstrated that *in* immunisation of recombinant replication-deficient adenovirus-based vaccine (serotype 5) expressing trimerised form of G2Na induced protective antibodies in mice [28, 74]. In another study, *in* administration in mice with a recombinant baculovirus displaying $G_{131–230}$ on the surface induced both protective IgG and IgA antibody responses [29]. These data confirmed and reinforced the potential of G2Na as a suitable RSV antigen candidate for mucosal delivery via live vectors.

5.6 Strengths and Weaknesses

An effective hRSV vaccine seems to be very difficult to develop. More than half of the century after the RSV discovery, no vaccine is still available. Recent advances in immunology and vaccinology have contributed to more progress in understanding how RSV escaped immune responses and how it caused pathology in humans. Animal models are available which should allow us to evaluate safety and efficacy of

various antigens. There are new hopes for non-replicating vaccines due to the development of alternative human adjuvants to replace Alum. Challenges are double: firstly, antigens should be very safe and immunogenic in humans, and secondly, the cost of the RSV vaccine production should be economically viable since this is a vaccine that WHO prioritises and it should be distributed and accessible worldwide.

In summary, for parenteral vaccines, our studies have clearly demonstrated that G2Na has many characteristics appropriate for a vaccine Ag against RSV in the elderly. In animal, it induced highly protective RSV-A Abs that also cross-reacted with RSV-B. This vaccine could be very useful to limit RSV transmission between health-care personnel (as for flu vaccine) and patients. For seronegative children, it should be carefully monitored in terms of immunopathology in animal models. G2Na could be associated with an RSV-B G peptide component or F protein derivatives and/or formulated with a specific immune response driving adjuvant (e.g. TLR agonists) to achieve both safety and protective efficacy. This G2Na antigen can be produced recombinantly with excellent yields. An industrial process (upstream and downstream) has already been optimised to provide a qualified human vaccine for injection.

The future challenge is to provide a safe, efficient, inexpensive and easily administered vaccine that can be immunised mucosally (*in*, *sl* or per os). Only live vectors could achieve these criteria. We have shown here that food-grade gram-positive bacteria could elicit protective local and systemic immune responses. Other groups have also proved and confirmed our results with other bacterial and viral vectors surface displaying the same G2Na antigen. However, there are still rooms for optimisations: increasing immunogenicity, stability and safety of a human mucosal RSV vaccine.

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Ebolavirus Vaccines

6

Thomas Hoenen

Contents

Abstract

Ebolaviruses cause a severe hemorrhagic fever in humans and non-human primates, with case fatalities of up to 90%. There are currently no approved therapeutics or vaccines available for these viruses. However, over the last decade there has been significant progress made in the development of experimental vaccines, which show great promise in non-human primates, the most stringent disease model for Ebolavirus hemorrhagic fever. This chapter will discuss experimental vaccines that are currently being developed, as well as the requirements for effective vaccination in different scenarios. Particularly, recombinant vaccines based on adenoviruses and vesicular stomatitis virus will be discussed in detail, as these are highly efficient in protecting non-human primates against Ebolavirus hemorrhagic fever.

6.1 Disease

Ebolaviruses cause Ebolavirus hemorrhagic fever (EHF), a disease first recognized during two concurrent outbreaks in the former Zaire (now Democratic Republic of the Congo) and former Sudan (now South Sudan) in 1976/1977 [1]. The disease is characterized by high case fatality rates, which can reach up to 90 %, depending on the virus species. Initial symptoms occur after an incubation time of, in general, 3–13 days (maximum 21 days) [2, 3] and are relatively nonspecific, including fever, headache, fatigue, myalgia

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and arthralgia, sore throat, and chest pain (reviewed in [2–4]). Within 2–7 days after the onset of disease, gastrointestinal symptoms, including diarrhea, nausea, vomiting, and anorexia, occur. A maculopapular rash has been described in about a third of cases [4], although this may be difficult to be perceived in dark-skinned patients. Hemorrhagic symptoms are most commonly found in the gastrointestinal tract and manifest themselves as melena, hematochezia, and hematemesis, although other hemorrhagic symptoms such as bleeding from venipuncture sites, the gingiva, and the conjunctivae can also occur [2–4]. In terms of laboratory parameters, thrombocytopenia, lymphocytopenia, elevated serum levels of alanine and aspartate aminotransferases, and prolonged coagulation times as well as an increase in D-dimer levels have been described [3, 4].

In fatal cases, death usually occurs in the second week of illness and is caused by a syndrome resembling septic shock [5]. In nonfatal cases, recovery is lengthy (up to 3 months) and sequelae can include arthralgia, myalgia, headaches, asthenia, and anemia [3, 4]. In addition, loss of hair, tinnitus, transient hearing loss, uveitis, conjunctivitis, and orchitis have been described in some survivors.

The pathogenesis of EHF is complex (reviewed in [5–7]). Macrophages and dendritic cells are considered to be early target cells, although a number of other cells types, including endothelial cells, are infected by ebolaviruses, albeit most likely later during the course of disease [5]. Ebolaviruses have been shown to dramatically impair the immune response. In particular, two viral proteins, VP35 and VP24, are instrumental in impairing the innate immune response. VP35 interferes with the interferon response and antagonizes the protein kinase R (PKR) activity [8, 9], while VP24 blocks both signal transducer and activator of transcription (STAT)-mediated and p38-mediated interferon signalling pathways [10, 11]. Further, dendritic cells infected with ebolavirus are impaired in their ability to support T-cell proliferation, and do not produce proinflammatory cytokines [12, 13].

Also, in terms of adaptive immunity, there seems to be dramatic differences between survivors and non-survivors of EHF, with survivors showing IgM antibodies as early as 2 days post-onset of symptoms and IgG antibodies 5–8 days after symptom onset, whereas only 30 % of non-survivors show IgM antibodies, and none develop an IgG response [14, 15]. Further, ebolaviruses have been shown to induce a massive loss of lymphocytes in patients even though these cells are not directly infected, presumably via bystander apoptosis [16].

Another hallmark of EHF pathogenesis is the production of high levels of proinflammatory cytokines and chemokines, sometimes referred to as a "cytokine storm" [16]. In addition, tissue factor is produced in increased amounts during ebolavirus infection [17] and is proposed to be involved in the development of disseminated intravascular coagulation (i.e., the systemic activation of the blood coagulation system causing fibrin deposition and microvascular thrombi), which is a prominent feature of EHF [18].

6.2 Pathogen

Ebolaviruses, together with the related Marburg viruses, belong into the family *Filoviridae*. There are currently five recognized species of ebolaviruses, *Zaire ebolavirus (ZEBOV)*, *Sudan ebolavirus (SEBOV)*, *Bundibugyo ebolavirus (BEBOV), Côte d'Ivoire ebolavirus (CIEBOV),* and *Reston ebolavirus (REBOV)* [19] (Table 6.1).

Since filoviruses contain a non-segmented, single-stranded RNA genome in negative orientation, they are grouped in the order of *Mononegavirales*. This genome is about 19 kb in length and contains seven genes, encoding for at least nine proteins (Fig. 6.1) [1]. Seven of these proteins are structural proteins and found in the virus particles, which have a characteristic thread-like appearance. Particles have an average diameter of 98 nm (not measuring the glycoprotein protrusions), and the majority of them have a length of about 980 nm [20].

The helical nucleocapsid can be found in the center of the virus particle and consists of the

Table 6.1 Overview of ebolavirus species

Species	*Zaire ebolavirus*	*Sudan ebolavirus*	*Bundibugyo ebolavirus*	*Côte d'Ivoire ebolavirus*	*Reston ebolavirus*
Abbreviation	ZEBOV	SEBOV	BEBOV	CIEBOV	REBOV
Case fatality rate[a]	44–90 % (avg. 79 %)	41–71 % (avg. 54 %)	25–49 % (avg. 32 %)	0 %	0 %
Case number	1388	785	214	1	0
Endemic countries	Gabon, RC, DRC	South Sudan, Uganda	Uganda, DRC	Ivory Coast	Philippines
Disease characteristics	Severe hemorrhagic fever	Severe hemorrhagic fever	Severe hemorrhagic fever	Severe hemorrhagic fever	Apathogenic in humans
Experimental vaccine platforms[b]	rAdV, rVSV, rhPIV, VLPs	rVSV, rAdV	rAdV[c], rVSV[c, d]	rVSV[c]	None

Characteristic features of the five recognized ebolavirus species are listed
Abbreviations: *avg.* average, *RC* Republic of Congo, *DRC* Democratic Republic of Congo, *rAdV* recombinant adenovirus, *rVSV* recombinant vesicular stomatitis virus, *rhPIV* recombinant human parainfluenza virus, *VLPs* virus-like particles
[a]Ranges of case fatality rates are based on outbreaks with more than one patient; average case fatality rates take into consideration all outbreaks
[b]Listed are only vaccine platforms that have been shown to be protective in nonhuman primates
[c]Cross-protection through vaccines directed at different ebolavirus species
[d]Only partial protection has been demonstrated until now

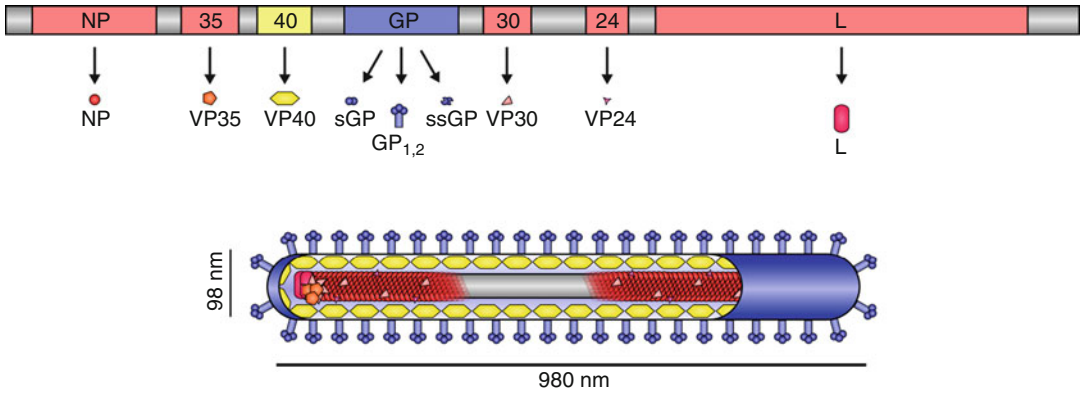

Fig. 6.1 Structure of an ebolavirus. The ebolavirus genome structure (*top*), as well as a schematic representation of an ebolavirus virion (*bottom*), is shown. Ribonucleoprotein complex proteins (NP, VP35, VP30, L, and VP24) are shown in *red*, the matrix protein VP40 in *yellow* and the glycoproteins (structural glycoprotein $GP_{1,2}$ and the nonstructural glycoproteins sGP and ssGP) in *blue*

RNA genome encapsidated by the nucleoprotein NP, and in association with the viral polymerase L, the polymerase cofactor VP35, the transcriptional activator VP30, and the accessory protein VP24, which is involved in nucleocapsid formation and primary transcription [20–22]. Surrounding the nucleocapsid is the matrix space containing the matrix protein VP40, which is responsible for morphogenesis and budding of new virions [23]. Virus particles are enveloped by a host cell-derived lipid bilayer, in which the surface protein $GP_{1,2}$ is embedded, which mediates entry and fusion with the host cell membrane [24].

Due to the compact genome size, many of the ebolavirus proteins serve multiple functions. For example, both the polymerase cofactor VP35 and the accessory protein VP24 also act as interferon antagonists [8, 11], and both VP24 and the matrix

protein VP40 are involved in the regulation of viral genome replication and transcription [25]. As a second strategy to maximize coding capacity, viral transcription of the GP gene primarily results in the production of a nonstructural soluble variant of the glycoprotein called sGP. However, during transcription non-templated adenosine residues can be incorporated into the nascent mRNAs by the viral polymerase at a poly-A editing site in the GP gene [26]. This leads to a shift in the open reading frame and production of either the structural, membrane-embedded $GP_{1,2}$ protein, or a second nonstructural GP variant called ssGP [27]. The functions of both nonstructural proteins (sGP and ssGP) are currently unknown.

The life cycle of ebolaviruses follows the same general principles as other non-segmented negative-strand RNA viruses. Ebolaviruses can attach to host cells via a number of cell surface molecules including various C-type lectins, TIM-1, and members of the Tyro3 receptor tyrosine kinase family (reviewed in [28]). Virus particles are internalized by macropinocytosis [24, 29] delivering them to endosomes where the mucin-like domain, as well as glycan caps, is cleaved from $GP_{1,2}$ by host cell proteases such as cathepsins [30]. This exposes the receptor-binding domain and allows $GP_{1,2}$ to interact with its receptor Niemann-Pick C1 (NPC1) [31, 32], ultimately resulting in fusion of the virus membrane with the endosomal membrane, thereby delivering the nucleocapsids into the cytoplasm where the rest of the replication cycle takes place. Initially, mRNAs are transcribed from the viral genome in a process called primary transcription, which involves only components brought into the cell within virus particles. Subsequently, secondary transcription occurs utilizing viral components newly synthesized in the host cells, and the viral genome is replicated via an antigenomic intermediate.

Viral genome replication takes place in viral factories called inclusion bodies, which might also be involved in other parts of the viral life cycle [33]. Newly formed nucleocapsids are transported to the cell surface, where VP40 facilitates budding in a process involving oligomerization of VP40 [34, 35] and usurpation of the cellular ESCRT machinery [36–38].

6.3 Transmission

Until now, all human EHF cases have originated in central Africa (Fig. 6.2). However, a number of imported infections with the closely related Marburg virus into the US and Europe highlight the potential for imported EHF cases worldwide [39, 40]. Also, the importation of ZEBOV from Gabon into South Africa, where it caused a fatal infection in a doctor from Johannesburg, demonstrates this risk [41]. REBOV, which represents the only known ebolavirus species outside of Africa, originates from the Philippines and has the ability to infect humans, although until now no human disease has been observed following infection with this species. Interestingly, some reports suggest that subclinical infections with ebolaviruses or related filoviruses might occur in areas outside of the recognized endemic areas [42, 43], based on serological evidence. Further, some reports suggest that subclinical or mild ebolavirus infections also occur in endemic areas with a much higher frequency than those that are recognized in outbreaks, again based on serological evidence [44–46].

EHF cases occur in association with outbreaks, which have a frequency of about one outbreak per year in the last decade (Fig. 6.2). For some outbreaks, contact between humans and nonhuman primates (NHPs) has been implied as a probable cause [47]. However, direct transmission from bats, which are generally considered the natural reservoir of ebolaviruses [48], to humans has also been implied as a probable source of infection [49]. Human-to-human transmission is mostly through close contact with sick or deceased patients, and particularly through contact with body fluids [50, 51]. Barrier nursing techniques are considered to be sufficient to minimize the risk of person-to-person transmission [51].

6.4 Diagnostics and Classical Therapy

Due to the nonspecific early symptoms of EHF, diagnosis is difficult, particularly before an outbreak is recognized. The most common diseases

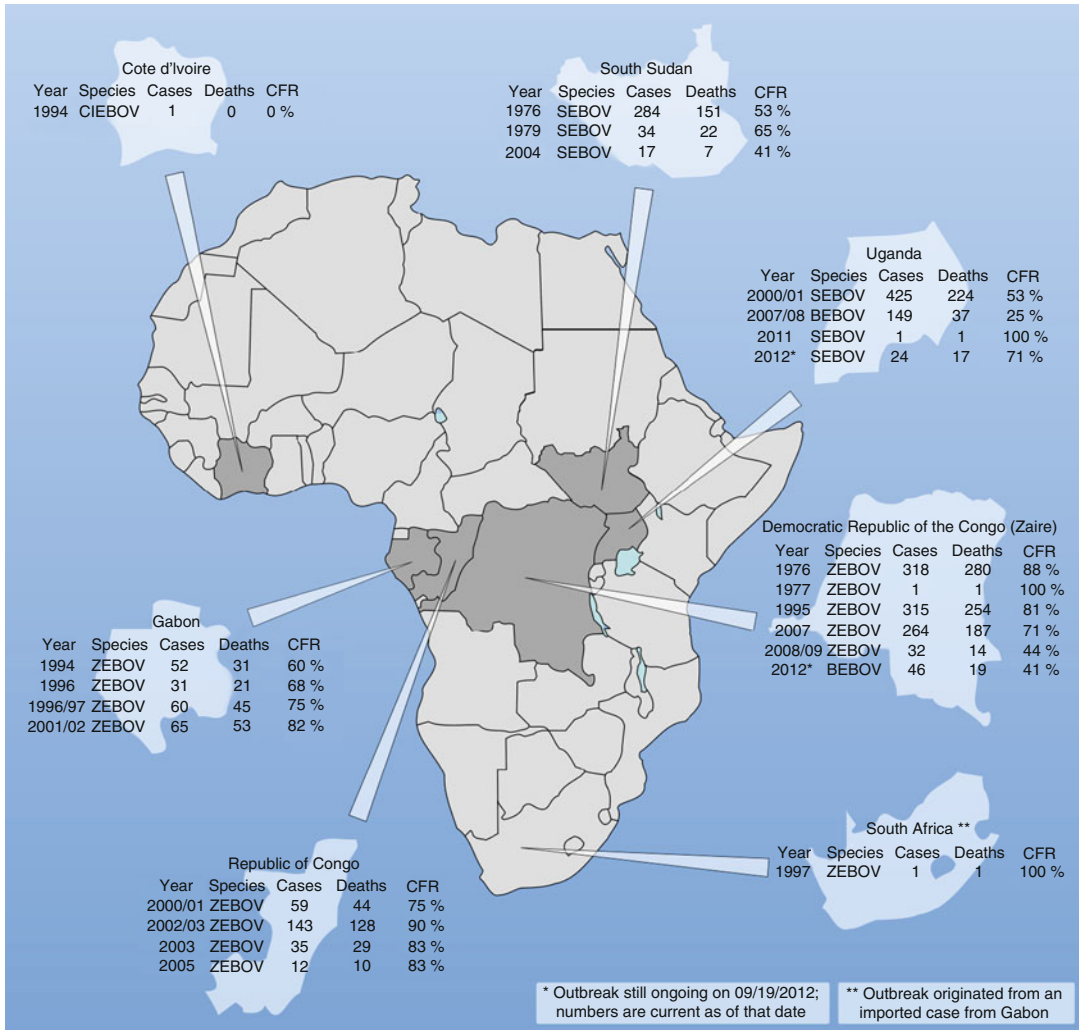

Fig. 6.2 Human outbreaks of EHF. All human outbreaks of EHF are listed. Countries from which outbreaks originated are shown in *dark gray*. The numbers of cases and deaths are listed according to the reports of the WHO, except for two outbreaks in 2012, for which numbers are based on the most recent reports in ProMED-mail (www.promedmail.org). *CFR* case fatality rate

causing similar clinical symptoms are malaria and typhoid fever, although a number of other infectious diseases also have to be considered [52]. For a definitive diagnosis, detection of viral material or detection of an immune response against ebolaviruses is required. Virus isolation is a sensitive method to detect ebolaviruses; however, it requires biosafety level 4 facilities and is comparatively slow. In contrast, RT-PCR is highly sensitive and rapid, and, depending on the specific assay used, viral nucleic acid can be detected in the blood of patients between 0 and 3 days after the onset of symptoms [53, 54].

Due to the chaotropic agents used as a first step in RNA-isolation, samples are inactivated, making RT-PCR a safe method without the need for containment laboratories. Also, RT-PCR is very amenable to field diagnostics, since this method can be implemented in the field by an outbreak response team with minimal requirements in terms of available infrastructure [52]. RT-PCR has been used as the basis for diagnosis in numerous outbreaks [53–56]. However, RT-PCR has the potential to produce false-positive or false-negative results, so that a confirmatory assay such as an antigen capture-ELISA,

or if these are not available, confirmatory RT-PCR assays, ideally using an independent target gene and/or independent samples, should always be performed [52, 53].

Antigen capture ELISAs have been established and have also been used as a diagnostic method in a number of outbreaks [53–57]. While they generally seem to be somewhat less sensitive than RT-PCR, particularly early after disease onset [53, 54], they are invaluable to complement RT-PCR-based assays. Finally, serology aimed at detecting IgM is also used for diagnostic purposes, and IgM antibodies can be detected in some patients as early as 2 days post infection, although others do not develop a significant IgM response, or do so only late in disease, so that this method should only be used in conjunction with other diagnostic methods.

After recognition of an outbreak, the most important issue is to minimize transmission by establishing isolation procedures, ensuring that proper barrier nursing techniques are used while attending patients, and to initiate contact tracing to identify suspected EHF cases as early as possible. Unfortunately, to date there is no specific therapy for EHF available, so that treatment is restricted to supportive care, including rehydration and provision of analgesics [2, 58]. However, in NHPs, which represent the gold standard animal model for EHF, several experimental treatment strategies have been highly successful.

The most efficient experimental treatments today are antibodies [59, 60] and siRNAs [61], both of which protected 100 % of infected animals from an otherwise lethal challenge. Other strategies aimed at modulating the host response also show promise, even though they could not fully protect NHPs. These include recombinant nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation [18], and activated protein C [62], which showed 33 and 18 % protection in NHPs, respectively.

Unfortunately, despite promising results in animal models, moving specific EHF therapies forward into classical (pre-)clinical trials faces many challenges, including a lack of commercial interest, insufficient available infrastructure in the affected areas, and the sporadic nature of outbreaks and comparatively low cases numbers, such that alternative approaches might be necessary to bring these therapies into the field [63]. Also, evaluation of drugs already approved for other medical conditions in terms of their suitability to treat EHF might be a very prudent approach, since this would significantly speed up the approval process and reduce the costs for drug development.

6.5 Vaccines

Since their discovery more than 35 years ago, many different vaccine approaches against ebolaviruses have been developed and evaluated. For evaluation of these vaccines, a number of animal models are currently available, including mice, hamsters, guinea pigs, and NHPs. While all of the rodent models require the use of rodent-adapted virus strains, NHPs are susceptible to nonadapted viruses. They also represent the model closest to human EHF, exhibiting both the characteristic pathological and clinical features seen in human infection, although disease seems to progress faster and outcome is actually worse in NHPs than in humans, so that they can be considered an extremely stringent model for vaccine and drug evaluation [64, 65].

Ebolavirus vaccines can be broadly divided into replicating and non-replicating vaccines, as well as into inactivated vaccines, subunit vaccines, and vector-based vaccines (Fig. 6.3). Early vaccination approaches using inactivated ebolaviruses, replicons expressing ebolavirus proteins, or recombinantly expressed ebolavirus proteins, while promising in mice and guinea pigs, were not successful in NHPs (reviewed in [66]) and will not be discussed here. In contrast, a number of more recent approaches have been shown to be 100 % protective in NHPs, and should be considered as vaccine candidates for human use. These include a recombinant adenovirus-based vaccine, a vaccine based on recombinant vesicular stomatitis virus (VSV), a vaccine using virus-like particles, and a vaccine based on a recombinant human parainfluenza virus (Fig. 6.3). Unfortunately, currently none of these vaccines is approved for use in humans, and only the adenovirus-based vaccine has been advanced into clinical trials. However, the VSV-based vaccine

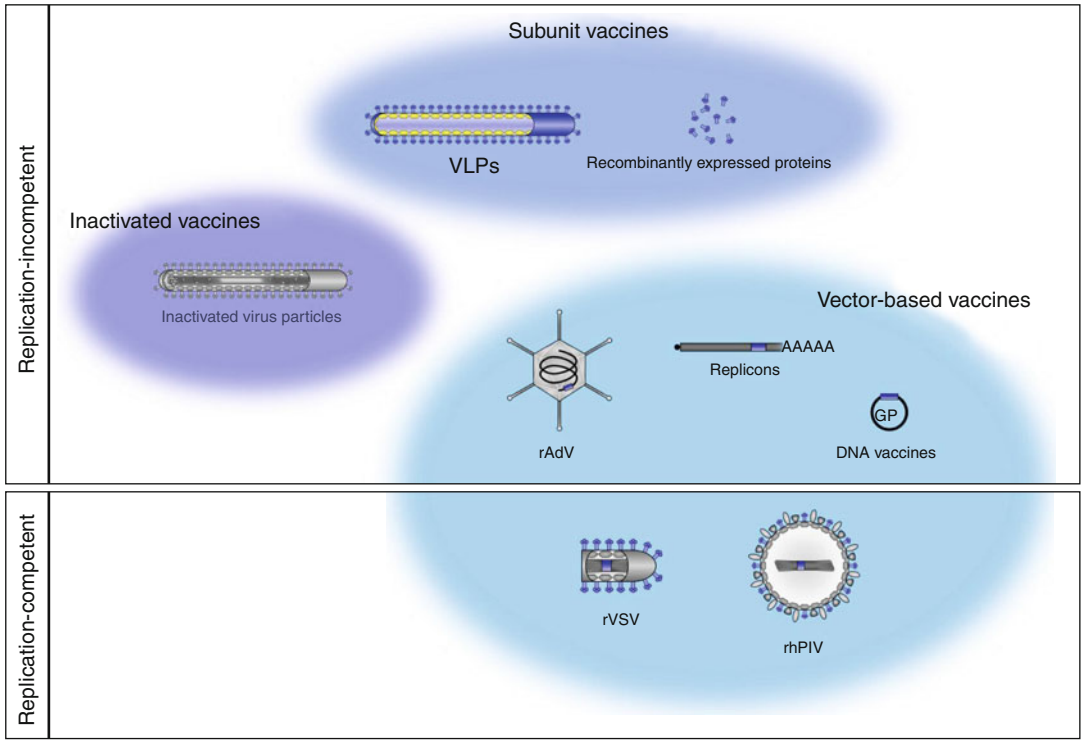

Fig. 6.3 Overview of EHF vaccines. Vaccines against EHF can be broadly divided into replication-competent and replication-incompetent vaccines. Further, they can be divided into inactivated vaccines; subunit vaccines, which comprise recombinantly expressed proteins as well as virus-like particles (*VLPs*); and vector-based vaccines. Vector-based vaccines include DNA vaccines and replicons, as well as recombinant viruses such as recombinant adenoviruses (*rAdVs*), recombinant vesicular stomatitis viruses (*rVSVs*), and recombinant human parainfluenza viruses (*rhPIVs*). Native ebolavirus components are shown in *color*, whereas inactivated ebolavirus components and vector components are shown in *gray*

has been used in one human on an emergency basis after a laboratory exposure to ZEBOV. As such, this chapter will focus on these two vaccines, which represent the most extensively studied ebolavirus vaccines, and for which there is at least some experience in humans.

6.5.1 Recombinant Adenovirus-Based Vaccine

The first vaccination to fully protect NHPs against lethal ebolavirus challenge was a recombinant adenovirus (rAdV), which was given in combination with a DNA vaccine [67] (Fig. 6.4). The rAdV vaccination platform was chosen because recombinant viruses, albeit not adenoviruses, had been shown to boost immunity generated by DNA vaccines to a higher level than could be achieved with DNA vaccines alone. Similar to the DNA vaccine component, the rAdV vaccine component served as gene delivery vehicle and contained a gene encoding for an ebolavirus antigen, which was expressed within cells of the vaccinee, but was not physically present on the surface of the adenovirus. Initially, the DNA/rAdV vaccination strategy encompassed a total of 4 immunizations (3 with the DNA component and 1 with the adenovirus component) and required more than 6 months between the first immunization and challenge. However, in a follow-up study it was shown that a single vaccination with the rAdV alone, in the absence of any DNA component, conferred 100 % protection to NHPs 4 weeks after vaccination [68].

The rAdVs used for ebolavirus vaccines carry deletions in their E1 and sometimes also their E3 and E4 regions and are, therefore, replication

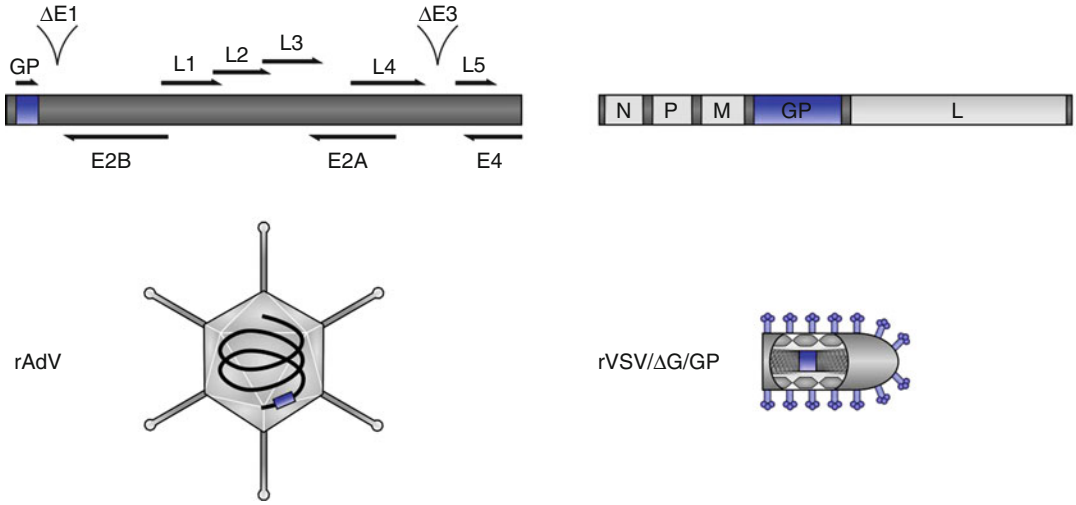

Fig. 6.4 Structure of recombinant adenovirus and vesicular stomatitis virus-based EHF vaccines. The genome structure (*top*) and a schematic representation of a virus particle (*bottom*) for the recombinant adenovirus (*rAdV*) and vesicular stomatitis virus (rVSV/ΔG/GP)-based vaccines are shown. Ebolavirus components are depicted in *color*, and vector components are depicted in *gray*. Deletions in the rAdV genome are indicated (ΔE1 and ΔE3)

incompetent. For this reason, comparatively high doses of the vaccines have to be used (1×10^{10} virus particles are required to achieve 100 % protection in NHPs), and the vaccination dose has been shown to be critical for vaccine efficacy [69]. In mice, it was possible to lower the required vaccination dose by using optimized expression cassettes, but this has not been evaluated in NHPs [70].

In terms of serotype, human adenovirus serotype 5 (HAdV-5) is usually used for recombinant ebolavirus vaccines. However, since there is a high seroprevalence against this serotype, with 60–90 % of adults being seropositive, depending on their country of origin [71], preexisting vector immunity is a potential concern. Indeed, it was shown that in rodents [72–74] as well as in NHPs [75] preexisting immunity can significantly impair the efficacy of the HAdV-5 vaccine platform, and that in humans antibody responses in individuals with preexisting immunity were lower than in HAdV-5-naive individuals [76].

Several approaches have been developed to address this problem. rAdVs based on different human serotypes (i.e., HAdV-26 and HAdV-35) were shown to be fully protective in NHPs, although a prime-boost approach was required to attain protection, and to overcome the problem of preexisting immunity [75]. Further, vaccines based on simian serotype SAdV-21 and SAdV-24, to which no or only little preexisting immunity should be present in humans, were shown to be protective in mice and guinea pigs [73, 77]. Similarly, changing the route of administration to a mucosal route increased protection of mice with preexisting immunity against the adenoviral vector [72, 78], and increased the immune response in guinea pigs with preexisting immunity against HAdV5 [74]. Finally, increasing the number of vaccination has been shown to overcome preexisting immunity in NHPs [79].

Initial studies used a blend of different rAdVs expressing two antigens, i.e., $GP_{1,2}$ and NP [68], but subsequently it was shown that NP can be omitted from vaccination without loss of protective efficacy [69]. Also, rAdV vectors have been developed that allow the expression of several antigens from a single rAdV and have been used to vaccinate NHPs against multiple ebolavirus species using a single vaccine [69, 80]. Another approach to confer protection against multiple species has been the use of blended vaccines, which was also shown to be successful [69, 81]. In fact, using a blended approach with rAdVs

containing GPs from two ebolavirus species, ZEBOV and SEBOV, it was possible to protect NHPs from challenge with a third species, BEBOV, demonstrating that this approach can induce broad immunity against ebolaviruses [81].

Some theoretical concerns have been brought forward as to a potential toxicity of GP, since expression of this protein at high levels causes cytotoxicity in vitro [82]. In order to address these concerns, a point mutant of GP (E71D) was developed, which is no longer cytotoxic, but remains immunogenic and can induce protective immunity in NHPs, although at least in combination with NP it induced a less robust protection [69].

rAdV vaccines induce a strong ebolavirus-specific $CD8^+$ T-cell response [68, 83]. However, neutralizing antibody responses are also observed after vaccination with these vaccines [68, 84] and antibody titers have been shown to correlate with protection [69, 85]. In order to investigate the mechanistic contributions of cellular and humoral responses to protection, antibody transfer and T-cell depletion studies were performed. Interestingly, transfer of antibodies from rAdV vaccinated animals alone did not confer protection to recipient NHPs. This was despite antibody titers in the recipients surpassing the titers seen in vaccinated animals and the fact that the antibodies showed neutralizing activity [84].

Similarly, depletion of B-cells did not reduce efficacy of the vaccine in NHPs, further speaking against a major role of antibodies for this vaccination approach. In contrast, depletion of $CD8^+$ T-cells significantly impaired the protective efficacy of the rAdV vaccine, with only 20 % survival of vaccinated $CD8^+$ T-cell-depleted animals, compared to 100 % survival in vaccinated controls. However, time to death in the $CD8^+$ T-cell-depleted animals was prolonged, suggesting that immune responses other than $CD8^+$ T-cells still play some role in protection. Interestingly, two other recent studies have shown that under some circumstances antibodies can be sufficient to confer protection in NHPs from otherwise lethal ebolavirus challenge [59, 60], indicating that mechanisms of protection from EHF might differ between different vaccine and treatment approaches and that correlates and mechanisms of protection have to be established for each vaccine platform separately.

Ebolavirus outbreaks are sporadic and unpredictable, rendering classical clinical efficacy trials impossible. In such circumstances, it is possible to attain FDA approval for a novel vaccine under the animal rule, which requires that efficacy is shown in animal models and that safety and immunogenicity indicative of a protective effect is shown in humans [85].

In order to obtain such safety and immunogenicity data, a phase I clinical trial was performed for the rAdV ebolavirus vaccine, which showed overall safety in 22 individuals [76]. This trial, which was designed to evaluate the rAdV vaccine as one component of a combined DNA/rAdV vaccination, also assessed the immunogenicity of the rAdV. After a single vaccination with 10^{10} particles of rAdVs expressing $GP_{1,2}$ from ZEBOV and SEBOV with the E71D mutation, all of the vaccines developed antibodies against SEBOV GP, and more than half developed antibodies against ZEBOV GP. Unfortunately, $CD8^+$ T-cell responses, which have been suggested as the main mechanism for protection with the rAdV-based ebolavirus vaccine, were lower, with only 9 % of vaccines showing $CD8^+$ T-cell responses to SEBOV GP and 21 % of vaccines to ZEBOV GP [76], suggesting that further studies aimed at increased immunogenicity, and possibly involving wild-type GP, will be required for this vaccine platform.

6.5.2 Recombinant Vesicular Stomatitis Virus-Based Vaccine

A second vaccine platform that is highly effective in NHPs is based on a recombinant vesicular stomatitis virus (rVSV). Vesicular stomatitis viruses belong to the family *Rhabdoviridae* and cause self-limiting disease in livestock, whereas infection of humans is rare and either asymptomatic or accompanied by mild flu-like symptoms [86]. The rVSVs used for ebolavirus vaccination have their glycoprotein gene replaced with the ebolavirus GP gene encoding $GP_{1,2}$ (rVSV/ΔG/GP)

(Fig. 6.4). In nonhuman primates, rVSV/ΔG/GP conferred 100 % protection after a single vaccination 4 weeks prior to challenge, with no detectable ebolavirus viremia after challenge [87].

In contrast to the rAdV used for ebolavirus vaccination, the rVSV remains replication competent, so that a relatively low dose (10^7 PFU rVSV vs. 10^{10} particles rAdV) is required for successful vaccination of NHPs [87], and at least in mice, it is possible to confer complete protection with as little as two PFU [88]. However, the replication-competent nature of the rVSV has also raised concerns with regard to vaccine safety. Indeed, a transient VSV viremia can sometimes be observed in vaccinated animals; however, in more than 100 vaccinated NHPs, no adverse effects were observed [66]. In animal models of VSV infection, pathogenicity, and particularly neurovirulence, has been linked to the G gene [89, 90], which is no longer present in rVSV/ΔG/ GP.

Consequently, this virus does not exhibit neurovirulence in NHPs, even when administered intrathalamically [91]. To further confirm the safety of the rVSV vaccine platform, NHPs infected with simian human immunodeficiency virus (SHIV) were vaccinated with rVSV/ΔG/GP, and despite their immunocompromised status, they did not develop any signs of clinical illness, fever, or local reactions at the vaccination site [92]. This study was of particular relevance not only because it demonstrated the safety of the rVSV but also because there is a high HIV prevalence in regions where ebolavirus outbreaks occur. In addition to being safe in the SHIV-infected NHPs, the rVSV vaccine was also able to protect 4 out of 6 SHIV-infected NHPs against lethal ebolavirus challenge [92], despite their immunocompromised status. Finally, no significant vaccine shedding has been observed in NHPs [86].

rVSV vaccines for a number of ebolavirus species have been developed and tested in NHPs [93, 94], and similar to the rAdV platform, blended rVSV vaccines have been shown to protect against multiple species in a single vaccination. In particular, a blend of three rVSV/ΔG/GP encoding glycoproteins from ZEBOV, SEBOV, and the related Marburg virus conferred protection not only to these virus species but also against challenge with CIEBOV [95].

In contrast, development of a single (non-blended) rVSV/ΔG/GP cross-protective among several species has been more challenging. Vaccination with an rVSV/ΔG/GP encoding for ZEBOV does not protect NHPs from lethal challenge with SEBOV [87], although it was partially protective against challenge with *Bundibugyo ebolavirus* [93]. Current attempts to produce a cross-protective rVSV-based ebolavirus vaccine focus on the generation of second-generation rVSVs, which encode several antigens, e.g., $GP_{1,2}$ and VP40 [96]. This approach shows promise in the guinea pig model; however, it has not been evaluated in NHPs, and currently several vaccinations are required in order to confer complete protection against lethal challenge.

The mechanism and correlates of protection for the rVSV vaccine are less well studied than for the rAdV vaccine. However, studies have shown that rVSV/ΔG/GP induces a strong antibody response in vaccinated NHPs, even though these antibodies are mostly non-neutralizing [97]. Also, both IFN-γ and IL-2 producing effector cells (T-cells and/or NK-cells) are produced in response to vaccination.

In mice it has been shown that $CD8^+$ T-cell depletion does not inhibit protection from a lethal ebolavirus challenge in rVSV/ΔG/GP-vaccinated animals, whereas serum transfer from vaccinated mice into naïve mice protected 80 % of these animals from an otherwise lethal challenge. This suggests that, at least in mice, the mechanism of protection for the rVSV/ΔG/GP vaccine is due to the humoral immune response, and independent of the cellular immune response. However, similar studies in NHPs are thus far lacking and will be required to give a more definitive answer as to what the mechanism and correlates of protection for this vaccine platform are.

One very remarkable feature of the rVSV vaccine is that it can be used for postexposure protection. In NHPs at least 50 % of animals were protected against an otherwise lethal ZEBOV challenge, and 100 % against an otherwise lethal SEBOV challenge, if they were vaccinated with

an rVSV vaccine expressing the homologous GP 30 min after challenge [94, 98].

For the related Marburg virus, postexposure vaccination was even possible 48 h after challenge, with 33 % of challenged animals surviving, whereas 83 or 100 % of animals survived Marburg virus challenge if the postexposure vaccination was performed 24 h or 30 min after challenge, respectively [99]. Similar to preventative vaccination, the mechanism of postexposure protection is not well understood. However, it was observed that there were marked differences in ebolavirus viremia between survivors and non-survivors, as well as in the humoral immune responses [94, 98]. Survivors showed only low-level viremia, whereas non-survivors developed viremia greater than 10^4 PFU/ml (with peak titers reaching 10^8 PFU/ml). Interestingly, this mirrors the situation in humans, where viral load has been shown to be predictive for the outcome of EHF [53, 100]. In terms of humoral response, survivors showed IgM titers about 6 days after challenge, and IgG titers 10 days after challenge, whereas non-survivors did not develop a detectable antibody response [94, 98].

In contrast to the rAdV vaccine, the rVSV vaccine has not yet progressed into clinical studies. However, based on its ability to confer postexposure protection in NHPs, it has been used as an experimental postexposure vaccine in one human laboratory worker after a needle-stick accident with an ebolavirus-contaminated needle in a BSL4 laboratory [101]. The patient received 5×10^7 PFU of the vaccine about 48 h after the incident and developed a light fever and myalgia 1 day after vaccination. These symptoms abated within less than a day, and simultaneously with them low-level viremia of the rVSV-vaccine was detectable by RT-PCR. Other than that no adverse effects were observed, and most importantly at no point did the patient develop any detectable ebolavirus viremia. While it is impossible to definitively determine whether they were infected, and completely protected by the vaccine, or whether the exposure did not actually lead to an EBOV infection, this incident serves as first indication that the rVSV vaccine is safe in humans.

6.6 Strengths and Weaknesses

Outbreaks of EHF occur sporadically and often in remote areas of Africa. Compared to other diseases, the overall case numbers are relatively low, although the effect on the public health system is often higher than anticipated based on case numbers alone, due to the closure of affected health facilities and the fact that health care providers are often heavily affected by EHF, particularly before outbreaks are recognized. Also, ebolaviruses are considered potential bioterrorism agents and are perceived by the public as a significant threat. Based on these properties, there are a number of possible exposure scenarios, each of which has unique requirements for an ideal vaccine [66].

Preventative vaccination of the complete population in the endemic area, while in theory the most rigorous solution, is not practical due to the size of the affected area, as well as logistical challenges. However, preventative vaccination of high-risk individuals such as health care providers, outbreak response personal, or laboratory staff working with ebolaviruses should be more feasible. In this scenario, a long lasting and broad immunity against as many ebolavirus species as possible (as well as the related Marburg virus) is of paramount importance. However, rapid protection is only of secondary importance, since vaccination would most likely occur long before a potential exposure. Also, preexisting immunity against the vaccine vector is less of an issue, since current data suggest that a prime-boost approach is able to confer protection even in the presence of vector immunity [79].

In contrast, during an outbreak situation (or a bioterrorist attack), vaccination of people in the affected areas in order to contain the outbreak by limiting person-to-person-spread would require rapid protection, ideally after a single vaccination. Under such circumstances preexisting vector immunity would constitute a significant problem. In contrast, a broad protection would be only of secondary importance, since it would most likely be possible to determine the exact virus species causing the outbreak. The only exception to this would be if an outbreak is

Table 6.2 Characteristics of recombinant adenovirus (rAdV) and recombinant vesicular stomatitis virus (rVSV) vaccines

	rAdV	rVSV
Replication competent	No	Yes
Required vaccination dose	10^{10} particles	$\leq 10^7$ PFU
Time required between vaccination and challenge for NHPs/guinea pigs/mice	4 weeks/4 weeks/4 weeks	4 weeks/3 weeks/1 day
Impaired by preexisting immunity	Yes, but multiple vaccinations overcome preexisting immunity	No
Protection against multiple species	Yes, single vaccine vector	Yes, blended vaccine mixture
Cross-protection against other/new ebolavirus species	Yes, depending on vaccine formulation	Yes, depending on vaccine formulation
Postexposure protection	No	Yes
Mechanism of protection	Mainly T-cell mediated	Unknown
Produced under GMP conditions	Yes	No
Development stage	Phase I clinical trials	Not progressed into clinical trials

Abbreviations: *PFUs* plaque forming units, *NHPs* nonhuman primates, *GMP* good manufacturing practice

caused by a new ebolavirus species, for which no species-specific vaccine would be available, so that the only way to achieve rapid protection would be through cross-protective properties of already existing vaccines.

Both vaccine platforms discussed here have properties that make them amenable for these scenarios (Table 6.2). The rAdV-based vaccine confers a broad protection, and preexisting immunity against the vector can be overcome using a prime-boost approach. This vaccine has advanced the furthest in terms of clinical trials, has been produced under GMP conditions, and experience with rAdV vaccines for other viral diseases has shown that it is possible to produce this vaccine in large quantities. It, therefore, represents a very feasible candidate for a preventative vaccine, although the results from the first clinical trials suggest that further optimization of this vaccine might be required in order to obtain maximum immunogenicity.

The rVSV-based vaccine provides a very rapid protection, and can even be used postexposure, making it extremely well suited for outbreak control or to be used in the case of a laboratory exposure. Preexisting immunity is of no concern, and rVSV-based vaccines for all relevant species of ebolaviruses are available. Unfortunately, this vaccine has not yet progressed into clinical trials, which might be partially due to a perceived safety risk, despite no adverse effect in more than 100 NHPs, including immunocompromised animals, as well as one human. Also, the mechanism and correlates of protection for this vaccine have not yet been elucidated, which will be necessary prior to moving this vaccine into clinical trials.

Overall, several very promising vaccine candidates for preventing and treating EHF are now available, and while further optimization and study of these vaccines is certainly necessary, the most important obstacles to overcome now in order to move these vaccines into clinical trials, and then further into the field, are of political, regulatory, and economic nature.

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Experimental Dengue Vaccines

7

Sathyamangalam Swaminathan and Navin Khanna

Contents

Abstract

Nearly half the global population is at risk of dengue, a viral disease transmitted to humans by *Aedes* mosquitoes. Each of four closely related dengue viruses of the *Flaviviridae* family causes a wide spectrum of disease ranging from mild dengue fever to potentially fatal dengue hemorrhagic fever and shock. Unambiguous clinical diagnosis of dengue disease is difficult and needs laboratory confirmation. Though a wide range of diagnostic tools has been developed, including antigen detection tests in recent times, the lack of well-characterized sera panels has precluded their validation. Once diagnosed, the only treatment option available to severe dengue patients is symptomatic and supportive medical care. The failure of vector control measures and the lack of vaccines and drugs have made dengue a significant public health problem worldwide.

Vaccination against dengue offers an effective way to arrest the spread of dengue. Increased awareness in recent years has helped intensify ongoing vaccine development efforts by multiple players. As a result, a multitude of vaccine candidates are in various stages of development. Of these, live attenuated viral vaccines are in advanced phase of clinical development, triggering expectation that a dengue vaccine is imminent. The need to protect against all four serotypes in order to avoid immune enhancement of disease and the lack of a preclinical animal model to reliably

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predict efficacy in humans have made dengue vaccine development a challenging task.

Recent efficacy trial data underline the need to understand better the role of the immune system in pathogenesis and protection to be able to design potentially efficacious dengue vaccines.

Dengue which is spread to humans by mosquitoes represents the most important arboviral disease of the twenty-first century [1, 2]. The prevalence and incidence of dengue have undergone a dramatic expansion in recent decades [3]. This appears to be the combined result of multiple factors that include massive unplanned urbanization, overpopulation, increasing global travel, and importantly the failure to eradicate mosquitoes [4]. Currently dengue is endemic to over a 100 countries in the Asia-Pacific region, the Americas, the Middle East, and Africa. World Health Organization (WHO) estimates suggest that more than >2.5 billion people live in dengue-endemic areas with about 50 million infections each year, 500,000 of which are severe, and lead to >20,000 deaths [5]. More recent studies indicate that the global dengue burden is probably much higher [1]. For example, about one-fifth of China falls within tropical latitudes and is potentially conducive to dengue transmission. Though this country reported dengue outbreaks to the WHO in the 1980s and 1990s, it has stopped reporting since. Outbreaks have been reported in the USA. Imported dengue fever from returning travelers and the identification of one of the dengue mosquito vectors in Europe portend the future spread of dengue to this continent as well [3].

Though dengue has not been historically considered as a significant public health problem, the realization that it can spread beyond the borders of the tropical world has spurred recent interest in understanding dengue and developing vaccines for its prevention [2]. This chapter will seek to provide concise background information on dengue followed by an overview of current vaccine development efforts outlining the issues and challenges that must be met and resolved.

7.1 Dengue Disease

Dengue infection results in a range of symptoms. Traditional WHO case definitions, based on four criteria, namely, fever, hemorrhage, thrombocytopenia, and plasma leakage, recognize the following distinct clinical conditions: classic dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [6]. Rather than discrete clinical entities, these three conditions likely represent progressively severe stages of a continuous spectrum of dengue disease (Fig. 7.1, *left panel*).

Classic DF is characterized by a sudden onset of high fever, which typically lasts for about 5–7 days. This febrile phase which coincides with viremia is associated with a host of symptoms. Usually a full recovery follows in most cases. A small fraction of DF patients may rapidly progress to DHF. As the fever falls, there may be sweating, restlessness, and signs of circulatory disturbance. Plasma leakage is the hallmark that distinguishes DF from DHF. When such leakage is substantial, it leads to life-threatening circulatory failure (hypovolemic shock) seen in DSS. Factors that predispose a DF patient to severe disease are not known. Clinically, defervescence (when fever subsides) is recognized as a critical phase. Symptoms such as bleeding, severe thrombocytopenia, and ≥20 % hemoconcentration, accompanied by severe and continuous abdominal pain, restlessness, persistent vomiting, sudden hypothermia with profuse perspiration, weakness, and fainting, herald the imminence of shock. Compared to DF, the viremia in DHF/DSS is an order two higher [7, 8]. A recent WHO classification, designed to identify severe dengue based on a single parameter, has replaced DF, DHF, and DSS with the following three entities: dengue without warning signs, dengue with warning signs, and severe dengue [9]. While the revised classification allows better case capture [10], it

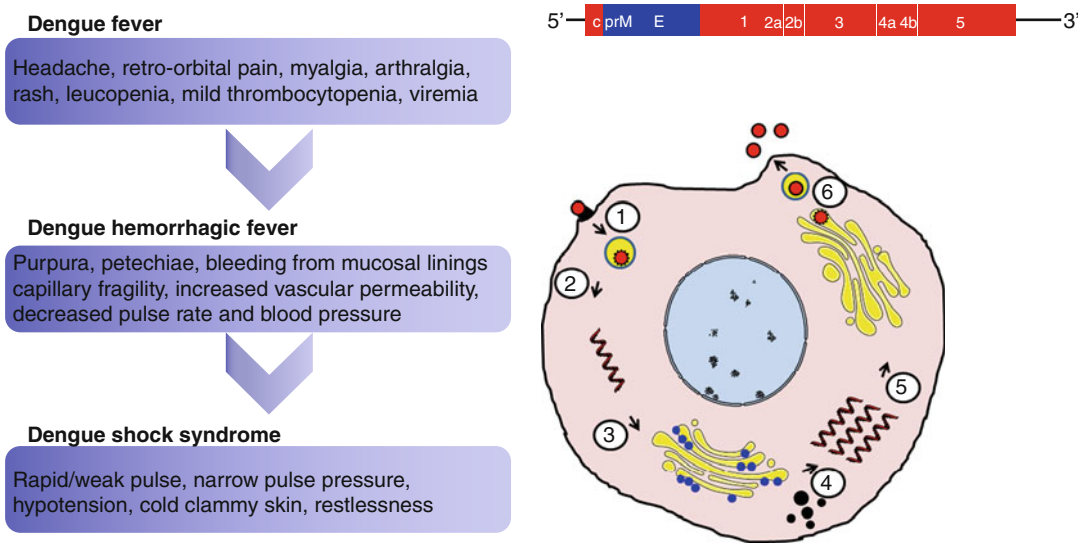

Fig. 7.1 Dengue disease, the virus, and its life cycle. The *left panel* shows a schematic representation of the symptoms through dengue disease progression. Shown at the *top* of the *right panel* is the DENV genome. The short *black lines* at either end of the genome denote the 5′and 3′ NTRs. The ten viral proteins encoded by single ORF are shown by the *red* (capsid and NS proteins) and *blue boxes* (*prM* and *E* proteins). The *bottom part* of the *right panel* depicts the DENV life cycle. The steps in the life cycle are shown by the encircled numbers: (*1*) receptor recognition and entry; (*2*) viral/host membrane fusion and uncoating; (*3*) translation of genomic RNA at the rough endoplasmic reticulum; (*4*) genomic RNA replication using NS proteins/further translation; (*5*) virion assembly and maturation; (*6*) final maturation and exit from the cell

has been felt that it can overload health-care facilities during outbreaks [10, 11].

7.2 Dengue Viruses

Dengue disease is caused by any of four closely related, but antigenically distinct dengue viruses (DENV-1, -2, -3, and -4). The DENVs belong to the genus *Flavivirus* of the family *Flaviviridae*. The mature DENV particle is about 50 nm in size and consists of a nucleocapsid core surrounded by a host-derived lipid bilayer coated with the viral structural proteins [12]. The DENVs as well as other members of the *Flavivirus* genus that include yellow fever (YF), Japanese encephalitis (JE), and tick-borne encephalitis (TBE) viruses share a similar genetic organization [13]. The flaviviral genome consists of an ~11 kilobase, 5′ capped, poly A tail-lacking, single-stranded plus (+) sense RNA. This RNA is comprised of a single open reading frame (ORF), flanked by 5′ and 3′ non-translated regions (NTRs), ~100 and ~450 nucleotides (nt) long, respectively (Fig. 7.1, *top right*). The NTRs contain unique sequence and structural elements that mediate genomic RNA circularization and RNA/protein interactions which are critical for the virus life cycle [2, 14–16]. The ORF encodes ten viral proteins, three of which are structural [capsid, C, membrane, M (which is synthesized as the larger precursor, prM), and envelope, E] and the rest nonstructural (NS) proteins, NS1, 2a, 2b, 3, 4a, 4b, and 5 [13].

The E protein which is anchored on the lipid bilayer represents the major structural antigen of the virus. Ninety homodimers of E form a "herringbone" array covering the entire virion surface [12]. It binds to an as yet unidentified host cell surface receptor, mediates membrane fusion, and serves as the major target of the host neutralizing antibody response [13, 17]. Each E monomer is comprised of three distinct domains [18], of which the carboxy-terminal envelope domain III

(EDIII), which protrudes out from the virion surface [12], is implicated in host receptor binding [19], and contains multiple serotype-specific conformational neutralizing epitopes [17, 20]. Following receptor binding and internalization, the E homodimer rearranges to a homotrimer, in response to the acidic environment of the endosome. This is necessary for membrane fusion and release of the viral genomic RNA into the cytoplasm [21].

The single ORF of the viral RNA directs the synthesis of a >3,000 amino acid (aa) polyprotein precursor. The combined action of viral and host proteases processes this precursor into the mature viral proteins listed above [13]. Viral RNA replication, which also takes place in the cytoplasm, proceeds through a complementary minus (–) RNA intermediate and is mediated predominantly by NS3 and NS5 which together provide viral RNA replicase functions. Following viral RNA and protein synthesis, virion morphogenesis takes place to initially produce immature virion particles covered with prM-E heterodimers [22]. The association of prM with E is believed to help prevent premature membrane fusion of the latter during virus maturation through the acidified sorting compartments. Just prior to release by exocytosis, host furin-mediated cleavage of prM triggers the final maturation step concomitant with release of mature virus particles [21] (Fig. 7.1).

7.3 Transmission

DENVs are transmitted to humans by *Aedes* mosquitoes, *Aedes aegypti*, *A. albopictus*, and *A. polynesiensis* [23]. The dense human population, the high prevalence of the mosquito vectors, and the co-circulation of multiple DENV serotypes, in many of the urban cities of the world, provide the ideal situation to sustain efficient dengue transmission between humans and mosquitoes [4] (Fig. 7.2). *A. aegypti* serves as the major dengue vector because it is highly adapted to the urban environment. DENV is transmitted by the bite of an infective female *A. aegypti* mosquito to a human host. Once in the human host, the virus initiates the intrinsic phase of its life cycle. Disease symptoms become evident after an incubation period of 4–7 days, when virus appears in the blood. The viremia which lasts for ~5 days coincides with the febrile phase which lasts 5–7 days. The human is thus an amplifying host.

The virus load at this viremic phase appears to correlate with disease severity [7, 8]. High viremia can facilitate efficient transmission. When an uninfected mosquito feeds on a viremic host, it acquires the virus, which now undergoes the extrinsic phase of its life cycle in the mosquito host. At the end of this phase (~8–12 days), the mosquito becomes infective for the rest of its lifespan. The cycle continues when this mosquito bites another human host to refeed. A single infective mosquito which bites several times during a blood meal can transmit the virus to several individuals in a short time span [4].

7.4 Diagnosis and Treatment

As clinical diagnosis may not be unambiguous, laboratory diagnosis is essential for confirming dengue infection. Infection may be confirmed based on the identification of the DENV, its genomic RNA, its antigens, or the antibodies it elicits [2, 4, 24] (Fig. 7.2). Currently there is no antiviral therapy available for dengue. Symptomatic and supportive medical care is the only means available for the treatment [6].

7.4.1 Laboratory Diagnosis

DENV isolation by mosquito inoculation or cell culture and its identification is possible if patient sera are collected during the febrile phase. Viral RNA can be detected and serotype identified using either coupled reverse transcription polymerase chain reaction (RT-PCR) [25–27] or isothermal RNA-specific amplification [28]. Antigen detection tests in multiple formats, designed to detect the viral NS1 protein which appears early in the bloodstream [29, 30] and may serve as a predictive marker of severe disease [31], and NS1-specific IgM and IgG tests

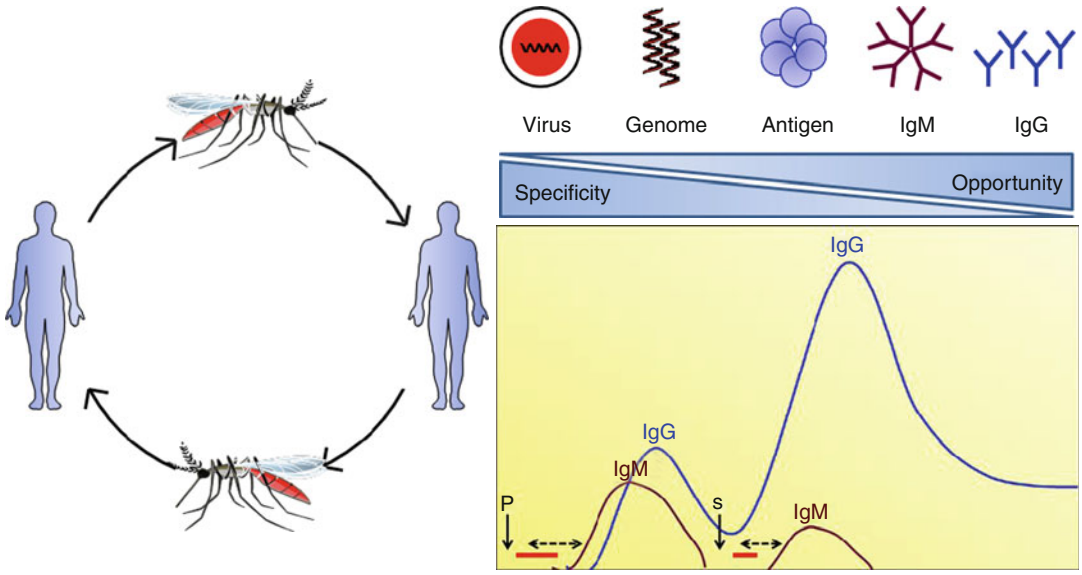

Fig. 7.2 Aspects of dengue transmission, diagnostics, and antibody response. The *left panel* depicts the urban epidemic dengue transmission cycle by *A. aegypti*. As humans serve as the amplifying host, DENV transmission between humans and mosquitoes proceeds efficiently without the need for a sylvatic amplification host. The *top part* of the *right panel* is a schematic depiction of the relative pros and cons of laboratory diagnosis of DENV infection using direct (the virus, its genome, and the antigens it encodes) and indirect (the antibodies elicited by DENVs) markers. Direct markers can be detected only in a limited time window. IgM and IgG are more practical markers for diagnostic confirmation (the loss in specificity arises from flavivirus cross-reactivity). The *bottom part* of the *right panel* is a pictorial representation of the qualitative nature of the antibody response (the vertical and horizontal axes represent antibody levels and time after DENV infection, respectively). First infection (*P*) of a flavivirus-naïve individual elicits a primary antibody response, characterized by the induction of IgM antibodies at ~3–5 days of illness, which peaks at ~2 weeks and declines over the next couple of months. IgG antibodies appear shortly thereafter and persist for life. During a repeat infection (*S*), a secondary antibody response is mounted wherein high-titer IgG antibodies appear either before or along with a comparatively muted IgM antibody response. The window of viremia (indicated by the *solid red bar* just above the horizontal axis) coinciding with the febrile phase is abbreviated during the secondary infection. The NS1 antigen detection window (indicated by the *dashed double-headed arrow*) overlaps with viremia and is also truncated in secondary infections

have been developed recently [24]. Commercial NS1 antigen detection tests in different formats have become available a few years ago [32, 33].

Historically, the hemagglutination inhibition (HI) test, which is the WHO standard test for confirming recent DENV infection, measures the ability of antibodies in paired acute and convalescent sera to inhibit virus glycoprotein-mediated erythrocyte agglutination. A ≥4-fold difference in HI titers of the paired sera is diagnostic of recent infection [6]. The plaque reduction neutralization test (PRNT) [34] and other ELISA [35] and flow cytometry-based [36] virus neutralization assays, which are useful in estimating neutralizing antibody titers and identifying DENV serotypes, are used mainly in vaccine research.

Routine serological tests for dengue utilize ELISA-based antibody detection as these are relatively inexpensive and easy to perform [4, 24]. As IgM antibody titers tend to be relatively lower (Fig. 7.2, *lower right*), their identification is based on capture using antihuman IgM-specific antibodies bound to a solid phase, followed by detection using a mixture of the four serotypes of DENV antigens. A major limitation is the scope for false positivity arising from DENV-specific IgG and cross-reactivity with other flaviviruses [4, 24]. IgG antibodies on the other hand can be detected in indirect ELISA format, using the same DENV antigens. IgG ELISA on paired sera can be used to confirm dengue infection. Due to extensive cross-reactivity within the flavivirus

serocomplex [17], the IgG ELISA lacks DENV specificity. A novel strategy to eliminate this problem is based on creating synthetic multi-epitope protein (MEP) antigens that incorporate carefully chosen DENV-specific epitopes. MEP antigens have shown promising results in initial studies of their diagnostic utility [37–40]. The ratio of IgM to IgG antibodies, both detected using a capture format, can be used to distinguish primary from secondary DENV infection [41]. Several commercial DENV antibody detection kits of varying sensitivity and specificity are available commercially. The lack of well-defined sera panels from primary and secondary DENV infections from different endemic regions, as well as from non-DENV flavivirus infections and other febrile infections, has precluded meaningful validation of these kits.

7.4.2 Clinical Management

For uncomplicated DF cases, the treatment consists of bed rest, oral rehydration, and paracetamol as an antipyretic and analgesic. Aspirin is not recommended as it can contribute to platelet dysfunction. Both clinical and laboratory monitoring are essential from fever day three onwards, around defervescence. Clinical signs that signal progression to serious disease include cold limb extremities, low pulse, low urine output, signs of mucosal bleeding, and abdominal pain. A rising hematocrit (≥20 %) and a falling platelet count (10e6/ul) strongly indicate DHF. If any of these signs are detected, immediate hospitalization is necessary.

The key component of clinical management of DHF patients is based on intravenous fluid therapy to maintain effective circulation during plasma leakage plus careful clinical monitoring of hematocrit, platelet count, pulse rate and blood pressure, temperature, urine output, fluid administered, and other signs of shock. As the rate of plasma leakage can vary, it is necessary to adjust fluid therapy accordingly. Many patients recover quickly after 12–48 h of fluid therapy. Prolonged fluid therapy is risky as it can cause pulmonary edema when the lost plasma is reabsorbed. In case of DSS the key to saving the patient is early recognition that the patient is in shock. Treatment consists of immediate and aggressive fluid therapy with colloids to restore an effective circulating volume, meticulous and extensive monitoring, and management of any complications. Poor clinical condition of the patient coupled to falling hematocrit, despite adequate fluid therapy, is indicative of internal hemorrhage and must be treated with whole blood transfusion. Timely and optimal clinical care can reduce case fatality rates significantly [6].

7.5 Vaccine Development

The four DENV serotypes are co-prevalent in several countries around the world, and each of these is capable of causing dengue disease. Immunity to any one DENV is durable and serotype specific, with only transient cross protection against the remaining three DENV serotypes [42]. Importantly, DENV infections tend to be serious in those with prior immunity to a different DENV serotype, acquired either passively through maternal transfer or actively through a primary infection [43–45]. The very high viremia seen in severe DENV infections [7, 8] has been implicated in triggering a vigorous cytokine storm that is believed to underlie endothelial damage and capillary leakage [2, 17, 46]. It has been hypothesized that cross-reactive antibodies can bind to heterotypic DENV and facilitate its increased uptake into Fc receptor-bearing cells, such as monocytes and macrophages, believed to be the in vivo sites of DENV replication. This phenomenon, termed antibody-dependent enhancement (ADE), may be responsible for increased virus replication resulting in severe disease [17, 45].

Recent in vitro and in vivo studies have uncovered a role for highly cross-reactive antibodies to prM protein in mediating enhancement and causing severe disease [47, 48]. Original antigenic sin, causing the expansion of low-avidity preexisting memory T cells incapable of effectively clearing heterotypic virus, may be another factor contributing to increased virus load in secondary

Table 7.1 DENV strains used in the replicating viral vaccines[a]

DENV[b]	Mahidol[c]	WRAIR/GSK[d]	CYD[e]	DENVax[f]	TetraVax[g]
1	16007 (PDK-13)	45AZ5 (PDK-20/27)	PUO-359	16007	West Pac
2	16681 (PDK-53)	S16803 (PDK-50)	PUO-218	16681	New Guinea C
3	16562 (PGMK-30)	CH53489 (PDK-20)	PaH881/88	16562	Sleman/78
4	1036 (PDK-48)	341750 (PDK-20/6)	1228	1036	814669

[a]The strains indicate DENVs used for the empirically attenuated Mahidol and WRAIR/GSK vaccines, for CYD, DENVax, and TetraVax vaccines; the strains indicate the donor virus from which *prM* and *E* genes were used for vaccine virus construction
[b]This column indicates DENV serotype
[c]The passage number of the attenuated strain is indicated in brackets; DENV-1, -2, and -4 were passaged in primary dog kidney (PDK) cells, while DENV-3 was passaged in primary green monkey kidney (PGMK) cells as it failed to grow in PDK cells (data from ref [50])
[d]The passage number of the attenuated strain is indicated in brackets; DENV-1/PDK-20 was replaced by DENV-1/PDK-27, and DENV-4/PDK-20 was replaced by DENV-4/PDK-6 in the lead formulation (#17)
[e]Sanofi's chimeric yellow fever 17D vector-based dengue vaccine
[f]CDC/Inviragen intertypic chimeric vaccine based on Mahidol DENV-2/PDK-53 vector backbone; intertypic chimeric constructs specific to DENV-1, -DENV-3, and DENV-4 carry the *prM* and *E* genes of the corresponding Mahidol vaccine strains
[g]NIH vaccine based on Δ30 mutant vector backbone

DENV infections [49]. Thus, a dengue vaccine that confers incomplete immunity may sensitize a vaccine recipient to severe dengue disease, as all four DENV serotypes co-circulate globally. This safety concern underlies the opinion that a dengue vaccine must be tetravalent, capable of affording durable protection against infection by all four serotypes simultaneously.

7.5.1 Experimental Dengue Vaccines

Many different strategies are being pursued with experimental vaccine candidates in a range of preclinical to clinical phases of development [2, 24, 50–54]. But for a few exceptions, the majority of these are based on creating monovalent vaccines, targeting each of the DENV serotypes and then mixing these together into a single tetravalent dengue vaccine formulations. Historically, empirical attenuation has been the major focus of dengue vaccine efforts. Two groups, one in Mahidol University in Thailand [55] and the other at Walter Reed Army Institute for Research (WRAIR) in USA [56], independently developed monovalent DENV vaccines for each of the four serotypes, by serial passaging in primary dog kidney (PDK) cells (Table 7.1). Both of these vaccines, which are not being pursued anymore, have given way to reverse genetic-based approaches to attenuation that exploit infectious clone technology [57]. Recombinant technology has also spawned vaccine candidates that are based on DENV structural antigens either encoded by genetic vectors or produced as recombinant antigens using heterologous host expression systems.

7.5.1.1 Replicating Viral Vaccines

Using reverse genetics, scientists at Acambis Inc., USA, have replaced the *prM* and *E* genes of the empirically attenuated yellow fever vaccine virus (YF17D) vector with corresponding DENV genes and developed four chimeric constructs, each carrying the *prM* and *E* genes of one DENV serotype [58–60]. These chimeric yellow fever-dengue (CYD) vaccines are frontrunners in the dengue vaccine development pipeline. The use of reverse genetics has revealed that alterations in the 3′ NTR can lead to attenuation.

For example, this approach led to the identification of a 30-nucleotide deletion mutant of DENV-4 [61]. This deletion (Δ30 mutation) which removes nucleotides 10,478–10,507 in the 3′ NTR provided an acceptable balance between attenuation and immunogenicity in monkeys [61]

and humans [62]. Subsequent work by investigators at the US National Institutes of Health (NIH) revealed that while the Δ30 mutation conferred attenuation on DENV-1 [63], but not on DENV-2 [64] and DENV-3 [65], for unknown reasons. Using the antigenic chimerization strategy devised earlier for CYD vaccine creation, the *prM* and *E* genes of DENV-4 Δ30 mutant have been replaced with the corresponding genes of DENV-2 [66] and DENV-3 [65]. The latter two variants are intertypic chimeric constructs whose serotypes correspond to those of the cognate *prM*+*E* gene donors. Scientists at the US Center for Disease Control and Prevention (CDC) have developed intertypic chimeras based on the use of the DENV-2 PDK-53 Mahidol vaccine virus as the backbone to carry the *prM*+*E* genes of DENV-1, DENV-3, and DENV-4 [67]. As the parent DENV-2 PDK-53 virus does not carry any of its attenuating mutations in its *prM* and *E* genes [68], it can directly serve as the DENV-2 vaccine candidate.

A new approach to develop host-range DENV mutants has been reported recently [69]. These mutants which have deletions in the transmembrane domain of the E protein show preferential growth in insect cells and impaired infectivity in mammalian cells and retain the capacity to induce virus-specific neutralizing antibodies.

7.5.1.2 Vectored Vaccines

This class of experimental dengue vaccines is based on the use of either a plasmid [70, 71] or a virus [72–75] vector encoding DENV antigen genes. Though attenuated pox virus vectors encoding DENV antigens have been described in the past [72], more recently West Nile (WN) virus [75] and human adenovirus type 5 (hAd5) [74] vectors have been used for expressing DENV antigens. Though the plasmid-based vaccines eliminate the safety risk of the replicating viral vaccines above, they do not induce a robust immune response. This shortcoming is addressed by replacing the plasmid vector with viral vectors with built-in safety features. For example, the WN vector mentioned above is programmed to undergo one cycle of infection in the vaccinated host by deleting its capsid gene, while the hAd5 vector is rendered replication-defective by deleting its early region one which is essential for replication.

Many of the plasmid and virus-vectored vaccine candidates encode both *prM* and the *E* genes and possess the ability to generate virus-like particles (VLPs) in vivo and are based on physical mixtures of four monovalent vaccines to develop a tetravalent formulation [71, 75], similar to the approach being adopted for the replicating viral vaccines. However, some are based on unique approaches to elicit immunity against all DENV serotypes. For example, one plasmid-based strategy utilizes a single shuffled E gene containing epitopes representing all four DENV serotypes [70], while another utilizes two replication-defective hAd5 vectors, each encoding the *prM*+*E* genes of two DENV serotypes [74]. Plasmid- [76, 77] and virus-vectored [78–80] vaccines encoding EDIII-based antigens have also been reported in the literature. Some of these are based on a single tetravalent construct incorporating EDIIIs of all four DENV serotypes [77, 80, 81].

7.5.1.3 Non-Replicating Vaccines

Apart from the plasmid vaccines above, a conventional approach to developing non-replicating dengue vaccines is based on the use of inactivated virus preparations. Purified inactivated virus (PIV) as a vaccine offers the advantage of conferring immunity without the risk of infection. However, it is essential to formulate it in a good adjuvant to elicit robust immunity. It was shown in the past that DENV-2 can be inactivated with low concentrations of formalin without compromising its immunogenicity [82]. Until recently, concerns regarding low growth titers and potential adverse effects of inactivation on vaccine antigenicity had relegated PIVs to the background [83]. However, in recent years there has been renewed interest in developing DENV PIVs [84, 85]. The WRAIR in collaboration with GSK has accelerated the development of PIVs for all four DENV serotypes (Table 7.2).

Recombinant subunit vaccines offer another noninfectious alternative like PIVs. Similar to PIVs, these also need adjuvants to augment their

Table 7.2 Summary of tetravalent dengue vaccines in clinical trials

Vaccine[a]	Dose[b]	Trials[c]	Phase	Trial site	Current status (end date)
CYD (Sanofi)	3 (0, 6, 12)	6 (>33,000)	III	Australia, Latin America, and SE Asia	Ongoing (Aug 2016)
		1 (~4,000)	IIb	Thailand	Interim results published (Mar 2014)[d]
		11 (~3,600)	II	USA, Australia, Latin America, SE Asia, and India	5 completed,[e] 6 ongoing (Jun 2015)
TetraVax-DV (NIH)	2 (0, 6)	1 (300)	II	Brazil	Yet to start (May 2018)
		3 (374)	I	USA	Ongoing (May 2015)
DENVax (CDC-Inviragen)	2 (0, 3)	1 (344)	II	Latin America and SE Asia	Ongoing (Oct 2016)
		4 (416)	I	Colombia and USA	1 completed; 3 ongoing (Sept 2013)
LAV (WRAIR/GSK)	2 (0, 6)	7 (~1,100)	I/II	USA, Thailand, and Puerto Rico	All completed (Apr 2010) [6] On hold
TDENV-PIV (WRAIR/GSK)	2 (0, 1)	2 (200)	I	USA and Puerto Rico	Ongoing (Dec 2015)
TVDV (WRAIR)	3 (0, 1, 3)	1(40)	I	USA	Ongoing (Dec 2012)
V180 (Merck)	3 (0, 1, 2)	1 (120)	I	Australia	Ongoing (Dec 2014)

[a]Names of the vaccine as referred to by the developers (who are shown in parenthesis): *CYD* chimeric dengue vaccine viruses in YF17D backbone, *TetraVax-DV* dengue vaccine viruses carrying DENVΔ30 deletion, *DENVax* dengue vaccine viruses in DENV-2 PDK-53 background, *LAV* dengue vaccine viruses empirically attenuated by serial passaging, *TDENV-PIV* purified inactivated virus vaccine, formulated in ISCOMATRIX or alum, *TVDV* plasmid DNA vaccines, and *V180* recombinant E ectodomain (insect cell-expressed) subunit vaccine

[b]Number of vaccine doses (the numbers in parenthesis indicate the months at which these doses are given)

[c]Number of trials for each vaccine candidate (the total number of participating trial subjects is indicated in parenthesis)

[d]Interim data published in *ref*. [86]

[e]Data for one trial published in *ref*. [87]

immunogenicity, but eliminate the yield limitation of PIVs, as they can be produced to high levels in heterologous host expression systems. For a variety of reasons enumerated earlier, the E protein and EDIII have been the focus of recombinant vaccine efforts. Recombinant DENV E proteins have been expressed in yeast [88–90] and insect [91, 92] expression systems, purified to varying degrees of homogeneity and analyzed as immunogens in mice and monkeys.

All these studies have focused on the DENV E amino-terminal 80 % of the molecule known as the ectodomain. Of these, the DENV E ectodomain antigen of the four serotypes produced using the *Drosophila* expression system [92] has the backing of an industrial developer (Table 7.2). Recombinant antigens based on DENV EDIII have been explored by different groups using *E. coli* and yeast expression hosts. Recombinant EDIII antigens expressed either independently or fused to different carriers such as maltose-binding protein [93] and the *Neisseria meningitidis* p64k protein [94–96] have demonstrated their potential in evoking anti-DENV immune responses. Some groups have incorporated the EDIIIs of the four DENV serotypes into a single construct to design tetravalent immunogens [97, 98]. The enthusiasm to develop EDIII-based vaccines has been tempered down recently by reports that in natural DENV infections the fraction of antibodies targeted to EDIII is very small [99].

A newly emerging approach to recombinant DENV subunit vaccines is based on the virus-like particle (VLP) platform. Recombinant structural antigens of many viruses possess an intrinsic propensity to assemble into VLPs [100]. These VLPs which display an ordered repetitive array of epitopes are powerful immunogens [101, 102]. The prM and E proteins of DENVs co-expressed in heterologous hosts co-assemble into VLPs. Reports in the literature show that mammalian [103], insect [104], and yeast [105, 106] cells can host the production of DENV VLPs.

From the perspective of VLPs for vaccine use, the yeast system may be more suitable, as it has the potential for higher yields. Recent work indicates that yeast-expressed DENV E ectodomain forms VLPs in the absence of prM [53]. Another approach is based on displaying the DENV EDIII on VLPs formed by hepatitis B virus surface or core antigens [53, 107] or on Qβ VLPs [53].

7.5.2 Current Status of Dengue Vaccines in Development

For most dengue vaccine approaches, the standard strategy has been to develop the four monovalent candidates separately and evaluate them singly first and then in combination. As a result dengue vaccine development has turned out to be much more of a long-drawn process. Several candidate vaccines have reached clinical testing phase [108], and those being tested as tetravalent formulations are summarized in Table 7.2. All of these vaccines are expected to require at least 2 doses to achieve adequate seroconversion to all 4 DENV serotypes.

Of the three replicating viral vaccines in development, Sanofi's CYD vaccine is the frontrunner undergoing as many as six different phase III trials in multiple dengue-endemic locations involving several thousands of trial volunteers. Several phase II studies have been completed, and results from a pediatric trial in Peru concluded that a 3-dose regimen of the tetravalent CYD vaccine had a good safety profile in children with prior YF vaccination, eliciting a balanced tetravalent immune response [87]. Interim data from a proof-of-concept CYD vaccine efficacy trial involving ~4,000 children in Thailand, a dengue-endemic country, revealed an overall efficacy of ~30 % [86]. The CYD vaccine failed to protect against DENV-2 infection, prompting serious debate in the dengue vaccine research community.

DENVax, the attenuated vaccine based on DENV-2 PDK-53 backbone, showed protective efficacy against DENV-1 and DENV-2 in the AG129 mouse challenge model [109]. AG129 mice which carry interferon-α/β and γ-double receptor knockout mutations manifest dengue-like symptoms upon challenge with certain virulent DENV strains [110]. DENVax has undergone phase I testing in one location and has progressed to a phase II trial in Latin America and Southeast Asia. Multiple monovalent Δ30 vaccine viruses, directed against each of the DENV serotypes, have been extensively evaluated in clinical trials to devise different tetravalent formulations [111], referred to as TetraVax-DV, which are currently in phase I trials.

It is to be noted that GSK which was actively codeveloping the empirically attenuated replicating viral vaccine with WRAIR up to phase II has stopped further development. The WRAIR/GSK vaccine phase I/II trial data show that the lead tetravalent formulation (#17), after two doses at 0 and 6 months, elicited 54 and 63 % tetravalent seroconversion, respectively, in infants [112] and adults [113]. The non-replicating vaccines, which are in phase I, include WRAIR's inactivated virus (TDENV-PIV) and plasmid DNA (TVDV) vaccines and Merck's recombinant protein vaccine (V180). All other vaccine candidates are in preclinical development with the hAd5-based tetravalent vaccine apparently in preclinical efficacy testing in nonhuman primates.

7.6 Challenges

Dengue vaccine development faces several significant hurdles. Unlike the YF, JE, and TBE vaccines, which target single flaviviruses [17, 83], a dengue vaccine needs to be tetravalent, targeting all four DENV serotypes. Antibodies are

involved in protection as well as enhancement. Developing a safe and efficacious vaccine that is able to simultaneously protect against all four DENVs without sensitizing the vaccine recipient to severe disease is a major challenge. The interference between monovalent replicating vaccine viruses when mixed into a tetravalent formulation has been an unexpected hurdle. First seen more than a decade ago with the Mahidol vaccine [114], it also plagued the WRAIR/GSK vaccine [115, 116].

After several attempts that involved replacing some of the monovalent vaccine viruses and several readjusted tetravalent formulations [113, 115–120], both of these vaccines have been shelved. That the challenge of viral interference is not unique to only the empirically attenuated virus vaccines is evident from the susceptibility of the replicating virus vaccines created through reverse genetics approach. The CYD [121], TetraVax-TV [111], and DENVax [109] vaccines have all revealed the potential for interference. Understanding and resolving interference is critical to the success of these replicating viral vaccines.

Another challenge is to identify the correlates of protective immunity so that efficacy of experimental vaccines can be assessed reliably. Neutralizing antibodies are assumed, but not established definitively, to be indicators of a vaccine's protective efficacy. Several lines of evidence suggest the importance of neutralizing antibodies in protection against dengue. For example, the resistance of infants to DENV infection in dengue-endemic areas, during the early months of their lives, has been correlated to the presence of maternal DENV-neutralizing antibodies in their circulation [122]. Much less is known about the contribution of cell-mediated immune responses to protection. Finally, the lack of a predictive animal model has been a very significant hurdle in the preclinical assessment of vaccine candidates. Neither the mouse nor the monkey model used in preclinical assessment of experimental vaccines manifests dengue disease and is of limited value in predicting a vaccine's efficacy in humans. This has been underscored by recent CYD vaccine phase IIb trial data [86].

7.7 Conclusion

Dengue is prevalent in more than a 100, predominantly tropical/subtropical, countries around the world. The potential for this disease to spread beyond the tropical world has spurred intense efforts to develop vaccines for its prevention. The growing vaccine pipeline and the advance of many live viral vaccine candidates to clinical trials have contributed to the expectation that a dengue vaccine could be available in the near future. However, vaccine initiatives face immense challenges stemming from the unique biology and epidemiology of the DENVs that cause this disease. Recent efficacy trial results from a leading live attenuated vaccine candidate reveal that prediction of vaccine immunogenicity may not necessarily be predictive of the vaccine's protective efficacy. This has stimulated renewed interest in several non-replicating vaccine approaches. However, it is becoming increasingly apparent that there are huge knowledge gaps in the current understanding of the mechanisms underlying immune enhancement, viral interference, and protection against infection. With humans representing the only model for testing dengue vaccine candidates, it is necessary to gain a better understanding of these phenomena to enable rational design of vaccines with greater chance of conferring protection.

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Virus-like Particle Vaccines for Norovirus Gastroenteritis

8

Qiang Chen

Contents

Abstract

Gastroenteritis (GE) and its associated diarrheal diseases remain as one of the top causes of death in the world. Noroviruses (NoVs) are a group of genetically diverse RNA viruses that cause the great majority of non-bacterial gastroenteritis in humans. However, there is still no vaccine licensed for human use to prevent NoV GE. The lack of a tissue

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culture system and a small animal model further hinders the development of NoV vaccines.

Virus-like particles (VLPs) that mimic the antigenic architecture of authentic virions, however, can be produced in insect, mammalian, and plant cells by the expression of the capsid protein. The particulate nature and high-density presentation of viral structure proteins on their surface render VLPs as a premier vaccine platform with superior safety, immunogenicity, and manufacturability. Therefore, this chapter focuses on the development of effective NoV vaccines based on VLPs of capsid proteins. The expression and structure of NoV VLPs, especially VLPs of Norwalk virus, the prototype NoV, are extensively discussed. The ability of NoV VLPs in stimulating a potent systemic and mucosal anti-NoV immunity through oral and intranasal delivery in mice is presented.

The advantages of plant expression systems as a novel production platform for VLP-based NoV vaccines are discussed in light of their cost-effectiveness, production speed, and scalability. Recent achievements from the first successful demonstration of NoV VLP production in plant expression system under the current Good Manufacture Practice (cGMP) regulation by the US Food and Drug Administration (FDA) are detailed.

Moreover, results of human clinical trials demonstrating the safety and efficacy of insect and plant-derived NoV VLPs are also presented. Due to the diversity of capsid protein among different NoV strains and its rapid antigenic drift, we speculate that vaccine development should focus on multivalent VLP vaccines derived from capsid proteins of the most prevalent strains. With the very recent approval of the first plant-made biologics by the FDA, we also speculate that plant-based production systems will play an important role in manufacturing such multivalent VLP-based NoV vaccines.

8.1 Gastroenteritis

Gastroenteritis (GE) is a worldwide health problem that affects people of all ages. As its name implies, GE is characterized by inflammation of the gastrointestinal tract and often associated with symptoms of diarrhea, nausea, vomiting, and abdominal cramping and pain. GE and its associated diarrheal diseases remain as one of the top causes of death in the world especially in developing countries and in young children with an estimated death toll of four to six million per year [1, 2]. GE is also the most common disease and the leading cause of morbidity in the developed world. For example, it is suggested that in the United States (USA), everyone is likely to suffer from viral GE at least once a year, 610,000 will be hospitalized, and at least 4,000 will die from this disease [1]. GE can be caused by a variety of pathogens including viruses, bacteria, and parasites and by ingestion of noninfectious toxins or medications, with viruses as the most common offending agents [1]. Norovirus (NoV) and rotavirus are the most common viruses that cause viral GE, while adenovirus, astrovirus, coronavirus, and parechovirus are also known to cause GE in humans [1–3].

8.2 Noroviruses

NoVs are a group of genetically diverse RNA viruses that belong to the genus of *norovirus* in the *Caliciviridae* family [4, 5]. They were first discovered and characterized in their prototype virus, the Norwalk virus (NV) in 1972 [6, 7]. Studies of NV revealed that NoVs are nonenveloped viruses with an RNA genome surrounded by a round capsid protein shell approximately 38 nm in diameter [8]. Classification of NoVs, however, has proved difficult and controversial until the recent development of molecular biology tools. Since there is no culture system available to grow these viruses in vitro, accurate serotyping based on neutralization is not possible [9]. Instead, classification of NoV had to rely on cross-challenge studies in

Table 8.1 Classification of noroviruses

Genogroups	Animal hosts	Genotype clusters	Representative virus strains
GI	Human	GI.1	NV-USA93, Wtchest-USA, KY89-JPN
		GI.2	SOV-GBR93, C59-USA, FB258-JPN
		GI.3	DSV-USA93, LR316USA, VA98115-USA
		GI.4	Chiba-JPN00, Valetta-MLT, NO266-USA
		GI.5	Musgrov-GBR00, AB318-USA, SzUG1-JPN
		GI.6	Hesse-DEU98, CS841-USA, WUG1-JPN
		GI.7	Wnchest-GBR00
		GI.8	Boxer-USA02
GII	Human and Pig	GII.1	Hawaii-USA94, Miami81-USA, DG391-DEU
		GII.2	Msham-GBR95, CF434-USA, SMV1-USA
		GII.3	Toronto-CAN93, MD102-USA, NLV2004-SWE
		GII.4	Bristol-GBR93, Minerva, NT104-JPN
		GII.5	Hilingd-GBR00, MOH99-HUN, NO306-USA
		GII.6	Seacrof-GBR00, SU3-JPN, Miami292-USA
		GII.7	Leeds-GBR00, GN273-USA
		GII.8	Amstdam-NLD99, SU25-JPN
		GII.9	VABeach-USA01, Idafall-USA
		GII.10	Erfurt-DEU01
		GII.11	SW918-JPN01, SWVA34-USA, SW43-JPN
		GII.12	Wortley-GBR00, U1GII-JPN, Pirna110-DEU
		GII.13	Faytvil-USA02, KSW47-JPN,
		GII.14	M7-USA03
		GII.15	J23-USA02, Mex7076-USA
		GII.16	Tiffin-USA03, Fayett-USA, Tonto-USA
		GII.17	CSE1-USA03
GIII	Cattle	GIII.1	BoJena-DEU98
		GIII.2	BoCH126-NLD00, BoNA2-GBR, BoCV95-USA
GIV	Human	GIV.1	Alphatn-NLD99, FLD560-USA, SCD624-USA
GV	Murine	GV.1	Murine1-USA03

Table is generated based on results of phylogenetic analysis of capsid protein VP1 sequence in reference [13]

volunteers and immune cross-reactivity analysis with electron microscopy [10–13], which lacks accuracy and reproducibility due to the cross-reactivity of antibodies [14].

Recent development of sophisticated molecular methods including reverse transcription polymerase chain reaction (RT-PCR) has allowed a more reliable NoV classification based on the amino acid sequence of the major capsid protein [15]. In this classification system, NoVs are divided into five genogroups and 29 clusters with eight clusters in genogroup I (GI), 17 in GII, two in GIII, and one each in GIV and GV (Table 8.1) [15]. Within the five genotypes, GI and GIV strains are found to infect humans exclusively, and GII are found in both humans and pigs, while GIII and GV strains are animal viruses that infect cattle and murine species, respectively [15, 16]. Currently, strains in cluster 4 of GII (GII.4) are the most prevalent NoVs in human population [17, 18].

The genome of NoVs, which was first characterized in NV, contains a single-stranded positive sense RNA of 7.5–7.7 kb with three open reading frames (ORFs) and a poly A tail at its 3' end [19]. ORF1 encodes a polyprotein that is processed by viral protease 3CLpro into the RNA-dependent RNA polymerase and approximately five other

nonstructural proteins including p48, the nucleoside triphosphatase, p22, VPg, and 3CLpro [20, 21]. The two structural proteins, the major (VP1) and minor (VP2) capsid proteins, are encoded by ORF2 and ORF3, respectively [19, 22]. Structural analysis of NoV has revealed that each viral capsid is composed of 90 dimers of VP1 in a $T=3$ icosahedral symmetry [8, 23]. VP1 folds into two domains: a shell (S) domain that is responsible for initiating capsid assembly and icosahedral contacts and a protruding domain (P), containing two subdomains of P1 and P2, that enhance the stability of the capsid by providing intermolecular contacts between VP1 dimers [24].

In addition to providing the sole structural shell component of NoV capsid, VP1 has to perform other functions including immunogenicity and infectivity. It was suggested that the P domain may contain cellular receptor binding sites and viral phenotype or serotype determinants [23, 25, 26]. The P2 subdomain has the most variable sequence among different NoV strains and is found to protrude outside the capsid surface, suggesting that it is responsible for the immune recognition and cellular interactions [27, 28]. Studies of NV also indicate that the VP2 protein enhances the expression level of VP1 and stabilizes the VP1 in the viral capsid [24].

8.3 Transmission and Infection Cycle of NoVs

NoVs are one of the leading causes of GE in people of all age groups and are responsible for more than 95 % of viral GE in adults [29]. With the development of new diagnostic methods, it is now recognized that the impact of these viruses had been significantly underestimated as they cause far more outbreaks and infections than previously realized [30, 31]. NoVs require an extremely low infectious dose, as challenge studies with NV have suggested that the probability of infection with a single virus particle is approximately 50 % among susceptible human populations [32]. They can be transmitted by several routes including fecal-contaminated food or water, direct person-to-person contact, indirect exposure through droplets, or contaminated objects of infected persons [33].

The high environmental stability of NoVs further facilitates their spread among human populations. For example, NoVs are stable at a wide range of temperatures from subzero to 60 °C and at pHs even below 2.7 and are resistant to treatment with chlorine (0.5–1.0 mg/L), ethanol, quaternary ammonium compounds, or detergent-based cleaners [34, 35]. As a result, NoVs are highly contagious, spread rapidly, and their outbreaks commonly occur in various social places and settings where people share common food and water sources or are in close physical proximity, such as cruise ships, schools, military units, nursing homes, daycare centers, hospitals, restaurants, and catered events [29, 36–39].

Viral shedding may start before symptoms even occur and continue after they have disappeared. This prolonged shedding period (several weeks) of NoVs further enhances the secondary attack rate and usually results in large-scale outbreaks [38, 40–42]. Consequently, these outbreaks often lead to the infection of thousands of people and the closure of facilities and businesses [43, 44]. For example, NoV has been identified as one of the most common pathogens for hospital ward closures [45]. Outbreaks of NoV GE are distributed worldwide and year-round, although as its old name “winter vomiting disease” implies, there are more outbreaks during the winter season of the year [46]. It was suggested that this seasonality is due to climatic conditions that promote a closer person-to-person contact and favor the survival of the viruses [47]. Further studies demonstrated that various NoV strains have different seasonal outbreak periodicities as GII strains mostly occur in winter, while GI viruses are more evenly spread over the year [48–50].

Due to the routes of transmission, young children, elderly nursing home residents, students, military personnel, travelers, and immunocompromised people are particularly vulnerable to NoV infections. Recent studies indicate a possible genetic basis of susceptibility to NoV infection. For example, susceptibility in a human

population is found to be associated with an individual's ABH histo-blood type, mucosal cell-expressed carbohydrates, secretor status, and strain binding preferences of histo-blood group antigen (HBGA) receptors [26, 51–55]. Furthermore, P2s of different viral genotypes have been found to have specific affinity for certain ABO HBGA, with GI NoVs preferentially binding to group antigens A and O, and GII to group antigens A and B [56, 57]. Consequently, it is suggested that HBGA may serve as putative receptors for NoVs [54, 55, 58–60]. These findings may help to explain the phenomena of "asymptomatic infections" in certain individuals who develop NoV-specific antibody response and shed virus, but do not display any typical symptoms of disease after NV infection [52]. In addition to acute GE, chronic NoV infection has also been documented in immunocompromised patients [61, 62].

The life cycle of NoV has not been fully understood due to the lack of an in vitro cell culture system and a small animal model of infection. However, a study by Asanaka and colleagues has demonstrated that an expressed NV genomic RNA has the ability to replicate, to be transcribed into NV subgenomic RNA, and subsequently translated into VP1 in mammalian cells [63]. Moreover, the expression of VP1 was found to generate virus particles filled with NV genomic RNA in this particular cell [63]. These results clearly demonstrate the ability of mammalian cells in supporting the replication of NoV genomic RNA and the assembly of viral RNA into virus particles. Thus, the failure of NoV replication in mammalian cell cultures is not due to the lack of host factors to support intracellular expression of NoV RNA. Instead, the problem may lie in the steps of viral binding to cellular receptors, virus entry into cells after receptor binding, or uncoating of virus particles for releasing genomic RNA into the cytoplasm.

After ingestion of viral particles, symptoms of vomiting and diarrhea usually appear after a 12–48-h incubation period. These symptoms persist during the course of the illness (12–72 h) and are often accompanied by nausea, abdominal cramps, and occasionally by malaise, chills, muscle aches, headaches, and low-grade fever [34]. The molecular and cellular mechanism of how diarrhea and vomiting are induced by NoV has not been fully revealed. Furthermore, the target cell(s) for human NoVs has yet to be conclusively identified. However, it has been observed that NoV starts to multiply within the small intestine upon infection and cause histopathologic lesions in the jejunum and reversible broadening and blunting of the jejunal villi in both symptomatic and asymptomatic individuals [64]. Moreover, cytoplasmic vacuolization and infiltration with a unique CD8+ lymphocyte population in the epithelium have also been observed [65, 66]. It is speculated that these changes may be involved in the blunting of the villi. While it is still not possible to visualize NoV particles in biopsies from challenge studies, in vitro binding experiments suggested that viral binding occurs mostly at the villi level [67].

Although NoV-caused acute GE is usually mild and self-limiting and can be treated with hydration fluids, instances of necrotizing enterocolitis and mortality due to dehydration do occur. It has been estimated that NoVs cause up to 200,000 deaths in young children in developing countries and 300 deaths in the US per year [68–70]. Furthermore, the shear frequency and scale of NoV outbreaks have become a major burden on the health-care system and caused tremendous economic loss in the developed world [45]. Since the emergence of a new NoV variant GII.4 in 2002, the number of outbreaks has increased significantly, and large epidemics have occurred worldwide almost every 2 years [71, 72]. As a result, NoVs have been considered as emerging pathogens. Furthermore, because of the recent realization of their prominence in causing GE and their potential of causing rapid and large water-, food-, and possibly airborne outbreaks with as few as 10–100 viral particles, NoVs have been also classified as class B biodefense pathogens [73]. Currently, there are no effective drugs or vaccines available for treating or preventing NoV infections. Therefore, development of vaccines and therapies for these viruses is urgently needed.

8.4 Current Diagnostic Methods and Therapeutics for NoV GE

Various diagnostic methods have been developed for NoV [74]. While serum antibodies to NoV can be readily detected, this method has little clinical relevance due to the cross-reactivity of antibodies. Since there is no culture system available for NoV, the detection of virus in stool samples has become the preferred method of diagnosis. Traditionally, NoV infection was diagnosed by detecting the virus by immune transmission electron microscopy (TEM) [11]. TEM offers the advantage of direct visualization of any potentially responsible virus particles in stool samples. However, it does have the disadvantage of requiring sophisticated and expensive equipment and highly specialized technicians for its operation.

Moreover, the concentration of intact virus particles in the samples has to be at least 10^5–10^6 particles/ml in order to be detected. TEM can provide rapid diagnostic results for individual samples, usually within 3 h of sample delivery [75]. However, since operators have to spend substantial amount of time in examining each specimen, TEM is not a high-throughput assay for rapidly processing a large number of samples. TEM relies on the identification of viral particles with characteristic NoV morphology, therefore, does not allow reliable speciation within the NoV genus, which lowers its specificity and may hinder diagnosis. These challenges may have contributed to the historic underestimation of NoV epidemics.

Several enzyme-linked immunosorbent assays (ELISAs) that detect NoV antigens were later developed for NoV diagnosis [76, 77]. Antigen-based ELISAs are high-throughput, relatively easy to operate and can rapidly provide diagnostic results within one working day [78]. Consequently, they are usually the method of choice in situations where large numbers of specimens are required to be screened rapidly and economically. Studies have shown that NoV antigen-detecting ELISAs have high specificity (94–96 %) but poor sensitivity (40–60 %), most likely due to the antigenic diversity of NoV strains [4, 79]. As a result, they are useful for specific detection of certain NoVs, but not sensitive enough for applications where a detection of a broad spectrum of NoVs is desired. The employment of pooled antibodies against multiple antigens or multivalent antibodies against a wider range of recombinant viral antigens has, however, improved the sensitivity of ELISAs [80].

With the cloning of the NV genome in 1990 and the development of molecular biology methods, RT-PCR with stool samples (which detects the viral RNA) has increasingly become the popular assay for NoV diagnosis [81–83]. Similar to ELISA-based assays, RT-PCR is rapid and robust, because it can process large numbers of samples simultaneously and results can be obtained within a working day. However, it requires RNA extraction from fecal samples and needs expensive equipment and skilled workers to operate. Therefore, RT-PCR is more labor intensive and less economical than ELISAs. Using primer pairs designed for either highly conserved regions or strain-specific regions of the genome, RT-PCRs are versatile and offer both high specificity comparable to ELISAs and much higher sensitivity. The high sensitivity of this assay allows the detection of NoV in samples in which the concentration of virus is too low to be detected by other methods. These include clinical specimens with low viral load and environmental samples such as food and water [83].

Another advantage of RT-PCR is that, when combined with subsequent nucleotide sequencing, it allows genotyping to trace the source of outbreaks [84]. On the other hand, the high sensitivity of the assay may produce false-positive results from nonspecific amplification or potential sample cross-contamination. This challenge has been addressed by designing more specific primers to avoid amplification of nonspecific RNAs and by implementing more stringent procedures to prevent cross-contamination of samples. Due to the extraordinary sequence diversity among viruses in this genus, there is no single universal primer pair that can detect all strains of NoV. However, several primer pairs have been developed that can detect more than 90 % of all

strains in GI and GII [85]. The robustness of RT-PCR-based diagnostic methods is being further optimized by employing faster and more sensitive assays such as real-time quantitative RT-PCR, which aims to rapidly detect even low copy number NoV in a large number of stool samples during outbreaks [86]. These studies demonstrated that TaqMan based real-time PCR offers more sensitive real-time diagnosis of NoV in both sporadic cases and the outbreak setting, and its results are easy to interpret and available within the working day [87].

Overall, TEM, ELISA, and RT-PCR-based methods all have their advantages and challenges. TEM allows detecting all responsible viral agents, but is expensive and low throughput. In contrast, ELISA is high throughput, economical, and easy to use, but requires improvement in sensitivity. RT-PCR combines the advantage of both sensitivity and specificity and enables molecular epidemiological studies. The three methods detect different components of the virus and therefore are complementary to each other.

For a particular diagnostic application, three factors should dictate the choice among TEM, ELISA, and RT-PCR. They include (1) the type of diagnosis such as epidemic investigations vs. clinical diagnosis, (2) the sensitivity and specificity of each assay, and (3) the speed, robustness, and ease of use of the assay. It is also crucial to remember that the heterogeneous and complex nature of stool samples and their storage conditions may often cause changes in the integrity of virus particles and/or RNA and consequently affect their reactivity in enzymatic and immunological assays. For example, the low sensitivity of ELISA-based methods will result in a significant number of false-negative samples if they are used as the only diagnostic method for clinical samples. Instead, they should be used only as "screening" assays for clinical diagnosis to take advantage of their speed and availability to be performed at a location close to the patient. However, the high specificity of ELISAs makes them an appropriate assay in outbreak investigations because the availability of multiple samples improves their reliability to confirm the cause of an outbreak [79, 80].

Nevertheless, RT-PCR should be used in conjunction with ELISAs in both clinical diagnosis and outbreak investigations to confirm positive findings and assess negative samples. In contrast, if the goal of the diagnosis is to track down the point-source of infection in epidemiology studies, RT-PCR followed by DNA sequencing should be the method of choice due to its high sensitivity and specificity [88, 89]. TEM has been accepted as a "gold standard" for the diagnosis of NoV. However, as more comparative results become available, it has been proposed that either RT-PCR or a positive result in two of the three assays discussed above should be regarded as the "gold standard" [78, 79, 90]. Regardless, it is recommended that at least two of the three methods among TEM, ELISA, and RT-PCR should be combined to achieve a reliable clinical diagnostic result, while all three methods are applicable for epidemiological investigations for NoV outbreaks depending on the number of samples available [79].

Currently, there is no specific and effective vaccine or therapy available for preventing or treating NoV gastroenteritis [34]. General clinical management includes oral administration of rehydration solutions containing essential electrolytes and sugar to treat diarrhea. Parenteral fluid and electrolyte replacement may be required for patients with significant dehydration symptoms or those who cannot tolerate oral fluids replacement [4]. Current efforts are focused on the development of effective vaccines and possible attachment inhibitors for anti-adhesion therapy through glycomimetics [91].

8.5 Vaccine Development for NoV

8.5.1 Immunology of NoV Infection

The immunological knowledge of NoV is mostly obtained from human challenge studies and natural outbreaks due to the lack of small animal models. These studies showed that infected volunteers did develop immunity after a NoV challenge [13, 92, 93]. However, immunity to one strain did not provide complete protection from

challenge of heterologous strains, and symptomatic individuals could often be reinfected when exposed to the same NoV strain 2–4 years later, indicating their immunity seems to be strain or genogroup specific and short lived [13, 92–94]. Challenge studies did not yield conclusive results regarding long-term immunity, which may be confounded by preexposure of volunteers to various circulating NoV strains [71]. Observations of repeat infections in adults suggest the scarcity of long-term immunity against these viruses [38, 84]. However, other studies showed that close to 50 % of the genetically susceptible subjects were not infected by NoV challenge, which support the possibility of long-term immunity [95]. Furthermore, community cohort studies indicate that the duration of symptoms were generally decreased with age, suggesting the development of at least partial protection against NoV [38, 93].

While no vaccine is currently available to prevent NoV GE in humans, these studies support the feasibility of developing vaccines that can induce protective immunity and reduce the disease burden of these viruses.

8.5.2 Virus-like Particles as an Effective Vaccine Against NoV

The lack of a tissue culture system also impedes the development of vaccines against NoV. Fortunately, the discovery of the spontaneous assembly of expressed VP1 into virus-like particles (VLPs) that are morphologically and antigenically similar to the native viruses has facilitated vaccine development [73]. VLPs combine the best traits of whole-virus and subunit antigens for vaccine development. VLPs are noninfectious, therefore, safer than inactivated or attenuated virus due to the lack of viral nucleic acid genome. At least in theory, the immunogenicity of VLPs can even be enhanced over that of the native virus by excluding immunosuppressive viral proteins in their composition. Moreover, an inactivation process is unnecessary for VLP production. Consequently, no unintended epitope modification would occur, further ensuring the VLP's immunogenicity. Importantly, VLPs can induce potent cellular and humoral immune responses without adjuvants and are more effective vaccines than other subunit antigens because their architectures mimic infectious viruses. VLPs can be produced by recombinant technology in heterologous expression systems without requiring the ability to support viral replication [31]. This is particularly important for NoV because no such culture system has been developed to support the growth of these viruses [96].

The particulate nature and the dense repetitive array of epitopes on their surface make VLP far more immunogenic than other subunit vaccines. VLPs can effectively induce T-cell-mediated immune responses through interaction with antigen-presenting cells (APCs), especially dendritic cells (DCs). Specifically, VLPs can mimic the natural viral infection process by being specifically recognized and taken up by DCs and subsequently processed and presented to cytotoxic T cells to trigger their activation and proliferation [97]. Studies have demonstrated that viruses and corresponding VLPs have a particle size ideal for DC and macrophage uptake to initiate antigen processing [98, 99]. Thus, the particulate nature of VLPs favors their targeting to relevant APCs for optimal induction of T-cell-mediated immune responses.

VLPs can also be presented efficiently to B cells and induce strong antibody responses. Like live viruses, the quasicrystalline surface of VLPs, with its arrays of repetitive epitopes, presents a prime target that vertebrate B cells have evolved to specifically recognize [100]. This recognition triggers the cross-linking of surface membrane-associated immunoglobulins (Ig) on B cells [101–103] and leads to their proliferation and migration, T helper cell activation, antibody production and secretion, and the generation of memory B cells [101]. Thus, VLPs can directly activate B cells at much lower concentrations than other subunit antigens and induce high titer and durable B-cell responses in the absence of adjuvants.

Since NoV is an enteropathogenic virus, a potent vaccine should also induce NoV-specific gut mucosal immunity such as through oral delivery. In general, oral delivery of subunit protein

vaccines for gut immunity may not be effective due to the possibility of denaturation and degradation of antigens by stomach acid and digestion enzymes, poor transport to the gut-associated lymphoid tissue (GALT) for antigen processing and presentation, and potential stimulation of systemic immune tolerance [102]. However, VLPs' compact and highly ordered structures allow them to be more resistant to degradative enzymes in the digestive tract than other protein vaccines. The resemblance of VLPs to authentic viral particles may also present a "danger signal" that overcomes the perception of gut antigens as benign and thus prevents the development of immune tolerance [55]. Both of these characteristics are especially true for VLPs of NoV because their cognate viruses are natural gastrointestinal pathogens. Moreover, they are also naturally recognized and efficiently transported into GALT [4]. Thus, the challenges of oral vaccine delivery can be potentially overcome by the unique structure of VLPs that allow them to elicit a potent gut immune response.

These inherent advantages of VLPs have made them one of the most successful recombinant vaccine platforms. For example, five VLP-based vaccines for hepatitis B virus (HBV) and human papillomavirus (HPV) have been commercially licensed, and all have demonstrated excellent safety profiles and long-term protection against infection in humans [31]. These successes and the potential of evoking a gut mucosal immune response upon oral delivery have encouraged the preclinical and clinical development and testing of VLP-based vaccine candidates for NoV.

8.5.3 Characterization of NoV VLPs

VLPs of NoVs were first produced in insect cells using baculovirus vectors [23, 104] and then in plants using tobamovirus [101] and geminivirus [102] vectors and in mammalian cells using the Venezuelan equine encephalitis (VEE) replicon system [105]. These studies demonstrated that expression of the major capsid protein VP1 alone can drive the self-assembly of VLPs that morphologically and antigenically resemble native virus particles (Fig. 8.1). VLPs generated by all three expression systems are similar to each other.

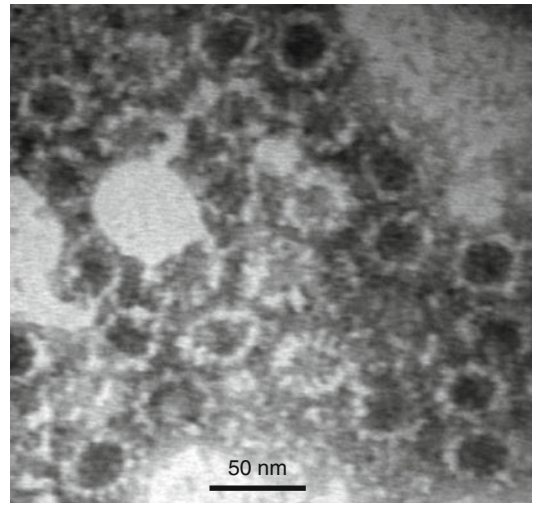

Fig. 8.1 NVCP VLPs produced in plants. NVCP was expressed in lettuce leaves with a geminiviral replicon vector. NVCP VLPs were purified from plants, negatively stained with 0.5 % uranyl acetate, and examined by electron microscopy. Bar = 50 nm. One representative experiment of three is shown

The structure of NoV VLPs is exemplified by the VLP of NV capsid protein (NVCP). Studies of insect cell-baculovirus-derived NVCP VLPs by cryo-electron microscopy and X-ray crystallography reveal that the NV capsid is a 38 nm icosahedral arrangement of 180 copies of the 58 kDa capsid protein VP1 organized into 90 dimers in a $T=3$ symmetry [104]. While all dimers are formed from two identical NVCP monomers, two different dimer configurations are required to correctly form the complete assembled capsid [106, 107].

As in native NV particles, the NVCP also folds into two distinctive domains in VLPs, with S domain forming the inner core of the shell and P domain protruding out from the capsid [108]. Similarly, the P2 subdomain is also the most surface exposed region in NV VLPs and may contain HBGA and neutralizing antibody binding sites and determinants of strains specificity [27, 56, 108–111]. The similarity between VLPs of NV and other NoVs including GII.4 viruses has been demonstrated [112].

8.6 Preclinical Development of NoV VLPs

8.6.1 Insect Cell-Baculovirus Vector Produced NVCP VLPs

As discussed earlier, it is desirable for NoV vaccines to elicit gut mucosal immunity. Oral delivery is a feasible strategy for NVCP VLPs to induce such immunity as evolutionary selection for enteric infection has allowed them to be stable in the oral-gastrointestinal environment and efficiently transport to GALT for antigen processing and presentation [113]. Consequently, various oral doses of NVCP VLPs in the range of 5–500 μg were given to mice to examine their ability to elicit a systemic and mucosal antibody response.

It was shown that four oral doses of as little as 5 μg NVCP VLPs without any adjuvant triggered serum NV-specific anti-IgG response in the majority (8/11) of VLP-fed iCD1 outbred mice [106]. Systemic IgG response was observed after two oral dosages, and the highest titer was induced by four doses of 200 μg VLPs. Moreover, mice in the 200 μg dosage group developed NV-specific intestinal IgA in a level up to 0.1 % of total IgA. Inclusion of the mucosal adjuvant cholera toxin (CT) did not significantly change the number of positive responders of serum IgG or intestinal IgA, but significantly enhanced the amplitude of serum IgG response, especially for higher doses of VLPs [106]. Thus, NVCP VLP is clearly a potent oral immunogen and can induce both systemic and gut mucosal antibody responses.

The success of oral delivery of NVCP VLPs encouraged the exploration of their delivery through alternative mucosal routes. A study by Guerrero and colleagues demonstrated that intranasal (IN) delivery was more effective than oral delivery at provoking NVCP-specific serum IgG and intestinal IgA responses by low doses of VLPs [114]. For example, IN delivery of two 10 μg doses of insect cell-derived NVCP VLPs in the presence of a mucosal adjuvant (mutant *E. coli* heat-liable toxin LTR192G) elicited anti-NVCP titers equivalent to that of two dosages of 200 μg orally delivered adjuvanted VLPs [114].

In addition to intestinal IgA in fecal samples, a strong anti-NVCP IgA response was also detected in vaginal washes. Furthermore, these mucosal IgA responses were long lasting and could be detected a year after the IN immunization [114]. These data not only demonstrate that NVCP VLP is a potent mucosal antigen in stimulating systemic and local mucosal antibody responses, but also indicate its ability in eliciting antibody response at distal mucosa. These findings clearly demonstrated the ability of insect cell-derived NVCP VLPs in eliciting systemic and mucosal B-cell responses to potentially neutralize NV and inhibit its infection and also suggest their application as carriers of heterologous epitopes to combat sexually transmitted infections (STI). For example, chimeric NVCP VLPs that are decorated with epitopes of STI pathogens can potentially induce the production of neutralizing IgAs in the reproductive mucosa.

8.6.2 Mammalian cell-VEE Replicon Produced NoV VLPs

VEE, an alphavirus, has been developed as a replicon vaccine vector. To express a specific vaccine, the coding sequence for the antigen of interest is cloned in place of the VEE structural gene just downstream from the 26S promoter in VEE replicon cDNA to drive its high expression levels [105]. Cotransfection of the cDNA replicon construct with another construct carrying VEE structural genes into mammalian cells will allow the recombinant viral RNA to pack in the VEE viral capsid to form virus replicon particles (VRPs) [57, 105, 115]. These VEE VRPs can infect mammalian cells and accumulate large amounts of vaccine proteins [116]. Because the VEE structural genes are provided in trans for VRP formation and are not part of the recombinant genome, the infection of cells by VEE VRPs is a one-hit event [116].

To produce NVCP VLPs, the gene for NV VP1 was cloned in VEE replicon cDNA and expressed in mammalian cells [105, 115, 117, 118]. VEE-VRP/mammalian cell-derived VLPs can then be purified and delivered through

parental or mucosal routes as immunogens. In addition, the recombinant VRPs can be used as vaccines themselves to produce VLPs in vivo. Therefore, this system can be used in two ways to induce immunity and potentially be advantageous, as VRPs can be directly used to infect permissive mammalian host target cells in which a large quantity of NVCP VLPs are assembled for antigen presentation to B and T cells [117]. For example, direct subcutaneous inoculation of two doses (10 [7] infectious units per dose) of recombinant NVCP-VRP through the footpads in mice elicited strong systemic IgG and intestinal IgA responses against NV VLP, as well as heterotypic responses to VLPs of another GI NoV [117]. Furthermore, the serum and intestinal immune responses in mice that were inoculated with two doses of NVCP-VRPs are substantially stronger than those in mice that were given two oral doses of either 75 or 200 μg of VEE-derived NVCP VLPs [117].

Multivalent NoV vaccine candidates have also been produced in the VEE-VRP system and tested in mice [115, 119]. These studies demonstrated that inoculation of a cocktail of three or four VRPs that express strain-specific VLPs in mice not only induced strong serum antibody response against all inoculum strains but also elicited receptor-blocking heterotypic antibody response against novel strains [115]. Moreover, coadministration of a panel of eight human NoV VLPs that cover more than 95 % of all NoV infections revealed that inclusion of VLPs from both genogroups (GI and GII) in the vaccine cocktail did not detract from either genogroup-specific response, but induced receptor-blocking antibodies against intergenogroup strains [119].

Compared with the insect cell-baculovirus system, the VEE VRP system can be cumbersome and more expensive due to the need of cotransfection and BSL-3 facilities for VRP production. However, parenteral VEE VRP inoculation has been shown to target DCs and stimulate potent humoral and cellular immunity including protection at mucosal surfaces from infection [113, 120, 121]. Furthermore, VRPs have been shown to possess inherent adjuvant activity most likely due to stimulation of immune cells by a single round of viral RNA replication in mammalian cells [119, 122]. Overall, these advantages may allow VEE VPR to be a superior system over the baculovirus system when VRPs are directly used as vaccines for the induction of potential protective immunity against NoV.

8.7 Production and Immunogenicity of Plant-Derived NoV VLPs

8.7.1 Plants as Production Platform for NoV VLPs

Even though NoV VLPs or VRPs have shown promising results as NoV vaccine candidates, production systems based on insect and mammalian cell cultures have several limitations that may hamper the commercial development of these vaccines [54]. For example, the coproduction of baculovirus particles with NoV VLPs in the baculovirus/insect cell system may create problems in VLP purification, immunogenicity, and regulatory approval. Since residual baculovirus may alter the overall immunogenicity of NoV VLP preparation and raise safety concerns, they and their infectivity have to be removed or inactivated by cumbersome and expensive purification processes or chemical treatments. These procedures not only increase the overall production costs but may also impair the quality of the resulting VLPs [31].

The production cost with the VEE/mammalian cell system is significantly higher than that of insect cell cultures. In addition, it also requires heavy capital investment to construct a BSL-3 manufacturing facility [123–126]. Both insect and mammalian cell-based production systems also share challenges in scalability as new fermentation tanks and facilities have to be built for larger-scale production. These challenges may hinder the full realization of the health-benefit potential of NoV VLPs especially in the developing world. As a result, plants have been explored as a safe, cost-effective, and scalable production platform for NoV VLPs.

Plants are considered as an alternative protein vaccine production system because they can produce high levels of vaccine protein at low cost, and biomass production does not require expensive investment in cell culture facilities or facility duplication for scale-up production [124, 127]. As a result, the flexibility and capital efficiency for plant biomass generation and scale-up are far more superior to current fermentation-based technologies [128, 129]. In addition, plants possess eukaryotic processing machinery for proper posttranslational modification and assembly of proteins and have low risk of introducing adventitious pathogens to humans [124, 127]. Despite these potential advantages, earlier production of protein vaccines using stable transgenic plants resulted in slow and low levels of target protein accumulation [54, 126]. The long time frame (several months to a year) to generate transgenic plants, the lack of strong promoters and the position effects from the random insertion of the transgene are responsible for these problems that reduce the cost-saving benefit of plants as a production system [102].

The challenges of VLP production speed and yield has been overcome by the development of plant virus-based transient plant expression systems [130, 131]. For example, our group and others have reported that the cloning and high-level transient expression of plant-derived VLPs can be achieved quickly in 1–2 weeks of vector infiltration with the MagnICON system, which is based on a tobacco mosaic virus (TMV) RNA replicon system or a geminiviral DNA replicon system derived from bean yellow dwarf virus (BeYDV) [126, 132–134]. These improvements in the speed and yield of VLP expression also provide plant expression systems, an additional advantage in versatility for producing VLP vaccines against NoV and other viruses that have rapid antigenic drift and multiple genogroups and strains with unpredictable epidemics around the world.

An effective NoV vaccine needs to be produced in the shortest achievable time frame after strain identification in order to halt the spread of the new strain, preferably by low-cost platforms that allow affordable vaccine manufacturing in locations including the developing world. A platform based on transient plant expression is likely to address such cost and time issues and provide the critical versatility that allows the rapid production of strain-specific vaccines to control potential NoV outbreaks in a timely manner.

8.7.2 Plants as Delivery Vehicle for NoV VLP-Based Vaccines

VLP vaccines produced from insect or mammalian cell cultures are purified products that require expensive downstream processing, cold storage, and transport temperatures [102]. The successful induction of systemic and mucosal antibody responses by oral delivery of insect cell-produced NoV VLPs suggests that oral immunization may also be achieved by ingesting edible plants parts containing NoV VLPs [124]. This is an attractive approach as it may reduce the need for the costly purification steps and may circumvent logistic challenges to allow implementation of immunization programs in regions where refrigeration and other medical supplies are limited.

This approach is most likely to be successful with NoV VLPs as NoV naturally infects the gastrointestinal system and, therefore, is resistant to denaturation and digestion in the oral-gastrointestinal tract [31, 107]. Moreover, their resemblance to the native virus allows them to be efficiently sampled by the “M” cells of the gut epithelium and transported into GALT for antigen processing and presentation [102]. Thus, VLPs produced in edible plants represent a novel and cost-effective approach to establishing gut mucosal immunity by oral delivery [135–137]. As a vaccine is required to have a defined dosage unit, this strategy may face regulatory hurdles for commercialization in developed countries [102, 123, 124]. However, this strategy may eventually offer a feasible option for commercial vaccine delivery by oral route, especially as more consistent VLP accumulation per unit of plant tissue is being achieved by the new generation of expression vectors.

Overall, current plant expression systems offer advantages far beyond the traditional proper

eukaryotic protein modification and assembly, low cost, high scalability, and increased safety. For example, they allow VLP production at an unprecedented speed to control potential epidemics or pandemics.

8.7.3 NoV VLPs Derived from Stable Transgenic Plants

Our laboratory has investigated the expression and assembly of VLPs in plants and successfully produced several non-enveloped and enveloped VLPs including ones based on NVCP, HBV core antigen (HBcAg), and enveloped protein of West Nile virus [31]. VLP based on NVCP is one of the most investigated VLPs in plants and has been successfully expressed in many plant species including tobacco, potato, *Nicotiana benthamiana*, tomato, and lettuce by our group and collaborators [103, 129, 132, 133].

As for other vaccine proteins, NVCP was first expressed in transgenic tobacco and potato plants [103]. It usually took several months to generate and select transgenic tobacco and potato plants that expressed NVCP. Expression of NVCP in these transgenic plants was approximately 10 μg/g fresh tissue weight, which is rather low compared with the insect or mammalian cell-based expression system [101]. However, assembled virion-sized icosahedral VLPs that resemble insect cell-derived VLPs or native NV particles were observed in transgenic tobacco leaves and in potato tubers [101]. In another experiment, the VP1 gene of NV was codon optimized and expressed in transgenic tomato plants [138]. Comparative studies indicated that VLP yield and assembly varied depending on the codon usage, host plant species, and possibly the target tissue of NVCP accumulation [101]. For instance, NVCP expression level was low, and only 25–50 % of them was assembled into VLP in potato tubers, while at least ten-fold higher expression and more efficient VLP assembly were achieved in tomato fruits [101].

To achieve gut mucosal immunity against NV, four doses of 4 g uncooked potato tuber containing 40–80 μg NVCP were fed to mice at days 1, 2, 11, and 28. This immunization regime induced specific serum IgG and intestinal IgA responses in mice [101]. When four doses of 50 μg purified NVCP VLPs were orally delivered to mice in the same immunization regime, serum anti-NV response was four-fold higher than that of mice fed with NVCP-expressing-tuber [101]. The humoral immune response can be further enhanced to 16-fold higher when adjuvant (CT) was co-delivered [101]. Perhaps, the enrichment of assembled VLPs in the purified samples is responsible for the enhancement of humoral immunity because only ~50 % of the NVCP in the potato tubers was assembled into VLPs [31]. It is also possible that NVCP was not efficiently released from the potato tissue to be delivered to GALT for antigen processing and presentation.

Tomato fruits present a more feasible plant material for developing oral NoV vaccines as they are more palatable than raw potato tubers, and their production and processing have been well established by the food industry [31]. Oral delivery of four doses of 0.4 g freeze-dried tomatoes (containing 40 μg VLP) stimulated strong serum anti-NVCP IgG and intestinal mucosal IgA responses in more than 80 % of mice [139]. Furthermore, 100 % of mice developed strong systemic and mucosal antibody responses when a higher dosage (0.8 g per dose) of transgenic tomato was used [138].

The same study also showed that NVCP fed in freeze-dried tomato was more immunogenic than that in freeze-dried potato tubers [138]. The less oxidative environment or the unique tissue structure in tomato fruits may allow better stability or more efficient release of VLPs and, in turn, better immunogenicity. It was also noticed that ingestion of NVCP air-dried tomatoes elicited more potent serum IgG and intestinal IgA responses than that NVCP freeze-dried tomatoes [138]. It is possible that the freeze-drying process altered the assembly status of VLPs in tomato fruits. Alternatively, air-drying may result in better VLP stability by preserving the architecture of VLPs and the tissue structure so that VLPs are better protected from the digestive enzymes in the oral-gastrointestinal tract or

more efficiently released from the tissue for uptake by GALT. Overall, these data support the development of oral vaccines for NoV in tomato plants.

8.7.4 NoV VLPs Produced by Transient Expression with Plant Virus-Based Vectors

The major challenges up to this point for NoV VLP production in plants were the slowness of obtaining VLP-expressing plant lines and poor VLP yield. One of the strategies to overcome these challenges was the use of transient expression systems based on vectors developed from plant viruses. In these systems, transgenes are not integrated into one of the plant genomes, but instead, they are present in the plant nucleus transiently while being transcribed, and later, the transcripts are transported into the cytoplasm, and the transgenic proteins are translated [140, 141].

These systems are focused on production speed and yield and gain the flexibility of nuclear gene expression with the speed and expression amplification of viruses. For example, the MagnICON transient expression system based on replication-competent TMV and potato virus X (PVX) allows high levels of recombinant protein production within 7–10 days of vector delivery [141, 142]. Indeed, our results show that the MagnICON system allows us to produce fully assembled NVCP VLPs at a level of 0.8 mg/g of fresh leaf weight (FLW) within 12 days of infiltration in *N. benthamiana* plants, at least an 80-fold greater production than in transgenic tobacco and tomato [133].

When delivered through the oral route, the partially purified VLPs (100 μg per dose) from transiently expressed plant material provoked potent and balanced systemic IgG1/IgG2a response in the absence of any adjuvant [133]. Significant NVCP-specific vaginal and fecal mucosal IgA responses were also detected in 100 % of immunized mice by the same immunization regime [133]. Moreover, a significant enhancement in NVCP-specific immunity was achieved by the inclusion of adjuvant CT in the oral immunization [103]. Thus, the "destructed" viral vector-based transient expression system has enabled us to overcome the challenges associated with transgenic plant systems and rendered a robust plant system for NoV production.

To further optimize the transient expression systems for commercial production of NoV VLP vaccines, we developed another robust expression system based on geminiviral BeYDV DNA replicon vectors and commercially available lettuce [129, 143]. There are two major advantages of this transient expression system: the noncompetitive nature of BeYDV DNA replicon vector and the use of commercially produced lettuce. In contrast to the MagnIcon system, which can produce proteins with maximally two different hetero-subunits, the geminiviral vector allows the production of VLPs with up to at least five hetero-subunits [143].

Lettuce is cultivated readily and rapidly produced in large quantities in well-established commercial greenhouses. Unlike tobacco and related species of *Nicotiana* such as *N. benthamiana*, lettuce is a palatable plant and can be consumed raw for oral delivery of NoV VLPs. When NVCP was expressed in *N. benthamiana* plants with BeYDV replicon vectors, assembled VLPs were accumulated up to 0.4 mg/g FLW in leaves [132]. Our data also indicate that the BeYDV replicon system allows a high level of NVCP VLP expression and assembly in lettuce as those driven by the MagnICON system in *N. benthamiana* [129].

Actually, VLP accumulated to its highest level at day four after vector introduction, indicating that this expression system can produce similar levels of VLPs in lettuce but in a much shorter time frame than the MagnICON system in tobacco [129]. Importantly, this study is one of the first demonstrations of using commercially produced lettuce for rapid and high-level production of VLPs [129]. This represents a significant milestone for the eventual commercial production of NoV VLP-based vaccines, as this would allow our production system to have access to unlimited quantities of low-cost material from existing commercial sources. Coupled with the almost unlimited nature of

lettuce production, the high efficiency and scalability of our BeYDV replicon vectors provide us a new NoV VLP production platform that is low cost, robust, safe, and amenable to large-scale manufacturing.

8.8 Production of Plant-Derived NoV VLP Vaccines Under Regulatory Conditions

Despite the successes in expressing NVCP VLPs and a variety of other subunit vaccines in plants and the recent improvement of vaccine production levels by transient expression systems, no plant-derived vaccine has been licensed yet for human use. This lack of commercial success lies in several technical and regulatory barriers that remain to be overcome. These challenges include the lack of scalable downstream processing procedures, the uncertainty of regulatory compliance of production processes, and the lack of demonstration to date of plant-derived vaccines that meet the required standards of regulatory agencies in identity, purity, potency, and safety [123, 124, 127].

While immunization by ingesting plant tissue still presents a viable approach to orally delivering plant-produced VLPs, product regulatory concerns have necessitated the development of downstream processing technologies to produce purified VLP vaccines with a defined unit dosage [123]. Therefore, for the promise of plants as an alternative for vaccine production to become a reality, the technology must be able to produce sufficient quantities of VLPs at a relevant scale and with product qualities that meet all required standards of regulatory agencies such as US Food and Drug Administration (FDA).

8.9 Establishment of Facilities for Plant Production of NoV VLPs Under FDA Regulation

To overcome these remaining challenges, the regulatory compliance of facilities, materials, and procedures for production of NoV VLPs under current Good Manufacture Practice (cGMP) has to be addressed first. Our group has led the effort in this area and established the first cGMP-compliant bioprocessing facility in academia for the production of NoV VLPs in plants [126].

As shown in Fig. 8.2, the purposefully built facilities in our university permit biomass generation and bioprocessing of plant-derived NoV VLP vaccines under cGMP Quality Management System (QMS). For example, separated rooms for buffer preparation, incoming plant samples, chromatography, and sterile fill are purposefully designed in the central bioprocessing suite (Fig. 8.2a).

As indicated by the arrows in the same figure, the suite is operated under differential pressure with the sterile fill room being a Class 100 environment and designed to have a separated unidirectional flow of human workers and biological material, so that the products meet the requirements of both US and European Union (EU) regulatory agencies for manufacturing of human pharmaceuticals. As a part of the QMS, the quality control (QC)/Quality Assurance (QA) laboratory is adjacent to the bioprocessing suite for product testing and quality management (Fig. 8.2a). A 3,600 ft^2 biosafety level 2 (BSL-2) greenhouse facility has also been built for plant biomass production under cGMP (Fig. 8.2b).

The BSL-2 greenhouse is equipped with advanced technology for containment of plants or pathogens, which is necessary to maintain the integrity of the plant-derived VLPs and to minimize the risk of accidental genetic contamination of the external environment.

The established bioprocessing facilities and QA/QC laboratories have also been certified for producing human vaccines, which laid a solid foundation for the cGMP compliance of VLP production procedures and the final VLP vaccine product.

8.9.1 A. tumefaciens and N. benthamiana Master and Working Banks

As part of the cGMP compliance of upstream processes, biomaterials including relevant

Fig. 8.2 cGMP production facilities for NVCP VLP vaccine. (**a**) The design of the central bioprocessing suite. The arrows indicate the unidirectional flow of in-process materials (*green*), purified final product (*red*), and people (*yellow*). (**b**) The BSL-2 greenhouse facility for plant biomass generation and NVCP expression. (**c**) The Plant Biopharmaceutical Center that houses the central bioprocessing suite, the QA/QC laboratory, and the Process Development laboratory (Adapted with kind permission from Springer Science+Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 1)

Agrobacteria strains and the *N. benthamiana* plant line have to be qualified by the QMS. This process starts with the establishment of master and working banks of *A. tumefaciens* strains that containing the NVCP expression vectors and a seed bank of wild-type *N. benthamiana* for growing biomass for transient expression. To generate plant material for cGMP-compliant production of NVCP VLPs, three *A. tumefaciens* master banks, each containing the expression cassette for VLP production in TMV 3' module, TMV 5' module, and integrase of the

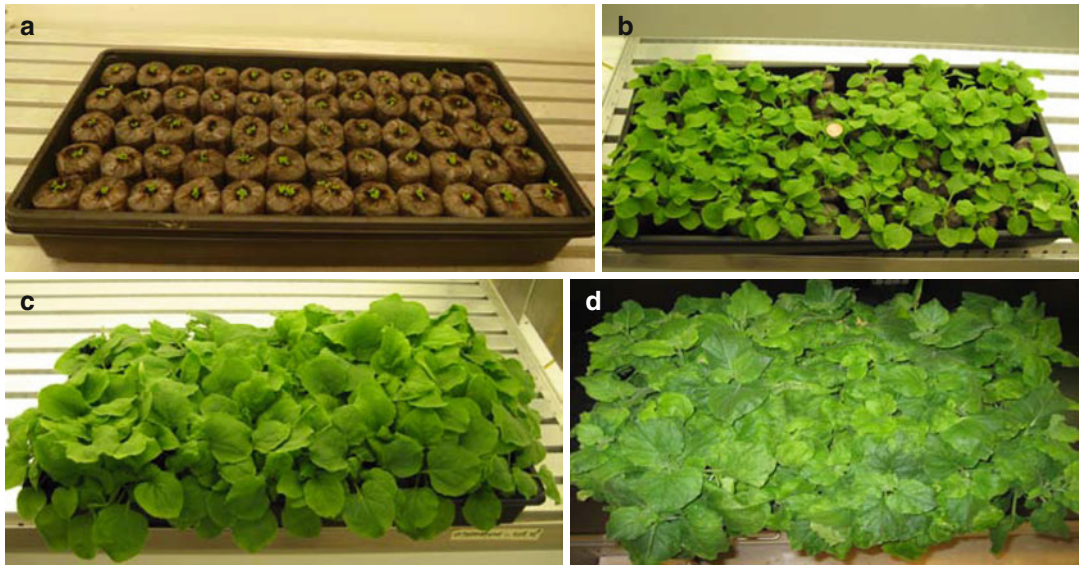

Fig. 8.3 Biomass generation under production conditions in this study. Wild-type *N. benthamiana* plants one (**a**), three (**b**), four (**c**), and five (**d**) weeks after planting (Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 2)

MagnICON system, [141] have been established in the bacterial strain of GV3101 [126]. The identity of the *A. tumefaciens* host strain and associated functional genes and vectors are fully validated. These banks have been shown to be genetically stable over a 4-year period [126]. A master seed bank for wild-type *N. benthamiana* was also established. Our experiment has shown that this seed bank has a germination rate of 99 % and produced uniform seedlings with typical *N. benthamiana* morphology even after a 4-year storage at 4 ± 2 °C (Fig. 8.3) [126]. These results collectively demonstrated that it is feasible to establish master and working banks of *A. tumefaciens* strains and *N. benthamiana* seeds that meet the qualification standards of identity and stability for cGMP production of NoV VLPs.

8.9.2 Biomass Production and Infiltration at Pilot Scale

In this part of the upstream process, the goal is to optimize conditions that permit the maximal production of leaf biomass and VLPs per square meter of green house space. Studies by our group and others demonstrated that temperature, light source and intensity, plant age, and incubation time after leaf infiltration all have significant impacts on biomass generation and VLP accumulation [126]. For example, *N. benthamiana* grown under nature light produces more biomass, but accumulates much less NVCP VLPs than plants grown under artificial light (Fig. 8.4a). Furthermore, leaves grown under natural-light also produce more solid debris during VLP extraction, causing problems for VLP purification [126]. Our data also indicate that a 16-h light/8-h dark cycle at 25 °C is the optimal condition to generate biomass under such artificial lighting [126]. When *N. benthamiana* plants were grown under these conditions and sampled at various ages for NVCP VLP expression, it was founded that 5-week-old plants provided the optimal material for VLP production, because they already accumulated an adequate amount of biomass and produced the highest level of VLPs (Fig. 8.4b). Younger plants produce insufficient biomass, and older plants are too tall for infiltration and start to produce more secondary metabolites that complicate VLP purification.

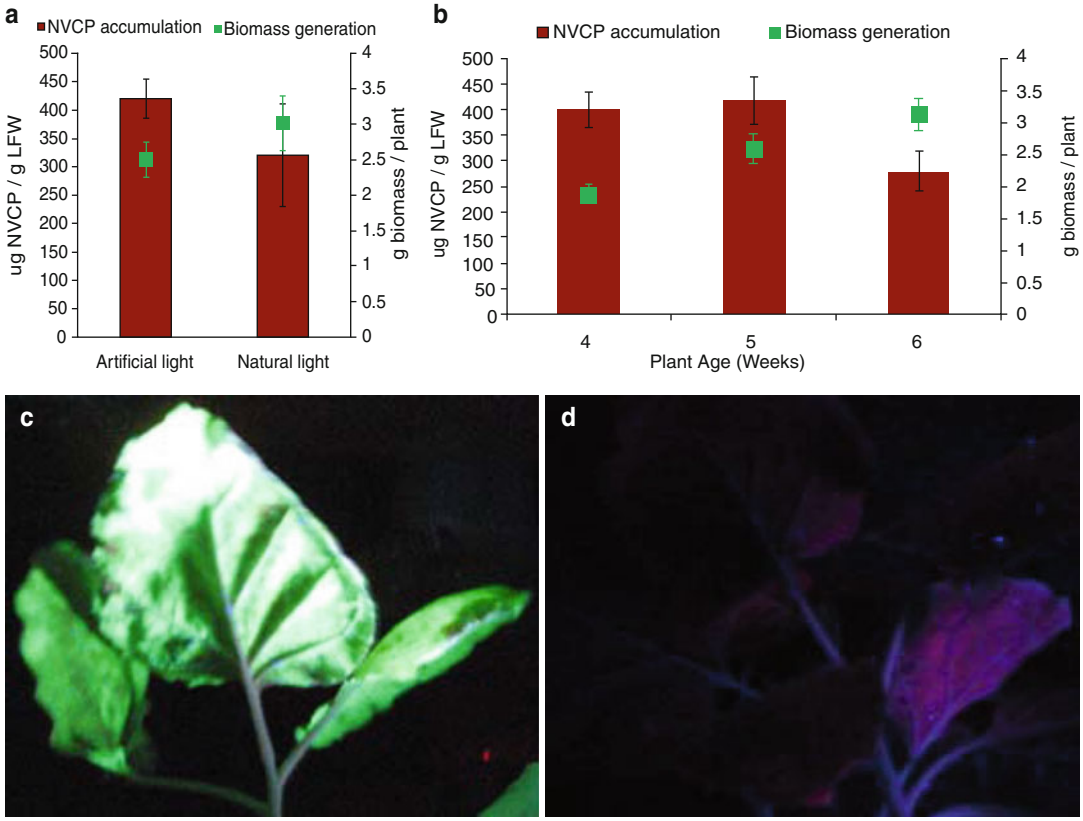

Fig. 8.4 Optimization of biomass and NVCP VLP accumulation. (**a**) Biomass and NVCP yield under natural and artificial light. *N. benthamiana* plants were growth either under natural or artificial light for 5 weeks. Leaf biomass (*green square*) and NVCP expression level (*red column*) were measured by weighing and ELISA, respectively. (**b**) Temporal pattern of biomass yield and NVCP expression. Plants were grown under artificial light for 5 weeks, and biomass production (*green square*) and NVCP accumulation (*red column*) were examined. For both (**a**) and (**b**), mean ± standard deviation (SD) of samples ($N > 10$) from three independent infiltration experiments is presented. (**c**) and (**d**) visualization of GFP expression in agroinfiltrated leaves. *N. benthamiana* plants were infiltrated either with GV3101 cultures that carry the three MagnICON vectors for GFP expression (**c**) or with infiltration buffer as a negative control (**d**). Leaves were examined and photographed 7 dpi under UV light. One representative of at least three independent experiments is shown (Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 3)

Consequently, 5-week-old *N. benthamiana* optimally balance the combined need for biomass generation, filtration operation, and VLP accumulation. Our data demonstrated that under these optimized conditions, biomass for producing sufficient amount of purified NVCP VLPs for a Phase I clinical trial can be generated in 5.6 square meters of green house space in a 5-week period [126]. The horizontal space requirements for this type of production could be significantly reduced by vertically stacking layers of growth trays vertically.

Methods for efficient introduction of *Agrobacterium* into leaves by vacuum infiltration were also developed [126]. As illustrated by the expression of the GFP and NVCP VLPs, plants infiltrated based on these methods under scale-up conditions allowed efficient infiltration of vector-carrying *Agrobacteria* in the entire leaf area (Fig. 8.4c) and accumulated the highest level of NVCP VLPs in leaves 7 dpi (Fig. 8.4a, b). Moreover, the yield and temporal expression pattern of NVCP VLPs under these scale-up conditions are similar to that of our previous smaller

bench-scale experiments [126, 133]. These results clearly indicate that the upstream process for NV VLP production in plants is scalable and can be further scaled up for commercial production.

8.9.3 Downstream Processing of NoV VLPs

While eating unprocessed or partially processed NoV VLP-containing plant tissue remains a viable option for vaccine delivery, purified VLPs with a well-defined unit dosage are most likely to be the first generation of successful vaccine candidates for commercialization due to regulatory requirements [123, 124]. Therefore, it is absolutely necessary to have a robust and scalable downstream process that can effectively recover and purify VLPs in place in order for plant-produced NoV VLP vaccines to become commercial reality. An optimized downstream process should be able to preserve the integrity of VLPs and produce highly purified VLPs with a high recovery rate and a minimal number of processing steps.

It should also be scalable, economical, and compliant with the FDA's cGMP regulations. The most common practice for NoV VLP purification is density gradient ultracentrifugation [106, 108, 133, 144]. While these methods are useful for characterizing VLP size and assembly [107, 133, 144], they are not practical for commercial VLP manufacturing as they are difficult to scale-up and time-consuming [143, 145]. They may also be problematic for preserving the integrity of VLPs because the hyperosmotic gradient agents and severe centrifugation force can often shear VLPs [146]. To meet the demand for more scalable and robust methods of NoV VLP processing, a new trend has started toward more sophisticated methods like filtration and chromatography [31]. For example, our group has recently developed a robust and scalable downstream processing scheme for recovering NVCP VLPs from plants (Fig. 8.5).

In this three-step process, plant extracts are processed by low pH precipitation, ultrafiltration and diafiltration (UF/DF) with tangential flow filtration (TFF) membranes, and ion-exchange (IEX) chromatography [126]. We showed that low pH and UF/DF eliminated most plant host proteins and IEX chromatography purified NVCP VLPs to >95 % purity (Fig. 8.6, Lane 5) [126]. Production runs with various scales of plant biomass demonstrated that this downstream process is highly scalable and can consistently yield highly pure NVCP VLPs with high recovery rates [126]. In contrast to gradient centrifugation that is laborious and time-consuming, the new method is robust and more scalable and can be completed within ≤12 h instead of several days [126, 143]. Importantly, this new process is fully cGMP compliant and has produced cGMP grade VLPs for a human clinical trial [31].

New technologies such as microarrays have also been employed to further optimize NoV VLP purification from plants [143]. For example, our lab and its collaborators have identified peptide ligands with specific affinity to NVCP VLPs from a microarray. When conjugated to chromatography beads, these affinity ligands allow the recovery of highly purified NVCP VLPs from *N. benthamiana* plant extracts [31]. This approach is advantageous over the traditional affinity chromatography because our affinity ligands are entirely synthetic and therefore insensitive to ligand denaturation or degradation.

These advantages along with the rapid ligand discovery process and the low cost of peptide production would allow its application to large-scale NoV VLP manufacturing.

8.9.4 Quality Control of Plant-Derived NoV VLPs

One of the remaining challenges for the commercialization of plant-derived vaccines is the lack of examples that meet the required standards of regulatory agencies in identity, purity, potency, and safety [123, 124, 127]. As part of the efforts to overcome this challenge, our laboratory has identified and developed analytical assays to monitor the in-process samples and to ensure that

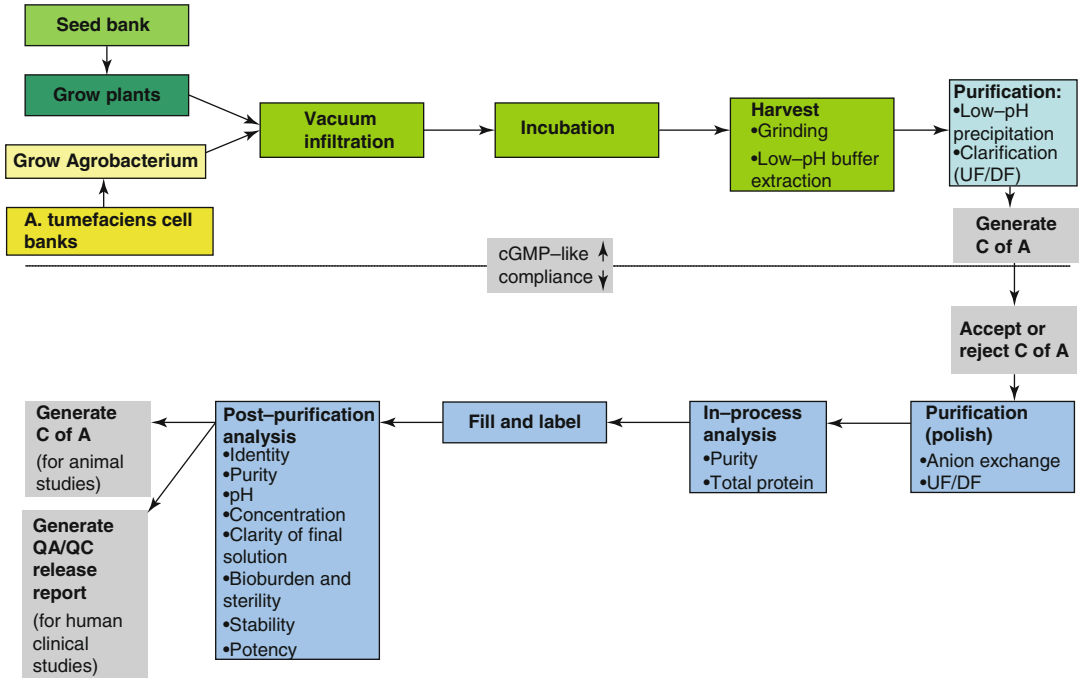

Fig. 8.5 Process flow for the production of NVCP VLP vaccine under cGMP regulations. C of A, certificate of analysis; *UF/DF* ultrafiltration and diafiltration (Adapted with kind permission from Springer Science+Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 4)

the final VLP product meets preset specifications for the release of human pharmaceuticals in identity, purity, concentration, tertiary structure, functionality, and in concentrations of host contaminating molecules essential for the cGMP compliance (Table 8.2). Our analyses showed that the identity and assembly of the final VLP product were confirmed and its purity, concentration, appearance, residual host DNA concentration, and stability all conformed to the insect cell-derived reference standard [126]. The final VLP product elicited strong NVCP-specific systemic, local, and distal mucosal immune responses in mice, demonstrating its potency [126]. These results have provided the first example of plant-derived NoV VLPs from a scale-up process that meets the predetermined release specifications.

In summary, production studies by our laboratory have successfully developed upstream process for plant biomass generation, infiltration and robust NoV VLP accumulation, and a novel downstream process for efficiently recovering VLPs from plant tissue [126]. Moreover, these processes have been successfully operated under cGMP regulations and produced high-quality VLPs that meet all preset release specifications in identity, purity, potency, and safety [126]. Hence, these studies provide the first precedent in an academic setting of producing a plant-derived vaccine at scale and under cGMP regulations and are an important step for plant-produced NoV VLP vaccines to become a commercial reality.

Ongoing research by our group and collaborators is evaluating the systemic and mucosal immunity of the cGMP-purified VLPs when co-delivered mucosally with various adjuvants [31]. Preliminary data suggest that mucosal immunization with several adjuvants evoked stronger serum IgG and mucosal IgA responses than with VLP alone [31]. We anticipate that the purified NVCP VLPs from our cGMP plant production runs and the adjuvants defined in our current studies will be used in a new Phase I human clinical trial in the near future.

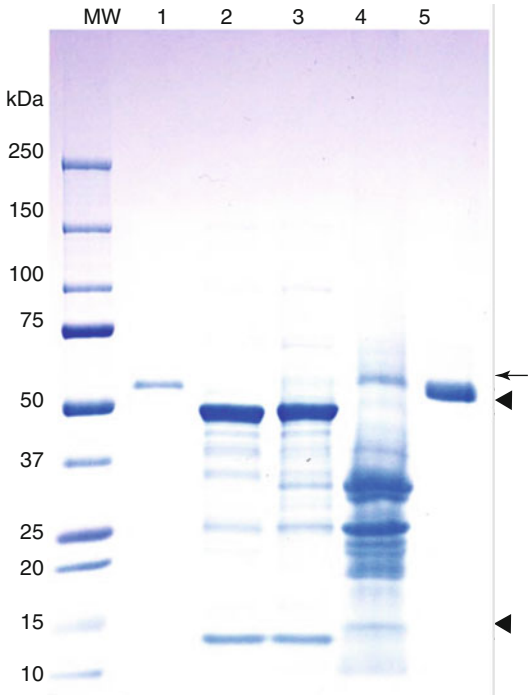

Fig. 8.6 Purification of NVCP from *N. benthamiana* plants. Leaf protein extract was purified and analyzed on a 4–20 % SDS-PAGE gel under reducing condition. *Lane 1* insect cell-produced NVCP as a reference standard, *Lane 2* clarified leaf extract from uninfiltrated plants, *Lane 3* clarified leaf extract from NVCP-producing plants, *Lane 4* supernatant of low-pH precipitation, *Lane 5* purified plant-derived NVCP from DEAE anion-exchange chromatography. ◀: RuBisCo large and small subunits; ←NVCP (Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 5)

Table 8.2 Analytical assays for NVCP VLP in-process samples and the final purified product

Product attribute	Analytical method
Product identity	Western blot
Product identity	N-terminal sequence with Automated Edman sequencing
Purity and molecular	SDS-PAGE/profile Coomassie and Silver staining
Protein purity and concentration	RP-HPLC
Protein concentration	Absorption at wavelength of 280 nm (A280)
Protein concentration	Amino Acid Analysis (AAA)
Mass	ESI-TOF mass spectrometry (ESI-TOF MS)
Quaternary structure	SEC HPLC multi-angle laser light scattering (MALLS)
Quaternary structure	Sucrose gradient centrifugation, TEM
Determination of pI	Isoelectric focusing (IEF)-PAGE
Glycan analysis	MALDI-TOF MS
Endotoxin	Limulus amebocyte lysate
Bioburden	Turbidity and colony formation
Residual host DNA	PicoGreen and PCR
Small molecules, nicotine	LC-MS/MS
Residual host cell protein	Polyclonal Abs to wild-type *N. Benthamiana* plant protein

Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen [126], Table 2.)
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis, *RP-HPLC* reverse phase high-pressure liquid chromatography, *ESI-TOF* electrospray ionization time of flight, *TEM* transmission electron microscopy, *MALDI* matrix-assisted laser desorption-ionization, *PCR* polymerase chain reaction, *LC-MS/MS* liquid chromatography-tandem mass spectrometry

8.9.5 Human Clinical Trials with NoV VLPs

The successful demonstration of strong systemic and mucosal immunogenicity in preclinical studies has led to several clinical trials to exam the safety and immunogenicity of NoV VLP-based vaccines in humans (Table 8.3)

Insect cell-derived NVCP VLPs were initially tested in two Phase I trials [144, 147]. In the first trial, 20 antibody-positive adult volunteers were given two oral doses (days 1 and 21) of 100 or 250 μg of VLPs formulated in water without adjuvant. It was observed that serum IgG responses were dose dependent and 100 % of vaccinated subjects who received 250 μg of VLPs increased their NV specific titers at least four-fold [147]. Fifteen out of 18 participants responded in serum IgG titers after the first VLP dose and showed no increase after the second dose [147]. Importantly, no side effects were observed in vaccinated volunteers [147].

In the second trial, the safety and immunogenicity of NVCP VLPs were further tested in 36 adult seropositive healthy individuals between 18

Table 8.3 Phase I human clinical trials with NVCP VLP-based vaccines

Production system	Number of volunteers	Formulation	Number of doses and dosage range	Delivery route	Immunogenicity
Insect cell/ baculovirus	20	Water, no adjuvant	Two doses of 100–250 μg at days 1 and 21	Oral	Serum IgG and IgA responses were dose-dependent and NV-specific tilters increased at least four-fold in 100 % of volunteers in the 250 μg group. 83 % responded in serum IgG titers after the first VLP dose and showed no increase after the second dose [147]
Insect cell/ baculovirus	36	Water, no adjuvant	Two doses of 250, 500, and 2,000 μg at days 1 and 21	Oral	Increase of NV-specific IgG titers was detected in 90 % of participants in the 250 μg group. No further increase in the rates of seroconversion or IgG titers for the 500 and 2,000 μg groups. Increase of NV-specific IgA ASC numbers in 100 % of vaccinated volunteers was observed and 30–40 % of volunteers developed salivary, fecal, or genital fluid IgA antibody [144]
Insect cell/ baculovirus	28	Dry power with chitosan, MPL as adjuvant	Two doses of 5, 15, and 50 μg at days 0 and 21	IN	Serum IgG and IgA responses were dose dependent with their titers increased 4.7 and 4.5 folds, respectively, for the 50 μg group. 53 % of subjects developed rises in NV-specific IgA ASCs [148]
Insect cell/ baculovirus	61	Dry power with chitosan, MPL as adjuvant	Two doses of 50 and 100 μg at days 0 and 21	IN	63 and 79 % of vaccinated subjects in the 100 μg group increased IgG and IgA titers by 4.8- and 9.1-folds, respectively. The IgG and IgA titers of the 100 μg group are higher than the 50 μg group, but not statistically different. All vaccinated individuals in the 50 and 100 μg groups developed IgA ASCs. Homing molecules to mucosal and peripheral lymphoid tissues were detected on these ASCs [148]
Transgenic potato	20	Raw potato tuber[a]	Two or three doses of 215–751 μg in 150 g potato tuber[a] at days 0 and 21 or at days 0, 7,and 21	Oral	20 and 30 % of vaccinated volunteers developed NV-specific serum IgG (12-fold rise) and fecal IgA (17-fold increase), respectively. 95 % of subjects developed NV-IgA ASCs [149]

IN intranasal, *ASCs* antibody-secreting cells
[a]Each 150 g of raw potato tuber contained between 215 and 751 μg variable amounts of NVCP antigen

and 40 years of age with two oral doses of increasing amounts (250, 500, and 2,000 μg) of antigens without adjuvant using the same immunization regime [144]. Significant increase in the number of NV-specific IgA antibody-secreting cells (ASC) was observed in all vaccinated volunteers and approximately 30–40 % of volunteers developed salivary, fecal, or genital fluid IgA antibody. An increase of NV-specific IgG titers was also detected in 90 % of participants

who received 250 μg of VLPs [144]. However, further increase in VLP doses (500 and 2,000 μg) did not increase the rates of seroconversion or IgG titers [144].

While the results from the first two trials were encouraging, however, the maximal serum IgG titers elicited by oral immunization were lower than those after experimental NV infection. Consequently, two additional Phase I studies were conducted to investigate whether the immunogenicity of NVCP VLPs can be further enhanced by using mucosal adjuvants [148]. Insect cell-produced NVCP VLPs were formulated in a dry power containing an TLR4 agonist adjuvant, monophosphoryl lipid A (MPL), and the mucoadherent chitosan and delivered by IN route to healthy subjects [148].

Study 1 was a stepwise dosage escalation trial with 5, 15, and 50 μg of NVCP VLPs. Study 2 was a dose comparison study of the two highest dosages (50 and 100 μg) VLPs. These studies indicate that IN delivery of NVCP VLPs in a dry power formula with MPL and chitosan was well tolerated and no vaccine-related serious adverse events occurred [148]. In study 1, dose-dependent serum IgG and IgA responses were observed with their titers increased 4.7- and 4.5-fold, respectively, for the 50 μg group [148]. In study 2, the 100 μg group developed higher titers of IgG and IgA (4.8- and 9.1-fold increase, respectively) than that of the 50 μg group, but the differences were not significant in statistical terms [148].

All vaccinees in the 50 and 100 μg groups developed IgA ASCs. Furthermore, expression of homing molecules targeting to mucosal and peripheral lymphoid tissues was detected in these ASCs. Compared with the previously described oral nonadjuvanted VLPs, IN delivery of MPL-adjuvanted VLPs induced a higher number of these ASCs [148]. While the correlates of protective immunity against NV disease are still unknown, these mucosally primed ASCs in combination with the serum IgG and IgA antibodies may contribute to protection.

A Phase I/II trial has been initiated to assess safety and immunogenicity of NVCP VLPs administered by the IN route, followed by a live virus challenge to determine the effectiveness of this approach in preventing or limiting NV infection in humans [103].

To demonstrate the safety and efficacy of plant-derived NoV VLPs, a Phase I clinical trial was conducted with potato-produced NVCP VLPs. In this trial, two or three doses of 150 g uncooked NVCP-transgenic potato tubers (215–751 μg VLPs per dose) were orally ingested by 20 human subjects on days 0 and 7, or on days 0, 7, and 21 [149].

All but one of the volunteers responded with a rise in NV-specific IgA ASCs, while the majority responded following the first dose. Four of the volunteers developed NVCP-specific serum IgG, and six developed specific intestinal IgA with a mean titer rise of 12- and 17-fold, respectively [149]. The incidence rates of nausea, vomiting, mild cramps, fever, or diarrhea were similar among volunteers who ate recombinant or control tubers, indicating that the ingestion of VLP-producing potato tubers appeared to be safe [149].

Together, these results indicate the immunogenicity and safety of using edible VLP-containing plant parts as oral vaccines of NoV in humans. However, the overall antibody response was not as strong as that obtained by orally delivered purified NVCP VLPs (250 μg per dose) produced in insect cells [144]. Perhaps, the variable effective VLP dosage due to inconsistent NVCP content and poor VLP assembly in potato tubers may cause this weak antigenicity. The potency of the plant-derived VLPs may also be further reduced by their poor release from the potato tissue in the gut lumen. In light of this and the results from recent human clinical trials with insect cell-derived VLPs, in the near future, we are planning a new human clinical trial with purified NVCP VLPs from our cGMP runs and the adjuvants defined in our current studies (see Quality control section above) [126].

Overall, these clinical studies have demonstrated that NoV VLP-based vaccine candidates produced in insect cells and plants are safe and immunogenic in humans. Since the model of NV infection in human volunteers has been established recently [40, 150], the efficacy of adju-

vanted NVCP VLP vaccines through IN and oral delivery should be further examined in this human challenge model.

8.10 Strengths and Challenges of VLP-Based NoV Vaccines

For uncultivable NoV, capsid protein-based VLPs possess the best properties in immunogenicity, safety, stability, and manufacturability as vaccine candidates to prevent NoV GE. Like other VLP-based vaccines, NoV VLPs have been shown to induce potent cellular and humoral immune responses without adjuvants due to their resemblance to infectious viruses. Since NoV naturally causes infection in the gastrointestinal tract, the VLPs are stable at low pH and resistant to digestive enzymes. As a result, they can be administered orally and elicit a potent mucosal antibody response and systemic response in mice and humans. For example, oral delivery of NVCP VLPs induced robust production of anti-NV serum and intestinal antibodies and interferon-γ in peripheral blood mononuclear cells [144].

Similarly, potent systemic and mucosal immune responses were provoked in mice and humans by IN delivery. The ability to induce NoV-specific gut mucosal immunity by mucosal delivery provides VLPs a distinguish advantage as vaccines for these enteropathogenic viruses. Moreover, the safety of NoV VLPs have been demonstrated in several human clinical trials. The stability of NoV VLPs also favors them as commercial vaccines as they can be lyophilized or stored at 4 °C in simple buffers for many years without degradation [73]. Significantly, studies by our group and others have demonstrated that NoV VLPs can be robustly produced by recombinant technology in plants at large scales with low cost [31].

The development of a novel downstream process and the successful production of NVCP VLPs that meet all regulatory release specifications in identity, purity, potency, and safety by our group provide an additional step for NoV VLP vaccines to become a commercial reality [126]. These successes have demonstrated the potential and feasibility of VLPs as NoV vaccines and suggest they can serve as an excellent model for developing effective strategies of mucosal immunization with non-replicating antigens.

There are still remaining challenges for the development of efficacious NoV vaccines. For example, the question of whether VLP-based vaccines can protect humans against a live NoV challenge still remains unanswered. Moreover, it is difficult to predict the level of protection that new vaccine candidates can provide due to the lack of a complete understanding of the immune correlates of protection. This problem is further intensified by the lack of culture systems to cultivate NoV in vitro and the lack of small animal models of NoV pathogenesis. Furthermore, the lack of complete cross-protection among diverse genotypes and genogroups of viruses and the rapid evolution of new variant strains further hamper the development of efficacious vaccines that are protective against multiple NoV strains.

Overall, the potent systemic and mucosal immunogenicity, the robust and low-cost cGMP manufacturability in plants, and the safety profile in human clinical trials all support the further development of VLP-based vaccines for the prevention of NoV-related GE. Due to the diversity of capsid protein among different NoV strains and its rapid antigenic drift, vaccine development should focus on multivalent VLP vaccines that are derived from capsid proteins of the most prevalent strains. Furthermore, vigilant epidemiological surveillance must be coupled with NoV vaccine production to identify this moving target and to include the most prevalent circulating strains in the formulation for optimal protection. New data from ongoing and planned human clinical trials, particularly challenge trials, should shed new light on the efficacy of VLPs in preventing or limiting NoV infection in the next few years. They may also provide clues for the immune correlates of protection. This knowledge will facilitate the development of efficacious NoV vaccines.

A lingering criticism of plant-based production platforms has been the absence of approved human products in the USA after 25 years of

active research and development [125]. Excitingly, this last barrier has been overcome by the recent approval of a plant-produced glucocerebrosidase (commercial name: ELEYSO™) by the FDA for treating Gaucher disease, heralding a new era in the field of plant-made pharmaceutics [31]. We speculate that plant-based production systems will offer a superior scalability, safety, time and cost-saving benefits for NoV VLP manufacturing.

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Toward a New Vaccine Against Measles

9

Alexander N. Zakhartchouk and George K. Mutwiri

Contents

Abstract

Measles is a highly contagious disease caused by measles virus (MV). Once the live attenuated vaccine was licensed in 1963, the incidence of measles dropped over 98%. However, measles remains a major cause of child mortality in developing countries. Partially, this is due to several limitations of the licensed vaccine which include thermal instability and low protective efficacy in young infants. Therefore, we are working on developing a novel and improved vaccine against measles based on the part of the recombinant MV haemagglutinin (H) protein (globular head domain). The primary function of the H protein is to bind to the host cellular receptors, and the majority of MV-neutralizing antibodies direct to the MV H protein. The recombinant protein was produced in stably transfected human cells and purified from the cell culture medium. We have chosen stably transfected human cells as a vaccine antigen production platform because of the absence of safety issues associated with use of viruses and viral vectors. In addition, posttranslational modifications of proteins expressed in human cells most closely resemble natural modifications of the viral proteins. In a feasibility study, we demonstrated that two subcutaneous immunizations of mice with the purified recombinant protein formulated with adjuvants resulted in the production of high MV-specific neutralizing antibody titers in serum. The inclusion of a polyphosphazene adjuvant into the vaccine formulation increased Th-1-type

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immune response, which is important to avoid a MV vaccine complication called atypical measles. Furthermore, our data indicate that the purified recombinant protein is thermally stable. More studies are needed to test immunogenicity and protective efficacy of our new vaccine in monkey model and in the presence of MV-specific (maternal) antibodies.

9.1 Disease

Despite the availability of live attenuated vaccines and reaching global measles vaccination coverage of >80 % of individuals, measles remains one of the leading causes of death in children under 5 years of age. Measles is one of the most contagious human diseases. Early clinical signs of the disease are mild fever, cough, coryza, conjunctivitis, and photophobia. These symptoms precede the Koplik's spots which are the small white spots inside the cheeks. The spots appear and disappear quickly within 24 h before the rash develops. The typical maculopapular rash starts usually on the neck and cheeks and within 3 days spreads over entire body. With the appearance of the rash, the body temperature usually rises to 39–40 °C and subsides rapidly in uncomplicated cases. The rash lasts for 3–4 days, and fading of the rash proceeds downward in the same sequence as its appearance [1]. Clinical recovery begins shortly after development of the rash.

Measles virus causes lymphopenia and a temporary suppression of T-lymphocyte responses that last several weeks, leaving the infected person susceptible to other infections. Therefore, secondary bacterial and viral infections are responsible for much of the measles-related morbidity and mortality [2].

Complications occur in up to 40 % of measles cases. The main complications of measles are otitis media, pneumonia, and encephalitis [3]. Infections of the lung lead to an interstitial lesion which is pathologically recognized as giant cell pneumonia. Post-measles encephalomyelitis complicates 1 in 1,000 cases [4], mainly in older children and adults. The most severe, but rare complications are measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). They are caused by a persistent MV infection of the central nervous system.

9.2 Pathogen

Measles virus (MV), the causative agent of measles, is a member of the *Morbillivirus* genus of the *Paramyxoviridae* family. It is a spherical, non-segmented, single-stranded, negative-sense RNA virus. The MV RNA genome comprises about 16 kb nucleotides and is enclosed in a lipid-containing envelope. The genome encodes eight proteins, two of which (V and C) are non-structural proteins and are alternatively translated from the RNA, coding for the phosphoprotein (P). Of the six structural proteins, P, large protein (L) and nucleoprotein (N) make the nucleocapsid that encloses the viral RNA. Hemagglutinin protein (H), fusion protein (F), and matrix protein (M), together with lipids from the host cell membrane, form the viral envelope.

The H and F proteins are responsible for virus attachment and fusion of the viral envelope with the host cell membrane. Receptor-binding residues have been mapped to the head domain of H [5–7], indicating that the primary function of the H protein is to bind to the host cellular receptors. To date, two of them (CD46 and SLAM, also called CD150) have been identified [8, 9], and another, the putative epithelial cell receptor (EpR), has been proposed [9]. The CD46 protein, which was originally identified as the vaccine strain receptor, seems to be of minor relevance for wild-type MV infection.

9.3 Transmission

Respiratory droplets from an infected person function as vehicles of MV transmission. The virus infects lymphocytes, dendritic cells, and macrophages in the respiratory tract where it replicates and then spreads to the regional lymph nodes. During viremia, the virus disseminates to various organs including the lymph nodes, skin,

kidney, gastrointestinal tract, and liver. Finally, MV infects polarized airway epithelial cells and leaves the host. The current model of MV infection postulates that systemic spread of wild-type MV depends only on infection of CD150-expressing lymphatic cells, without first virus amplification in the respiratory epithelium. The virus crosses the respiratory epithelium only when it leaves the host [6, 10].

9.4 Diagnostic, Therapy, and Current Vaccine

Clinical diagnosis of measles is based on clinical symptoms including fever, cough, coryza, conjunctivitis, and Koplik's spots followed by a generalized maculopapular rash lasting at least 3 days. However, the clinical diagnosis needs to be confirmed by laboratory testing. Specimens for laboratory testing include blood, nasopharyngeal swab, and urine.

The "gold standard" for laboratory diagnosis of measles is the demonstration of specific serum immunoglobulin M (IgM) antibodies within 7 days after the onset of rash. If the results of the acute (initial) serology in a person with clinical symptoms show low or negative IgM titers, a second blood sample drawn 7–10 days later is analyzed for measles-specific IgM and IgG titers. A significant increase in serum immunoglobulin G (IgG) antibody titer between acute and convalescent sera is indicative of infection.

Other analyses that can be performed in a laboratory include reverse transcriptase (RT) PCR and virus isolation. Monkey kidney Vero cells stably expressing human SLAM (Vero/hSLAM) [11] are now commonly used for MV isolation.

There is no cure for an acute measles infection. Young children may be treated with a preparation of human immunoglobulin. Fever may be controlled by temperature reducers, and secondary bacterial infections, such as pneumonia or an ear infection, may be treated with antibiotics. Vitamin A may lessen the severity of measles.

The best protection against measles is vaccination. Several live attenuated measles vaccines are available worldwide, either as single-virus vaccines or in combination with other vaccine viruses (commonly rubella and mumps, MMR). Most of these vaccines were derived from the Edmonston strain of MV isolated in 1954.

The measles vaccine induces both humoral and cellular immune responses. Circulating antibodies first appear between 12 and 15 days after vaccination, peak at 21–28 days, and then persist for years. Vaccination also induces MV-specific cellular immune responses [12]. The proportion of children who develop protective antibody titers following measles vaccination depends on the presence of maternal antibodies: approximately 85 % of children develop protective antibody titers when the measles vaccine is administered at 9 months of age and 90–95 % of children have a protective antibody response after vaccination at 12 months of age. As a result of these data, the CDC recommends a two-dose vaccination with MMR, with the first dose at 12–15 months and the second at 4–6 years of age. For developing countries, the WHO advises vaccination of children at 9 months of age.

9.5 New Vaccine

9.5.1 Rationale for This Vaccine

Despite the public health benefits of measles vaccination, the licensed vaccines have a number of limitations. First, live attenuated measles vaccines are easily inactivated by light and heat; therefore, a cold chain must be maintained. Second, measles vaccines must be injected subcutaneously or intramuscularly, necessitating trained healthcare workers, needles, syringes, and the proper disposal of hazardous waste. Third, maternally acquired antibodies reduce the protective efficacy of the live vaccines in early infancy. Fourth, live attenuated measles vaccines have the potential to cause severe adverse outcomes, such as lung or brain disease, in severely immunocompromised individuals. Fifth, two doses of the vaccine must be administered to achieve sufficient

levels of population immunity to interrupt MV transmission, though recent data suggest that even two doses may not be enough [13]. Finally, live attenuated measles vaccine cannot be used in the general population after global eradication of the wild-type virus. Some fundamental characteristics of an ideal anti-measles vaccine are summarized in Table 9.1.

Therefore, we [14, 15] and others (see Table 9.2) are working on developing a new anti-measles vaccine. Four main categories of MV vaccine have been or are in the process of being developed [16]: (i) immune-stimulating complexes (ISCOMs) containing the viral glycoproteins, (ii) Protollin-adjuvanted split MV vaccine, (iii) DNA vaccines, and (iv) viral-vectored vaccines. We have chosen stably transfected human cells as a vaccine antigen production platform because of the absence of safety issues associated with the use of viruses and viral vectors. In addition, posttranslational modifications of proteins expressed in human cells most closely resemble natural modifications of the viral proteins.

Table 9.1 Characteristics of an ideal anti-measles vaccine

Only requires administration of a single dose
Safe in immunocompromised individuals
Confers protection of young infants and newborns
Protection begins soon after vaccination and is long-lived
Administered without a needle and syringe
No need for a cold chain
Works in combination with other vaccines
Affordable for low-income countries

9.5.2 Antigen

The antigen that we used in our research is the globular head domain of MV H protein (residues 156–617). The MV H protein is a 617 amino acid (78-kDa) type II transmembrane glycoprotein (Fig. 9.1a) which is comprised of an N-terminal cytoplasmic tail, a membrane-spanning domain, and an extracellular membrane-proximal stalk region connected to a large C-terminal globular head [28]. The MV H protein plays a vital role in viral tropism, receptor binding, and the hemagglutinating activity. Since the majority of MV-neutralizing antibodies direct to the MV H protein, they also play a crucial role in induction of protective immunity against measles [29].

The full-length MV H forms dimmers [30], and the globular head domain was also crystallized as a dimer [31]. Analysis of the crystal structure of the globular head domain revealed that it covered with N-linked sugars [31, 32]. Therefore, a recombinant protein needs to be produced in mammalian cells as its antigenicity may depend on its glycosylation [33].

Table 9.2 Recombinant measles vaccines that demonstrated protection in primates

Vaccine	Mode of delivery	References	Notes
ISCOMs	i.m.	van Binnendijk et al. [17, 18]	Immunogenic in the presence of MV-specific antibodies
Vaccinia virus	i.d., i.m.	van Binnendijk et al. [17]	Not immunogenic in the presence of MV-specific antibodies
Modified vaccinia virus	i.m., i.n.	Stittelaar et al. [19, 20]	Immunogenic in the presence of MV-specific antibodies, safe in immunosuppressed animals
Alphavirus replicon particles	i.m., i.d.	Pan et al. [21, 22]	Protects juvenile and infant macaques
DNA	Gene gun, i.d.	Polack et al. [23–25]	Limited protection in newborns and in the presence of MV-specific antibodies
Alphavirus replicon DNA + adjuvanted protein	i.d, i.n.	Premenko-Lanier et al. [26]	Protects juvenile and infant macaques

IM intramuscular, *ID* intradermal, *IN*, intranasal
More comprehensive overview of the studies on experimental measles vaccines can be found elsewhere [16, 27]

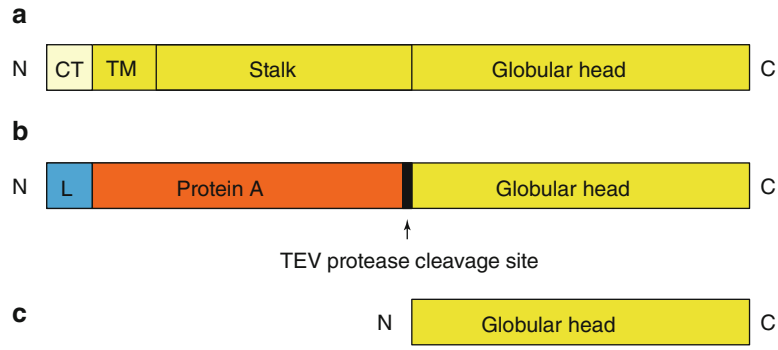

Fig. 9.1 Schematic presentation of proteins. (**a**) The MV H protein (78-kDa). CT, cytoplasmic tail; TM, transmembrane domain; N and C, N- and C-terminus, respectively. (**b**) The recombinant ProtA-MV-H156/617 protein (95-kDa) that was produced by stably transfected HEK 293 T cells. L, leader sequence; TEV, tobacco etch virus. (**c**) The recombinant H 156/617 protein (60-kDa) that has been used as an antigen in the feasibility study

9.5.3 Antigen Production

A codon-optimized gene encoding globular head domain of MV H (residues 156–617) was cloned into the expression vector, pPA-TEV [34], in frame with the transin [35] leader, the protein A purification tag, and the tobacco etch virus (TEV) protease cleavage site resulting in pProtA-MV-H156/617. Next, human embryonic kidney (HEK) 293 T cells (ATCC CRL-1573) were transfected by the calcium phosphate method, a bulk culture resistant to puromycin (5 μg/ml) was expanded, and the media were assayed for secreted protein levels by Western blot analysis using an anti-ProtA antibody.

The recombinant ProtA-MV-H156/617 protein (Fig. 9.1b) was produced in HEK 293 T cells grown in DMEM/F12 (50:50, Invitrogen) containing 3 % FBS (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mg/L puromycin, and 1 mg/L aprotinin. The media were filtered and concentrated ten-fold on a TIFF prepscale concentrator (Millipore). The concentrated medium was incubated with IgG sepharose beads overnight at 4 °C. The beads were then washed with buffer (50 mM Tris pH 7.5, 150 mM NaCl) followed by an overnight on-column cleavage with TEV protease to release the H protein fragment (H 156/617, Fig. 9.1c). The H protein fragment was exchanged into 50 mM Tris pH 7.5, 50 mM NaCl and purified on a HiTrap Q (GE Healthcare) column. The resulting protein was concentrated (to 1 mg/ml) and exchanged into sterile PBS using a 10 K concentrator (Millipore) for the immunization studies. The purified protein yield was ~ 1.8 mg per 2 L of harvested cell culture medium.

9.5.4 Animal Models and a Feasibility Study

Nonhuman primates have been used as the model of choice for the development and evaluation of MV vaccines and in the solution of many questions in MV pathogenesis. MV infection in cynomolgus [36] and rhesus [37] monkeys produces a disease with a number of similarities to that in infected humans. Experimentally infected cynomolgus monkeys develop viremia, cellular immune responses to the virus, and evidence of immune suppression but often lack clinical signs of measles. Macaques experimentally infected with MV by respiratory inoculation develop a maculopapular rash, infection of PBMC, lymphopenia, and immunosuppression.

Cotton rats are another model for MV, as these animals can be infected with MV via the intranasal route and show evidence of subsequent immune suppression [38–40]. Peak pulmonary titers have been found to occur on day 4 after infection with scattered areas of inflammation observed interstitially in lung sections. Leukocytes are the primary target cells for infection, and

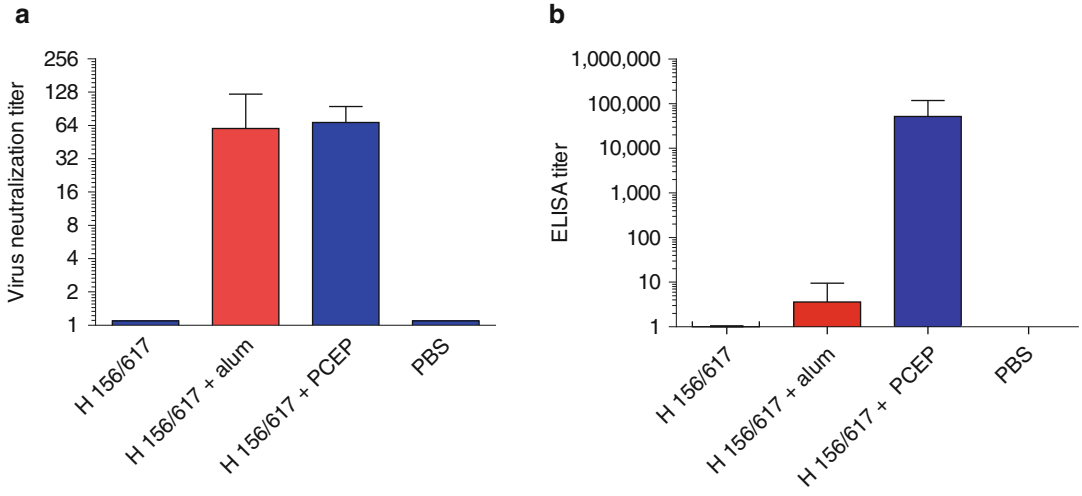

Fig. 9.2 (**a**) MV neutralization titers in mouse sera after two immunizations. (**b**) Titers of MV H-specific IgG2a antibodies in the sera of control and vaccinated mice. Error bars represent the S.D. of the mean of five mice per group

extrapulmonary virus dissemination is common. Although cotton rats cannot replace nonhuman primates as the best model, they provide a more practical model for the initial assessment of MV vaccine candidates.

In our feasibility study, we did not check the protective efficacy of the vaccine; therefore, we used a mouse model to study the immunogenicity of the vaccine. In this experiment, C57Bl/6 mice were randomly allocated to four groups of five animals each and vaccinated twice subcutaneously at a 4-week interval. The individual groups of mice were either immunized with phosphate-buffered saline (PBS), antigen alone (3 μg of the H 156/617 protein per dose), the same dose of protein formulated with alum, or the same dose of protein formulated with poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP). As shown in Fig. 9.2a, high levels of virus-neutralizing antibody titers were elicited after two vaccinations with the H 156/617 protein plus alum and the H 156/617 protein plus PCEP. The H 156/617 protein alone did not induce any neutralizing antibody titers at this dose.

In children, neutralization titers of 120 or more measured in a plaque reduction neutralization (PRNT) assay were shown to be protective [41]. Since PRNT titers are tenfold higher than neutralizing titers, PRNT titer 120 corresponds to neutralizing titer 12. In our experiment, vaccinated mice showed average neutralizing titers of about 60 which are five times the protective amount determined for children.

A Th2-type dominated immune response may be the basis for atypical measles, the severe disease primed by vaccination with a formalin-killed MV vaccine [42]. Since alum has been noted to favor a Th2-type bias [43], we tested a PCEP adjuvant in the vaccine formulation which is known to promote mixed Th1/Th2-type responses [44]. To characterize the type of immune responses generated, MV H-specific IgG2a titers in the mouse sera were assayed by ELISA. As shown in Fig. 9.2b, vaccination with the protein formulated with PCEP induced significantly higher ($P<0.05$) IgG2a titers compared to the alum-adjuvanted protein; therefore, as expected, PCEP enhanced a Th1-type immune response.

9.6 Strengths and Weakness

Use of a human cell line as an antigen production system is the strength of our vaccine developing strategy. Human cells offer the potential for proper posttranslational modification of viral

proteins, such as glycosylation that has been shown to be important in processing and antigenicity of the MV H protein [33]. Other advantages of this system are safety because it is free from viruses and viral vectors and also scalability and productivity.

In our feasibility study, we used HEK 293 T cells to establish a stable cell line expressing a secreted fragment of the MV H protein. In the future, another cell line, PER.C6®, has to be used. One of the great advantages of PER.C6® cells is that they grow in higher density than other cell lines, which means that more biological product can be harvested from smaller bioreactors. In 2008, a record yield of biological product (27 g per liter) was reported using these cells (www.crucell.com). In addition, the PER.C6® cell line has an excellent safety record, streaming from numerous preclinical and clinical studies. These data, together with a full description of the origin and development of the PER.C6® cell line, are included in a Cell Substrate Biologics Master File lodged with the FDA.

One of the limitations of the live measles vaccines is their dependence on cold-chain maintenance. After reconstitution, they lose about half of their effectiveness if stored at 20 °C for 1 h [45]. Our data [15] indicate that the recombinant H 156/617 protein is stable on storage at 37 °C for at least 2 weeks. Therefore, our new vaccine has an excellent thermal stability and cold-chain provision that is so important for current measles vaccines, may be avoided.

The history of measles vaccine development is overshadowed by unfortunate event of introduction of a formalin-killed MV vaccine in 1963. This vaccine provided transient protection, and children exposed to MV latter often developed atypical measles, a serious illness characterized by hemorrhagic rash and pneumonitis. Today, there is increasing evidence that atypical measles is caused by priming of non-protective Th2 cells [46]. Therefore, the induction of Th2-skewed responses must be avoided in measles vaccination. One of the approaches to overcome this problem is to use a Th1-type adjuvant in the vaccine formulation. We tested one of these adjuvants, PCEP, and demonstrated that it enhanced IgG2a antibody production which is an indicator of Th1-type immune response. However, the possible risk of atypical measles needs to be assessed using rhesus monkey model of the disease.

Primary protection against infectious diseases at birth is provided by maternal antibodies transferred through the placenta and with colostrum. These maternal antibodies are waning during the first months of life while the neonate's own immune system develops. However, maternal antibodies impede successful immunization with the live measles vaccines, and infants are unprotected after maternal antibodies have waned until they are vaccinated at 9 or 15 months of age. Therefore, the development of a vaccine that would induce protective immunity in the presence of maternally derived MV-specific antibodies in young infants is desirable. More experiments are needed to evaluate immunogenicity and protective efficacy of our new vaccine in the presence of MV-specific (maternal) antibodies. These experiments can be conducted using a monkey model [17] or a cotton rat model [47].

Another issue that requires increasing attention is the safety of vaccine injection devices and the safe disposal of hazardous waste. Current measles vaccines are introduced through needle injections. In our feasibility study we also vaccinated mice subcutaneously using needles and syringes. In the future, we will assess a mucosal route of the vaccine administration. Mucosal immunization will overcome the necessity of needles and may also be effective in the presence of maternal antibodies.

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Development of Subunit Vaccines Against Shigellosis: An Update

10

Francisco J. Martinez-Becerra, Olivia Arizmendi, Jamie C. Greenwood II, and Wendy L. Picking

Contents

Abstract

Shigellosis is a gastrointestinal disease with a significant morbidity and mortality rate; infants living in developing countries are the most-at-risk population. The disease is caused by *Shigella* spp., a genus comprising 4 species with 50 different serotypes. While several formulations have been proposed, a broadly protective vaccine is not yet available. The generation and characterization of subunit vaccines against *Shigella* represents a promising opportunity for vaccine development.

Shigellosis remains an important cause of morbidity and mortality, with about 90 million episodes occurring each year and about 100,000 deaths per year. About 60 % of deaths occur in infants under 5 years of age living in third world countries. The search for a *Shigella* vaccine has spanned over years, and while several advances have been achieved, a broadly protective vaccine for prevention of shigellosis is still not available. Four different species (*S. flexneri*, *S. sonnei*, *S. boydii*, *and S. dysenteriae*) have been described so far, along with more than 50 O-antigen serotype variations.

The presence of such a large number of serotypes is an important concern that raises questions about the efficacy of serotype-restricted vaccines, such as live-attenuated or LPS-based preparations. A promising approach is the use of proteins conserved across multiple

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serotypes as part of vaccine formulations. The inclusion of conserved proteins in vaccine compounds potentially solves the issue of serotype specificity, thus allowing the generation of a highly desirable pan-*Shigella* vaccine. In addition, recombinant proteins usually have increased safety profiles. In this chapter we will review the advantages of a recombinant protein vaccine against *Shigella* spp, analyze the protein candidates tested so far, and discuss the advances that have been made in this expanding field.

10.1 Shigellosis

Childhood diarrhea is one of the leading causes of mortality in children under 5 years old, with 759,000 deaths attributed to it annually [1]. This represents about 11 % of all infant deaths occurring yearly worldwide. Among the pathogens involved in diarrheal disease, *Shigella* spp are important because of their high incidence in children. Shigellosis is characterized by bloody diarrhea, tenesmus, vomiting, intestinal cramps, and fever; however, symptoms and their severity vary greatly from individual to individual. For instance, a multicenter study performed between 2000 and 2004 in Asia found that ~60–70 % of all patients positive for *Shigella* infection presented watery stools, while fever was only present in ~40 % of all cases [2]. Fatalities aside, these episodes lead to profound nutritional disadvantages with infected children often suffering stunted growth and cognitive development – adding yet another degree of severity to this disease [3, 4]. In addition, some infections lead to chronic arthritis, probably due to MHC class I-mediated presentation of self-reactive peptides [5].

Burden calculations performed in 1999 set the number of *Shigella*-mediated episodes at 165 million with 1.1 million deaths [6]. Sixty-nine percent of these episodes and 61 % of these deaths were in children under 5 years old, highlighting this age group as the most susceptible to *Shigella* disease. Recently, a decrease in global diarrheal burden has been described [7, 8]. These newer estimates set the number of episodes at 90 million episodes and 108,000 deaths [9]. These statistics, however, likely underrepresent the actual number of *Shigella* infections since some of these new analyses have excluded *Shigella* outbreaks (which have a significant impact on epidemiology), and they did not estimate the number of infections that are not treated at a hospital. Such omissions would be expected to have a large impact on the perceived burden of shigellosis in the developing world. Unfortunately, these decreased numbers mask the urgent need for a protective vaccine.

Surveillance of ten states in the USA during 2010 revealed 1,780 shigellosis cases [10] with most of them present in children under 5 years old with an incidence of 16.4 cases per 100,000 individuals. This incidence puts *Shigella* infection as the third most frequent laboratory-confirmed pathogen behind *Salmonella* and *Campylobacter*. Shigellosis is particularly important in certain high-risk populations, such as refugees, where large outbreaks have been described. Recent analysis of *Shigella* outbreaks in refugee camps situated in central Africa have described the attack rates (ranging from 6.3 to 39.1 %) and mortality (up to 9 %) associated with *Shigella* infection [11]. Another susceptible group is deployed military personnel with *Shigella* being the most isolated pathogen in the military of several countries. Diarrheal symptoms are also highly predominant in military personnel abroad with reported incidences of up to 10 % in several countries [12, 13].

10.2 *Shigella* spp as Causative Agent of Shigellosis

First defined as a causative agent of bacillary dysentery by Shiga in Japan, *Shigella* is a Gram-negative bacillus that is noncapsulated and nonmotile. Diagnosis is generally based on symptoms [14] since bloody, mucoid stools are indicative of *Shigella* infections. However, because several diarrheal infections caused by other microorganisms share these symptoms (enteroinvasive *E. coli* and *Campylobacte*r, among others), the sole analysis of symptoms is insufficient for an accurate diagnosis. Therefore,

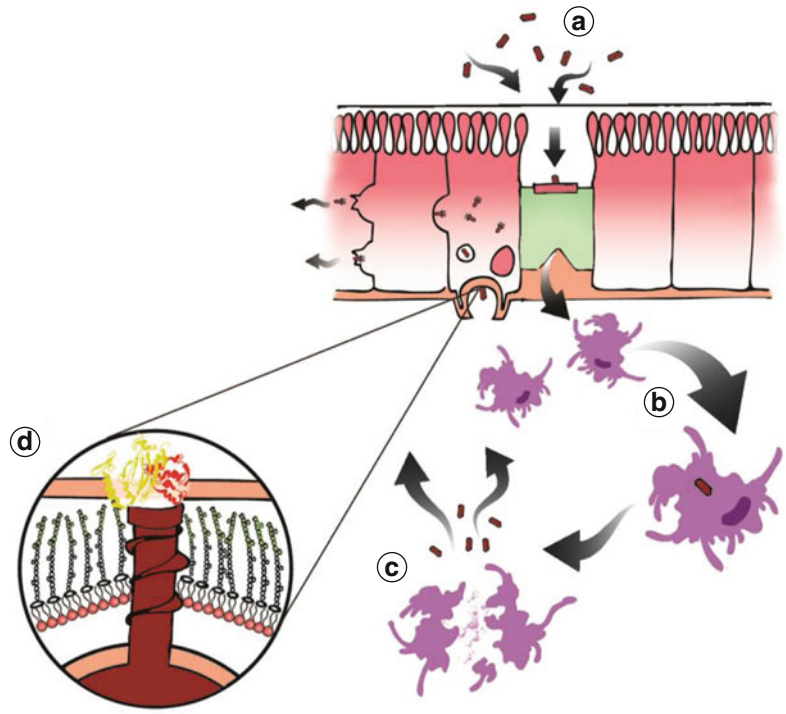

Fig. 10.1 Current model for invasion of epithelial cells by *Shigella*. *Shigella* reaches the lumen of the intestine and is taken up by M cells (**a**) and released to the basal side. Subsequently, *Shigella* is phagocytized by macrophages residing under the M cells (**b**). After escaping by inducing apoptosis (**c**), *Shigella* invades epithelial cells using its type three secretion system (T3SS) (**d**)

clinical diagnosis must be complemented with microbiological isolation from culture.

There are several difficulties associated with culture since in nature *Shigella* does not survive for extended periods of time outside a human host. Nevertheless, cultures can be grown aerobically in selective media such as MacConkey and Hektoen agar. More recently, several research groups have proposed the use of molecular techniques to confirm a *Shigella* infection [15–17]. For example, PCR amplification of the *ipaH* gene has increased the number of diagnoses of *Shigella* infection by 45 % in dysentery patients. An analysis performed in rural China demonstrated that 58 % of culture negative samples were positive for *ipaH* amplification by PCR, while 97 % of the *Shigella* culture positive samples were positive by PCR amplification. In contrast to culture methods, this PCR-based method does not lose sensitivity after the use of antibiotics, increasing the time frame for proper diagnosis without compromising patient treatment.

Thus, the application of molecular methods to *Shigella* diagnosis could give a more accurate characterization of the magnitude of *Shigella-mediated* disease worldwide.

10.3 *Shigella* Invasion and Pathogenesis

Shigella is transmitted through the fecal-oral route by consumption of contaminated food and water. Following ingestion, the acid-tolerant *Shigella* passes through the stomach and small intestine into the large intestine [18] (Fig. 10.1a). Here, they are taken up by M cells, transcytosed to the basolateral face of the colonic epithelium and presented to resident macrophages (Fig. 10.1b) wherein IpaB of the type three secretion system (T3SS) induces apoptosis by caspase 1 activation, thereby escaping killing by the macrophage (Fig. 10.1c) [19]. *Shigella* then invades epithelial cells using its T3SS to create a translocation pore in the host cell membrane to initiate an orchestrated flow of effectors into the host cell cytoplasm to induce actin rearrangements that ultimately result in uptake of bacteria (Fig. 10.1d).

Once inside, *Shigella* quickly escapes its vacuole, replicates, and moves about the cytoplasm via actin-based motility. In a T3SS-dependent manner, the *Shigella* then forms a protrusion into a neighboring uninfected cell with the resulting vacuole being quickly lysed to complete the process of intercellular spread.

The genes associated with the T3SS are encoded on a 220-kB plasmid which is highly conserved among the *Shigella* species. At the heart of the T3SS is the type three secretion apparatus (T3SA) which is composed of a basal body similar to that of flagellar systems and an extracellular needle [20]. Invasion plasmid antigen D (IpaD) is a 37 kDa protein that forms a pentameric ring at the tip of the needle [21]. It controls secretion of effector proteins and is the environmental sensor for mobilization of IpaB to the T3SA tip complex [22]. IpaB is a 64 kDa translocator that forms a ring atop the IpaD ring and is responsible for host cell contact. This contact is required for mobilization of IpaC to the needle tip [23] and formation of a complete unidirectional conduit from the bacterial cytoplasm to the host cell cytoplasm. The initiation of inflammation and invasion processes occurs exclusively at the basolateral side of host cells, highlighting the importance of the previous steps of macrophage subversion in *Shigella* colonization of the gut.

10.4 Serotype Variability and Antibiotic Resistance: Highlighting the Need for a Vaccine

Shigella spp comprises four different species or serogroups: *S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*. These four groups are further divided by O-antigen variability which generates over 50 different serotypes (14 in *S. flexneri*, 1 in *S. sonnei*, 20 in *S. boydii*, and 15 in *S. dysenteriae*) [24]. Epidemiological studies have revealed a large variability in the disease burden of each species and serotype based, in part, upon a particular geographical localization [2, 6]. A meta-analysis of case studies revealed that in Asia and Africa during the 2007–2009 time frame, *S. flexneri* was responsible for the majority of the infections described (83.5 %), while *S. sonnei* was second with 14.9 % of all cases.

In America and Europe, the frequencies of the two main serogroups were reversed: *S. sonnei* was the leading serogroup with 62.4 % and *S. flexneri* second with 31.7 %. *S. dysenteriae* and *S. boydii* are usually underrepresented with less than 4 % [25]. Another variable found to affect the prevalent serotype is the age group of the affected individuals. In Israel, a study spanning a 2-year period found that *S. flexneri* serotype 2a was responsible for the majority of *Shigella* infections in children 1–4 years old, while *S. flexneri* serotype 6 was the most isolated serotype in individuals 5–14 years old.

Serotype variability is dictated by modifications of the O-antigen portion of LPS. O-antigens vary in the number of oligosaccharide unit repeats, the types and distribution of carbohydrates, and the intra- and intermolecular linkages [26]. In *S. flexneri*, these genes are encoded in the bacterial chromosome. In contrast, *S. sonnei*, which shows no serotype variability, expresses plasmid-encoded O-antigen modification enzymes. The O-antigen is one of the major immunogenic components of *Shigella* and is a virulence factor, in part, due to masking the exposure of type three secretion apparatus [27].

Furthermore, modifications in these molecules can emerge by acquisition of genes that encode glucosyl transferases and acetyltransferases after phage infection and recombination [28]. These modifications play a key role in immune evasion [27]. Thus, O-antigen variability is an important factor that needs to be taken into consideration for vaccine development since it is the basis for serotype-specific protection – exposure to a particular serotype protects against subsequent infections by the same serotype, but not against others, highlighting the immunodominant role of LPS during infections. This dominance is so strong that *Shigella* exposure generates a majority of serotype-restricted mucosal responses masking heterologous recognition [29].

The other phenomenon in *Shigella* epidemiology that prompts vaccine development is the increasing frequency of antibiotic resistant strains. Antibiotic resistance is continually rising for this pathogen [30]. Recent studies have demonstrated that over 30 % of all *Shigella* analyzed

are resistant to ciprofloxacin, the current first-line treatment for shigellosis [25]. This continuous increase of resistant strains amplifies the urgency for generating novel *Shigella* vaccine candidates that protect against a broad number of serotypes and are safe in the infant population – the most at risk segment of our society.

10.5 Testing Vaccine Candidates: Animal Models

Shigellosis is strictly a human disease. While the basis of this restriction is unknown, it complicates the ability to investigate the pathogenesis of *Shigella*. Several animal models, however, have been developed to study the pathogenesis of *Shigella*, the resulting immune response against *Shigella* antigens and the protection efficacy of candidate vaccines against shigellosis.

Mouse Lethal Pulmonary Challenge: Many of the recent vaccine development efforts have been tested in the mouse lethal pulmonary model. In this model, a high dose of virulent *Shigella* is administered to mice intranasally. *Shigella* then invades the epithelial cells of the lung and, after a few days, the mice succumb to the infection [31, 32]. Though an unnatural model, it takes advantage of the ever-increasing breadth of knowledge of the mouse immune system as well as the sizeable pool of reagents and protocols available for analyzing the immune response. Conversely, a significant drawback is the largely different infection location, which raises questions pertaining to our ability to extrapolate the protection observed in vaccines tested in this model to humans.

Mouse Colonic Infection: Another mouse model more recently developed involves the use of streptomycin to clear the intestinal commensal bacteria. After such treatment, *Shigella* is able to colonize the colon with viable *Shigella* being isolated from fecal samples for up to 30 days [33]. Infiltration of neutrophils mediated by CXCL8 is an important immune response present in humans that is missing in these animals; however, administration of CXCL8 in conjunction with *Shigella* mimics the pathological profile described in bacillary dysentery [34].

Sereny Test: The Sereny test model [35] has long been used to test the invasive capabilities of *Shigella*. In this model, guinea pigs are inoculated in the eye with *Shigella*, which induces a keratoconjunctivitis. This model allows examination of *Shigella* invasion and the protective efficacy of candidate vaccines. As with the pulmonary model, however, the site of infection does not relate directly to the human intestinal tract.

Rabbit Cecal Ligation Model: A first approach to a rabbit model of shigellosis consisted of long periods of fasting and treatment with antibiotics and toxic agents, followed by oral administration of virulent bacteria. This infection, however, was usually restricted to the small intestine and symptoms did not always resemble human shigellosis. Later, a rabbit colon infection model was developed, which consisted of ligation in the cecal area (cecal bypass), followed by direct inoculation of virulent *Shigella* into the colon [36]. In this model, diarrhea is used as an indicator of infection and disease. Although this model can be used to characterize the interactions of *Shigella* and the intestinal mucosa at the natural site of infection, it has the difficulty that is introduced by surgery in laboratory animals.

Nonhuman Primate (NHP) Models: NHP models have been used to define the ability of vaccines to elicit immune responses and protection (rhesus and cynomolgus monkeys) [37, 38]. The main advantage of this model is that *Shigella* is able to colonize the large intestine and generate symptoms that these bacteria generate in human infection. This model would also allow a comparison of highly similar immune systems. The use of this model, however, requires highly trained personnel and specialized animal facilities. A further drawback to this model is the very high cost of experiments with NHPs.

10.6 The Search for a Vaccine: Subunit Vaccine

Two main approaches have been used in the search for a viable vaccine candidate against shigellosis: (1) generation of attenuated strains and (2) subunit vaccines. Several vaccine candidates

are based on attenuated strains where mutations have been designed to reduce virulence. The principle behind this approach is that administration of live, yet nonpathogenic, bacteria is the best vaccine to mimic the naturally occurring infection by the virulent counterpart. Mutations in one or more genes involved in *Shigella* virulence, like *virG* and *ShET1/2*, or in metabolic pathways (*aroA*, *guaBA*) [39, 40] have been generated and have demonstrated good protective efficacy in animal models. When tested in humans, however, many of these attenuated strains were either too reactogenic or poorly immunogenic. Subunit vaccines are therefore a preferred option over attenuated strains due to better safety profiles and usually better long-term stability in comparison to live, oral vaccines [24, 41].

10.7 Finding Targets for Vaccine Development

Several studies have focused on finding targets for use in subunit vaccines. For instance, analysis of the immunoproteome of *S. flexneri* revealed both soluble and membrane-bound proteins that are recognized by sera either from infected individuals [42] or from mice immunized subcutaneously with formalin-inactivated bacteria [43]. Unfortunately, at the time these studies were performed, there were no direct hits identifying *Shigella*-sequenced proteins. The hits that were observed tended to identify membrane proteins and a flagellar protein found in other microorganisms. These novel immunogenic proteins are therefore an interesting alternative for vaccine development. Further analysis and identification of these proteins could elucidate their function in *Shigella*.

Another interesting vaccine candidate is the OspE family of effector molecules. The ability of *Shigella* to bind to colonic epithelial cells [44] is increased after treatment with bile salts (which mimics the host intestinal environmental signals). Microarray experiments demonstrated that upregulation of OspE1 and OspE2 occurs after bile salts exposure. In addition, bile-induced adherence is abolished in an *ospE* deletion mutant. The fact that these molecules mediate the first adhesion to target cells makes them an interesting target for a vaccine. An interesting protein capable of immunomodulation is the major outer membrane protein (MOMP) [45], a protein with porin activity from *S. dysenteriae*. MOMP is able to activate T cells via Toll-like receptors (TLR) [46], leading to T cell proliferation and release of proinflammatory cytokines and chemokines, which could be important effectors in an immune response against *Shigella*. The further characterization of these and other molecules implicated in pathogenesis broadens the available targets for vaccine development. Other vaccine candidates have already been tested in animal models where they have been evaluated for both safety and protective efficacy. Some of them have also been characterized in clinical trials [47, 48].

O-antigen/Proteosome: As discussed above, O-antigen represents the variable portion of *Shigella* LPS (Fig. 10.2a). Administration of LPS or O-antigen alone in animal models is not enough to elicit immune responses, making them ineffective immunogens. To solve this limitation, these molecules have been used in conjunction with different proteins as carriers. Several variants of LPS/O-antigen mixtures have been developed and characterized. One of these protein combination approaches uses *S. flexneri* and *S. sonnei* LPS complexed with *Neisseria meningitidis* outer membrane protein proteosomes [49–51] (Fig. 10.2b). LPS is extracted from *S. flexneri* or *S. sonnei* by hot phenol extraction and mixed with detergent extracted outer membrane proteins from *N. meningitidis*. The complex was then separated from free LPS present in the mixture by gel filtration chromatography. The concept behind this vaccine is that the proteins present in the *N. meningitidis* proteosome are able to act as carriers for T cell stimulation, thus allowing the recognition of LPS.

When this complex is administered orally and intranasally to mice and guinea pigs [49], serum IgG and mucosal IgA in intestines and lungs are generated, and protection is demonstrated in the Sereny test, as well as in the mouse lethal pulmonary mode [51]. While this vaccine has the advantage of using *N. meningitidis* proteosomes as a carrier/adjuvant system allowing recognition of carbohydrate antigens, the response is serotype restricted. Accordingly, no cross protection was observed when proteosome/LPS mixtures

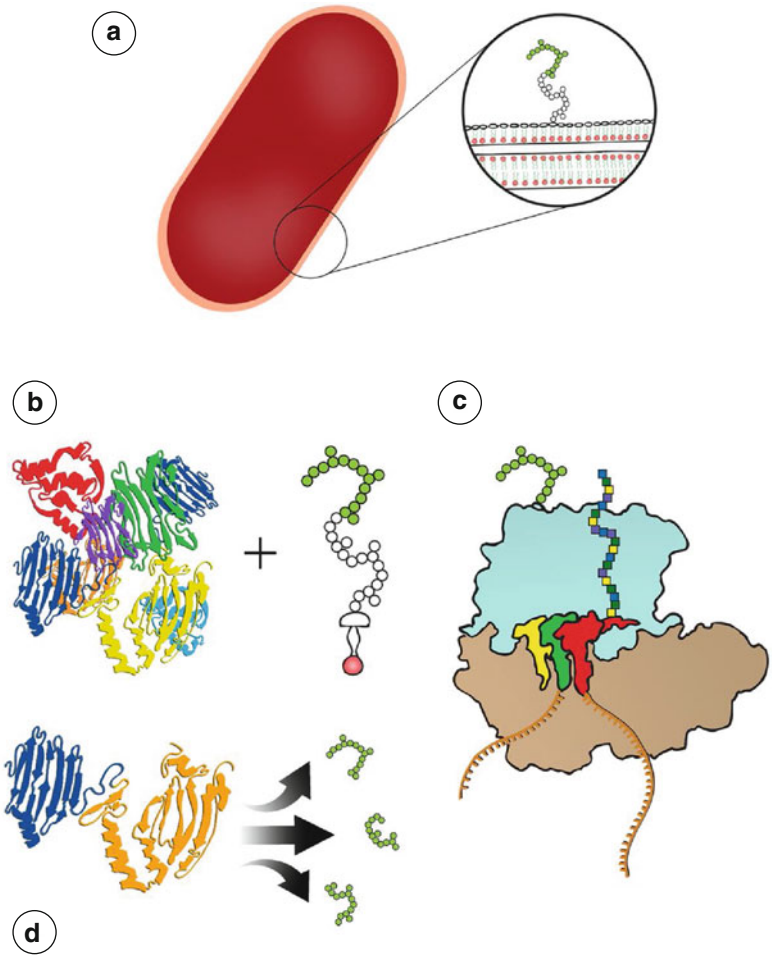

Fig. 10.2 Depiction of LPS/O-antigen-based vaccines. LPS (**a**) extracted from *Shigella flexneri* or *sonnei* is admixed with protein preparations from *N. meningitides* and used as a vaccine compound (**b**). O-antigen purified from LPS is delivered in combination with exoprotein A from *P. aeruginosa* (**c**). Finally, O-antigen from different *Shigella* serogroups is combined with ribosomes from *Shigella* is depicted in (**d**)

were administered in mice, and a heterologous organism was used for challenge.

The proteosome/LPS vaccine candidate has been tested in a phase I clinical trial in adult human volunteers using an intranasal administration. The vaccine was well tolerated with some dose-unrelated symptoms such as headache and muscle pain and mild dose-related reactions such as nasal discharge. The serum IgG antibody responses against *Shigella* LPS were statistically significant in the two highest doses (1 and 1.5 mg), but the influence of dose was not clear when mucosal IgA was analyzed [52]. During the clinical trial, the immune responses that were measured were qualified as mild in general. Finally, the safety of LPS-containing vaccines is often questioned in the field.

rEPA-LPS: Another LPS carrier includes recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA), an ADP-ribosyltransferase with adjuvant properties [53]. For this vaccine, O-antigen was generated by acid hydrolysis of bacterial LPS followed by DNAse, RNase, and protease treatment. Finally, size-exclusion chromatography separation is performed. Reactive forms of O-antigens from *S. flexneri*, *S. sonnei*, and *S. dysenteriae* were generated, conjugated to rEPA, and purified by chromatography (Fig. 10.2c). The O-antigen conjugates were delivered to mice by subcutaneous injection and the serum IgG against O-antigen was measured. The antibody levels were higher in mice immunized with the O-antigen/rEPA conjugates delivered with alum [53].

When the conjugates were administered to adult volunteers, there was an elevation of serum IgG against the O-antigen [53]. The safety and immunogenicity of this vaccine was evaluated in another study, again showing strong serum IgG

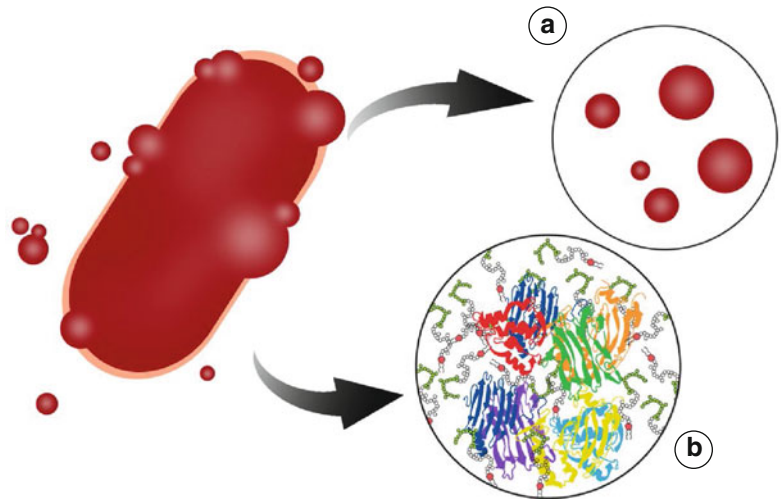

Fig. 10.3 Depiction of vesicle-based vaccines. (**a**) A vaccine preparation consists of outer membrane vesicles purified from *S. boydii*. These vesicles are naturally generated by all *Shigella* spp. (**b**) Another conjugate known as Invaplex consists of water extracts from *Shigella flexneri* or *sonnei*. This is composed primarily of LPS and proteins, in particular IpaB and IpaC

and IgA responses [54]. An efficacy trial in young adults demonstrated that this vaccine has a protective efficacy of 74 % against *S. sonnei* infection and that serum antibody levels correlated with protection [55]. This vaccine was tested in children 4–7 years old with only mild, transient adverse effects being seen. O-antigen-specific antibody levels remained after 6 weeks post vaccination [56] (or 2 years after immunization in children 1–4 years old [57]). While this vaccine has been demonstrated to be safe and protective in adult volunteers, it is of great relevance that this vaccine managed to elicit immune responses in children and generated only mildly adverse reactions. Serotype restriction, however, remains a possible drawback for this vaccine, but further development and generation of conjugate mixtures could solve this problem.

LPS-Ribosomes: Ribosomes from *Shigella* have also been employed in conjunction with O-antigen (Fig. 10.2d). The exact mechanism of adjuvant activity of these particles is not clear; however, it has been proposed that they act as carriers of weaker antigens [58]. The ribosomes are isolated from cytoplasmic extracts of avirulent *S. flexneri*. These preparations have been shown to be protective in guinea pigs, monkeys, and mice [59, 60]. Strong serum IgG responses were generated after intranasal administration of this vaccine [61]. Mucosal IgA secretion was also demonstrated in multiple mucosal compartments. Mucosal administration that elicits an IgA response was found to be required for protection generated by the ribosomes/O-antigen mixture. Parenteral administration was found not to be protective.

De Novo Synthesis: An alternative used to improve the safety profile of O-antigen-based vaccines is the synthesis *de novo* of O-antigens of different chain lengths and compositions. These molecules are generated by multistep chemical synthesis and are coupled to a maleimide-activated tetanus toxoid to generate an immunogenic conjugate [62–64]. This approach allows selecting the minimal antigenic portion of this molecule, aiming to maximize the immune recognition, while avoiding any side effects of using the full length molecules.

Mice immunized with the conjugates via the intramuscular route generated antibody titers specific for the LPS on which the synthetic O-antigen is based. One of the advantages of chemically synthesized analogues of O-antigens is to avoid the growth and processing of *Shigella*. This avoids contamination with complete, toxic LPS and other contaminants. A disadvantage is the lack of heterologous protection; however, O-antigen portions used in this vaccine can be combined to form multiple serotype vaccines. In addition, the cost of the synthesis of these analogues could hinder its use.

Shigella Outer Membrane Vesicles: Outer membrane vesicles (OMVs) are particles composed of LPS, proteins, and nucleic acids. In a proposed vaccine formulation, these particles were purified from liquid cultures of *S. boydii* by centrifugation with subsequent filtering (Fig. 10.3a). The precise identity and amount of the proteins included in this preparation is not currently known,

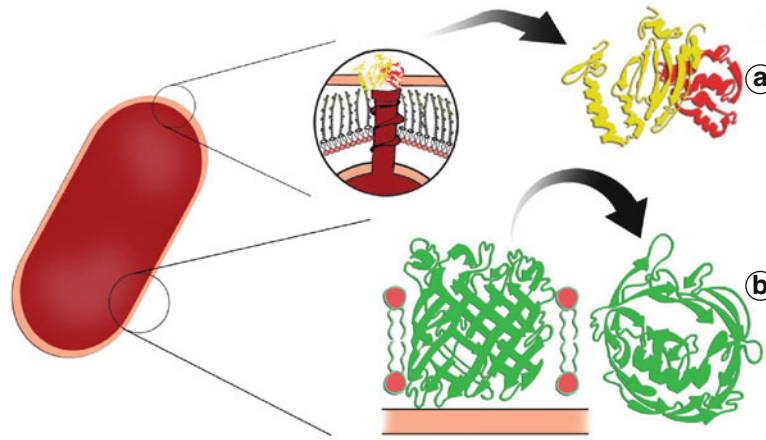

Fig. 10.4 Depiction of *Shigella* recombinant protein vaccines. IpaB and IpaD, key components of the T3SS of *Shigella* spp, are expressed in *E. coli* and administered with the adjuvant dmLT (**a**). The outer membrane protein OmpA is expressed in *E. coli* and delivered without additional adjuvants (**b**)

although the presence of proteins having the same mass as IpaB, IpaC, and IpaD suggests its composition includes these proteins. When these OMVs are administered orally to mice, antibodies are generated against OMV lysates.

Immunity is passively transferred from vaccinated female mice to offspring mice, which are then able to survive an oral infection of *Shigella*. Interestingly, heterologous protection is achieved in these immunized animals [65]. This is possibly due to the presence of proteins that are conserved across serogroups in the vesicles. These vesicles have been tested using the intranasal, oral, and ocular administration routes and are found to be effective in the mouse lethal pulmonary model, alone or combined with polymer-based nanoparticles [66]. This vaccine has the advantage of heterologous protection (as shown by challenge against strains from each *Shigella* serogroup) and the absence of adjuvant dependency. In addition, immunity can be passively transferred to offspring, suggesting that the protective mechanism involves antibodies and raising the possibility that this vaccine can be used in infants, which is the main target population for a *Shigella* vaccine. The use of live, fully virulent *Shigella* during its formulation process, the presence of LPS, and lot-to-lot consistency are possible downsides of this preparation.

Invaplex: Another vaccine candidate that uses T3SS proteins and LPS as part of the formulation is the Invaplex [67]. These complexes are obtained by aqueous extraction followed by ion exchange chromatography (Fig. 10.3b). The precise composition of these extracts has not been completely characterized but includes LPS, IpaB, and IpaC [68]. These complexes are able to elicit IgG and IgA responses against Ipa proteins as well as LPS in both mice and guinea pigs. In addition, they are protective against the *Shigella* species/serotype used for extract generation [69] in the mouse and guinea pig challenge models.

Two phase one studies have been performed using the Invaplex vaccine on adult volunteers [47, 48] and showed no major side effects to delivery of intranasal doses of up to 690 μg. The highest dose employed in these studies generated an Antibody-secreting cells (ASC) response to LPS in 58 % of the volunteers. An advantage of this approach is that, other than the Invaplex itself, no additional adjuvants need to be administered. A drawback of this vaccine consists in a challenging production process that includes cultures of virulent *Shigella* as well as the presence of bacterial LPS products in the intermediate steps and final formulation. Another possible caveat is the uniformity of protein composition in these complexes through manufacturing lots.

Finally, this vaccine was not designed to protect against multiple serotypes. A solution for this possible drawback, however, is the generation of formulations that include Invaplex complexes generated from more than one particular serotype, which increases an already difficult manufacturing process. This would allow the generation of vaccine formulations specific for the serotypes prevalent in a particular region.

Recombinant T3SS Proteins: A vaccine candidate that targets conserved *Shigella* virulence proteins includes some of the T3SS Ipa proteins (Fig. 10.4a). Recombinant IpaB and IpaD can be expressed in *E. coli* at high levels. IpaD is then

easily purified from the *E. coli* cytosol while IpaB must be purified as a complex with its cognate chaperone IpgC. The chaperone is needed to maintain the hydrophobic IpaB in a soluble state and to provide stability for IpaB from proteolytic degradation. IpaB can then be further purified after separation from IpgC in low concentrations of detergent. Analyses have indicated that IpaB is more than 90 % pure following this scheme. In its final formulation, this Ipa-based vaccine also contains a double mutant of heat-labile enterotoxin from E. coli (dmLT) [70] as an adjuvant. The mechanism of protection for this vaccine has not yet been worked out. Nevertheless, it was tested in the mouse lethal pulmonary model [71] where it exhibited over 90 % homologous protection (against *S. flexneri*) and greater than 60 % heterologous protection (using *S. sonnei* during the challenge experiments). IgG and mucosal IgA were generated after intranasal administration along with antigen-specific IFN-γ secreting cells.

Antibodies against IpaB and IpaD have been used to directly block invasion by *S. flexneri in vitro*, so this vaccine could be making use of such a mechanism; however, other mechanisms such as generation of cytokine secreting cells or activation of phagocytes cannot be excluded. The main advantage of this vaccine is that it targets the main virulence mechanism that is conserved across all pathogenic *Shigella*, thereby allowing protection against *Shigella* spp, unrestrictive of serotype. Another advantage of this vaccine is the use of two different proteins as targets. This approach reduces the possibility that mutations arise in one of the proteins that would allow the bacteria to avoid recognition of the immune response elicited by this vaccine. Among the disadvantages is the use of detergent mixture during protein production, as well as possible costs related to recombinant protein expression and purification.

OmpA: A 34 kDa outer membrane protein (OMP) was purified from *S. flexneri* 2a using ion exchange chromatography (Fig. 10.4b). Incubation of macrophages with this 34 kDa protein induced the production of nitric oxide and increased production of IL-12 and TNF-α. This protein was delivered parenterally five times in rabbits, giving protection against challenge by *S. flexneri* in the rabbit cecal ligation model [72]. Subsequent work using a recombinant protein purified by affinity chromatography identified this 34 kDa OMP as OmpA, part of a family of immunomodulating proteins present in numerous Gram-negative bacteria. This protein showed high protective efficacy in the mouse lethal pulmonary model [73] where it elicited serum IgG and mucosal IgA.

In addition, this protein is able to directly activate TLR2 and induce Th1 responses [74]. Interesting advantages of the use of this protein as a vaccine include the absence of LPS during the vaccine preparation steps and OmpA's inherent immune activation activity that allows it to be used without the addition of an adjuvant. It will be interesting to determine whether this vaccine confers heterologous protection, not only across different *Shigella* serotypes but heterologous across other pathogenic bacteria that express closely related OmpA orthologues.

10.8 Closing Remarks

Shigellosis remains an important global public health problem, especially in the developing world where children under 5 years of age are especially vulnerable to multiple episodes of shigellosis. The high variability of *Shigella* serotypes and increasing incidence of antibiotic resistance underscore the need for increased efforts in vaccine development. Ideally, this vaccine should protect against multiple serotypes and have a solid safety profile due to the high incidence of shigellosis in infants, the group to whom vaccine efforts should be directed.

The use of subunit vaccines represents an important effort toward the development of such a vaccine having a broad range of serotype protection and improved safety when compared to attenuated *Shigella* strains. This is especially valid for the recombinant protein vaccine candidates. Currently, several recombinant protein vaccines and O-antigen-containing preparations have been tested in animal models and some of them are in clinical trials where they are showing good immunogenicity and protective capacity [24].

Unfortunately, the full scope of immunological protective mechanisms as well as how they are able to block disease in each of the different animal models is still unclear for many of these vaccine candidates. Knowledge generated during the study of *Shigella* pathogenic activities will further stimulate vaccinology efforts with identification of novel targets for vaccine development. Moreover, the full analysis of the protective mechanisms of these compounds will allow us to further our understanding of the interactions of this pathogen with its host, thereby enabling us to apply this understanding to other complex pathogens.

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Development of Subunit Vaccines for Group A Streptococcus

11

Colleen Olive

Contents

Abstract

There is currently no available vaccine to prevent infection with *Streptococcus pyogenes* (also known as group A streptococcus, GAS), which can lead to the life-threatening diseases rheumatic fever (RF) and rheumatic heart disease (RHD). RF and RHD occur worldwide with many populations of developing countries and indigenous populations of developed countries experiencing particularly high rates of these diseases. The majority of research efforts to develop a GAS vaccine are based on targeting the bacterial surface antigen called M protein. However, since the M protein contains host tissue cross-reactive B and T cell epitopes, whole M protein antigen vaccine approaches are not a viable option because of the potential to induce autoimmunity. This chapter will discuss the various subunit vaccine approaches based on the M protein that are being investigated towards the development of a highly efficacious GAS vaccine that includes the different delivery technologies, formulations and adjuvants used to administer these vaccine candidates in preclinical studies, and clinical development.

11.1 Disease

Group A streptococcus (*Streptococcus pyogenes*) (GAS) is an important human mucosal pathogen that is responsible for a wide spectrum of diseases with varying clinical manifestations and

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Table 11.1 Major clinical manifestations of GAS infections

Non-invasive diseases	Invasive diseases	Post-infectious diseases
Pharyngitis	Cellulitis	Rheumatic fever
Tonsillitis	Pneumonia	Rheumatic heart disease
Impetigo	Necrotising fasciitis	Post-streptococcal glomerulonephritis
Pyoderma	Toxic shock syndrome	
Scarlet fever	Erysipelas	
Otitis media	Meningitis	
	Septicaemia	

severity [1, 2] (Table 11.1). Pharyngitis (strep throat) is a common minor complication of GAS infection, but left untreated can lead to life-threatening diseases including the autoimmune sequelae rheumatic fever (RF) and rheumatic heart disease (RHD). Of the 616 million new cases of GAS pharyngitis diagnosed each year, it is estimated that up to 3 % can give rise to RF and potentially progress to RHD [3, 4]. RF affects mainly the joints, brain and heart, leading to symptoms of polyarthritis, chorea and carditis [5]. RHD results in permanent damage to the heart tissues and valves.

GAS infections cause >500,000 deaths each year mostly in developing countries and indigenous populations within developed nations where poor socioeconomic conditions and overcrowding contribute to the high rates of GAS diseases [3]. In developing countries, RF is the leading cause of heart disease among children [6]. There is currently no available vaccine to prevent infection with GAS and consequently prevent GAS diseases.

The development of a safe and effective GAS vaccine that induces mucosal immunity to eradicate GAS from the upper respiratory tract is the best strategy to assuage the global GAS disease burden and would be a major public health contribution. Immunity at mucosal surfaces requires the induction of secretory IgA antibodies [7].

In addition to antibodies, Th17 responses have recently been reported to be critical for host defence against mucosal GAS infection [8]. A successful mucosal GAS vaccine would therefore need to stimulate the appropriate humoral and cellular immunity for protection against GAS infection (Fig. 11.1). This is especially difficult due to a lack of human compatible mucosal vaccine adjuvants that are essential to boost immune responses. Researchers have therefore focused mainly on parenteral GAS vaccine delivery approaches, for which suitable adjuvants are available, designed to provide protection against systemic infection via the induction of opsonic IgG antibodies.

GAS that breaches the physical barrier of the mucosal epithelium of the nasal-associated lymphoid tissue, functionally analogous to human tonsils, is purported to be transported to the underlying lymphoid tissue via association with membranous (M) cells. Cells of the innate immune system sense GAS and produce cytokines and chemokines to contain the infection to the mucosa. GAS antigens are delivered to antigen-presenting cells such as DCs and B cells. IgA-committed B cells are activated and initiate antigen-specific IgA responses. DCs play a fundamental role in the development of immunity to GAS and present antigen to T cells to induce a Th17 response that is integral along with IgA for mucosal defence against pharyngeal GAS colonisation. Mucosal vaccination is designed to mimic these responses and effectively clear GAS from the mucosal surface upon infection to prevent GAS colonisation and carriage. GAS that escapes the host's defence mechanisms can disseminate into the lymphatics and blood, leading to systemic infection. Mucosal vaccination is also able to induce a systemic immune response characterised by the induction of opsonic IgG antibodies, which destroy the pathogen by opsonophagocytosis. Parenteral GAS vaccination induces serum IgG but is not able to induce mucosal immunity

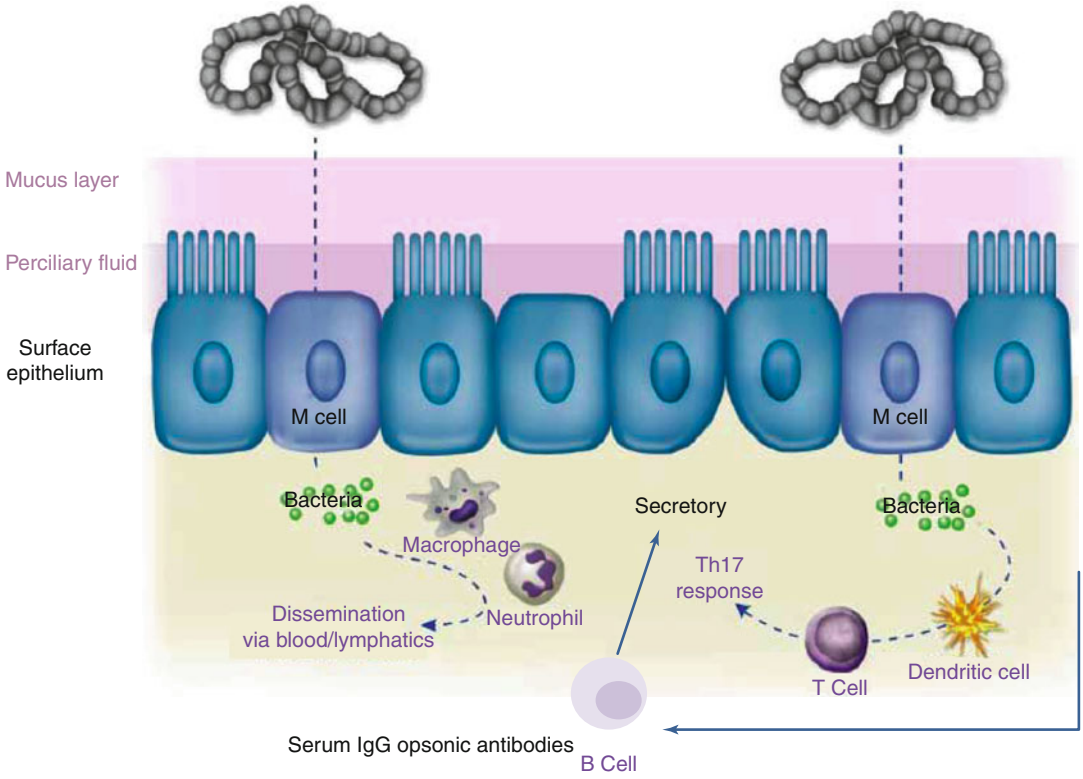

Fig. 11.1 GAS vaccination approaches

11.2 Vaccine Delivery

An effective GAS vaccine needs to have broad antigenic reach because of the many different GAS strains (>150 different M types) circulating in a population, and should not induce immune responses that are potentially cross-reactive with self tissue proteins. The GAS M protein is the major protective antigen and an ideal target for vaccine development; however, it contains heart tissue cross-reactive epitopes particularly in the conserved region [9–11]. Evidence suggests that cross-reactive T cells especially play a pivotal role in the pathogenesis of RHD [12].

The M protein is an α-helical coiled-coil surface protein consisting of a hypervariable amino-terminal region and a highly conserved (>98 % sequence identity) carboxy-terminal C-repeat region [13] (Fig. 11.2). Functionally, the M protein is important in preventing bacterial clearance by complement-mediated phagocytosis, which limits host defence mechanisms [14]. Previous studies indicate that protective immunity to GAS can be evoked by opsonic antibodies to serotypic epitopes at the amino-terminal region that are M type-specific [15].

Antibodies elicited to C-repeat region epitopes are generally less opsonic and may exert protection against GAS that is non-serotypic via blocking bacterial cell adhesion and colonisation of mucosal surfaces [16]. Researchers have focused on using M protein fragments or peptides containing protective epitopes as potential subunit GAS vaccine candidates and concomitantly identifying suitable delivery strategies.

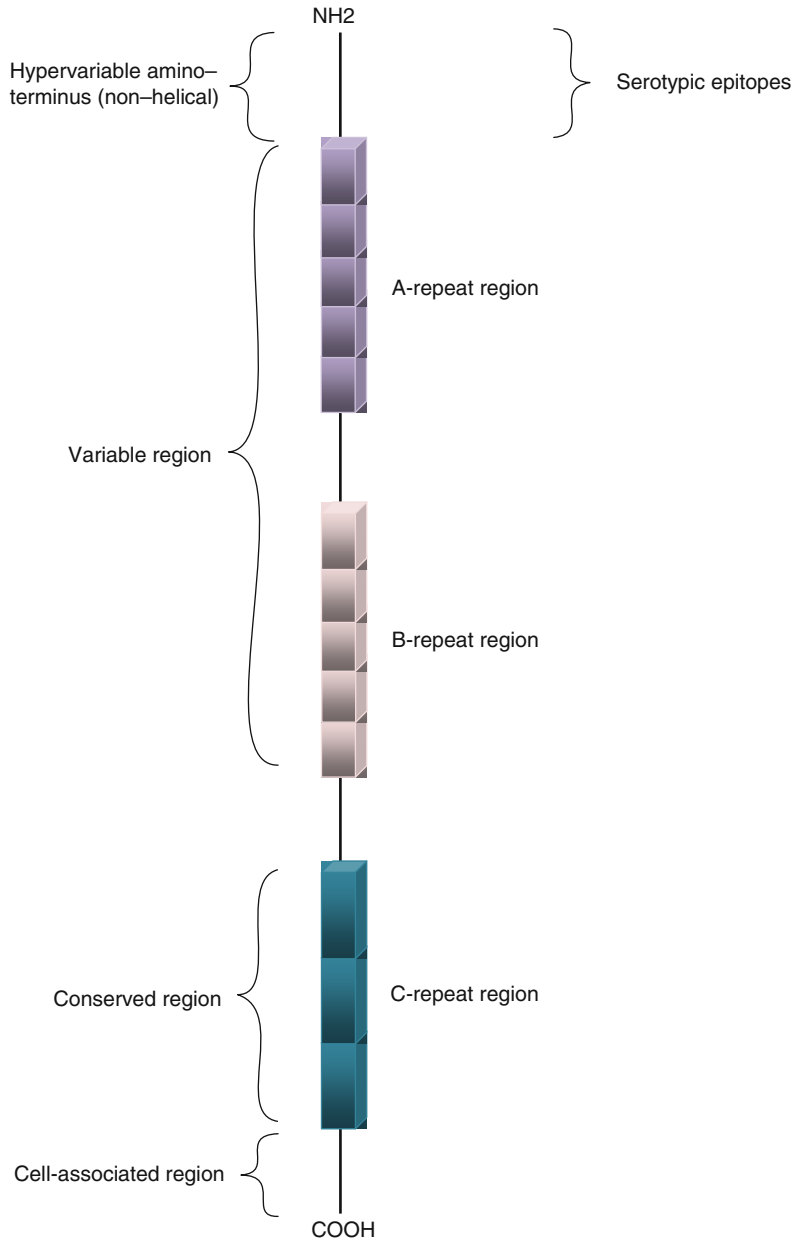

Fig. 11.2 Diagrammatic representation of the GAS M protein illustrating the different repeat regions (*A*, *B* and *C*), location of serotypic epitopes and increasing conservation from the amino-terminal to the carboxy-terminal region

11.3 Formulation/Chemistry of Adjuvant

Subunit vaccines, while offering greater safety, are intrinsically poorly immunogenic, and immunopotentiators are essential to boost the activation of immune responses. The current vaccine adjuvants licensed for use in human vaccines are limited to alum, the alum-monophosphoryl lipid A (MPL) combination adjuvant system (AS)04, oil-in-water emulsions MF59 and AS03 and reconstituted influenza virosomes [17]. The function of an adjuvant is to increase uptake of antigen by antigen-presenting cells (APCs) and to stimulate pattern recognition receptors (PRRs) on cells of the innate immune system, thereby

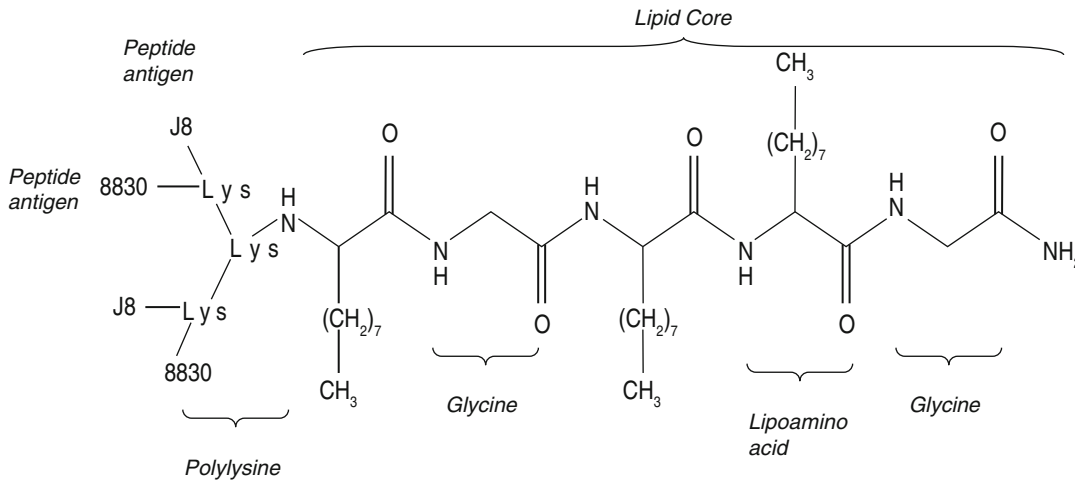

Fig. 11.3 The Lipid-Core-Peptide (LCP) System

initiating an innate immune response. Dendritic cells (DCs) translate the appropriate signals from the innate to adaptive immune system to stimulate B- and T-cell responses, and as such play a pivotal role in vaccine efficacy.

11.4 Microtechnology/ Nanotechnology

Combined vaccine/adjuvant delivery systems offer the potential of mucosal vaccine delivery. For example, the lipid-core-peptide (LCP) system is a novel, synthetic, self-adjuvanted vaccine delivery system that incorporates the adjuvant (PRR agonist), carrier and antigenic peptides of a vaccine into a single molecular entity [18] (Fig. 11.3). This system has been previously shown to efficiently deliver GAS vaccines and induce immunity [19]. Evidence suggests the adjuvant activity of LCP involves the induction of DC activation [20, 21].

11.5 Working Mechanisms

Alum is widely used in human vaccination but its mechanism of action remains unclear. Reports have suggested the adjuvanticity of alum is mediated by activation of the NLRP3 inflammasome [22–24], alteration of membrane lipid structures [25] and by host cell DNA released as a result of cell death [26]. The adjuvanticity of MF59 appears to be independent of the NLRP3 inflammasome [27] and the mechanisms of action of AS03 and virosomal adjuvants are less understood. Unlike alum, which promotes Th2 responses [28], MPL modulates the quality of the immune response towards a balanced Th1/Th2 response [29, 30]. MPL is a detoxified derivative of LPS from *Salmonella minnesota* and is the first of a new generation of defined vaccine adjuvants to achieve widespread use in human populations since the approval of alum [17].

MPL enhances adaptive immunity without causing excessive inflammation. The mechanism by which MPL enables potent but safe adjuvanticity appears to be a result of biasing cell signalling pathways [31, 32].

The LCP system contains an adjuvant component (lipid core made of lipoamino acids) and a polylysine carrier onto which peptide epitopes are attached. The example shows a GAS vaccine candidate containing J8 and an amino-terminal serotypic epitope called 8830. The adjuvant has three 2-amino-dodecanoic ($n=9$) lipoamino acids separated by glycine amino acid spacers

It has also recently been reported that MPL fails to activate caspase-1 leading to defective production of the proinflammatory cytokine IL-1β and the impairment of NLRP3 inflammasome activation [33].

11.6 Animal Model and Feasibility Study

It has been demonstrated in murine passive transfer experiments that M protein-specific secretory IgA but not IgG protected mice against mucosal GAS infection, as indicated by a reduction in nasopharyngeal GAS colonisation [34]. These findings indicate the importance of a local IgA immune response in preventing GAS colonisation of mucosal surfaces. Vaccination by the mucosal route such as intranasal delivery is therefore a logical approach to prevent GAS infection. Mucosal GAS vaccination would also elicit systemic immunity by inducing opsonic antibodies.

11.7 Preclinical Development

Three approaches are currently being investigated in the development of a subunit GAS vaccine based on the M protein. A multivalent approach employs a combination of amino-terminal protein fragments representing different M types and is designed to target prevalent GAS strains in a population. Using this approach, a recombinant multivalent GAS vaccine containing M protein peptides from 26 different GAS serotypes prevalent in North America was demonstrated to evoke opsonic antibodies in animals [35]. From epidemiological data, the 26-valent vaccine would cover the majority of pharyngitis and invasive GAS diseases, including RF, invasive fasciitis and toxic shock syndrome. Recently, a new 30-valent GAS vaccine was shown to be immunogenic in rabbits and evoked opsonic antibodies against "non-vaccine" serotypes [36] potentially creating a vaccine with much broader coverage. This type of vaccine is population-specific and therefore may not be effective universally. It may also need to be redesigned periodically to reflect changes in the epidemiology of GAS infections.

A GAS vaccine that employs peptide epitopes from the conserved C-repeat region of the M protein is the second approach and has the potential in theory for greater coverage of M types. Immunisation of mice with a C-region peptide GAS vaccine candidate called J8 conjugated to the carrier protein diphtheria toxoid (dT) and co-delivery with an appropriate adjuvant led to protection against systemic and mucosal GAS infection [37–39] (Fig. 11.4). J8 also elicited protective immunity against GAS when linked to lipopeptides [40, 41]. Other studies have shown that intranasal immunisation of mice with C-region peptides conjugated to the experimental mucosal adjuvant cholera toxin B subunit (CTB) evoked protective immunity against GAS at the mucosal level [16, 42, 43]. CTB could possibly enter olfactory regions of the central nervous system and cause neuronal damage following intranasal delivery [44], and therefore is not suitable for human use. Vector delivery approaches have included expressing the C-repeat region on vaccinia virus [45], the commensal bacterium *Lactococcus lactis* [46, 47] or *Streptococcus gordonii* [48].

The third combination vaccine approach uses both serotypic and conserved M protein peptide epitopes. Initially, a heteropolymer GAS vaccine construct was synthesised by free radical-induced polymerisation of acryloyl peptides to combine seven serotypic epitopes and a highly conserved C-region peptide epitope called J14 [49]. The M types that were targeted in the heteropolymer represented GAS infections prevalent in the Northern Territory of Australia – a region highly endemic for GAS. Immunisation of mice with the heteropolymer demonstrated excellent immunogenicity and protection against homologous and heterologous GAS strains, indicating its potential to provide broad coverage [49, 50]. However, batch-to-batch variation led to altered immune responses (C. Olive, unpublished data), which limited its applicability for human use. The vaccine also required the addition of an adjuvant to be effective, further limiting its use as a mucosal vaccine due to a lack of safe and effective mucosal adjuvants. Later, multiepitope GAS vaccine candidates were synthesised based on the LCP system that induced highly opsonic antibodies following parenteral delivery to mice [51, 52], as well as protection against mucosal GAS infection following intranasal immunisation [53] (Fig. 11.4).

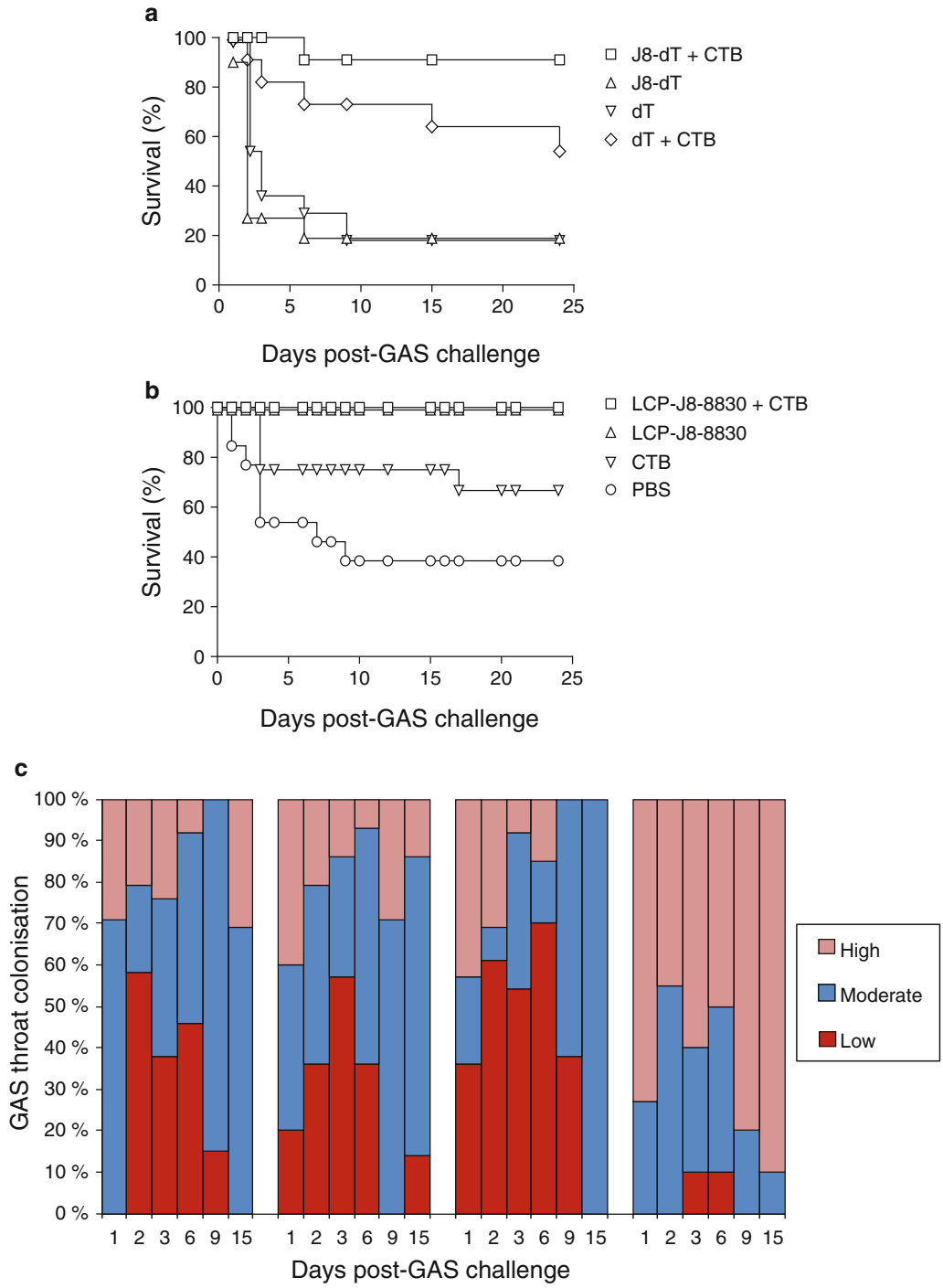

Fig. 11.4 Preclinical evaluation of GAS vaccine candidates. Intranasal immunisation of mice with the J8-dT vaccine candidate led to significantly greater survival after intranasal challenge with GAS versus control groups but the mucosal adjuvant CTB was essential for protection (**a**). A multiepitope LCP-based GAS vaccine candidate elicited protective immunity against mucosal GAS infection even in the absence of CTB (**b**). The LCP-GAS vaccine candidate and J8-dT were also effective in reducing the burden of GAS detected in throat swabs but unlike J8-dT the LCP-GAS vaccine candidate did not require CTB for its efficacy (**c**) (Reprinted from Olive et al. [39], with permission from Elsevier. (**b**) Adapted from Olive et al. [53], with permission from Oxford University Press. (**c**) Reprinted fromOlive et al. [53], with permission from Oxford University Press. Olive)

11.8 Safety and Efficacy

The main concern when using large regions of the M protein in a GAS vaccine is the potential for inducing an autoimmune response due to immunological cross-reactivity with host proteins. It is therefore important to identify protective antigenic determinants and to separate the biological relevant epitopes from those that are host tissue cross-reactive and potentially harmful. Epitope mapping studies were used to identify the conserved GAS vaccine candidate J8, which contains a conformational protective B-cell epitope and was designed to lack a human heart cross-reactive T-cell epitope [54, 55].

11.9 Clinical Development

Early human clinical trials used the whole M protein in mineral oil emulsion administered into adult patients that had various chest complications [56]. Although antibody responses were induced, which supported the feasibility of an M protein-based vaccine strategy, the formulations were highly reactogenic and a number of individuals experienced either considerable pain, soreness or stiffness for several days. Immunisation of healthy adult volunteers with a purified type 1 M protein induced serum opsonic antibodies and prevented clinical illness following mucosal challenge with GAS but had no effect on GAS colonisation of the upper respiratory tract [57]. Conversely, administration by aerosol spray of purified type 1 M protein into the nasopharynx of 21 healthy adults, prior to mucosal challenge with GAS, prevented both GAS colonisation and clinical illness [58].

Similarly, intranasal administration of purified M proteins type 3 or type 12 but not parenteral delivery in alum into healthy individuals led to a reduction in GAS throat colonisation [59]. While no sequelae of GAS infection occurred in these studies, Massell et al [60]. reported two cases of RF and one case of probable RF among 21 children vaccinated with partially purified type 3 M protein. This high rate of RF when compared to unvaccinated individuals raises the possibility that vaccination with M protein may lead to RF [60]. Therefore, the use of the entire M protein is not considered a viable option for a GAS vaccine and has been superseded by the development of subunit vaccines that contain only protective epitopes.

Dale' s multivalent subunit GAS vaccines made from the terminal fragments of the surface M protein are designed to produce protective antibody responses against different GAS strains. Clinical trials showed that subjects vaccinated with the 26-valent StreptAvax™ (ID Biomedical Corporation) developed antibody responses to the vaccine, and none of these antibodies cross-reacted with human tissues [61, 62]. Serum of vaccinated subjects demonstrated significant killing of all 26 GAS serotypes included in the vaccine. StreptAvax™ demonstrated broad immunogenicity and was well tolerated by the volunteers.

Clinical trials are planned with J8-dT formulated in alum (personal communication, Good MF).

11.10 Strengths and Weaknesses

The mucosal administration of vaccines is highly desirable for inducing mucosal immunity against mucosal pathogens but is rather a difficult task. Poor immunogenicity due to inefficient antigen uptake, and the identification of potent mucosal adjuvants that can be tolerated in humans, are key parameters that need to be overcome for this approach to be feasible.

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Vaccination Against Malaria Parasites: Paradigms, Perils, and Progress

12

Noah S. Butler

Contents

Abstract

Malaria, caused by infection of humans with *Plasmodium* spp., remains a global health emergency with >200 million new cases and hundreds of thousands of deaths annually. Although naturally acquired resistance against severe malarial disease can develop with age and following repeated exposures to the parasite, current control of *Plasmodium* infection still relies heavily on the use of anti-malaria chemotherapies. However, the continued selection of drug-resistant parasites continues to confound efforts to effectively manage the incidence and prevalence of the disease caused by this major human pathogen.

Thus, the development of an efficacious vaccine against *Plasmodium* remains a major goal for improving global public health. Despite decades of significant effort, currently no licensed vaccine for malaria exists. Several factors contribute to the difficulties with developing anti-malaria vaccines, most notably the complex multi-host, multistage developmental life cycle of *Plasmodium* parasites. Additionally, our limited understanding of the immunologic requirements necessary for the host to control, or clear, *Plasmodium* parasites remains poorly defined. Importantly, recent work has improved our understanding of host-*Plasmodium* parasite interactions and has provided critical insight into new strategies for enhancing anti-malarial immunity via prophylactic vaccination.

Based on these new concepts and paradigms, exciting progress has been made in the

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areas of subunit, vectored, and whole-parasite vaccination against liver-stage and blood-stage *Plasmodium* infection. Herein, we review the biology and pathogenesis of *Plasmodium* parasite infection and highlight recent progress and illustrate remaining hurdles for vaccination against malaria parasites.

12.1 Introduction

Malaria develops following infection with *Plasmodium* parasites. The disease remains a global health crisis with more than 650,000 deaths and >215 million new clinical cases annually [1, 2]. To date, more than 80 % of all malaria-associated deaths occur in sub-Saharan Africa [2]. Effective tools to combat the infection exist, including drugs that can cure malaria and insecticides to control mosquito vectors responsible for transmission of *Plasmodium* parasites. However, the widespread use of these agents has continued to select for drug-resistant parasites [3] and chemical-resistant mosquitoes [4], further complicating efforts to control malaria [5]. For these reasons the international community is embracing the long-term goal of eradicating malaria through the generation of a safe and effective vaccine.

Vaccines have proven effective against a multitude of disease-causing microbes including the agents that cause smallpox, rubella, and whooping cough [6]. Indeed, a licensed anti-malaria vaccine would be a powerful weapon against this devastating disease and significantly close the gap left by the use of therapeutic and prophylactic drugs, insecticides, and bed nets. Given that nearly 50 % of the world's population lives in regions where malaria transmission is endemic [2], it is easy to appreciate how even a modestly effective vaccine would protect hundreds of thousands of people from malaria each year.

Clearly, the motivation for developing a anti-malaria vaccine is strong. However, despite more than 50 years of effort, our world is still without a fully licensed vaccine that affords sterilizing immunity against *Plasmodium* parasite infection. Achieving the goal of malaria eradication cannot happen without the generation of a vaccine that completely prevents the infection or the transmission of the parasite. The current lack of an effective vaccine against malaria is likely due to many factors, including inadequate funding, the complexity of the parasite life cycle [7, 8], and extensive antigenic variation among field isolates [9]. While these are significant obstacles, perhaps the largest hurdle to developing a anti-malaria vaccine is our incomplete understanding of the complex relationship between the parasite and the immune system of the vertebrate host. However, with the advent of *Plasmodium* molecular biology and reverse genetics tools, coupled with the development of new approaches to anti-malarial vaccination, we are moving closer to an effective, licensed anti-malaria vaccine. In this chapter we will focus our attention on the relationships between the biology/life cycle of the parasite, malaria pathogenesis and disease, and how an interconnected understanding of these processes has both identified pitfalls and enhanced strategies for anti-malarial vaccination.

12.2 Plasmodium Life Cycle and Transmission

Malaria parasites belong to the phylum *Apicomplexa* and genus *Plasmodium*. The defining characteristic of all *apicomplexan* parasites, including *Cryptosporidium* and *Toxoplasma*, is the presence of the "apical complex," which comprises the biological machinery that mediates host cell invasion [10]. *Plasmodium* parasites can exist in two hosts: a vertebrate intermediate host, in which reproduction is asexual, and a definitive host, *Anopheles* mosquitoes, in which sexual reproduction takes place [11]. Female *anopheles* mosquitoes are also responsible for host-to-host transmission and therefore function as the vector of malarial disease. When transmitted by *anopheles* mosquitoes during blood meal feeding, *Plasmodium* sporozoites are first deposited in the dermis of the vertebrate host [12] (Fig. 12.1).

Only several dozen to a few hundred sporozoites are inoculated during a mosquito bite [13], so parasite burden is relatively low following initial transmission. Of note, this numerical bottleneck

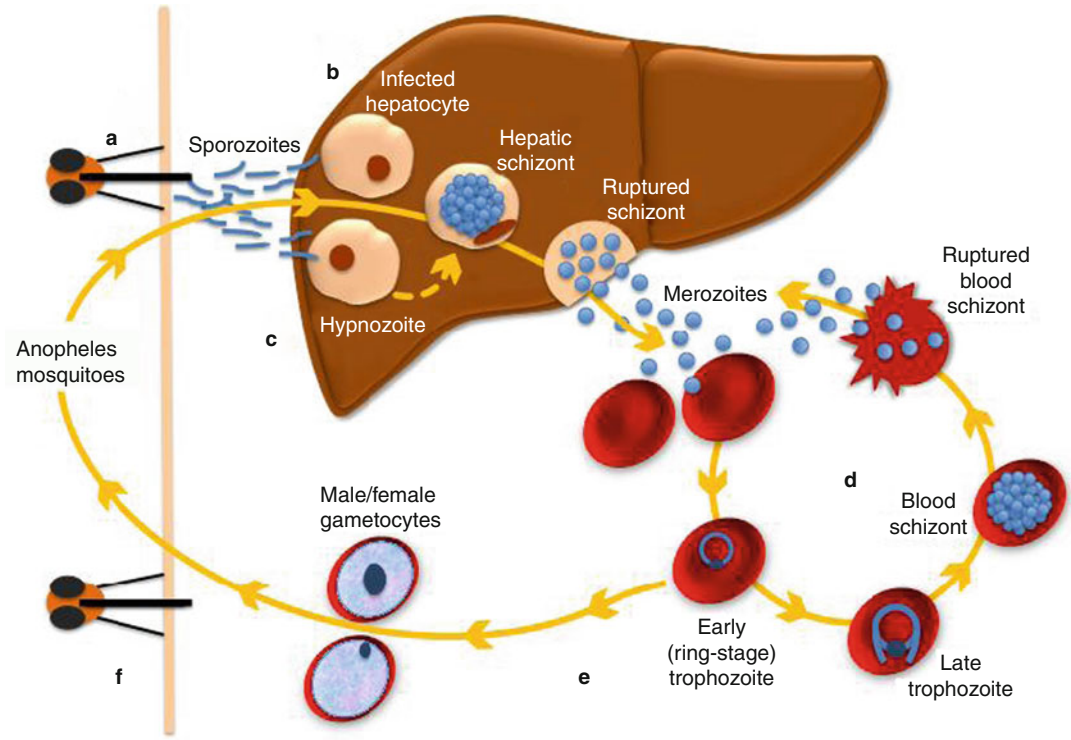

Fig. 12.1 The life cycle of *Plasmodium*. (**a**) During blood meal feeding, *Plasmodium*-infected *Anopheles* mosquitoes inoculate sporozoites into the dermis of the vertebrate host. (**b**) Sporozoites migrate from the dermis into the bloodstream and then passively transit to the liver where they invade hepatocytes. Parasites undergo a phase of enormous expansion in numbers, form multinucleated schizonts, and differentiate into merozoites. (**c**) Hypnozoites are a quiescent liver-stage form that only develop during *P. vivax* or *P. ovale* infections. Liver-stage *Plasmodium* infection does not cause clinical symptoms, but with reactivation and release into the circulation, late-onset or relapsed disease can occur up to many months after initial infection. (**d**) The schizonts rupture and release merozoites into the circulation where they invade red blood cells to cause clinical disease in vertebrate hosts. (**e**) Some merozoites differentiate into male or female gametocytes. (**f**) If each type of gametocyte is ingested by a new *Anopheles* mosquito during feeding, the cells will fuse to form an oocyst and subsequently mature and further differentiate into sporozoites in the midgut. Midgut sporozoites are noninfectious to vertebrates. However, once they leave the mosquito midgut, they mature into infectious forms as they migrate to the salivary glands. This *Anopheles* mosquito is now competent to complete the transmission cycle in a new vertebrate host

underlies much of the rationale for efforts to stop or limit the infection by means of vaccinating against *Plasmodium* sporozoites or sporozoite-expressed antigens (discussed below). In the absence of preexisting humoral immunity, sporozoites transit unimpeded to the liver through the circulation. Upon reaching the liver, parasites engage and "glide" along the fenestrated liver sinusoids [14, 15] (open endothelial networks). Sporozoite gliding activity is mediated by a specific interaction between two major anti-malaria vaccine candidate antigens, circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP), and heparan sulfate proteoglycans that protrude from the sub-sinusoidal extracellular matrix through the fenestrations within endothelial cell network [16, 17].

Of note, CSP is a dominant and highly expressed surface protein that is shed from sporozoites as they traverse host tissues. Because of this, CSP has been a leading target protein for an anti-liver-stage vaccine for decades (Tables 12.1, 12.2, and 12.3). When a gliding sporozoite encounters liver resident macrophages, known as Kupffer cells (KC), the parasite arrests and then proceeds to actively traverse KC [14] via mechanisms that involve an additional vaccine antigen target, cell-traversal protein for ookinetes and

Table 12.1 Major *Plasmodium* candidate vaccine antigens currently under clinical evaluation

Liver-stage antigens	Gene function	Immune mechanisms	Ref.
CSP	Sporozoite motility, infectivity	CD4 T cells and antibodies	[108, 109]
TRAP	Sporozoite motility	CD4 T cells and antibodies	[110]
CelTOS	Sporozoite cell traversal, infectivity	CD8 T cells and antibodies	[19]
Blood-stage antigens			
AMA-1	RBC binding	Antibodies	[25, 26, 108, 109, 111]
MSP-1	RBC binding/infectivity	Antibodies	[27, 28, 112, 113]
MSP-3	RBC binding/infectivity	Antibodies	[29, 30, 114]
EBA175	RBC infectivity	Antibodies	[31, 115]
SERA5 (SE36)	RBC egress	Antibodies	[116]
GLURP/MSP-3 (GMZ2)	RBC infectivity	Antibodies	[117, 118]

Table 12.2 Severe *Plasmodium* infection-induced disease manifestations

Disease	Clinical features	Mechanisms
Cerebral malaria	Coma, impaired cognitive function, unconsciousness, long-term neurological sequelae	Cerebral parasite sequestration, occlusion of the CNS microvasculature, endothelial cell activation, cytoadherence, inflammation, neutrophil and T cell recruitment and activation
Placental malaria	Low birth weight, premature delivery, loss of fetus	PfEMP-1 (VAR2CSA)-mediated cytoadherence to placental endothelium, inflammation, neutrophil and T cell recruitment and activation
Severe anemia	Lethargy	Suppression of hematopoiesis, destruction of RBC, loss of hemoglobin, noninfected erythrocyte clearance
Acidosis	Respiratory distress, hypovolemia	Widespread parasite sequestration, high parasite burden and associated parasite glycolysis, decreased gluconeogenesis, increased vascular permeability, anemia

sporozoites (CelTOS) [18, 19]. This first step of KC traversal was recently shown to potentiate sporozoite infectivity of hepatocytes, the terminal target cell of *Plasmodium* sporozoites. Interestingly, the sporozoite traverses several hepatocytes before finally taking up residence in the liver. Each productive infection of a hepatocyte by a single sporozoite can result in the generation of tens of thousands of merozoites, a differentiated form of the parasite that is infectious to red blood cells [20] (RBC). Thus, the liver stage of *Plasmodium* infection represents a period of enormous expansion in parasite numbers, which for *Plasmodium* species that infect humans lasts 6–16 days [21] and is wholly asymptomatic [22].

Infected hepatocytes eventually rupture or "bleb" off multiple packets of host cell membrane encasing several hundreds of merozoites [23, 24]. These so-called merosomes enter the circulation and appear to prevent exposure of newly released merozoites to host immune clearance pathways [24]. The release of merosomes and merozoites from the liver marks the start of the clinical phase of *Plasmodium* infection and the disease malaria. Each merozoite can attach to and infect a single RBC. These interactions are again mediated by specific receptor ligand

Table 12.3 Current molecular anti-malaria vaccines currently under clinical evaluation

Subunit vaccines	**Targeted life cycle stage**	**Antigen**	**Adjuvant**	**Routes**
RTS,S/AS01	Liver stage	CSP, recombinant protein (viruslike particle)	AS01[a]	i.m.
PfCelTOS FMP012	Liver stage	CelTOS, recombinant protein	GLA-SE[b]	i.m.
EBA175 RII	Blood stage	EBA175, recombinant protein	Aluminum phosphate	i.m.
FMP2.1/ASO2A	Blood stage	AMA-1, recombinant protein	ASO2A[c]	i.m.
GMZ2	Blood stage	GLURP + MSP-3, recombinant protein	Alum, DDA-TDB[d]	i.m.
$MSP\text{-}3_{181–276}$	Blood stage	MSP-3, synthetic peptide	Alum, Montanide ISA 720[e]	s.c.
SE36	Blood stage	SERA5, recombinant protein	Aluminum hydroxide gel	s.c.
JAIVAC	Blood stage	MSP-1 + EBA175, recombinant proteins	Montanide ISA 720	i.m.
AMA1-C1/ Alhydrogel® + CPG 7909	Blood stage	AMA1-C1, recombinant protein	Alhydrogel®[f] CPG 7909[g]	i.m.
BSAM-2/ Alhydrogel® + CPG 7909	Blood stage	MSP-1 + AMA-1, recombinant protein	Alhydrogel® CPG 7909	i.m.
CSP, AMA-1 virosomes	Liver and blood stage	CSP + AMA-1, mimotopes in virosomal platform	N/A	i.m.
DNA vaccines	**Targeted life cycle stage**	**Antigen(s)**	**Adjuvant**	**Route**
Polyepitope DNA EP1300	Liver stage	CSP, TRAP, LSA-1, EXP-1 multiple epitopes with linkers	N/A	i.m. (electroporation)
NMRC-M3V-D/Ad-PfCA (prime-boost)	Liver and blood stage	CSP + AMA-1, DNA prime + adenovector boost (Ad5 serotype)	N/A	i.m. (jet injection)/i.m.
Vectored vaccines	**Targeted life cycle stage**	**Antigen(s)**	**Adjuvant**	**Route**
Adenovirus (Ad35) CS and RTS,S (prime-boost)	Liver stage	CSP, recombinant protein, and adenoviral vectored	N/A	i.m./i.m.
ChAd63/MVA ME-TRAP	Liver stage	TRAP, CSP, LSA-1, LSA-3, STARP, EXP-1 chimpanzee adenoviral prime and modified vaccinia virus Ankara boost	N/A	i.m./i.m.
Ad35 vectored CS	Liver stage	CSP, replication-deficient adenovirus serotype 35	N/A	i.m.
Ad35 CS prime Ad26 CS boost	Liver stage	CSP, replication-deficient adenovirus serotype 35 and 26 prime-boost	N/A	i.m.
ChAd63/MVA MSP-1	Blood stage	MSP-1, chimpanzee adenoviral prime and modified vaccinia virus Ankara boost	N/A	i.m.

(continued)

Table 12.3 (continued)

Attenuated sporozoite vaccines	Targeted life cycle stage	Antigen(s)	Adjuvant	Route
PfSPZ	Liver stage	Whole sporozoite, irradiation attenuated	N/A	s.c. or i.d. (more recently i.v. trials)
Pf GAP p52-/p32-	Liver stage	Whole sporozoite, genetically attenuated	N/A	Mosquito bite

[a]Mixture of liposomes and immunostimulants MPL and QS21: MPL (3-0-desacyl-4′- monophosphoryl lipid A), QS21 (glycoside purified from bark of *Quillaja saponaria*)
[b]Synthetic Toll-like receptor (TLR) 4 agonist formulation, glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE)
[c]Monophosphoryl lipid A and QS21 in a water-in-oil emulsion
[d]Squalene-based water-in-oil adjuvant formulation
[e]Dimethyldioctadecylammonium (DDA) and trehalose dibehenate (TDB)
[f]Alhydrogel® is a sterilized aluminum hydroxide wet gel suspension
[g]CPG 7909 (VaxImmune™), a CpG oligodeoxynucleotide (ODN)

interactions between the host and the parasite and largely dictate whether a specific species of parasite can replicate and cause disease in a given species of vertebrate host. Not surprisingly, many of the merozoite-expressed RBC-binding proteins are also major vaccine candidate antigens currently under evaluation in clinical trials, including apical membrane protein-1 (AMA-1) [25, 26], merozoite surface protein-1 (MSP-1) [27, 28], MSP-3 [29, 30], and erythrocyte-binding antigen 175 (EBA175) [31] (Tables 12.1, 12.2, and 12.3). Following invasion of the RBC, the merozoite matures into a ring-stage form, then trophozoites, and then finally divides into daughter merozoites. This maturation and division generally takes 48 h, after which the RBC will rupture, releasing merozoites, and this cycle of asexual reproduction repeats. Through processes that are less well understood, some ring-stage trophozoites develop into sexual-stage parasites, male and female gametocytes. It is important to note that gametocytes express a unique array of cell surface antigens, such as Pfs230 and Pfs48/45 [32] or Pfs25 [33], relative to the asexual merozoites. Accordingly, several sexual-stage antigens could potentially serve as candidate vaccine antigens designed to limit transmission of malaria by preventing gametocyte survival or fusion in the mosquito host [34]. Finally, if both male and female gametocytes are taken up by a single mosquito during blood meal feeding, the sexual stage can commence. The sexual stage involves the fusion of male and female gametocytes to form an ookinete in the gut wall of the mosquito. The ookinete eventually matures into an oocyst, followed by the development of sporozoites, which ultimately migrate from the gut to the salivary glands of the mosquito effectively completing the *Plasmodium* life cycle.

12.3 Clinical Disease and Pathogenesis

There are five species of *Plasmodium* that infect humans and cause clinical disease. Whereas *P. malariae*, *P. ovale*, and *P. knowlesi* are less pathogenic, establish fewer clinical infections, and are less of a burden on public health, *P. vivax* and *P. falciparum* cause significant morbidity. *P. vivax* is often associated with acute febrile illness with or without anemia, but this infection is generally not fatal. Despite this, geographically more people are at risk for developing *P. vivax* malaria as opposed to *P. falciparum* malaria [35]. In contrast, *P. falciparum* is responsible for the most severe forms of clinical illness (severe malaria) and is the major cause of *Plasmodium*-associated mortality [2].

As illustrated above, *Plasmodium* parasites exhibit a complex multi-host, multistage life cycle, and malarial disease is linked to the biology and life cycle of the parasite. The inoculation of sporozoites and their subsequent infection of the liver are wholly asymptomatic. However,

blood-stage merozoites, through their cyclic waves of infection and destruction of RBC, drive the entire spectrum of clinical disease. Moreover, *Plasmodium* species that cause morbidity in humans are known to replicate in a synchronous fashion, rupturing from erythrocytes approximately every 48 h. The synchronous rupture of RBC generally manifests as relapsing fever, malaise, chills, and sweats. There are also well-described relationships between the tropism of malaria parasites for various maturation stages of host RBC and clinical severity.

For example, *P. vivax* preferentially infects Duffy blood antigen-positive [36] reticulocytes (immature RBCs that comprise <1 % of all erythrocytes) and causes less severe disease, whereas *P. falciparum* infects both reticulocytes and normocytes (mature RBC) and causes much more severe disease [37].

Patterns and manifestations of disease also depend largely on the exposure history and immune status of the host. Indeed, following the majority of exposures in adults in *P. falciparum-endemic* areas, the parasites productively infect resulting in apparent blood-stage infection, yet the infection remains clinically silent [38]. This latter observation underscores that exposure-induced naturally acquired immunity (NAI) can develop with time. However, this form of immunity is widely appreciated to protect against severe disease, but not the infection itself [38–40]. In contrast, *P. falciparum*-naïve individuals are at greatest risk for developing severe illness, including single-organ, multiorgan, or systemic manifestations (Table 12.2). The most severe disease manifestations include metabolic acidosis, cerebral malaria, and severe malaria anemia.

The mechanisms of malarial disease are not uniform and vary widely. Metabolic acidosis is largely associated with the parasite's ability to derive energy from anaerobic glycolysis of glucose to lactic acid [41]. With parasite densities often exceeding 50,000 or more parasites/μl blood, it is not surprising that hypoglycemia and metabolic acidosis are common manifestations of severe malaria [42, 43]. High parasite densities are also a major factor driving the development of severe anemia in *P. falciparum*-infected individuals. Severe anemia during malaria infection is classically associated with the degradation of hemoglobin in infected RBC, a major energy source for the parasites. However, anemia also results from the clearance of both infected and noninfected RBC: noninfected RBC can acquire parasite glycosylphosphatidylinositol (GPI) effectively targeting them for clearance in the spleen [44]. The lysis of RBCs also releases parasite-derived glycosylphosphatidylinositol (GPI), which triggers systemic production of proinflammatory cytokines such as TNF and IL-1 [45–47]. These proinflammatory cytokines are known to suppress hematopoiesis, which further compounds the severity of anemia.

In addition to metabolic and physical modifications that parasites impart to host RBC, a large number of studies support a major role for cytoadherence and sequestration of infected red blood cells (iRBC) as a major mechanism of disease [48, 49]. Sequestration of parasite-infected RBC in specific target organs can lead to obstruction of blood flow, inflammatory cascades, and breakdown of the endothelium. Inflammation and ischemic occlusions within the central nervous system are responsible for the most deadly form of the disease, cerebral malaria. Cytoadherence between infected and noninfected RBC and vascular endothelial cells is mediated by a class of parasite-expressed proteins which includes *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1/VAR2CSA) [50]. PfEMP-1 proteins bind host proteins such as ICAM-1 on vascular endothelium, CD36 on endothelial cells and platelets, and CSA, which is largely expressed in the placenta. Indeed, pregnant women and fetuses are exceedingly vulnerable to the malarial disease, which contributes to anemia, low birth weight, premature birth, and significant numbers of infant deaths [51]. Targeting PfEMP-1 proteins through vaccination would seem a reasonable strategy to prevent severe pregnancy-associated malaria, but the extensive polymorphism of this family of proteins has confounded efforts [52]. Collectively, *Plasmodium* parasites induce a series of profound changes to RBC membranes rendering them less deformable [53, 54] and highly adherent, which compounds the problems associated with occlusion of the microvasculature and the development of cerebral malaria.

12.4 Diagnostic Criteria and Classical Therapy

Diagnosis of malaria can occur via four distinct modes: clinical diagnosis, microscopic diagnosis, antigen detection, and molecular diagnosis. Criteria for clinical diagnosis include patient presentation of symptoms that include fever, chills, sweats, headaches, muscle pain, and potentially nausea and vomiting. It is important to note however that the clinical signs of malaria are quite similar to symptoms of other childhood infections in malaria-endemic regions. For these reasons it is important that these clinical criteria are supported or confirmed by additional diagnostic tests.

Microscopic examination of Giemsa-stained thin and thick peripheral blood smears remains the gold standard for the diagnosis of malaria. However, this type of diagnosis requires both quality reagents and well-trained individuals who can both accurately identify *Plasmodium-infected* red blood cells and identify the particular species of *Plasmodium* within the cells. More recently, parasite antigen-based assays have been used with success to rapidly and accurately diagnose *Plasmodium* infection. These assays rely on the detection or "capture" of *Plasmodium* antigens in the blood of patients by antibodies that are immobilized on a small card [55, 56]. Although the antigen-based diagnosis takes only 15 min, the test is not inexpensive and therefore less widely available in malaria-endemic areas. Moreover, the sensitivity of the assay (i.e., ability to detect low numbers of parasites) remains a limitation, and a positive result must also be confirmed microscopically. In contrast to antigen assays, molecular assays represent a highly sensitive and specific test for the presence of malaria parasites. These assays rely on the use of the polymerase chain reaction (PCR) to detect and amplify parasite genomic DNA from a patient's blood [57]. Molecular assays are very sensitive and can also be used to distinguish between various species of *Plasmodium* parasites. But again, their widespread use is limited due to the requirements for specialized reagents, equipment, and training.

Regarding treatment, case management depends whether the disease has manifested as uncomplicated or severe malaria. Patients who have severe *P. falciparum* malaria are generally unable to take oral medications and are therefore treated with continuous intravenous infusions in a hospital setting. In contrast, patients who present with uncomplicated malaria are generally treated on an outpatient basis. There exist a variety of pharmacological agents with activity against key stages of the parasite life cycle. However, many of the drugs, including chloroquine, mefloquine, and pyrimethamine, are no longer effective in many parts of the world due to their over application, which has resulted in the selection of resistant parasite strains. Despite this, several drugs remain effective and are in widespread use today. Most effective are therapies that include artemisinin-containing combination treatments. Examples include artemisinin (or artesunate) with amodiaquine, atovaquone, doxycycline, or quinine. In order to extend the usefulness of artemisinin and its derivatives, the use of artemisinin monotherapies is not recommended [5, 58].

12.5 The Rationale for a Vaccine

Several lines of evidence suggest that a prophylactic anti-malaria vaccine for humans is feasible. First, nearly 40 years ago it was observed that sterilizing protection against sporozoite challenge could be achieved in human volunteers following vaccination with attenuated whole malaria sporozoites [59]. Secondly, as described above, NAI progressively builds up during the first two decades of life in people living in malaria-endemic countries [60, 61].

Thus, unlike many pathogens, including human immunodeficiency virus (HIV), *Plasmodium* infections induce "clinical immunity" or protection against severe disease. Finally, reductions in parasite burdens have been elicited by passive transfer of immune immunoglobulins from semi-immune adults to children with fever and high parasitemia [62]. Collectively, these observations argue that the induction of protective immunity against the malaria parasites is possible, which has lent significant hope for the eventual development of a licensed vaccine. Although substantial

progress has been made over the past several decades, as discussed below there is only a single candidate vaccine that has shown partial efficacy in clinical trial, RTS,S.

Nevertheless, new insight into *Plasmodium* pathobiology and a better understanding of NAI will continue to shape novel approaches to anti-malaria vaccine efforts. Currently, anti-malaria vaccine efforts are focused on developing three main types of vaccines: liver-stage, blood-stage, and transmission-blocking vaccines. Transmission-blocking vaccines have been reviewed in detail elsewhere [63]. Moreover, because transmission-blocking vaccines confer no direct protection to the vaccinated individual, our discussion will focus only on liver- and blood-stage anti-malaria vaccines. Of note, the World Health Organization maintains a useful, comprehensive, and up-to-date list of anti-malaria vaccines currently in preclinical or clinical trial [52].

12.6 Preerythrocytic (Liver-Stage) Vaccines

Stopping *Plasmodium* infections before parasites complete development and differentiation in the liver would completely prevent clinical disease. Vaccines directed against this life cycle stage have been designed to elicit potent antibody responses that block sporozoites from productively infecting hepatocytes or to trigger potent T cell immunity that can recognize and eliminate *Plasmodium*-infected hepatocytes. One potential drawback to these approaches includes the very high titers of antibodies that would be required to effectively target sporozoites during the very transient period during which they exist as extracellular parasites.

Moreover, even a single productively infected hepatocyte can lead to blood-stage malaria. Thus, unless all infected hepatocytes are efficiently cleared from the host, clinical disease will develop. Consistent with this, recent data suggest that exceedingly large numbers of parasite-specific T cells are necessary to eliminate *Plasmodium*-infected hepatocytes and prevent blood-stage infection [64]. Despite these potential limitations, it is widely appreciated that even a vaccine that only reduces the number of sporozoites or infected hepatocytes should decrease the incidence or magnitude of blood-stage parasite infections [65].

There have been several molecular liver-stage vaccine platforms developed over the years including the use of peptide- and recombinant protein-based vaccines, DNA vaccines, viral vectored vaccines, as well as continued development of whole-sporozoite-based vaccines [52] (Table 12.3). Here we describe several of these candidate approaches and highlight those having either shown significant promise in clinical trial or revealed important insight into ways to improve current anti-malaria vaccine approaches.

Recombinant Protein-Based RTS,S. More than 24 years ago, a novel vaccine platform was developed to specifically target the preerythrocytic stage of *Plasmodium* infection. The vaccine, termed RTS,S, is a formulation of subunits from the CSP of *P. falciparum*. The vaccine contains components of the central repeat (R) of CSP, with known B cell epitopes, linked to the C-terminal (T) region of CSP, which is known to contain T cell epitopes. These CSP polypeptides are fused to the hepatitis B surface antigen (S), giving rise to RTS. This construct is co-expressed in yeast with free hepatitis B surface antigen (S), giving rise to the viruslike antigen particle, RTS,S [66].

In preliminary phase II trials, it was determined that RTS,S induced protection in 30–50 % of malaria-naïve, healthy individuals [66, 67] but only when administered with potent adjuvants, specifically with the proprietary adjuvant AS01 [68]. These levels of efficacy were a significant step forward on the road to a licensed anti-malaria vaccine, and the overall efficacy of RTS,S has yet to be exceeded by another recombinant protein-based vaccine formulation. Since that time, RTS,S has progressed into large-scale phase III trials in seven countries in Africa, and recent published results show that the vaccine provided children aged 5–17 months protection against clinical disease (56 % efficacy) and against severe malaria (47 % efficacy) [69].

The mechanisms of protection elicited by RTS,S vaccination have yet to be fully defined, but it is clear that when administered in the

presence of the AS01 adjuvant, RTS,S vaccination elicits very high titers of anti-CSP antibodies that correlate with protection [70] and likely reduce the number of infectious sporozoites [65]. Importantly, antibodies are known to directly interfere with sporozoite infection of hepatocytes [71, 72] and are classically involved in opsonization of pathogens, with subsequent and enhanced effects on pathogen uptake by phagocytic cells such as macrophages and dendritic cells (DC). Thus, it is conceivable that in RTS,S-immunized individuals, pathways of opsonization also function to accelerate host clearance of sporozoites. In addition to antibodies, CD4 T cells targeting the C-terminal regions of CSP are also believed to contribute to protection. However, T cell responses measured in vaccinated individuals are modest, and evidence for the participation of CD4 T cells (or CD8 T cells) is only minimally correlative [73].

While RTS,S has become the current benchmark for experimental anti-malarial subunit vaccine efficacy, several questions remain. First, the large-scale clinical trials have thus far been limited to studies aimed at African children, and *P. falciparum* malaria is a global public health problem, particularly for South America and Asia. Therefore, it remains to be determined whether RTS,S is equally (or more) efficacious in other populations.

Second, the durability of protection is not yet clear; the phase III trials have thus far all had relative short follow-up periods (14 months) [69], so it will be critically important to determine how long the apparent protective capacity of RTS,S lasts. Finally, it will be important to understand how or whether RTS,S influences transmission rates among vaccinated populations. Indeed, even the modest protective efficacy of RTS,S could have significant beneficial impact on the rates of *Plasmodium* transmission. On the other hand, it is also possible that the RTS,S disrupts the development of NAI induced through natural exposure, thus potentially leaving older children at greater risk for severe disease later in life. Despite these questions and potential issues, hopes remain high that full licensure of RTS,S will help reduce the global incidence and prevalence of malaria.

Plasmid and Viral Vectored Vaccines. A second major approach to inducing liver-stage immunity is through the use of DNA-prime + viral vector-boost or viral vector prime-boost strategies (Table 12.3). Experimental plasmid DNA vaccines based on CSP were first developed in the late 1990s [74], but their relatively low stimulatory capacity necessitated a secondary booster immunization with non-replicating viral vectors encoding CSP [75], including poxvirus vectors (e.g., modified vaccinia virus Ankara or fowlpox) and adenoviral vectors.

Consistent with boosted immunogenicity, viral vectors are classically regarded for their ability to elicit potent T cell immunity against encoded transgenes expressed from the viral backbone. Indeed, vectored delivery of *Plasmodium* proteins expressed within infected hepatocytes remains a central strategy to generate large numbers of parasite-specific cytotoxic T cells that can eliminate those infected cells [76]. In addition to CSP, other sporozoite/liver-stage antigens have been clinically evaluated using DNA and/or viral vectored approaches, including TRAP, liver-stage antigen-1 (LSA-1), LSA-3, and exportin-1 (EXP-1) [52]. The efficacy of viral vectored approaches was initially promising when protection was evaluated in malaria-naïve human volunteers [65, 75, 77, 78]. Unfortunately, when clinical trials for several past formulations moved to an examination of protection among Gambian adults and Kenyan children, results were not encouraging [79, 80], and the trails have since been halted [52].

Despite these disappointing initial results, work continues on expanding the development of viral vectored approaches. For example, currently in trial are heterologous viral vector prime-boost strategies that incorporate antigens from both the liver (CSP, TRAP) and blood stage (AMA-1, MSP-1) [52] with the goal of eliciting cross-stage protection (Table 12.3). Moreover, efforts to enhance the immunogenicity of vectors continue to move forward. Indeed, one major obstacle to this approach is that vaccinated individuals rapidly develop or have preexisting anti-vector immunity.

Thus, any efforts to "boost" the magnitude of the immune response are countered by humoral

and cellular immune responses that effectively neutralize the vector and accelerate its clearance from the host upon secondary exposure. This rapid clearance of the vector subsequently dampens responses to the encoded immunogen. To enhance immunogenicity, other types and combinations of viral vectors are being investigated, including various poxviruses [81]. To counter preexisting immunity, researchers continue to evaluate the stimulatory capacity of chimpanzee adenoviruses and less commonly circulating human adenovirus serotypes, such as Ad26 and Ad35 [82–84].

Collectively and despite early poor clinical results and noted limitations, viral vector prime-boost approaches are likely to prove useful vaccine platforms that can be used in combination with other modalities (e.g., RTS,S) to enhance protection against *Plasmodium* infection. Indeed, the relative ease with which these platforms can be scaled up and deployed to regions of the world where malaria is endemic underscores our continued need for their further development and evaluation in larger clinical trials.

Live Attenuated Parasite Vaccines. Although not formally a molecular vaccine platform, a brief discussion of whole-parasite (sporozoite) vaccination approaches (Table 12.3) is warranted, as these platforms are the only experimental vaccines to have afforded very high levels of sterilizing immunity in man [85, 86]. For more than 40 years, it has been known that vaccination with large numbers of radiation-attenuated sporozoites (RAS) induces sterilizing liver-stage-specific immunity. RAS parasites, attenuated via radiation-induced, random DNA damage, undergo intrahepatocytic developmental arrest at the onset of replication. In preclinical models, RAS-induced protection is largely mediated by CD8 T cells, with activity directed against *Plasmodium*-infected hepatocytes [87–90].

One major concern relevant to translating RAS vaccination involves the necessity to attenuate *Plasmodium* sporozoites with a dose of radiation to induce DNA damage that is sufficient to both prevent parasite replication and differentiation (i.e., progression to blood-stage infection) and maintain sporozoite infectivity and immunogenicity [91, 92]. Despite the potential issues of safety and deployment, RAS have recently progressed to clinical evaluation [52, 93, 94] but have thus far shown little protective efficacy in humans [95]. The central conclusion of these preliminary trials was that limited efficacy was due to the poor immunogenicity of subcutaneously or intradermally injected RAS, whereas immunogenicity was high in nonhuman primates that were immunized via intravascular injection [95].

In comparison to RAS, genetically attenuated parasites (GAP), which are generated by targeted gene deletion(s), are an attractive alternative because parasites can be "designed" to arrest at specific points during liver-stage development [96], thereby exhibiting uniform biological activity, which could be beneficial for vaccination. GAP have been generated to arrest at an early stage of liver development prior to extensive parasite replication [97] or at a late liver (schizont) stage [98]. In preclinical models, late-arresting GAP, compared to early-arresting GAP, elicit higher levels of protection that correlate with larger CD8 T cell responses targeting a diversified array of parasite antigens [99]. Clinical evaluation of *P. falciparum* GAP as a potential anti-malaria vaccine has begun [52], and it will be of great interest to determine whether similar (or higher) levels of protection can be elicited by GAP, compared to RAS, when delivered via intradermal, subcutaneous, or mosquito bite inoculation.

In contrast to vaccination with relatively large numbers of attenuated RAS and GAP sporozoites stands infection-treatment immunization (ITI). In this experimental platform, low numbers of virulent *P. falciparum* sporozoites are used as immunogens that are delivered by mosquito bite. When administered to malaria-naïve volunteers concurrently taking chloroquine (to prevent replication of blood-stage parasites), the virulent sporozoites elicit high levels of protection against subsequent mosquito bite-delivered sporozoite challenge [100]. In the context of ITI, sporozoites productively infect the liver and parasites undergo full liver-stage development, with the eventual release of merozoites. However, in the presence of chloroquine, which is active only against blood-stage parasites, clinical disease is prevented and merozoite-infected red blood cells are rapidly

cleared. Protection in preclinical models is associated with T cell responses against liver-stage parasites, yet whether blood-stage immunity substantially contributed to protection of human ITI vaccines in the recent clinical trial remains an open and critically important question [100].

A second major question relates to the exceedingly high potency following inoculation with so few non-attenuated sporozoites. One hypothesis is that allowing the parasites to complete liver-stage development effectively increases parasite antigen biomass, which drives potent sterilizing immunity. Moreover and similar to late-liver-stage-arresting sporozoite GAP vaccines [99], it may be that an increase in the diversity of antigens expressed by the parasites as they mature into blood-stage merozoites contributes substantially to protection. Although no evidence for diversification of antigenic targets was provided in the human trials, these studies provide clear evidence that prolonged liver-stage differentiation and increases in antigen load correlate with enhanced protective immunity following sporozoite vaccination.

Clearly there are significant obstacles to translating and deploying live sporozoite vaccines to malaria-endemic populations. Nevertheless, these experimental vaccines are likely revealing important information about the interaction of *Plasmodium* parasites and the host immune system. Understanding the precise cellular and molecular basis for the potent immunogenicity of whole-sporozoite vaccines might reveal clues that could enhance the development of easily deployable subunit or viral vectored vaccines. For example, it is possible that live sporozoites expose antigens to the host immune system in a unique context that enhances their immunogenicity; antigen presentation, or T cell or B cell priming, might occur in fundamentally distinct ways that are not mimicked when the same antigens are administered via recombinant protein or vectored formulations.

Alternatively, it is possible that current subunit and vectored approaches are not targeting the most immunogenic or protective antigens or the correct combinations of antigens necessary for protective immunity (discussed below). Addressing these questions should provide critical information about the delivery of antigens during subunit malaria vaccination, and whole-sporozoite experimental vaccines should continue to prove useful tools for dissecting key mechanisms of immunity against malaria.

12.7 Erythrocytic (Blood-Stage) Vaccines

Vaccines targeting the blood stage of *Plasmodium* infection would be expected to decrease parasite burden and as a consequence reduce the severity of disease. Thus, in addition to preventing severe infection in malaria-naïve individuals, blood-stage vaccines might also act therapeutically to reduce morbidity and mortality in malaria-exposed persons. Similar to liver stage, the main vaccine platforms that have been utilized in an effort to induce protection against blood-stage infection include peptide, recombinant protein, and vectored technologies [52] (Table 12.3). Although there are relatively larger numbers of blood-stage vaccines currently in clinical trial, compared to liver-stage experimental vaccines [52], progress towards developing a blood-stage vaccine has been much slower, and success has been limited [101]. Because there are no candidates that stand out as having demonstrated potent efficacy against blood-stage infection, our discussion will instead focus on the potential reasons for our continued struggles to develop and license blood-stage anti-malaria vaccines.

There are likely several reasons why comparatively little success has been achieved in developing a blood-stage vaccine. First, blood-stage vaccines are focused on eliciting immunity against an exceedingly limited number of proteins expressed on the surface of the merozoite or the infected RBC. The list of candidate antigens currently targeted in clinical vaccine trials consists of only six proteins: MSP-1, MSP-3, AMA-1, EBA175, serine repeat antigen-5 (SERA5), and the glutamine-rich protein (GLURP) (Tables 12.1, 12.2, and 12.3). Of note, compared to liver-stage-expressed proteins, blood-stage proteins are highly polymorphic among *P. falciparum* field isolates [102].

Indeed, there appears to be a high degree of selective pressure placed on the parasites by antibodies targeting these proteins during natural

exposure and infection [103]. Second, recombinant proteins expressed and formulated for use in a subunit vaccine may not adopt the conformation necessary to mimic the protein as it appears to the immune system when expressed on the surface of the merozoite or RBC. Thus, vaccination with soluble recombinant or vectored malaria antigens may routinely fail to elicit protective immunity because the relevant B cell epitopes are conformation dependent. Third, current strategies often employ the administration of recombinant proteins in the presence of strong adjuvants (Table 12.3). Unfortunately, the mechanisms of action for most vaccine adjuvants are poorly understood. Moreover, currently available adjuvant formulations are generally poor inducers of antibody responses in malaria-exposed individuals [104]. Consistent with this, levels of vaccine-induced antibodies against some recombinant *Plasmodium* proteins are generally no higher than the responses detected in individuals living in malaria-endemic areas [104].

Clearly there are major issues to be resolved before an efficacious vaccine solely targeting merozoites or infected RBC can be developed. Despite the limitations, it is now becoming appreciated that multistage vaccines, those that target both the liver- and blood-stage parasites, might synergize to reduce parasite burdens and thereby reduce the incidence and prevalence of severe disease. Yet in this regard, only 3 of the current 29 anti-malaria vaccine clinical trials are focused on determining whether CSP and AMA-1 can be simultaneously targeted to improve protection [52] (Table 12.3). Although multistage vaccines present a potential opportunity to significantly impact disease burden, more work will be required to determine the best combination of candidate antigens and vaccine delivery platforms in order to achieve appreciable protection.

12.8 Conclusions and Perspectives

The burden of malaria is enormous and this disease remains a global threat. *Plasmodium* infections kill hundreds of thousands of people each year, with most clinical cases involving children. Malaria truly presents one of the most pressing public health problems for the modern world. Although control of the incidence and prevalence of malaria has been met with some recent successes, as bed netting programs continue to expand and novel drugs are identified, the complete eradication of this disease will depend on the development of an effective vaccine. A more complete understanding of the biology of the parasite in the vertebrate host has helped focus new strategies to combat *Plasmodium* infection and spread. Yet still, many critical obstacles stand in the way of developing effective next-generation anti-malaria vaccines.

Perhaps the largest hurdle lies in our incomplete understanding of the interplay between the parasite and the host immune system. Although it is clear that NAI can develop with time following repeated exposures, very little is known about how this type of immunity develops [105]. Future immunologic studies aimed at dissecting these pathways of protection should in turn help guide anti-malaria vaccine development. Indeed, whether mimicking NAI through vaccination should be the goal or whether NAI is largely dysfunctional (and therefore not sterilizing) remain major questions.

Another issue for vaccine development relates to the sheer complexity of the parasite. The parasite's diverse life cycle stages and expression of highly polymorphic proteins during blood-stage infection [103] raise the important question of whether the "best" antigens are currently being targeted through peptide, subunit, and vectored strategies. Consistent with this, it is well appreciated that induction of the most potent immunity follows vaccination with attenuated whole parasites. In this scenario, the immune system is presented with the entire complement of *Plasmodium* liver- and/or blood-stage antigens. It is critical to note that *Plasmodium* species encode >5,200 known or predicted genes [106]. Remarkably, only ~35 proteins (0.8 % of predicted proteins) have been evaluated as potential vaccine candidates for *P. falciparum* [52] or have been identified as targets of the host immune system [107]. Based on the limited number of candidate

Table 12.4 Animal models used for the study of *Plasmodium* pathogenesis or evaluation of anti-malaria vaccine efficacy

Rodent models	**Strengths: molecular and cellular dissection of disease pathogenesis and/or immunity is possible using inbred or gene-knockout mice**	**Weaknesses: laboratory mice are not the natural host (no coevolution)**
Plasmodium spp.[a]	Clinical features	Common uses of the model
P. berghei ANKA	Infects reticulocytes (immature RBC) and normocytes (mature RBC). Causes rapidly lethal pathologies associated with infection-induced CNS inflammation and neutrophil and T cell recruitment and activation	Model for studies of cerebral malaria, severe malaria, anemia, drug sensitivity, and innate immunity in C57BL/6 mice
P. yoelii yoelii 17XNL	Infects reticulocytes and causes high-burden parasitemias lasting several weeks	Model for vaccine efficacy, innate and adaptive immunity, pathogenesis, and severe anemia
P. chabaudi chabaudi AS	Infects normocytes and exhibits long-term, persistent infection (months), marked by recrudescence	Model for vaccine efficacy, adaptive immunity, parasite drug sensitivity, and pathogenesis and a model for low-grade parasite burdens of blood-stage *P. falciparum*, with variable recrudescence
P. vinckei vinckei	Infects reticulocytes and normocytes. Causes acutely lethal infections associated with high parasite burdens and anemia	Model for pathogenesis of severe malaria and parasite drug sensitivity
P. falciparum in "humanized" mice	Cotransfer of *P. falciparum* parasites and human erythrocytes to immunodeficient mice. Mice lacking aspects of adaptive and innate immunity will support human erythrocytes	Enables the study of pathogenesis or drug sensitivity of *P. falciparum* in a tractable system
Nonhuman primate models	**Strengths: regarded as a closer mimic of human *P. falciparum* infection, specific coevolution of parasite, and host species**	**Weaknesses: requires specialized facilities and expertise to conduct this research. Costs and ethical considerations also influence suitability**
Plasmodium spp.[b]	Clinical features	Common uses of the model
P. coatneyi	Long-lasting chronic infections in natural host primates but severe pathologies develop in Rhesus macaques	Cerebral sequestration of parasite-infected RBC
P. knowlesi	Rapidly lethal to Rhesus macaques. Exhibits a less common 24-h erythrocytic cycle	Model for antigenic variation of widely used to model anemia and mechanisms of RBC invasion
P. cynomolgi	Mimics *P. vivax* formation of hypnozoites and establishes chronic infections	Model for the study of hypnozoite formation and liver-stage vaccine and parasite drug efficacy
P. fragile	Long-lasting chronic infections marked by recrudescence in natural host monkeys, with high parasitemias and severe infections in most monkeys	Model for blood-stage vaccine efficacy
P. falciparum in chimpanzees and limited species of New World monkey	Acute infection with parasitemias that resolve rapidly (10–14 days)	Models of liver- and blood-stage vaccine efficacy

[a]Numerous laboratory strains and isolates exist for each species of rodent malaria and exhibit variable patterns of pathogenesis. For example, some laboratory strains of *P. yoelii* (e.g., *P. yoelii* strain YM or strain 17XL) cause rapidly lethal infections in mice, whereas other strains (e.g., *P. yoelii* 17XNL) generally resolve in mice. For simplicity, the general features of only the most widely studied strains of each *Plasmodium* species are noted

[b]Pathology and pathogenesis of infections caused by these species of *Plasmodium* largely depend on the species of the nonhuman primate host

antigens evaluated thus far, it is likely that many additional *Plasmodium* proteins including more conserved proteins expressed by clinically relevant *Plasmodium* species (*P. falciparum* and *P. vivax*) are recognized by the host immune system.

Finally, there remain dire needs to identify meaningful correlates of immune-mediated protection, develop new in vivo models, and expand the number of current clinical anti-malaria vaccine testing centers. The development of key functional assays that quantify the ability of antibodies to neutralize sporozoites and block invasion of liver cells would be a significant advance. Also, the development and adoption of standardized assays that evaluate the ability of serum antibodies from vaccinated individuals to block the invasion of merozoites into red blood cells would help researchers worldwide more accurately interpret and compare their data sets. Equally important, better in vivo models are needed. As *P. falciparum* and *P. vivax* do not infect small laboratory animals, the generation of new recombinant versions of rodent or nonhuman primate parasites expressing *P. falciparum* or *P. vivax* antigens would be highly valuable tools to help focus studies of immunogenicity and protective capacity of candidate antigens.

New in vivo models would also significantly expand the number of systems to evaluate pathogenesis and immunity (Table 12.4), several of which are hampered by significant biological limitations and disadvantages. Last, additional clinical testing facilities, similar to the recent testing that has begun at the Seattle Biomedical Research Institute and the Malaria Clinical Trial Center, are needed in order to expand anti-malaria vaccine research. Of course, all of these needed advances will come at a cost. Investment in malaria research and control programs has risen significantly over the past decade [2], but important gains against malaria cannot continue without further investment. Thus, if the global community is truly committed to the development of next-generation anti-malaria vaccines, several economic, biological, and immunologic hurdles still need to be overcome.

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TB Vaccines: State of the Art and Progresses

13

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Abstract

Tuberculosis is one of the oldest human diseases, which still killing about 2 million people a year around the world. It is believed that one third of the world population is infected with the bacillus *M. tuberculosis*, representing a huge and worrying reservoir of the pathogen. Different factors contribute to this disturbing scenario: the only available vaccine, BGC, is inefficient; treatment is too long, causing considerable side effects, generating large non compliance rates and favouring the development of resistant strains; the diagnosis is not always accurate. Although the host-pathogen interaction and immune response against tuberculosis are intensively studied, there are still open questions on this subject. Thus, the development of new diagnostics, vaccines and therapies are extremely affected and harmed.

In this chapter, initially will be provided to the reader an overview of all these tuberculosis aspects to support the detailed discussion on the development of new vaccines. The understanding of this complex panel is extremely facilitated, through segregating vaccines thematically from the different technologies used to develop each of them, explaining the objectives and rational behind their design and presenting the most relevant results available until the time of publishing this book, highlighting those of clinical testing phase. Enclosing the chapter it will be addressed prospects for the area, discussing

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challenging points in tuberculosis research that directly affect the development of new vaccines.

13.1 Disease Development and Immune Response

Tuberculosis (TB) is a serious worldwide public health problem involving social and economic aspects. But it is far from being a modern problem, as there are indications of infection dating back to the Neolithic period [1]. TB was responsible for one of the most serious episodes of infectious diseases, known as "white plague," in the seventeenth and eighteenth centuries in Europe. At this time, a quarter of all adult deaths were attributed to TB, and it is believed that virtually all the European citizens were infected [2].

Today, it estimated that about one-third of the world population is infected with the TB bacillus. Approximately 54 million people are infected every year, 9.4 million develop the disease, and 1.7 million die from this curable disease [3]. The TB bacillus kills more people than any other infectious agent alone. There are important international policies and support to diminish TB prevalence and incidence, assembled in the Stop TB Partnership and Millennium Development Goals – a network of international organizations establishing public–private partnerships. Thus, TB is mainly a disease of neglected people, living in poor housing and health conditions. Alcoholism and smoking also increase predisposition to contracting the disease. Most important, a factor that has elevated the cases and the predisposition to TB is the coinfection by human immunodeficiency virus (HIV) [4].

Worldwide, *Mycobacterium tuberculosis*, identified by Robert Koch in 1882, is the main etiological agent of human TB. In Africa, mainly in west, *M. africanum* also has epidemiological importance [2]. Hereafter, they will be referred only as Mtb. It belongs to the Mycobacteriaceae family, Actinobacteria group, genus Mycobacterium. Mtb is facultative intracellular pathogenic bacterium that requires oxygen to grow but is believed that they can survive in anaerobic environment in a low metabolism rate. Mtb has unique high lipid cell wall among the prokaryotes: peptidoglycan, around the plasma membrane, linked to the arabinogalactan polysaccharide and this latter attached to mycolic acids (long chain fatty acids). Free amphipathic lipids such as TDM, trehalose dimycolate or cord factor, and saccharides, such as glucan, complete this astonishing structure that confers virulence, resistance to drugs, and tools to infect the host and subvert their immunologic response and makes the bacilli resistant to Gram staining (acid-fast detection techniques are used instead) [5].

Mtb infection occurs when small droplets containing viable bacilli are expelled by an individual suffering from active TB disease during speech, coughing, or sneezing and are inhaled by other individuals. TB mainly affects the lower respiratory tract, and lungs are the primary sites for the establishment of bacilli, although there are forms of non-pulmonary TB. The multiplication of mycobacteria in the human host is slow, and symptoms may take months to manifest, including chest pain, intense and prolonged cough for more than three weeks, or hemoptysis (coughing up blood). The patient may present further episodes of fever, chills, night sweats, loss of appetite, weight loss, pallor, and fatigue [6].

It is estimated that after contact with the bacilli, only 5–10 % of individuals develop active TB. In the other 90–95 % cases, individuals develop an asymptomatic latent infection and do not transmit the bacillus. This happens because the immune response is not able to eliminate the bacillus but can control its spread and restrict their biological activities and access to other cells and organs. Latency can last the entire life of the individual, which constitute important reservoirs of the pathogen. This characteristic contributes to high rates of incidence and prevalence of TB, since at any time a factor that causes immunosuppression can lead to illness reactivation. Furthermore, the immune response against Mtb and consequently the development of active or latent form of TB may be associated with genetic polymorphisms [6].

Once at lungs, establishment of infection depends on the interaction of the pathogen with professional host phagocytes, mainly alveolar macrophages, which will capture them. At phagosome Mtb should be destroyed, but instead it activates mechanisms that allow its survival

and replication therein, such as change of the endosomal pH, impairment of phagosome and lysosome fusion, and inhibition of active forms of oxygen radicals and nitrogen generation, and decrease the production of inflammatory cytokine. However, there are many receptors and cells of innate immunity that, once activated, cooperate to generate an immune response capable of containing the pathogen inducing the formation of a typical inflammatory reaction, characterized by pathological tissue globular structures consisting of a wide range of system cells immune. These structures are called granulomas [7].

Granuloma, one of the characteristics used in the diagnosis of pulmonary TB, results from the migration of infected macrophages and dendritic cells to adjacent lymph nodes, where mycobacterial antigens are presented to lymphocytes. There begins the development of the adaptive immune response. Thereafter, the activated lymphocytes migrate to the site of infection. Thus, granulomas are composed mainly by lymphocytes B and lymphocytes T $CD4^+$ and T $CD8^+$ T located around infected macrophages and dendritic cells, besides other cells. Subsequently, the granuloma is surrounded by collagen fibers produced by fibroblasts and may restrict the spread of bacilli for a long time in an environment with low levels of oxygen and nutrients, where it survives decreasing your metabolism, characterizing the latency phase of infection [8]. Although there are differences, mainly due to the study design used, the granulomas are seen today as a place of dynamic interaction between cells and the pathogen, all in constant multiplication, death and renewal that can both protect the host as contributing to its colonization by the pathogen.

In fact, this containment system can be destabilized by factors that weak the immune system of the host, as already mentioned, leading to the reactivation of the disease, which is typically accompanied by necrosis, caseation, liquefaction of granulomas, and release of bacteria. Thus, the bacilli can spread to local lung or other organs via the circulatory system or may be released to the environment through the breath and cough, causing contamination of other individuals [9].

Therefore, the success or failure of immune response to TB depends on an intricate network of activation and repression mechanisms, whose components are still being established or known in more detail. In other words, it is a complex biological phenomenon and not understood in its entirety. Many factors are defined as "necessary" to control Mtb. However, knowledge about which host factors are "sufficient" to generate effective immune response in combating the pathogen or which factors are responsible for the development of the disease is still incomplete and has been subject of intense research. It is admitted, but not proved, that some individual could naturally eliminate Mtb.

The ideal immune response that could lead to this scenario evolves both innate and adaptive immune components, specially the T helper (Th) 1 type. Briefly, once that a phagocyte captures Mtb, it starts signalization by Toll-like receptor (TLR) and/or nucleotide-binding and oligomerization domain (NOD) that promotes translocation to nucleus of the factor nuclear kappa B (NF-κB) leading to the production of interleukin (IL)-12. In turn, one of the main actions of IL-12 is to stimulate the growth and activation of T $CD4^+$ cells, favoring the development of the Th1 response. The main events of Th1 response are the translocation of T-box transcription factor (T-bet) to the nucleus, which will control the hallmark cytokine production, interferon (IFN)-γ. Among multiple effects, IFN-γ increases antigen presentation and lysosome activity of macrophages and activates inducible nitric oxide synthase (iNOS), contributing to killing of Mtb. Moreover, IFN-γ also activates T CD8+ cells, which can produce molecules that kill infected cells, as perforins and granulysins, and are essential to overcome TB infection [7].

13.2 Diagnostic and Classical Therapy

Diagnosis of TB is usually established on the grounds of the clinical and epidemiological history of the patient. Chest X-ray and tuberculin skin test (TST) are routine tests with good sensitivity but poor specificity to confirm the etiology of disease. Since Mtb was defined as the etiological agent of TB in 1882, several techniques have been described for its detection [10].

After more than one century, the standard methods for the etiologic diagnosis of TB remain grounded on the same basic principles enunciated by Koch. Thus, smear microscopy with Ziehl–Neelsen staining is still the method most used worldwide to diagnose TB. A recent systematic review showed that although the sensitivity of smear microscopy might reach 70 %, it varies widely among different settings and is substantially lower (approximately 35 %) in areas with a high prevalence of TB/HIV coinfection [11]. In addition to its low sensitivity, smear microscopy is unable to supply information on mycobacterial species and the sensitivity of bacillus to anti-TB drugs.

Microbiological culture in a mycobacterium-specific medium is still the gold standard for diagnosing and assessing the resistance of Mtb to the drugs used in treatment. Although the sensitivity of culture is higher compared to smear microscopy and varies between 80 and 90 %, it is usually only performed at reference services that treat a larger number of and more complex cases. The main limitation of culture still remains the time needed for diagnostic confirmation, which might require up to eight to 12 weeks [12].

Nevertheless, TB diagnostic technologies made remarkable progress in the last three decades, and the translational potential for these research-based resources is enormous. The ultimate test developed to evaluate TB immune reactivity is the interferon-gamma release assays (IGRA). It happens to be a simple immune-based test, which evaluates the IFN-γ production by whole blood cells in culture stimulated by Mtb antigens. IGRAs have pros and cons; they are highly specific since prior BCG vaccination does not cause a false-positive result. However, they do not help differentiate latent tuberculosis infection from tuberculosis disease and are very expensive when we talk mainly about developing countries [13].

Further diagnostic alternatives are supplied by the development of serologic tests, the detection of specific Mtb proteins, and the measurement of inflammatory cytokines. These methods aim to distinguish active TB disease from latent TB infection. However, few biomarkers are available that can identify and/or distinguish among infection with Mtb, previous bacillus Calmette–Guérin (BCG) vaccination and active TB disease, although they might be highly relevant for the management of the cases requiring treatment. The application of nucleic acid amplification techniques to diagnose TB has attracted much interest, especially due to the possible shortening of the time needed to detect and identify Mtb in clinical samples [10].

Unfortunately, the time of development and implementation of new microbiological tests for TB have not caught up with the technological development of medicine or with the catastrophic spread of the disease associated with the AIDS pandemic, the marginalization of low-income populations to the outskirts of large urban centers, and the appearance of MDR strains [10]. The development of new diagnostic methods also depends on reliable determination of biomarkers in each stage of the disease, as we discuss below.

13.2.1 Treatment

Several protocols are available for anti-TB chemotherapy, which includes an initial, intensive phase aiming at the fast elimination of active bacilli, followed by a continuation phase that seeks to eliminate the latent bacilli [14]. Thus, at most countries, the first stage lasts two months, when a combination of rifampin (rifampicin), isoniazid, pyrazinamide, and ethambutol is given, and the second stage lasts four months, when only rifampin and isoniazid are given [14, 15]. This regimen is efficacious in more than 90 % of cases when appropriately performed, but its long duration, associated side effects, and improvement of symptoms before the full elimination of bacilli cause many patients to drop out before completion. In this situation, the risk of relapse increases and favors the appearance of bacilli resistant to first-line drugs, especially to rifampin and isoniazid, leading to MDR-TB or, in extreme cases, to resistance to all first- and some second-line drugs, a state known as extensively drug-resistant TB (XDR-TB) [15–17].

Although novel drugs that make treatment simpler and/or shorter are desirable, no new drug has been released to the market since the 1970s, when the US Food and Drug Administration

approved rifampin [14]. The latest WHO report [3] lists 10 new drugs currently undergoing clinical research, of which seven are in phase II and three in phase III.

Maybe, these new drugs will allow the development of effective regimens that are as short and have as few side effects as possible to achieve a positive impact on the patient's compliance with treatment. Some regimens lasting three or four months use both newer and older drugs, e.g., rifapentine combined with isoniazid and quinolones such as gatifloxacin and moxifloxacin. Although these regimes are up to 50 % shorter than those currently applied, they are still far from representing a solution to the problem. More recently, our group has investigated new therapeutic regimens combining chemotherapy and immunotherapy in animal models [18]. The results showed the potential of this approach to induce a quicker cure, complete elimination of bacilli, and reduction of the endogenous reactivation rate. Again, a further challenge is the development of markers indicating the efficacy of anti-TB treatment, preferentially as short as possible after it starts, which are currently unavailable.

13.3 Vaccine

Considering the immense reservoir of pathogens represented by the human population infected, vaccine development is fundamental to contribute for new cases prevention and to decrease the high rates of TB prevalence. Currently, BCG (bacillus Calmette–Guérin) is the only vaccine recommended by the WHO [19], with more than three billion doses were administered since its introduction in 1921. BCG is prepared from a strain of the attenuated live bovine tuberculosis bacillus, *Mycobacterium bovis*, which lost its virulence in humans by being specially subcultured in an artificial medium. However, although it protects newborns and children from severe forms of TB, its efficacy against pulmonary TB in adolescents and adults is far from optimal, with protection rates varying between 0 and 80 % according to the geographical area.

As TB represents a global emergence and there is urgency for a new vaccine, a global effort was put into motion during the last two decades, involving all strategic sectors. Modern immunologic tools focusing on specific cellular activation, molecule expression, and best peptides to be recognized and presented by antigen-presenting cells (APCs) and many other tools associated in a systems biology context are helping to achieve this goal.

13.3.1 But, What Features We Should Expect from a New TB Vaccine Exactly?

The landscape is complex, but some required elements are efficiency in newborns and adults, safety, and versatility. It means being better than BCG, eliciting long-lasting memory cells; offering risk to immune-deficient individuals, like HIV carries; and being useful in different conditions and situations, like naïve individuals, BCG-vaccinated, and Mtb-exposed individuals. Maybe the magic bullet idea will not fit the challenge, and we will need more than one simple solution.

Considering that, new vaccines and vaccination strategies are being developed including the use of attenuated live mycobacteria, recombinant microorganisms, and subunits; prime-boost strategies based on the successive administration of a certain mycobacterial antigen under two different vaccine vectors; and DNA vaccines. So far, on 2012 year, more than 12 vaccines are under clinical trials, most of them focusing at prevention of tuberculosis. More vaccine candidates are in the pipeline to enter in clinical trials, including vaccines for individuals with latent infection. These efforts can be summarized in two main rationales: replace BCG or boost BCG [20]. The main features of vaccines with potential use to combat tuberculosis are briefly described below and summarized in Table 13.1.

13.3.2 BCG Improvement/ Replacement

The development of a recombinant BCG (rBCG) is focused on deletion of undesirable genes, overexpression of the candidate ones, or introduction of foreign genes that favors a differential immune

Table 13.1 Mainly anti-TB vaccines under clinical trials

Name	Strategy	Design	Rational	Action	Development point	Reference
VPM1002	BCG improvement	Recombinant BCG	Insertion of a *Listeria monocytogenes* gene coding for the protein listeriolysin. Inactivation of BCG gene for urease C	Enables access to cytoplasm, promoting antigen presentation by MHC I, improving $CD8^+$ T response	Phase II clinical trial	21
rBCG30	BCG improvement	Recombinant BCG	Secretion of Ag85, a mycolyl transferase	The most abundant secreted protein in Mtb cultures induces $CD4^+$ and $CD8^+$ T effector cells	Phase I clinical trial completed, moving to next phase	22
MTBVAC01	BCG replacement	Live *Mtb* genetic attenuated strain	Removal of two independent virulence genes, fadD26 and phoP	Impairs Mtb ability of block phagosome–lysosome fusion	Phase I clinical trial	23
MVA85A/ AERAS-485	Boost of BCG immune response	Recombinant modified vaccinia virus Ankara (MVA)	Expression of Ag85A gene	Induces polyfunctional, long-lasting $CD4^+$ T cells	Phase IIb clinical trial completed	24–26
Crucell Ad35/ AERAS-402	Boost of BCG immune response	Replication-deficient adenovirus 35	Expression of antigens highly immunogenic Ag85A, Ag85B, and TB10.4 from Mtb	Induces $CD4^+$ and $CD8^+$ T cells activation and IFN-γ production	Phase IIb clinical trial	27
AdAg85A	Boost of BCG immune response	Replication-deficient adenovirus	Adenoviral vaccine-expressing Mtb Ag85A (AdAg85A)antigen	Increases numbers of gamma interferon-positive $CD4^+$ and $CD8^+$ T cells	Phase I clinical trial	28, 29
Hybrid 1+IC31 vaccine	Boost of BCG immune response	Recombinant protein and adjuvant	Combines antigens Ag85B and ESAT-6, adjuvanted by IC31 (or CFA01)	Induces IFN-γ $CD4^+$ T producing cells; long-lasting immunity	Phase I clinical trial/ phase II planned	30–32
SSI/SP H4-IC31 (or Hyvac 4)	Boost of BCG immune response	Recombinant protein and adjuvant	Fusion antigens Ag85B and TB10.4, adjuvanted by IC31	Induces robust and prolonged $CD4^+$ T cells response	Phase II clinical trial	33
GSK M72	Boost of BCG immune response	Recombinant protein and adjuvant	Fusion antigens Rv1196 and Rv0125, adjuvanted by AS01	Induces robust $CD4^+$ T cell response, high and persistent antibody levels	Phase IIb clinical trial	34
SSI H56-IC31vaccine	Boost of BCG immune response	Recombinant protein and adjuvant	Ag85B and ESAT6 fusion antigens with the addition of Rv2660c antigen, adjuvanted by IC31	Antigens from different stages of disease (active and latency) allow their use as a pre- as well as post-exposure vaccine	Phase I clinical trial	35–37
ID93+GLA-SE	BCG replacement	Recombinant fusion polyprotein and adjuvant	Mtb antigens associated with virulence or latency (Rv2608, Rv3619, Rv3620, and Rv1813), adjuvanted by GLA-SE	Induces antigen-specific Th1 type immune response, targets both active and latent TB, can be used as immunotherapy	Phase I clinical trial	38–40
RUTI	Therapeutic vaccine	Subunit and adjuvant	Detoxified, fragmented *M. tuberculosis* cells, delivered in liposomes	Induces polyantigenic cellular and humoral immune responses	Phase II clinical trial	41
Mycobacterium vaccae vaccine	Boost of BCG immune response; maybe immunotherapy	Inactivated strain	Heat-inactivated *M. vaccae*	Bets on shared antigens to induce durable cellular immune response and boost IFN-γ responses and lymphocyte proliferation	Phase III clinical trial completed	42–44

BCG, unique anti-TB vaccine currently approved to use around the world, based on attenuated live *M. bovis* strain, confers only partial protection (effective against miliary and meningitis TB in infants but fails against pulmonary TB in adults). Basically, candidate vaccines intend to replace or boost BCG

response against the BCG, conferring immunological memory and effective protection against TB.

VPM1002 is a live vaccine against TB based in rBCG that is in phase II clinical trial. This vaccine has 2 important modifications at gene level that together improved the immunogenicity of vaccine. One approach is the insertion of a *Listeria monocytogenes* gene coding for the protein listeriolysin (Hly). This protein is involved in the creation of pores in the phagosome after endocytotic uptake of the bacterium, permitting the bacterium to move into the cytosol. This approach was useful to overcome a property of BCG to conduct only MHC II antigen presentation. The escape to the cytosol allows the MHC I presentation and consequent T $CD8^+$ activation. Immunologically, this is the desirable pattern of response in the fight of Mtb. Another gene modification is the inactivation of BCG gene for urease C (ureC). Urease C catalyzes the hydrolysis of urea into carbon dioxide and ammonia, thus creating an alkaline environment. When the bacteria lost ureC, the inner environment becomes acid and favors the listeriolysin activity [21].

Another recombinant BCG strategy, named *rBCG30*, entered on phase 1 clinical trial on 2004. The vaccine passed on the safety study and now is "on hold" to move to next phase. This rBCG secretes a 30 kDa protein that is detected as the most abundant secreted protein by Mtb on culture. This protein, also called Ag85B, is a mycolyl transferase that conferred protection against TB on vaccinated guinea pigs and induces exuberant immune response in humans [22].

MTBVAC01 was designed to replace BCG. Instead of a recombinant BCG, it is boldly based on live *Mtb* genetic attenuated strain, resulting in the removal of two independent virulence genes, fadD26 and phoP. The phoP gene was greatly expressed in an epidemic strain responsible for a TB outbreak in HIV-infected patients with high mortality rate. FadD26 is related to the synthesis of the phthiocerol dimycocerosate, a cell wall lipid. Preclinical results showed a consistent protection against TB associated with the generation of polyfunctional cytokine-secreting T $CD4^+$ cells and long-term maintenance of T $CD4^+$ central-memory cells compared with BCG vaccination.

The rationale behind these interesting results seems to be due to impaired ability of MTBVAC01 to block phagosome–lysosome fusion compared with the parental *M. tuberculosis* strain. This candidate vaccine entered in phase I clinical trial quite recently and is the first vaccine of its kind to start clinical evaluation [23].

13.3.3 Viral Vectors

A viral vector carries the gene of the immunogen of interest and is used to improve the immune response against it, being extremely efficient to do that. On the other hand, often they are highly immunogenic which causes an undesirable exacerbate immune response against the vector. To circumvent this issue, several researchers are trying to work with viral vectors that humans virtually did not contact yet. Indeed, below we discuss some vaccines using viral vectors that meet this expectation.

An Oxford-Tuberculosis Consortium constructed a vaccine named *MVA85A/AERAS-485*, which passed the phase II on Gambia and South Africa. This vaccine uses a recombinant modified vaccinia virus Ankara (MVA) as delivery system, inducing the expression of Ag85A gene. Its administration induces the proliferation of high levels of antigen-specific $CD4^+$ T cells in children that are polyfunctional, persisting for 14 months following vaccination [24, 25]. However, a phase II b clinical trial to assess efficacy, as well as safety and immunogenicity, of MVA85A in infants was recently completed, in collaboration with University of Cape Town's South African Tuberculosis Vaccine Initiative (SATVI) in Worcester, South Africa. Results showed that this vaccine was well tolerated but induced modest cellular immune response [26].

Using adenovirus as delivery system the *Crucell Ad35/AERAS-402* is under phase IIb in South Africa, Kenya, and United States, and phase IIb with almost 4,000 infants from Mozambique, Kenya, and South Africa. This vaccine is composed of antigens Ag85A, Ag85B, and TB10.4 from Mtb that are highly immunogenic, inducing both $CD4^+$ and $CD8^+$ T cell activation and IFN-γ production. Furthermore, the use of adenovirus 35 is associated with an

increase of $CD8^+$ T cells, which is believed to be fundamental for a TB vaccine [27]. Using the adenovirus 5 and delivering only the Ag85A, there is a phase I clinical trial under way in Canada with a vaccine named *AdAg85A*, developed by Prof. Zhou Xing at McMaster University in Hamilton, Ontario, and acquired later by a Chinese company CanSino [28, 29].

13.3.4 Recombinant Proteins

The main rationale of recombinant proteins, including recombinant fusion proteins combining at least two immunodominant antigens, is also to elicit vigorous immune response against a pathogen without the risks of live or killed whole organism vaccines that could restrict their applications, especially in immunocompromised hosts, like HIV-infected people. As all protein-based vaccine, it must be administrated with a potent adjuvant to elicit sufficient and adequate immune response, e.g., polarizating T $CD4^+$ cells to Th1 profiles, triggering T $CD8^+$ cytotoxic cells, and activating dendritic cells and other antigen-presenting cells. Examples of this category of vaccines in the most advanced stage of study are listed below.

The Statens Serum Institut started in 2012 a phase II clinical trial in HIV-positive patients of the *Hybrid 1+IC31 vaccine* that combines antigens Ag85B (present in Mtb and BCG) and ESAT-6 (present in Mtb but not in BCG). This formulation is adjuvanted by IC31 (liposome formulation containing oligodeoxynucleotide and a cationic antimicrobial peptide) produced by Intercell Company. The immunogenicity and safety results of two doses of this vaccine are expected for 2013. It is intended yet to perform a phase II clinical trial in health adolescents. Preclinical and phase I clinical trials have demonstrated that this vaccine is safe; induce long-lasting immunity in different populations, including IFN-γ T $CD4^+$-producing cells; and is able to boost immune responses induced by BCG. The vaccine is also being tested with CFA01 adjuvant, also a liposome formulation but containing dimethyldioctadecylammonium (DDA) with trehalose 6,6'-dibehenate (TDB), a synthetic analogue of the mycobacterial cord factor trehalose 6,6'-dimycolate (TDM) [30–32].

The Aeras organization completed the phase I clinical trials of a similar vaccine, *SSI/SP H4-IC31* (*or Hyvac 4*), composed of fusion antigens Ag85B and TB10.4, adjuvanted by IC31. Previous preclinical results showed that this vaccine induced an increased and prolonged response consisting of $CD4^+$ T cells with an effector-memory and central-memory phenotype. In advantage, Hyvac 4 did not compromise ESAT-6-based diagnostics, what explains its replacement by TB10.4 [33].

GlaxoSmithKline (GSK) designed the M72 fusion protein, consisting of Mtb antigens Rv1196 and Rv0125, both expressed by Mtb as well as BCG. This vaccine has been tested with different synthetic adjuvants, and AS01 (monophosphoryl lipid A mixed with saposin QS21) has demonstrated better performance. Phase I/IIa clinical trials demonstrated that M72 has a clinically acceptable safety and reactogenicity profile and is highly immunogenic, inducing strong $CD4^+$ T cell response and high antibody levels that persisted for up to 3 years. The *GSK M72* (or *M72/AS01*) vaccine has thus been selected for further clinical development, phase IIb, as Aeras and (GSK) have just announced [34].

The *SSI H56-IC31vaccine* is composed basically by Hybrid I (Ag85B and ESAT6 fusion antigens) with the addition of Rv2660c antigen. As the name suggests, IC31 is the adjuvant. The main important difference here is that Rv2660c is a strong antigen, upregulated in latency period of disease, where Mtb faces starvation conditions and changes its gene expression profile significantly, which in turn impacts the availability and variety of "visible" antigens to the immune system. The advantage of this strategy, compared to the other preexposure vaccines, is the flexibility to be used as a pre- as well as post-exposure (also called multistage) vaccine, what is very interesting if we remember that one-third of world population is potentially infected. Preclinical studies using H56-IC31 as a boost to vaccination with BCG resulted in efficient containment of Mtb infection and improved survival rates of the animals compared with BCG alone.

Boosted animals showed reduced pulmonary pathology and extrapulmonary dissemination and protection correlated with a strong recall response against ESAT-6 and Rv2660c. Importantly, BCG/H56-vaccinated monkeys did not reactivate latent infection after treatment with anti-TNF antibody. By the way, treatment with anti-TNF-α is a common side effect of arthritis rheumatoid immunotherapy, when a patient reactivates a latent TB that has never been detected before. For this reasons, this vaccine is a good candidate and has entered in phase I clinical trial [35–37].

Recently, Aeras and the Infectious Disease Research Institute (IDRI) announced the start of the phase I clinical trial of *ID93 + GLA-SE vaccine*. ID93 is a recombinant fusion polyprotein comprised of Mtb antigens associated with virulence or latency (Rv2608, Rv3619, Rv3620, and Rv1813), and GLA-SE refers to the adjuvant glucopyranosyl lipid A – stable emulsion. The vaccine targets both active and latent TB, what makes it also an immunotherapeutic tool. Preclinical studies combining this vaccine to the conventional TB chemotherapy, using cynomolgus monkey as model, showed activation of pluripotent antigen-specific Th1-type immune response correlated to decreased bacterial burden. The authors also affirm that the duration of chemotherapy was reduced, what is an important feature once the long-lasting period required treating TB contributes to the reduction of patients' adherence to treatment to its end [38–40].

13.3.5 Therapeutic Vaccines

The possibility of using a therapeutic vaccine as an alternative strategy to treat TB, mainly if it could be associated to classical TB chemotherapy, has been studied. It could allow adopting a shorter period of chemotherapy and preventing reactivation of the possible lasting bacilli by restoring the potential of host immune response, as showed above.

RUTI is a therapeutic vaccine made of detoxified, fragmented *M. tuberculosis* cells, delivered in liposomes. In preclinical studies, RUTI was administrated after a short period of chemotherapy. Results showed that T $CD4^+$ and T $CD8^+$ cell response against secreted and structural antigens was boosted, a polyantigenic humoral response was induced, and this strategy was able to content the reactivation of infection. Based on these results, phase I clinical trial was completed, with a good safety and immunogenic profile, and a phase II clinical trial is almost finished [41].

Inactivated *Mycobacterium vaccae* Vaccine. The rationale behind the use of *M. vaccae* as vaccine was based on data showing that natural or vaccine-induced infection with nontuberculous mycobacteria confers protection against the development of TB. It occurs most likely through responses to antigens shared by mycobacteria. This vaccine completed the phase III clinical trial that showed for the first time the effective vaccine-based prevention of an opportunistic infection in HIV-infected adults. The vaccine boosts IFN-γ responses and lymphocyte proliferation to whole vaccine sonicate and antibody responses to lipoarabinomannan. In BCG-primed, HIV-positive, and HIV-negative subjects, this vaccine induced durable cellular immune responses.

These results support the development of a prime-boost immunization strategy using BCG and a whole-cell inactivated mycobacterial vaccine for preventing the most important opportunistic infection affecting HIV-infected persons in the developing world. Considering the immunogenic properties of *M. vaccae* vaccine, some studies wondered if this vaccine could also treat TB, as an immunotherapeutical tool. There are controversies among studies, trials, and meta-analysis whether *M. vaccae* would or not be a good immunotherapy to TB. It may be that different routes, assays, methodologies, or inclusion criteria have guided to apparently conflicting results. Importantly to note, these referred studies included different conditions like injected or oral formulations, association or not with chemotherapy, and the type of tuberculosis (drug sensible or resistant, already treated or not). But, taken together, these studies leave a confident impression that heat-killed *M. vaccae* vaccine, orally and daily administrated together with chemotherapy, could contribute to TB treatment [42–44].

13.3.6 Future Perspectives

Here we consider some techniques and approaches on vaccine development that potentially can accelerate TB control, including what our research group is performing to contribute in this area.

A technology that was originally developed to decontaminate plasma and platelet blood products, known as the INTERCEPT Blood System gave rise to another and interesting approach in vaccines field, called *killed but metabolically active* (*KBMA*) *vaccines* [45].

KBMA vaccines are whole pathogenic or attenuated organisms killed through photochemical inactivation with a psoralen cross-linking agent, impacting an absolute block to DNA replication and possible vaccine outgrowth. *In this process*, DNA alterations occur randomly and are limited, what allows that in a vaccine dose, containing million units of microorganisms, the expression of virtually all antigenic repertoire is achieve. What is most important in this strategy is that under these conditions the microorganisms retain enough metabolic activity to induce an immune response because they maintain the aspects relative to natural life cycle of the wild organism and are sensed as live for the host.

Through it all, the main applications of this tool are use KBMA as recombinant vectors encoding selected antigens and design KBMA vaccines from attenuated forms of a pathogen. In the case of TB, both approaches could be used. In the former, it would be possible, and also interesting, to use Mtb antigens that are expressed at the moment of bacilli reactivation, as a manner to block or prevent the progress of latent TB to active TB. The later strategy is particularly interesting because it allows the presentation of the whole antigenic repertoire of the pathogen, which is very desirable since the correlates of protection are unknown or poor understood. Also, been TB the major opportunistic disease of HIV-infected people, KBMA eliminates some issues related to safety that other approaches could bring. Preclinical studies showed that KBMA vaccines induce functional immune responses correlated with efficacy, but to this time there are no clinical trials conducted [45].

13.3.7 DNA Vaccines

Basically, a DNA vaccine comprises a bacterial plasmid that utilizes a promoter able to function in mammalian cells and a gene encoding an antigen. Once the vaccine is captured by the cell, the gene is translated in a protein, which can be presented by the MHC I pathway to CTL cells, the main feature sought when this strategy was designed. But, DNA vaccines showed competence to stimulate innate immunity and trigger the other arms of adaptive immunity: antibodies and helper T cells. Moreover, it has prophylactic and therapeutic properties. The bias of the generated response, e.g., Th1 or Th2, can be induced through the method chosen for vaccine delivery, like intramuscular or gene gun, respectively [46].

It is possible to improve its performance administrating DNA vaccines together with other vaccines, in heterologous prime-boost regimens, or by the addiction of molecules, e.g., cytokines, or delivering DNA vaccines to cells using formulations like polymeric microspheres and liposomes. Actually, efforts are concentrated to generate in humans the same effectiveness showed in preclinical models by improving DNA expression, delivery, and immunogenicity [47].

DNA vaccines efficacy and safety can be maintained once it dispenses to work with virulent pathogen or the use of deleterious pathogen antigens. Moreover, plasmid employment avoids safety issues of attenuated viruses or viral vectors. Thus, this strategy is attractive as an alternative for global vaccine programs, as such required for TB, considering its relatively simple and rapid construction/production and stability at room temperature [48].

Still regarding TB, it is reasoned that the variable protection conferred by BCG is due, at least in part, to its weak induction of MHC class I restricted responses. So that strategies seeking boost the immune response based in BCG or its mutants to prevent TB infection or activation may not fully deal with all aspects required and the use of plasmid DNA vaccine could offer a valuable partnership [49].

Box 13.1
DNA-hsp65

The first DNA vaccine for TB described in the literature was created in 1994 by our group [50]. Named DNA-hsp65, this vaccine includes a genetic sequence that encodes the intracellular expression of *M. leprae* antigen Hsp65. On the grounds of this original study, several researchers have developed other DNA vaccines containing genetic sequences for the expression of other Mtb immunodominant antigens, such as Ag85 [51], ESAT-6, and MPT-64 [52] and MPT-83 [53], many of which induce protection levels similar to BCG.

One of its most outstanding studies performed by our group showed that the genetic vaccine DNA-hsp65 has not only preventive but also therapeutic activity against Mtb infection by operating as an immunomodulator against the established disease. These initial results showing that DNA-hsp65 can treat chronic cases with systemic effects, latent TB, and MDR-TB were published in Nature in 1999 [54]. The vaccine further hinders reactivation of disease in immunosuppressed animals [18, 54], and its addition to chemotherapy significantly shortens the length of anti-TB treatment [18]. Among the several studies performed with DNA-hsp65, the main ones have focused on immunogenicity and protection of experimental Mtb infection models in mice, guinea pigs [55, 56], and nonhuman primates (Silva et al., unpublished data); optimization of PLGA microspheres formulation [57], liposomes [58], gene gun [59], and prime-booster systems [60]; technological development and scale up production; preclinical tests in animals [61, 62]; and phase I clinical tests in humans [63, 64].

Cationic Liposomes for DNA-hsp65 Delivery

Considering the promising results from the development of DNA vaccines and specifically, DNA-hsp65, the great challenge is the improvement of DNA vaccination in a single-dose administration. The vaccination efficiency of naked plasmid DNA-hsp65 against TB is achieved in mice when it is administered 4 times in a total amount of 400 μg via intramuscular route [58]. The number of administration doses is a consequence of different barriers that naked DNA has to overcome from extracellular matrix to the cell nucleus, requiring the use of carriers for proper DNA delivery [65]. One safe strategy is the use of nonviral carriers that are basically formed after DNA complexation with cationic molecules, such as chitosan and cationic lipids, among others [66]. Cationic liposomes were extensively investigated by our research group, and these colloidal systems are composed by amphiphilic lipids that self-assembly in bilayers, forming vesicles with interior aqueous lumen. The cationic characteristic is achieved by the use of synthetic lipids, conferring toxicity, and in our case we used 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and also the helper and neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). In order to overcome the cationic cytotoxicity, the liposomes were produced with egg chicken L-α-phosphatidylcholine [58, 67]. Among different parameters, the way of DNA incorporation into the liposomes influences the immunological response, reflecting in different nucleic acid release [67]. In order to assess this hypothesis, we prepared dehydrated-hydrated liposomes (DRV) ranging from 200 to 600 nm with DNA incorporated inside the nanostructures (conventional DRV encapsulation) or promoting electrostatic complexation between DNA and "empty" DRVs. The complexation protocol improved the prophylactic effect in mice, reducing administered dose in 16 times and using the noninvasive intranasal route.

Besides the great results, it is well known that cationic liposomes are effective in delivering nucleic acids into the cytosol [66, 68],

and the nuclear envelope is an additional barrier. To circumvent this problem, we designed a cationic and synthetic peptide that acts as a nuclear localization signal (NLS), helping DNA to cross the nucleus membrane [69]. NLS is basically a short peptide sequence that associates with specialized cargo proteins and provides nuclear transport [70]. The designed NLS was electrostatically associated with DNA-hsp65 forming a binary complex and then complexed with "empty" DRV liposomes (Fig. 13.1). This peptide-DNA-cationic liposome complex presented similar therapeutic effects against tuberculosis when compared to naked DNA treatment, but with four times less DNA, demonstrating the nonviral carrier protection.

The use of cationic liposomes and addition of NLS for DNA-hsp65 delivery reflects two approaches for TB prophylactic vaccination and also for treatment, suggesting the crucial role for combine different strategies to improve the efficacy of other vaccines

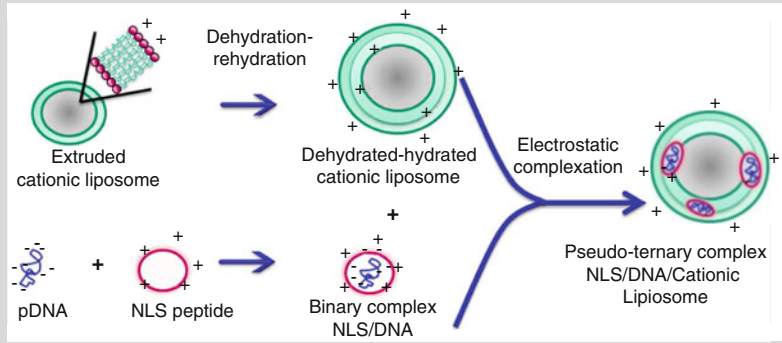

Fig. 13.1 Schematic representation of peptide-DNA-cationic liposome complex (Reprinted from Rosada [69], 8 pages, Copyright (2012), with permission from Elsevier)

13.4 Strengths and Weakness

Although the latest WHO report [3] is more optimistic than earlier ones, there are serious doubts regarding the goals of reduce until 2015 the TB prevalence and death rates by 50 % relative to 1990 and the elimination of TB as a global health problem by 2050 (program Stop TB Partnership). Important challenges must still be overcome in this regard.

As we highlighted, the relationship between TB and poor living conditions and poverty is well established. Several countries were able to control TB simply by improving the living standards of the affected population. In fact, factors such as poverty areas, high-risk groups, coinfection prevalence, low-quality control actions, and unstable access to healthcare services contribute to the current alarming scenario. However, even if the social and operational issues are solved, several crucial and challenging features must still be addressed to control TB in the twenty-first century.

Therefore, the need to investigate and develop new knowledge, strategies, and products to diagnose, treat, and prevent TB is urgent. In addition, it should be considered that TB research is usually performed with animal models, which results cannot be always transferred to human clinical practice. Moreover, the development of any new tool to treat, prevent, or diagnose tuberculosis must be supported by reliable biomarkers, and this information is lacking.

13.4.1 Coinfection

One of the weaknesses in TB vaccine development relates to coinfection with another pathogen. With the emerging of HIV infection since the 1980s, it became more evident.

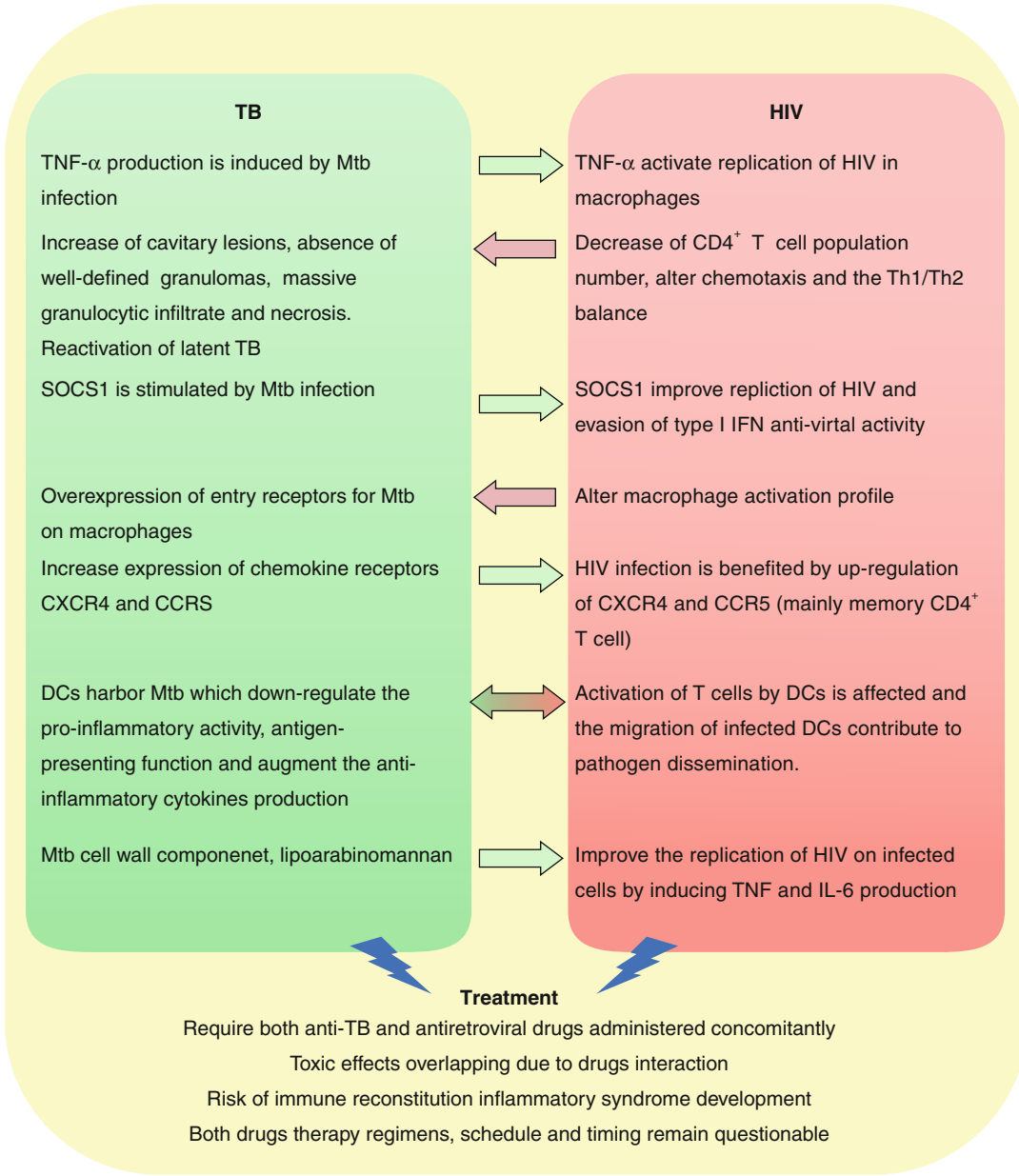

Fig. 13.2 TB/HIV interaction in coinfected host

It was observed that who harbors *M. tuberculosis* and HIV has potentiated both diseases, with fast weakening of immunological functions and premature death if they are untreated (Fig. 13.2). In this scenario, 14 million individuals worldwide are expected to be coinfected [71]. TB accounts to 26 % of deaths caused by AIDS [72], and 99 % of this cases are taking place in developing countries [73].

The systemic failure of immune response caused by HIV infection can easily abrogate the protection induced by some vaccine candidate. The virus kills effector T CD4+ cells, a hallmark character in the fight against Mtb. Memory T

cells are also affected by cytolytic effects of HIV, discrediting most of vaccines [74]. A good alternative to circumvent this scenario is to draw a combined TB/HIV vaccine, for example, a recombinant BCG carrying a combination of Mtb and HIV antigens [75].

Another weak point to consider in the development of a new TB vaccine in the context of HIV coinfection is the latency.

Implications of immunopathology and treatment. Yellow box represents host, and green box is related to TB and red to HIV. The arrows indicate how each disease can compromise the immune response to another [74, 76].

Actually, the most effective vaccine candidates in clinical trial or animal models can prevent active TB, but they are not successful in sterilizing the host from Mtb infection. Therefore, in a case of vaccinated people get coinfected with HIV, the immune response will fatefully fail, and the latency reservoir could be reactivated.

Moreover, not only viruses are responsible for this scenario, here we can cite parasites, which are highly endemic in poor countries, like helminths. At the poorest regions of the world, at least one-third of the population are infected with one or more helminths [77, 78]. Helminth infections are classically known as Th2 response inducers, and for these reason many researchers have been shown that helminthic parasites impaired Th1 immune responses induced by vaccines in preclinical trials [79–83]. Conversely, we previously showed that infecting BALB/c mice with *Toxocara canis* helminth did not alter susceptibility to subsequent infection with *M. tuberculosis* [84].

In contrast, *Schistosoma mansoni* infection can strongly enhance susceptibility to TB [82] and impair the protective effects of BCG vaccination [85]. These data provide evidence of peculiarities on TB/helminth coinfected host. Also, decrease in immunogenicity of BCG was observed in a study in Ethiopia where healthy or helminth-infected volunteers received anti-helminthic therapy or placebo and were subsequently vaccinated with BCG. Therefore, were observed in helminth-infected group immunized with BCG a reduced IFN-γ secretion and increased TGF-β production when the cells of patients were stimulated in vitro with PPD (purified protein derivative) [86]. These results show up potential elucidation for the perceptible failure of BCG to prevent pulmonary tuberculosis.

However, not all vaccines are influenced by helminths parasitism, as observed for DNA-hsp65. In experimental model of tuberculosis and schistosomiasis, or tuberculosis and toxocariasis, it was shown that therapeutic effects of DNA-hsp65 were not affected in the presence of an unrelated Th2 immune response induced by helminth infections [87]. Therefore, it has to be considered the pressure of helminth coinfection on the immune response during TB, influencing the development of new therapies and/or vaccination protocols against this disease.

13.4.2 Animal Models

The use of animals in TB research began when Robert Koch employed mice as a study model [88]. Since then, considerable advances have been made in the understanding of the dynamics of Mtb infection using animal models. The cellular immune response and cytokine production against Mtb are intense in mice, and they correlate with some parameters observed in humans infected with TB, such as the participation of $CD4^+$ T cells [89, 90], interleukin-12 (IL-12) [91, 92], and tumor necrosis factor-α (TNF-α) [93, 94]. In addition, the murine model is well characterized immunogenetically, and a wide scope of reagents is available to assess these features.

Despite the benefits of TB research in mice, some important differences with humans should be considered. From the immunohistopathologic perspective, *Mtb* infection in mice does not lead to formation of the typical granuloma in the lungs but rather induces a massive presence of lymphocytes at the center of the inflammation process, where the bacilli are located. Conversely, in humans, the inflammatory infiltrate resulting from infection exhibits the typical granulomatous configuration, with a ring of lymphocytes surrounding the infected macrophages, which occupy the

center of the structure [95, 96]. In addition, caseous necrosis and cavitation occurs in humans but not mice [97].

To overcome these problems, several teams have employed other experimental animal models, such as guinea pigs, rabbits, and monkeys, and compared their results with those obtained in mice, particularly regarding efficacy screening and/or development of new vaccines and drugs [98]. Nevertheless, due to the different genetic compositions, several host molecules involved in Mtb infection in mice are absent in other experimental models and humans [99, 100]. In addition, the immune response in the pulmonary environment, the site of Mtb infection in mice, monkeys, and other models, differs from the response that occurs in peripheral blood, making it difficult to correlate the data obtained from the lung in experimental models with findings in human peripheral blood [101–103]. Conversely, the immune response in the pulmonary environment of TB patients, assessed in bronchoalveolar lavage cells or lung biopsy samples, is not universally available due to ethical reasons and procedural difficulties, and the correlation of pulmonary features with data obtained from patients' peripheral blood is controversial [104, 105].

Therefore, although the use of animals in TB research improves our understanding of the dynamics of Mtb infection, the data thus obtained cannot be systematically transferred to human clinical practice. No study has globally shown which infection or disease markers are actually present in the several investigated animal species. Therefore, we may conclude that the techniques to assess TB clinical forms and efficacy of therapeutic interventions as well as to establish correlations between experimental and clinical data must be performed in samples of biological fluids, such as blood that is easy to harvest and the most commonly used source in clinical practice.

In this sense, reliable biomarkers of the different stages of disease are crucial to appropriately diagnose the several clinical forms of TB, as well as for analysis of therapeutic and vaccine efficacy. However, such biomarkers have not yet been identified.

13.4.3 Biomarkers

The first reference on the diagnosis of a disease using markers dates around 400 BC, when Hippocrates suggested that alterations in organic fluids, including blood, were associated with disease. From this time onwards, diseases were characterized by the alterations they induce in the components of our bodies. By the end of the twentieth and beginning of the twenty-first century, studies seeking to define the normal molecular identity of an individual and its modifications in response to diseases began to be performed in order to increase the accuracy of diagnosis. Currently, a biomarker is defined as a biological characteristic that is objectively measured and assessed as an indicator of normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention, supporting conclusions about the present or future status of individual's health, as well as the type or degree of induced pathology.

The complexity of the progression of Mtb infection and the immune response against it led to attempts to find diagnostic tools in one of the physiological components. Currently, lung X-rays and sputum cultures of patients with suspected TB infection are combined with the tuberculin skin test (delayed hypersensitivity reaction to Mtb antigens) to establish the diagnosis. More recently, attempts have been made to use cytokine levels in peripheral blood, the frequencies of different cell populations and the expression of cell surface molecules to distinguish the forms of TB. Although the possible combinations of constitutive components of an organism are countless, no specific set of elements has yet been established with enough sensitivity and specificity to distinguish the different forms or TB and to predict the success of current and possible treatments and preventive vaccines [12].

In the modern context of TB global emergency, biomarkers have different possible applications, from diagnosis, passing by prevention and treatment, up to outcome measurements. In each of these cases, there are important subdivisions in the use of biomarkers. It is clear that they will be

important catalysts of the advances in the diagnosis of different types of tuberculosis (e.g., antibiotics resistance), in the assessment of recovering or risk of disease reactivation, and in the development of new drugs and new prophylactic and therapeutic vaccines, attesting their effectiveness and providing endpoints for clinical trials. At the same time, research seeking for biomarkers will provide deep knowledge of disease per se [106].

Such biomarkers have not yet been identified, but several international research teams are devoting their efforts to finding them [15, 107]. A huge amount of studies have measured the interferon-γ-producing T cell frequencies, but it has been demonstrated that it poorly predicts the protective efficacy of vaccines. Equally, polyfunctional subsets of antigen-specific T cells that secrete several cytokines have been considered, without any final conclusion. Also, besides controversies remain the importance of antibodies in the host defense against mycobacterial infections has emerged from experimental and clinical studies [108]. If detection of specific antibodies will be useful as correlate of protection, it remains to be elucidated too. As immune response to TB is a very complex process, other components should be further investigated as potential correlates of protection [109].

While it is intuitive that correlates of protection should be available prior to new vaccines are tested, there is a great chance that these biomarkers will come from the randomized controlled clinical trials already in progress [17, 106]. The rationale behind this approach is to compare protected individuals from vaccine and placebo groups. The main problem here is setting the ideal groups in endemic infectious areas and the time-consuming character of this strategy. With the increase of vaccines entering clinical trials, there is an optimism to identify these correlates as faster as possible. Even better, comparing findings among different studies could be very useful to define a more universal set of markers.

But, *how to search for biomarkers or what are the best tools for reliable results*? Nowadays, biomarkers have been prospected at molecular level. The "omics" era, meaning genomics, proteomics, transcriptomics, and metabolomics, certainly has opened new perspectives, and it is possible that the association of data coming from two or more types of approaches will bring more robust answers, which we can call biosignature. However, adequate analyses of the huge amount of information generated by investigations using those technologies are equally challenging, and advances in bioinformatics have been fundamental, including the fruitful field of systems biology [110]. In support to this kind of approach, some repository datasets have contributed to accelerate researches.

The Tuberculosis Database (TBDB – http://www.tbdb.org/) is an integrated database providing access to TB genomic data and relevant resources to the discovery and development of TB drugs, vaccines, and biomarkers. In addition, TBDB provides access to software that allows comparing different datasets, constituting a very attractive platform for TB research [111]. Exploring and integrating the availability of pathogen-specific genomic information with functional datasets, TDR Targets Database (http://tdrtargets.org) is an online and versatile resource that facilitates the rapid identification and prioritization of molecular targets for drug development for different diseases, including TB [112].

The versatility and power of these tools to query the complex datasets are paramount because, besides some studies that have been relatively successful on populations geographically distant, it is important to keep in mind that this is maybe not the type of "one-fits-all" solution, and we must be aware of it an occasionally adjust the results regionally.

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Paracoccidioidomycosis: Advance Towards a Molecular Vaccine

14

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Contents

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Abstract

The P10 peptide is a conserved sequence in the gp43 diagnostic antigen of most isolates of *Paracoccidioides brasiliensis*. P10 is promiscuously presented by three different MHC class II molecules from mouse haplotypes, and also by 90 % of Caucasian HLA-DR antigens. It elicits an IFN-γ-dependent protective anti-fungal response and is a candidate for a peptide vaccine. Immunization with P10 of mice intratracheally challenged with virulent yeasts rendered significant protection measured by colony-forming units in the lungs. A predominant Th-1 immune response was obtained. Different adjuvants enhanced the activity of the peptide and P10-primed dendritic cells were highly effective in clearing fungal infection. Gene therapy with plasmids expressing P10 and IL-12 elicited a protective Th-1 immune response without causing hyperinflammation or fibrosis. In a long-term experiment, vaccination with pP10 and pIL-12 virtually eliminated all fungal elements and preserved a normal lung parenchyma.

Fungal diseases, which can affect a great number of individuals in endemic areas and be quite severe and even lethal in immunocompromised patients, have not so far a single approved vaccine, to be used in immunoprophylaxis or therapeutically in combination with chemotherapy. Progress is evident, however, in many research

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areas on pathogenic fungi, with the discovery of protective antigens, virulence factors, mutant strains, the complete genomics, and increased knowledge of the immunological response. Most promising are those vaccines that elicit fungicidal antibodies. Other vaccines aim at protective cellular immune responses to act even in patients with immunological deficiency and neutropenia. Presently, we review the advance towards a peptide vaccine or the equivalent gene therapy against systemic paracoccidioidomycosis.

14.1 The Disease

Paracoccidioidomycosis (PCM), previously named South American blastomycosis, is an endemic fungal infection caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. The disease was first described by Adolpho Lutz, in São Paulo 1908, and is the prevalent systemic fungal infection in Latin America (distribution from México to Argentina). The majority of cases are reported in Brazil (approximately 80 %), followed by Colombia, Venezuela, and Argentina. So far, no single case has been reported in Chile, Nicaragua, and the Antilles.

Non-autochthonous cases have been reported in the USA, some European countries, and Asia, all of them represented by individuals who had previously visited endemic areas in Latin America. Therefore, paracoccidioidomycosis may also be regarded as a disease of travelers who had lived for extended periods in endemic areas [1, 2].

Reporting on fungal diseases to Public Health authorities in Brazil and other Latin-American countries is not mandatory. As a consequence, data regarding the real incidence of fungal infections in endemic areas are scarce. In the 1996–2006 period in Brazil, 1,853 (51.2 %) of 3,583 confirmed deaths due to systemic mycoses were caused by PCM [3].

The infection is acquired through inhalation of airborne propagules (conidia) released by the mycelial form of the fungus, which develops in the soil of endemic areas. After the initial contact different progression states can take place known as: (1). PCM infection, (2). PCM disease (acute/subacute or chronic forms), (3). unifocal infection, (4). multifocal infection, and (5). residual clinical forms or sequels [4].

The primary pulmonary infection is usually unapparent or oligosymptomatic, and individuals may remain infected throughout life without ever developing progressive PCM. A few infected patients may develop a clinical disease weeks to months after inhalation of fungal conidia. A characteristic acute or subacute clinical form may represent 3–5 % cases of PCM mainly affecting children and teenagers of either gender. Predominant clinical signs include lymphadenomegaly, hepatosplenomegaly, digestive and/or osteoarticular involvement, and skin lesions [4, 5].

Most symptomatic patients develop the disease years after the acquisition of the infection, due to reactivation of quiescent foci (chronic form). Active disease is mainly reported among adult males (15:1), rural workers from endemic areas, ranging 30–60 years of age. When diagnosed by positive skin tests the infection is equally distributed between genders. β-Estradiol seems to protect women from developing the disease. The clinical presentation in the lung occurs in 90 % of patients. The disease may, however, involve other organs such as the oropharyngeal mucosa, skin, lymph nodes, adrenals, and the central nervous system [4, 6]. Despite the fact that PCM is an infection controlled by cell-mediated immunity, this mycosis has scarcely been reported among patients with AIDS, cancer, and patients submitted to organ transplantation [7, 8].

14.2 The Pathogen

The etiological agent of PCM is an eukaryotic dimorphic fungus only known in its asexual state (anamorph), called *Paracoccidioides brasiliensis* [9]. The characteristic features when examined in fresh preparations are the round cells, 5–25 μm in diameter, with well-defined refringent wall and multiple budding. The mycelial phase when observed by scanning electron microscopy shows hyphae with a smooth surface and tubular form with salient septal ring forms. Chlamydospores, arthroconidia, and conidia are found in the culture. The conidiogenesis is an asynchronous process and seems to be dependent on growth

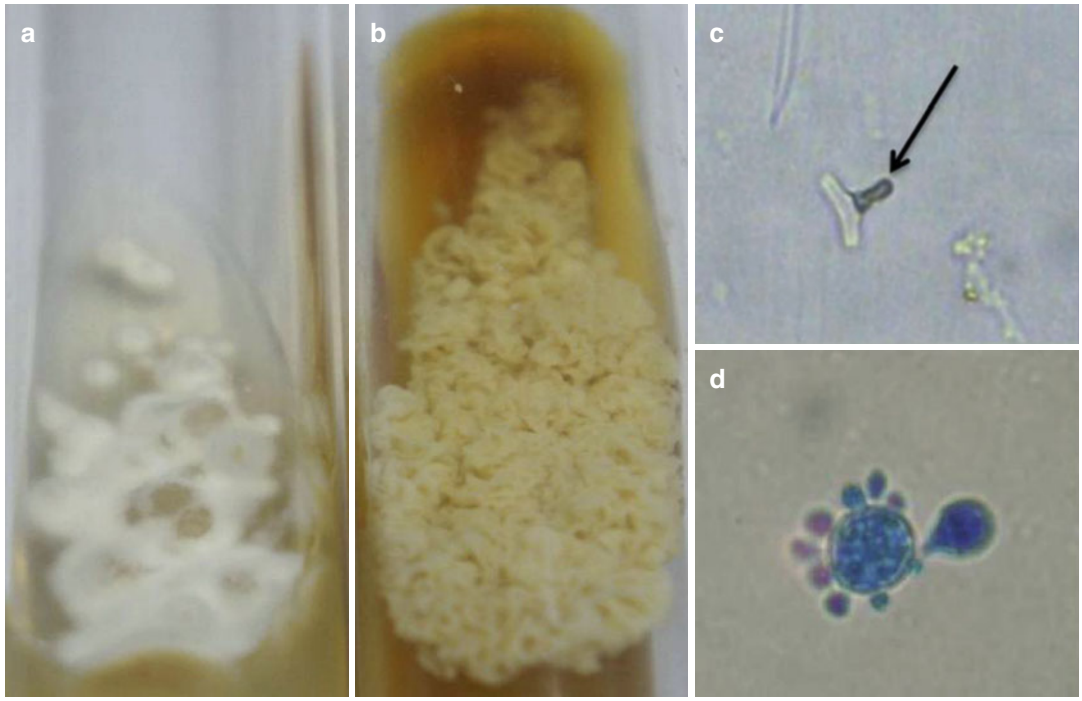

Fig. 14.1 *P. brasiliensis* culture at room temperature showing the mycelial form (**a**); culture at 37 °C showing the yeast form (**b**); hypha with emerging conidium (*black arrow*) (**c**); typical multi-budding yeast form. Lactophenol cotton blue staining (**d**)

conditions such as medium composition, incubation period, oxygen, and humidity [10]. There are no specific structures in the mycelial phase typical of *P. brasiliensis*, but the presence of yeast forms with multiple budding serves to diagnose the fungus. The colonies of the mycelial phase (environmental temperature – around 22 °C) are small, white, and irregular, and a brown pigment may cover by aerial mycelium of some isolates. In the yeast phase (37 °C), the colony is beige and cerebriform (Fig. 14.1).

Based on molecular phylogenetic studies *P. brasiliensis* is classified together with other dimorphic fungi (*Blastomyces dermatitidis* and *Histoplasma capsulatum*) in the following taxonomy: Kingdom *Fungi*, Phylum or Division *Ascomycota*, Class *Plectomycetes*, Order *Onygenales*, Family *Ajellomycetaceae*, Genus *Paracoccidioides*, and Species *brasiliensis*.

Using molecular systematics based on nucleotide sequencing of eight loci from 65 isolates, the existence of three phylogenetic species was identified: PS2 (Brazil and Venezuela), PS3 (Colombia), and S1 (Brazil, Argentina, Peru, Paraguay, and Venezuela) [11]. Phylogenetic analyses of 21 polymorphic loci in 21 *P. brasiliensis* isolates (14 of which were previously identified as belonging to either S1 or PS3) revealed an atypical isolate that is highly divergent from all others and clearly distant from the three described phylogenetic species [12]. Teixeira et al. [13] suggested that this isolate known as Pb01 as well as others referred to as "Pb01-like" could belong to a new phylogenetic species, named *Paracoccidioides lutzii*.

14.3 Transmission

Paracoccidioidomycosis is acquired through inhalation of spores released from the mycelium phase of *Paracoccidioides brasiliensis* probably present in the soil of endemic areas. The successful isolation of the fungus from these areas, however, has been a rare event, and there is difficulty in determining the precise fungal ecological niche (reviewed in Barrozo et al. [14]).

While *P. brasiliensis* has been seldom isolated from saprobiotic sources, it has been frequently

recovered from human clinical samples and from internal tissues of armadillos, dogs, two-toed sloth, and other wildlife fauna. More recently, Arantes et al. [15] associated nested PCR of rRNA coding sequence, comprising the ITS1-5.8S-ITS2 region with different techniques of culture. Although the pathogen could not be isolated by conventional culturing methods, the aerosol obtained from armadillo burrows and submitted to amplification of ITS sequences showed results with high homology indicative of the *Paracoccidioides* complex.

There are no reports of PCM outbreaks that can be related to environmental conditions, but a cluster of acute/subacute PCM cases has been identified in 1985 in the São Paulo State, Brazil [14], and tentatively associated with climate. The cluster comprised 10 cases, although 2.19 were expected ($p < 0.005$) in that area for that particular period. Authors observed that the soil water storage was atypically high in 1982/1983 due to the strong El Niño effects in the area and that the absolute air humidity in 1984 was much higher than normal. Such conditions might have favored, respectively, the fungal growth in the soil and conidia liberation in 1984, the probable year of patients' exposure to the fungus.

14.4 Diagnosis and Conventional Therapy

The definitive diagnosis of PCM can be made by direct examination of sputum, biopsy specimens, or crusts/pus from suppurated lymph nodes, which typically contain the yeast forms of *P. brasiliensis*. This is combined with culturing the fungi from any clinical specimen. Characteristically, the morphology of *P brasiliensis* fungal elements in its parasitical form shows large globose cells with narrow-necked multiple budding yeasts or mother cells with only two buddings (reviewed in Travassos et al. [16]). Routine methods for wet preparations use KOH treatment or calcofluor fluorescent stain. Histopathological preparations are usually stained with Grocott-Gomori stain or PAS for better identification of the fungal elements.

Culturing of *P. brasiliensis* from clinical samples is achieved on Sabouraud's agar or yeast extract agar containing chloramphenicol and cycloheximide. Recovery rates of the pathogen may be limited by the overgrowth of bacteria present on potentially contaminated organic fluids (e.g., sputum and skin lesions [16]).

Serological tests are important for the diagnosis of PCM including the double-immunodiffusion test, immunoenzymatic assays, and counterimmunoelectrophoresis. In clinical practice, detection of specific antibodies is used in the screening of patients suspected to be infected by *P. brasiliensis* as well as for monitoring the clinical response to therapy [16]. Patients infected with "Pb01-like" isolates (*P. lutzii*), however, may give false-negative serology, thus possibly requiring a pool of antigens for diagnosis [17].

Some authors have attempted to detect specific *P. brasiliensis* antigens in clinical specimens of patients with PCM. Detection of gp43 and gp70 specific antigens of *P. brasiliensis* was carried out using immunoenzymatic assays. Monitoring antigens of *P. brasiliensis* has a potential application in the diagnosis of PCM and may be useful as a tool for evaluating the clinical response to specific antifungal therapy [16].

Skin tests using both crude antigen preparations and purified antigen (gp43) from *P. brasiliensis* are used in epidemiological studies and have no diagnostic value.

PCR methods for DNA amplification have also been used to identify patients with PCM. *P. brasiliensis* DNA sequences of potential diagnostic use have been described by different authors, including the 5.8S rRNA gene and its ITS regions [18] as well as the gp43 gene [19]. San-Blas et al. [20] reported on specific primers designed on a 0.72-kb DNA fragment of *P. brasiliensis* that were useful for identification of this pathogen in sputum and cerebrospinal fluid of PCM patients. Due to the high costs, routine laboratories do not use molecular methods in the diagnosis of PCM.

Other laboratory exams and image detection such as X-ray, abdominal ultrasound, blood count, erythrocyte sedimentation rate, biochemical liver tests (transaminases, alkaline phosphatase), protein electrophoresis, and kidney metabolism (creatinine

and Na and K) are also needed for evaluation of patients. Those with involvement of the central nervous system, gastrointestinal system, adrenal dysfunction, respiratory failure, and lesions of bone or muscle require more complex exams [4].

Antifungal chemotherapy is required for PCM treatment, though even after treatment, there is no assurance of complete clearance of the fungus. Initial treatment lasts from 2 to 6 months and includes sulfonamides, amphotericin B, or azoles. Extended periods of treatment are often necessary, up to 2 or more years, with a significant frequency of relapsing disease [4, 21].

Currently, oral itraconazole is the drug of choice. In patients with severe forms intravenous amphotericin B or the association of sulfamethoxazole/trimethoprim is a more appropriate alternative. After the initial treatment of the disease, maintenance may involve sulfadimethoxine or sulphadoxine, for 2 years [4, 21, 22].

14.5 Vaccine

14.5.1 Rational for the Vaccine

Although PCM is generally controlled by chemotherapy, the efficacy of treatment is limited by the status of the host immune response. Immunocompromised individuals may develop the disseminated form of the disease with fatal cases. Fungal chemotherapy, therefore, as with other systemic fungal diseases, needs for a successful outcome to be complemented by an effective immune response to achieve long-term control of the infection. The long periods of treatment, relapses, fibrotic sequels, and severity of the disease are also a reflection of the patient's immunological defense mechanisms and inflammatory reactions.

A vaccine that could stimulate the immune system without undesirable effects of hyperinflammation should effectively complement chemotherapy, reducing the period of treatment and the occurrence of relapses. It could be a powerful agent in severe cases of PCM and in cases of poor response to chemotherapy. A prophylactic use of such vaccine might be considered in endemic areas with high incidence of PCM.

14.5.2 Antigen Characterization

The glycoprotein gp43, described in 1986 [23], is the major diagnostic antigen of *P. brasiliensis*. A high frequency of patients with PCM has antibodies against the gp43 and a decrease in the titers of anti-gp43 IgG, IgA, and IgM correlated with clinical improvement [24]. Different isoforms of the gp43 were recognized with pIs ranging from 5.8 to 8.5, but they were not equally reactive with patients' sera [25]. The B-cell epitopes eliciting high titer antibodies are peptidic in nature [26] and a monoclonal antibody (mAb 3E) to the gp43 reacts with peptide NHVRIPIGYWAV, a sequence shared with internal peptides of β-1,3-glucanases of *Aspergillus fumigatus* and *A. oryzae* [27]. In fact, the gp43 has 54–60 % homology and 50 % identity with exo-β-1,3-D-glucanases from several different fungal species without by itself expressing any enzymatic activity. The gp43 is found in intracellular vacuoles but then accumulates in the cell wall and is secreted in the supernatant where it represents almost 80 % of the exoantigenic protein. It can then be isolated by affinity chromatography using monoclonal antibody mAb 17c.

The gene of the gp43 has been cloned, sequenced, and expressed in *Escherichia coli* [28]; the recombinant protein has 416 amino acids with a leader sequence of 35 residues. The mature glycoprotein has a single *N*-glycosylation site with a high-mannose $Hex_{13}GlcNAc_2$ oligosaccharide [29]. A T cell epitope mediating delayed-type hypersensitivity reactions and sensitizing T-CD4$^+$ lymphocytes has been mapped to P10 ($gp43^{181–195}$), a 15-mer peptide with the sequence QTLIAIHTLAIRYAN [30], adjacent to the *N*-glycosylation site. Both the purified gp43 and the synthetic P10 peptide with amidated C-terminal exhibited protective effects in Balb/c mice intratracheally infected with yeast forms of a virulent strain of *P. brasiliensis* (Pb18) (Fig. 14.2). Although a polymorphism of the gp43 was shown, by examining the precursor genes from a number of isolates, the nucleotides encoding the P10 sequence were not mutated [31].

The gp43 can be processed for peptide presentation by dendritic cells or by B cells thus eliciting Th-1 or Th-2 immune responses and the

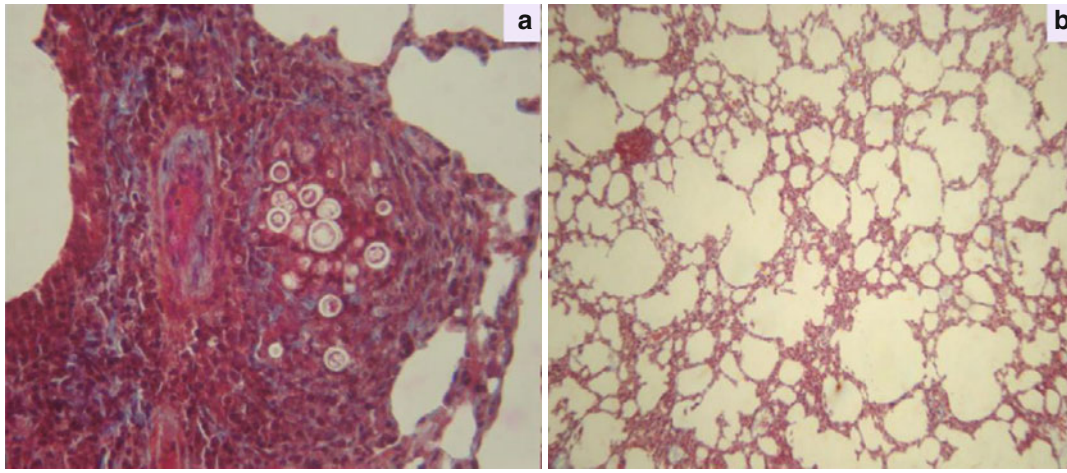

Fig. 14.2 Histopathology of murine lung sections from i.t. infected Balb/c mice immunized with P10 in CFA after 30 days infection. (**a**) Infected unimmunized control mice, 400×; (**b**) infected, immunized mice, 100×. Masson's trichrome staining. Blue staining shows type I collagen fibers

respective cytokines [32, 33]. A more favorable antifungal response is obtained with a predominant Th-1 response rich in IFN-γ and IL-12. It is also generally recognized that the inhibition of a Th-2 immunity and stimulation of Th-1 cytokines increases the efficacy of fungal chemotherapy [34]. We therefore concentrated our efforts on peptide P10 that elicited a Th-1 immune response and did not induce anti-gp43 antibodies. In the most aggressive forms of PCM, patients present high antibody titers and a severe depression of cell immunity. Although some mAbs to the gp43 are protective, some are not, and a predominant Th-2 immunity clearly does not protect against the infection.

14.5.3 Validation of the Peptide Vaccine

As said above, the P10 sequence is conserved in most isolates of *P. brasiliensis*. Moreover, MHC class II molecules from 3 mouse haplotypes present the synthetic peptide as inferred from its protective effects [30]. A direct study was then made with HLA-DR molecules. P10 and peptide ($gp43^{180–194}$) without C-terminal asparagine and with N-terminal lysine bound to the 9 prevalent HLA-DR molecules [35]. Using the TEPITOPE algorithm covering 25 Caucasian HLD-DR types, P10 and neighboring peptides in the gp43 were predicted to bind to 90 % of these molecules. The promiscuous nature of P10 in its presentation by human MHC-II molecules is thus a fundamental feature in the validation of this peptide as a vaccine.

A therapeutic vaccine with P10 should be used simultaneously with chemotherapy; therefore, the combination of antifungal drugs and P10 immunization was tested in different protocols. Balb/c mice infected intratracheally with 3×10^5 yeast forms of *P. brasiliensis* Pb18 were treated intraperitoneally with itraconazole, fluconazole, ketoconazole, or trimethoprim-sulfamethoxazole every 24 h or with amphotericin B every 48 h. Drug treatment started 48 h or 30 days after infection and was held for 30 days. One group of animals was immunized with P10 (20 μg) once a week for 4 weeks, the first emulsified with complete Freund's adjuvant and the 3 others with incomplete Freund's adjuvant. P10 immunizations were s.c. and i.p. (the last two). Although there was a significant protection measured by colony-forming units (CFUs) from lung homogenates, either in the drug-treated group or in the P10-immunized group, the combination of both chemotherapy and P10 immunization showed an additive effect [36]. Using the second protocol, after 60–120 days of infection the lung CFUs with the combined treatment were

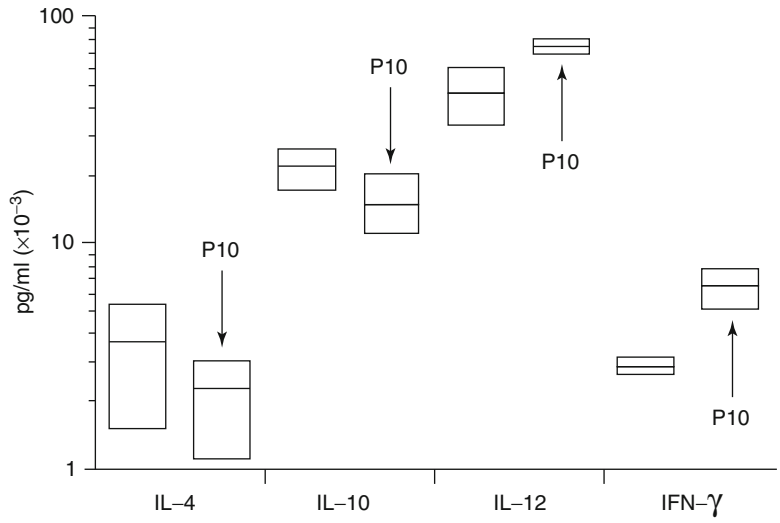

Fig. 14.3 Range of cytokine production in i.t. infected mice submitted to chemotherapy and with the same drugs and P10 vaccine. Internal lines are the average cytokine concentration considering all drugs tested

60–80 % fewer than those in untreated mice, and the lung cytokines showed a predominant Th-1 immune response (Fig. 14.3).

The immune protection by P10, which contains a T-CD4$^+$ epitope, promiscuously presented by MHC-II antigens, depends on IFN-γ production, and this was clearly shown by using mice homozygous for the null mutation of genes encoding IFN-γ, IFN-γ-R, or the IRF-1 transcription factor but not IFN-α-R or IFN-β-R. All knockouts that eliminated IFN-γ presence or function were extremely susceptible to *P. brasiliensis* intratracheal infection, with early mortality. Vaccination with P10 of these animals failed to induce immune protection. Infected mice deficient in IFN-γ do not form granulomas and had a heavy infiltrate of neutrophils along with the yeast forms of *P. brasiliensis* [37]. The increased susceptibility to this fungus of IFN-γ deficient mice has also been investigated by other groups [38, 39].

In face of the immunological requirement for P10 vaccination effectiveness, would this work in anergic animals? This issue was addressed by using Balb/c mice pre-treated with dexamethasone 21-phosphate in the drinking water. Within 30 days these animals became negative to delayed hypersensitivity tests, as previously determined in infected animals treated with 0.15 mg/kg of dexamethasone and sensitized with *P. brasiliensis* crude antigen. All anergic infected mice died after 70 days of infection. DTH-negative mice, paralleling PCM patients with severe disease and bad prognosis, were infected intratracheally with a virulent *P. brasiliensis* isolate.

With 15 days infection, mice were given trimethoprim-sulfamethoxazole or itraconazole with or without simultaneous immunization with P10. Also in this case, the P10 vaccine combined with chemotherapy significantly reduced the lung CFUs, largely preserved the alveolar structure and prevented fungal dissemination to the liver and spleen. P10 vaccination succeeded in inducing a Th-1 response with higher IL-2, IL-12, and IFN-γ and lower IL-4 and IL-10 than in the anergic, infected, but untreated animals [40]. Although significant, the partial fungal elimination was not higher because of the short time of the experimental design [41].

P10 vaccination had then to be tested without the use of CFA as an adjuvant, a regular procedure in early experiments. Different formulations allowed for human immunization had to be tested. In addition to other adjuvants discussed below, a multiple antigen peptide (MAP) construction with a branched lysine core was investigated (Fig. 14.4). Due to synthetic constraints only 13 of the P10 amino acids were allowed in the complex chains. This was named M10, to be compared with P10.

Control MAPs contained four chains of 5–10 aa P10-derived peptides. As little as 1 μg of M10 was needed to sensitize mice for lymph node cell proliferation stimulated by P10. The M10 vaccine

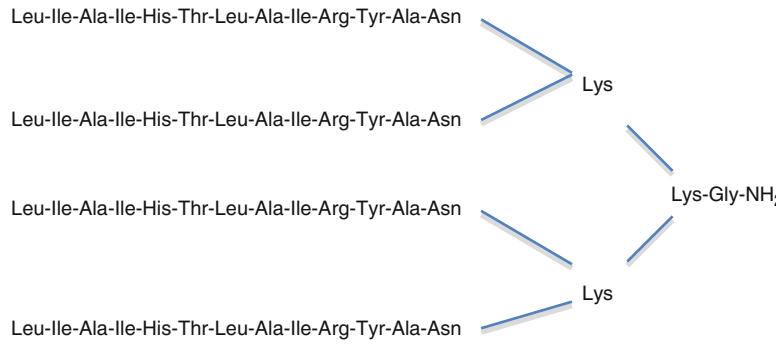

Fig. 14.4 MAP (M10) with truncated P10 tetramer linked to branched lysine core

with no adjuvant rendered significant protection in i.t. infected mice with fewer lung, spleen, and liver CFUs and also much fewer or no yeasts in the lung histopathological sections [42].

14.5.4 Animal Model

Most experiments on the immune protection by gp43 or by the P10 peptide were run in the medium susceptible to *P. brasiliensis* infection, Balb/c mice. Although the intraperitoneal and intravenous routes of infection have also been used in different laboratories, we have chosen as a standard method of infection the intratracheal route with properly anesthetized animals. P10 vaccination is subcutaneous. In a few cases the intranasal route was used when flagellin was the adjuvant. As shown [30], other mouse haplotypes were also protected, but Balb/c mice gave the most reproducible results with a consistent Th-1-mediated immune response. In the case of gene therapy (see below) a more stringent model was used with the highly susceptible to infection, B10.A mice. Macrophages from B10.A mice produce high and persistent NO levels as compared to resistant A/Sn animals [43]. These animals also synthesize high levels of IL-10 and did control fungal dissemination mainly with T-CD8$^+$ cells [44]. Nevertheless, they responded well to a long-term vaccination experiment with plasmids encoding P10 and IL-12 [45]. Other experiments of gene therapy were run in Balb/c mice, inoculated intramuscularly or intradermally [45, 46].

With the therapeutic DNA encoding P10 which is a promising candidate for a vaccine, 3 different protocols were used [45]: (a) for immunoprophylactic treatment, Balb/c mice received 4 weekly injections of the vaccine and were infected on week 7, and different groups sacrificed on week 11 or 15 (30 or 60 days after infection); (b) for short-term therapeutic treatment, Balb/c and B10.A mice were infected i.t and vaccinated on weeks 4, 5, 6, and 7 and sacrificed on week 8; (c) for long-term therapeutic treatment, B10.A mice were infected i.t. and vaccinated on weeks 4, 5, 6, and 7, then again on weeks 11, 15, and 19, and sacrificed on the 23rd week after infection. The fungus was virtually eliminated in the last long-term therapeutic protocol [45].

14.5.5 Gene Therapy

Immunization of Balb/c mice with the mammalian expression vector (VR-gp43) carrying the gene of gp43 induced a T cell-mediated immune response as well as antibodies, under the control of the CMV promoter [46]. The plasmid DNA was able to mount an effective, long-lasting cellular immune response, mediated by IFN-γ. The humoral and cellular specific responses were effective for 6 months after the last DNA inoculation. The immunization conferred protection against i.t. infection with virulent *P. brasiliensis* with significant decrease in the lung CFUs and reduced or no dissemination to the spleen and liver of immunized mice [46].

The effectiveness of plasmid vaccination with P10 and IL-12 inserts administered prior to or after challenge with virulent yeasts was tested in the murine pulmonary PCM disease model [45].

Table 14.1 Lung CFUs in B10.A susceptible mice infected with *P. brasiliensis* and treated with plasmid-P10 therapeutic vaccine

	Therapeutic protocol	
Gene therapy	Short term (30 days)	Long term (6 months)
Untreated control	14,630 ± 2,840	22,830 ± 2,232
pCDNA3	15,867 ± 204	18,214 ± 2,801
pCDNA3 + pP10	2,058 ± 381**	216 ± 25**
pCDNA3 + pIL-12	3,333 ± 111**	1,261 ± 71**
pP10 + pIL-12	372 ± 122**	18 ± 17**

Therapeutic protocols as previously described [45]. pCDNA3, empty vector; pP10 or pIL-12, vector with P10 or IL-12 insert. **$p < 0.001$, significant difference compared to empty vector

P10 nucleotide sequence was obtained with the sense PCR primer derived from the gp43 [28]. Plasmid pcDNA3 was used for cloning after adding a HINDIII site and an EcoRI site to sense and antisense primers, respectively. PCR reactions were carried out with cDNA and equal amounts of each primer. PCR products were purified, digested with the appropriate restriction enzyme, and cloned into pcDNA3. The resulting plasmid was called pP10. Plasmid pORF-mIL-12 was acquired commercially and used after confirmation of the insert [45]. For immunization, plasmid DNA was prepared in transformed *Escherichia coli* XL1Blue and DH5α cells and purified. A total of 100 μg of plasmid was inoculated in 100 μl by the intramuscular route (50 μg of pP10 + 50 μg of empty plasmid or 50 μg of pP10 + pIL-12).

In the immunoprophylactic treatment the association of p10 and pIL-12 achieved complete elimination of the fungus in the lungs measured as tissue CFUs [45]. A significant reduction of CFUs was observed with the plasmids alone without the inserts suggesting that unmethylated CpG motifs could have activated Toll-like receptor 9 of dendritic cells. The protective effect was then synergistic but clearly involving the predominant activity of pP10. In the therapeutic protocol in which both Balb/c and B10.A mice were infected and vaccinated 1 month afterwards, the empty plasmids showed no protective effect, and both pP10 and pIL-12 were active with maximal protection being achieved with the combination of both plasmids (Table 14.1).

Lungs of mice vaccinated with pP10 with or without pIL-12 showed reduced inflammation and lower or undetectable yeast cells. Finally, in a long-term therapeutic protocol, gene immunization started 30 days after infection and mice were sacrificed 6 months after infection. Vaccination schedule is indicated in the Sect. 14.5.4. Whereas the empty plasmid was not significantly protective in these conditions, vaccination with pP10 alone reduced the lung CFUs more than 100-fold, and the combination of pP10 and pIL-12 virtually eliminated all fungal elements. Comparing with epithelioid granulomas with giant cells and numerous yeast cells observed in the lungs of mice treated with the empty plasmid, pP10 vaccination rendered fewer granulomas and yeast cells with large areas of normal lung architecture. The combination pP10 + pIL-12 virtually recovered the lung parenchyma with barely detectable yeast forms [45].

These most encouraging results towards developing a vaccine against PCM were not the only attempts at gene therapy in this fungal disease. A cDNA encoding rPb27 [47] and *Mycobacterium leprae* DNAhsp65 plasmid [48] have also been investigated in infected Balb/c mice.

14.5.6 Feasibility and Preclinical Development

Both the P10 peptide and the plasmid encoding P10 or IL-12 are readily obtainable vaccines that can be prepared in high degree of purity and reproducibility. The costs of scaling up production and presentation in GMP conditions for human use are usually high and depend on direct investment, which may not be economically attractive considering the number and distribution of patients and the location of endemic regions. A pilot production of a successful antifungal peptide vaccine or the equivalent gene therapy may, however, be of governmental interest in endemic areas and stimulate similar procedures concerning other more universally distributed systemic mycoses.

So far, a complete registered preclinical study of the peptide vaccine, including primate toxicity, has not been carried out. The efficacy of the peptide vaccine has been repeatedly shown using the mouse

model of i.t. infection, mainly focusing on the issues of adjuvancy and delivery of the peptide. To replace complete and incomplete Freund's adjuvant for P10 immunization, different adjuvants were tested aiming at human immunization. In the Balb/c model previously infected with *P. brasiliensis* Pb18 strain, P10 was administered associated with different adjuvants 52 days after infection [49].

The association of P10 peptide and the cationic lipid dioctadecyldimethylammonium bromide (DODAB) provided the lowest numbers of viable yeast cells in the lungs along with granuloma clearance. Production of IFN-γ and TNF-α was high with low IL-4 and IL-10. Flagellin (FliC from Salmonella enterica [50]) was also effective but less than DODAB. Aluminum hydroxide was the least effective, as the association with P10 failed to clear large granulomas with numerous yeast cells and collagen fibers, similar to the untreated lungs, and high IL-4 production.

P. brasiliensis infection owing to persistent antigenic stimulation and active immune response is characterized by a granulomatous inflammation which increases the lung connective tissue rich in collagen types I and III leading to functional changes and fibrosis, a well-known sequel of PCM. It is noteworthy that the association of DODAB and P10 as well as gene therapy with pP10 resulted in a significant reduction of pulmonary fibrosis in infected mice.

14.6 Strengths and Weaknesses

The chief concern about a formulation consisting of a short-size natural amidated peptide is that of poor pharmacokinetics mainly due to plasma proteolysis and renal filtration. Nevertheless, we have shown that underivatized peptides, except for C-terminal amidation, exert remarkable antifungal and antitumor [51] protective activities in vivo administered subcutaneously or intraperitoneally in mice. Presumably, the bioactive peptides are rapidly taken up by dendritic cells, before spreading in the blood circulation, thus stimulating a cascade of events leading to immune protection. The use of adjuvants certainly enhanced the activity of the peptide probably through the activation of DCs. The efficiency of primed DCs in the immune protection against PCM has been investigated [52]. Intratracheally infected mice were treated on days 31 and 38 after infection with P10-primed DCs administered intravenously or subcutaneously. Mice were sacrificed 7 days after the second injection of DCs. A remarkable clearance of fungal elements was observed. Primed DCs were also highly effective in a prophylactic protocol.

Also, with a view on the pharmacokinetics of P10, another approach could be that of encapsulation of the peptide in a nanoparticle. This has actually been pursued using poly (lactic acid-glycolic acid) (PLGA) nanoparticles [53]. P10 entrapped within PLGA was more effective than free P10 emulsified in Freund's adjuvant, as tested combined with chemotherapy. Incorporating P10 into PLGA reduced the amount of peptide (1 μg/50 μl) necessary to significantly decrease the fungal load in infected animals and to avoid disease relapse. Interaction of PLGA with DCs presumably improved the immunomodulatory effects of P10.

Gene therapy in the form of expression plasmids is another way of delivering the peptide to the appropriate antigen-presenting cells while providing also CpG motifs for DC activation without another adjuvant. A combination of plasmids expressing the main P10 antigen and IL-12 achieved the right stimulus for a protective Th-1 immune response without causing hyperinflammation and fibrosis as a sequel of treatment. Although the present experiments are at the preclinical stage, they are promising as full protection was reached, with no simultaneous chemotherapy and no evident toxicity.

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Oral Vaccination of Honeybees Against Varroa Destructor

15

Sebastian Giese and Matthias Giese

Contents

Abstract

First reported in the USA, a mysterious so-called colony collapse disorder (CCD) decimated the bee colonies there between 50 and 90%. The huge global loss of honey bees as pollinators has a dramatic impact of agricultural pollination. About 130 crops, nuts, fruits, vegetables are pollinated by *A.mellifera*, with an overall value of more than $ 15 billion in the USA, and more than € 14 billion for the EU in 2005. In all CCD cases an overload of bloodsucking Varroa mites is detectable and Varroa is currently considered the major threat for apiculture. Honeybees possess a humoral and cellular immune system, e.g., multiple antimicrobial peptides and immune reactive enzymes. Varroa mites overcome the bee's immune response by massive suppressing some of these immune genes. We developed a DNA vaccine for oral application which is able to reconstitute the full function of the bee's immune system. This vaccine is absolutely biological safe, well-tolerated, show no side effects, and does not form any burden for the environment.

You read this and perhaps you may think we tell jokes. Have these guys nothing better to do? So many serious unmet needs in human health for vaccine developments and now a vaccine for bees? Intensive research and product development for a simple insect? And how shall this vaccine work if the oral delivery would be successful? And by the

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way, is this really a vaccine? We hope, we make you surprised just as we were surprised when we got the first magnificent results. Take time for this chapter, and enjoy.

15.1 Introduction

There are many environmental stressors and diseases which influence and seriously threat the life of European honeybees, *Apis mellifera*. The European honeybee is professionally managed worldwide for honey production and pollination. The bee was imported to the USA 400 years ago with the first European settlers and called "white man's fly" by the Native Americans, the Indians.

First reported in the USA, a mysterious so-called colony collapse disorder (CCD) decimated the bee colonies there between 50 and 90 %, first observed during the winters of 1995–1996 and then 2000–2001 and without interruptions up to now. A similar situation is also given in Europe. About 20,000–60,000 bees live in a colony.

An acceptable normal loss of winter bees ranges between 5 and 10 %. CCD is characterized by the abrupt disappearance of all adult worker honeybees in a hive while immature bees remain with a dramatic consequence for the next population. Even though the name colony collapse disorder is new, the phenomenon of extensive losses of honeybee colonies is very old.

The first description originated from 1950. In the early nineteenth century the colony losses were known in England as "Isle of Wight disease," and the Americans called this phenomenon "disappearing disease" in the 1960s, whereas these colony losses in France in the late 1990 were called "mysterious bee losses." Where have all the bees gone?

15.1.1 Economic Value

The huge loss of honeybees as pollinators has a dramatic impact of agricultural pollination. About 130 crops, nuts, fruits, and vegetables are pollinated by *A. mellifera*, with an overall value of more than $15 billion in the USA and more than €14 billion for the EU in 2005. A bee colony produces some 1 kg or 2,205 lb honey per day. In return, these bees have to pollinate 10–15 million flowers.

One should keep in mind that besides European honeybees, wild insects, among them 30,000 species of wild bees, have also a very great impact on pollination and seem to be more efficient in pollination as managed honeybees [1]. The industrial farming threatens also the natural biotope of wild insect pollinators.

15.1.2 Ecologic Value

The total global economic value of honeybee pollination was calculated in 2005 to more than €150 billion or $202 billion. The Food and Agriculture Organization of the United Nations (FAO) estimates that there are 65 million managed honeybee colonies worldwide. Besides this professional agriculture, honeybees are irreplaceable for the biodiversity. This organism appeared during the evolution of the first flower plants and existed since 100 million years ago as described in Table 2.5. After swine and cattle, bees are in Europe and North America the third important farm animal and since 2007 formally listed as farm animal in Switzerland. Therefore, CCD is not only an economical but also an essential global ecological problem which urgently must be solved in the future. "The bee is more than honey."

15.2 What Is Causing CCD?

The colony collapse disorder of the last years seems to differ from past outbreaks: The worker bees disappear instead of dying in place, leaving behind the queen and young bees. High levels of bacteria, viruses, and fungi are measured in the gut of the remaining bees. Collapses can occur within 2 days.

15.2.1 A Complex Problem

Different theories are discussed on what is causing CCD, such as pesticide contamination, hotly debated to interfere with the nerve system

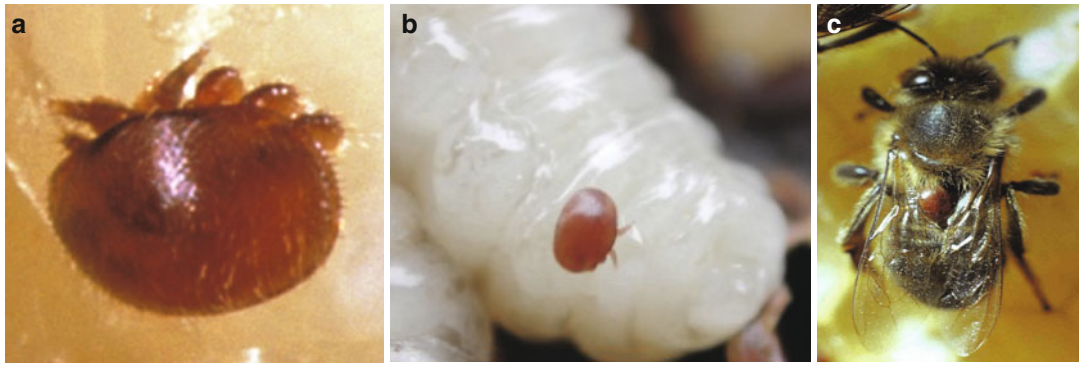

Fig. 15.1 (**a**) Varroa is reddish-brown in color, is flat-shaped, and is 1–1.8 mm long and 1.5–2 mm wide, with eight legs. (**b**) V. destructor mite on a bee larva. (**c**) V. destructor infests worker bees, drones. Varroa mites are important vectors for viruses and spread these viruses throughout the hive (Pictures kindly provided by Dr J. Mueller, Magdeburg, Germany)

affecting foraging behavior of bees, leading them to abandon their hives; fungal diseases, such as *Nosema* spp., known for big bee losses in Spain; monocultures or gene-manipulated crops; electro smoke (radio waves) caused by cell phones, destroying the bee's compass; and the rigors of travelling in trucks from crop to crop in the USA. Down from February, professional US beekeepers travel with their colonies through the country until December. Thereby the bees must relocate up to 15 times. In Europe the bee colonies begin the winter sleep around September. Aside from the climate change, the temperature sensitivity is discussed to have an impact on crop pollination. CCD is likely caused by a combination of factors [2–6].

15.2.2 Varroa Destructor

But in all CCD cases, an overload of bloodsucking Varroa mites is detectable and Varroa is currently considered the major threat for apiculture. The infection and disease is called Varoosis. *Varroa destructor* is an ectoparasite, has a reddish-brown color, is flat shaped, and is 1–1.8 mm long and 1.5–2 mm wide, with eight legs. *V. destructor* infest worker bees, drones, and its brood. The mite develops inside the brood cells. Varroa is a real colossus compared to the size of bees as can be seen in Fig. 15.1. Varroa mites belong to the scientific class of Arachnida, subclass Acari. There are 50,000 species described alone from mites. Some mites prefer carbohydrates as food such as meal or crops. The house dust mites feed on flakes of shed human skin. Varroa mites prefer fresh "blood," the hemolymph of bees, and can feed 0,1 mg or 0,0000002205 lb within 2 h.

Varroa is transported into the hives via piggyback by worker bees. The female mite enters broad cells, preferentially drone cells. Once the cell is capped, Varroa lays eggs on the larvae. The development from egg to insect takes 7 days. Bee larvae and mites hatch in about the same time and the newborn Varroa mites spread to other bees [7–9]. The lifetime of summer mites is 3–6 weeks, whereas fall mites can live for several months. Varroa can only reproduce in honeybees and thus are considered harmless to other insects. Varroa is more than a disease. Varoosis is a global pest having devastating effects on bees.

15.2.3 Cosmopolitan

The first traces of Varroa were found in Southeast Asia, in Java, in 1904. Varroa was a parasite of the Asian honeybee, *Apis cerana*. The Asian honeybee is partially resistant against Varroa. About 45 years later the mite spread over to the east of the former Soviet Union (SU). And probably, soldiers of the SU, hobby beekeepers based in Asian parts of SU were the door opener for the global distribution of Varroa. When they were ordered to a new garrison in the former German Democratic Republic (GDR) or other western

areas of SU, they relocated with their infested bee colonies and automatically distributed Varroa in the Western world. It's remarkable that Varroa was only found in the GDR in areas with SU army. A first Russian report on Varroa was published in 1971 [10]. Already some years later more and more emerging articles on Varroa infestation were released [11–15]. Over the next two decades only, Varroa has spread to every continent except Australia and central Africa. African honeybees are fully resistant to Varroa but not suitable for managing because of their aggressive behavior, also known as killer bees. Today, Varroa mite is a convinced cosmopolitan.

15.2.4 Varroa as Vector

Varroa may be not considered as an isolated agent for the disease. The mortality of adult bees and its brood must be considered in the context with secondary viral infections. At least 18 various viruses are able to infect honeybees, mostly ssRNA viruses. Eight viruses are known to be associated with Varroa mites: acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood bee virus (SBV), cloudy wing virus (CWV), and slow bee paralysis virus (SPV) [16–19]. The active transmission of viruses by Varroa is part of the horizontal transmission route, but note that also a vertical transmission from the queen to her offspring is possible. Viruses found in queens were also present in their eggs. But the vertical transmission could be a late consequence of Varroa infestation of drones. Infected males, with viruses in sperma, transmit viruses to females during mating. Therefore a control of Varroa mites results in an indirect control of viruses.

Several key factors can be identified summarizing some important health problems of honeybees causing CCD: chronic contamination with pesticides, especially the misuse of neonicotinoids [20, 21]; the overload with parasites and fungi (*Nosema* spp.); and the vectored transmission of lots of various viruses – too much for the honeybee.

15.2.5 Varroa Control

A number of natural and synthetic chemicals are commercially available for the control of Varroa infestations. The first compounds were bromopropylate, fluvalinate, or other pyrethroid insecticides [15]. And to make a long story short, Varroa mites became resistant not only against one product of a given chemical class; the resistance was against the entire class with several related synthetic products. Also, the use of natural products, such as formic acid, mineral oil, or thymol, is only partially and temporally effective and shows adverse effects [22]. There is no successful chemical treatment. Mites will quickly develop resistance to all chemicals.

15.2.6 Bees and Crop Pesticides

But the biggest problem are serious concerns over the contamination of honey by insecticides and pesticides resulting in human health hazards [23] and health problems for the bees themselves [24–26]. Pesticide contamination of bees can result in an increase of *Nosema* spp. Also, the bees' susceptibility to viral infections seem to increase after pesticide treatment [27]. Neonicotinoid systemic pesticides, among the most commonly used crop pesticides in the world, cause high mortality of honeybees due to homing failure, as recently published [28, 29]. The intoxication disrupts the bee's navigation system. Neonicotinoid pesticides also affect bumble bees by reducing the growth rate and dramatic reduction in production of new queens compared to untreated colonies [30]. Are these bee-friendly pesticides which in low doses may not directly kill the bees but cause serious behavioral difficulties?

15.3 The Immune System of Insects

As outlined in Chap. 2, the front line of defense in animals and in humans is primarily based on the innate immune response which activates and

triggers the acquired immunity. The evolutionary origin of TLRs of vertebrates is based on Toll receptors in insects, first discovered and analyzed in Drosophila [31, 32]. For this the Nobel Prize in Physiology or Medicine was awarded to J.A. Hoffmann in 2011.

The basic difference between insect and vertebrate immunity is the missing highly specific antigen response of the acquired immune system in insects. Nevertheless, in the 400 million years of evolution, insects developed a powerful defense strategy against bacteria, fungi, viruses, and parasites. Only protected by this "primitive" immune system, insects were so successful that they colonized all terrestrial ecosystems.

The innate immunity of insects shows many similarities to the vertebrate, to the human innate immunity, which is multifaceted and involves both humoral and cellular components [33]. Most insights of insect immunity are provided by Drosophila melanogaster research. The key mechanism is also observed in honeybees.

15.3.1 Humoral Response

The humoral and systemic response to bacterial and fungal infections is controlled by antimicrobial peptides (AMP). Fat body cells secrete into the hemolymph (blood of insects), upon stimulation, the following: (1) defensin (active against Gram-positive bacteria); (2) drosocin, diptericin, attacin, and cecropin (all active against Gram-negative bacteria) – adipaecins of bees are similar to drosocins of Drosophila; and (3) metchnikowin and drosomycin (active against fungi) [34]. Obviously, the humoral response can discriminate between different classes of microorganisms [35]. The expression of AMP genes is regulated by two distinct pathways, the Toll pathway which controls response genes to fungi and Gram-positive bacteria and the Imd pathway which controls AMP genes to Gram-negative bacteria [36]. There are circulating receptors sensing a danger signal and activating the Toll pathway whereas membrane-bound receptors activate the Imd pathway. Both pathways lead to the translocation of NF-kB-like transcription factors and the production of AMPs. NF-kB response elements can be detected in the promoter region of the diptericin gene.

15.3.2 Cellular Response

The cellular immune response is mediated by specialized blood cells, the hemocytes, plasmatocytes, crystal cells, and lamellocytes [37]. Plasmatocytes represent with 95 % the majority of hemocytes. These cells express phagocytic receptors and patrol through the body, clear microorganism and cell debris, and signal infections to the fat body cells. The fat body cells of insects are similar to the liver of vertebrates because they store nutrients and synthesize proteins, also AMP, lipids, and carbohydrates that circulate throughout the body. Crystal cells represent only 5 % of hemocytes. They produce enzymes and are involved in killing of parasites or microorganisms by melanization of these invaders. The third hemocyte type is lamellocytes, larger than the other blood cells, and are only induced and measurable during parazitations. They kill the invaders by encapsulation and enzymatic degradation [38].

15.3.3 Bee Immunity

The bee genome was completely sequenced in 2006 [39]. Compared to Drosophila, honeybees possess roughly only one-third of immune genes [40]. The reduction of the immune system remains still a matter of speculations. The bees are considered as "one" social organism which has developed a specific behavior to decrease the risk of infections to parasites and diseases. Grooming, hygienic behavior, aggression towards infected bees, and also nutrition play a central role in the defense against invaders and infections. How big the impact of nutrition on a healthy immune system is already discussed in this book on Chap. 2. This evolutionary ancient social immunity has clear limitations as can be seen by infestations with Varroa mites, and on an individual level, the low repertoire on immune

genes is additionally influenced by insecticides and pesticides and of course by pathogens.

A classical bee scientist is generally a conservative "colony thinker." He talks of the organism "bee" and means a big population of many, many thousands of individuals. His strategy to manage health problems is to consider only colonies. But from a perspective of a mite, Varroa doesn't give a damn of it. Varroa is hungry and looks for the next meal. And probably Varroa does not know anything from the colony, ignores the social immunity and the swarm intelligence. The mite attacks the individual bee and step by step, larvae by larvae, Varroa realizes that it has entered a paradise – a large colony.

15.3.4 Lame Duck

Some years ago, we were invited to contribute a proposal to the control of Varroa mites in Germany. It was a public call for innovation in agriculture, farming, and bee health. Until this call, most public bee institutes here preferred a classical breeding research aimed to create the Varroa resistant bee queen. And they do this research with millions of Euro since more than 10 unsuccessful years, a lame duck research. These classical "colony thinkers" will not accept that the repertoire of immune genes in bees is reduced to one-third in comparison to non-colony insects, and absolutely limited in numbers and functions.

We are no colony thinkers, we study the host-parasite interactions and look at the existing genes and their regulations, and we look for possibilities how to interfere. We do this on an individual level, like Varroa. The mite targets the individual bee and not the colony. Only when the individual bee is protected the spreading of Varroa to the colony can be prevented.

15.3.5 Disneyland

Our amazing project was evaluated by hand-picked and "special" reviewers, special because these reviewers were also applying in the same public call and consequently competitors for the same budget. And these reviewers were allowed to copy and paste our scientific program for their own use. And the end of this curiosity? That's a moot point to note that such innovations are not welcomed here – in the *land of ideas*.

15.4 The Bee Vaccine

A vaccine might be characterized as safe, no risk of reversion to pathogenicity, stable at field conditions, and effective. The production should be simple, standardized according to GMP, and inexpensive. Last but not least, in Europe this bee vaccine must fulfill the requirements of the EMA like all other animal vaccines.

We developed a DNA vaccine. Gene vaccines entail the direct, in situ inoculation of an expression vector that encode the sequence for an antigen and/or an immunoenhancer [41]. Figure 15.2 gives a schematic diagram of an expression plasmid used for vaccination.

15.4.1 Experimental Procedures

An expression plasmid was constructed with a CMV promoter. Surprisingly, no bee or other insect-specific promoter was essential to drive the expression of the protein. The enhanced green fluorescent protein (EGFP) was chosen as reporter gene and inserted into the multiple cloning sites, together with a SV40 enhancer element. The plasmid construct was produced in *E. coli* and highly purified by standard techniques.

European honeybees (*Apis mellifera*) were obtained from local beekeepers and cultivated under lab conditions. Varroa mites were collected from infested bees.

The oral vaccination of the EGFP plasmid was operated by feeding the bees with a mixed solution of sugar and plasmid DNA (vaccine sugar). Standard sugar solutions made by the beekeeper are the normal food for winter bees.

15.4.2 Results

Over 10 days after onset of feeding, we measured the expression of EGFP by immunofluorescence

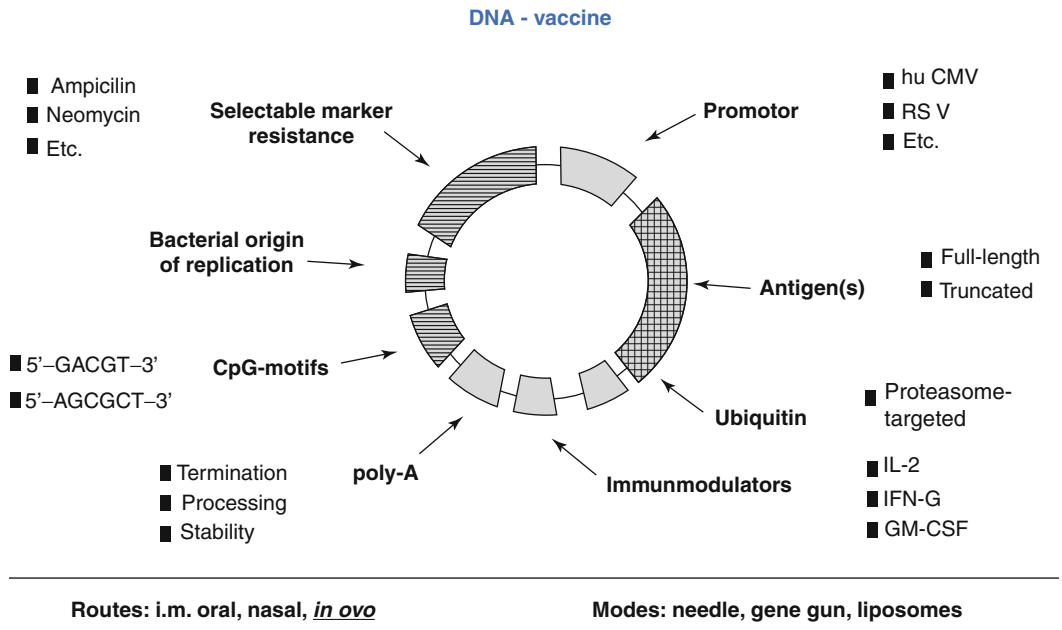

Fig. 15.2 Model of an expression plasmid used for DNA vaccination. Individual elements comprising functional expression cassettes. The encoded antigen, as full-length or truncated cDNA, is under control of a strong promoter/enhancer and polyadenylation sequences. Co-expression of cytokines will specifically enhance the immune response. Unspecific activation of the immune system can be initiated by CpGs. Vaccines focusing on a strong cellular response can be enhanced by co-expression of ubiquitin targeting the proteasome pathway. Various application routes and modes of administrations are possible (Giese [41])

and Western blot analysis with EGFP antibodies. Between days 3 and 10, a clear EGFP signal was detected in the thorax and especially in the Malpighian tubules. Control bees fed with DNA lacking the reporter did not show any signal. In parallel, control experiments with transformed *E. coli* were done to study the possibility of EGFP expression in gut bacteria instead of bee cells. No EGFP signal was detected in transformed bacteria.

Most surprisingly, we found the EGFP signal after 5 days in Varroa mites sucking hemolymph of bees which were fed by the vaccine sugar solution and no signals in control mites of infested control bees. Feeding of plasmid DNA results in expression of a reporter gene in different bee tissues over a period of several days, and finally Varroa absorbs this protein via bloodsucking. The bee blood is not carried by arteries and veins but flows loosely around the body. No EGFP signals were detected neither in the honey, in the stomach, nor in the feces. Figure 15.3 illustrates the EGFP passage through the bee body and towards the Varroa mite.

15.5 Perspective

The concept of our recombinant DNA vaccine is based on the blood sucking of Varroa and the transfer of macromolecular active biologicals via blood into the mite. This concept was first successfully demonstrated in cattle infested with ticks, *Boophilus* spp. [42]. See also Chap. 17 in this book.

Honeybees possess a humoral and cellular immune system, e.g., multiple antimicrobial peptides and immune reactive enzymes. Varroa mites overcome the bee's immune response by massively suppressing some of these immune genes [43]. We are developing a product which is able to reconstitute the full function of the bee's immune system. A lot of work remains to be done. So we had to learn that besides all serious

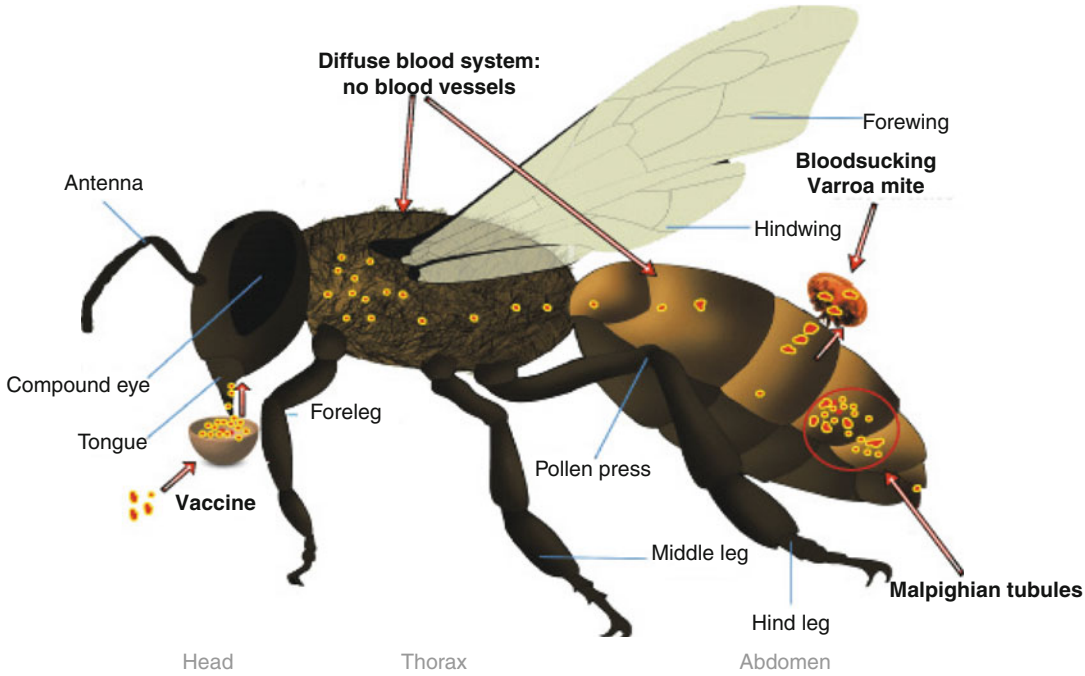

Fig. 15.3 Oral vaccination. Plasmid DNA encoding EGFP as place holder for a vaccine antigen is mixed to a standard sugar solution dissolved in water. Such sugar solutions are the nutrition for winter bees and are normally handmade by the bee keeper. The experimental DNA concentration was 500 µg DNA/ml sugar solution. DNA feeding was for 24 h. No booster feeding

scientific bottlenecks, resistance is given by a militant anthroposophy: Bees are "higher beings" with a purity of soul and are not allowed to be treated with gene technology – but with chemical insecticides and pesticides.

The first veterinarian DNA vaccines were developed for horses in 2001 in the USA and 2002 in Germany by our group [44, 45]. The first licensed DNA vaccine worldwide was a DNA fish vaccine for salmons against infectious hematopoietic necrosis (IHN) virus, a common viral pathogen of both wild and farmed salmonids, registered in 2005 in Canada by Veterinary Biologics Section (VBS), Animal Health and Production Division, and Canadian Food Inspection Agency (CFIA). A DNA vaccine for dogs against canine melanoma was recently licensed in the USA [46]. All these vaccines are absolutely biologically safe and well tolerated, show no side effects, and are effective. DNA vaccines for human health are in clinical trials.

Conclusion

How fascinating biology is! We started with the simple idea that the biochemistry in eukaryotic cells remains the same, irrespective the organism. A difference is given in the configuration of the immune system. That means, an insect can successfully fight against parasites and infections but with different weapons – no T cells, no B cells, and consequently no antibodies and no memory. We are able to stimulate targeted immune genes of bees and to measure an insect's typical immune response. A standard plasmid DNA vaccine, first developed for horses, bridges the evolution from fish to insects to mammals. No other vaccine type is able to do this job. How fascinating biology is!

Nothing in biology makes sense, except in the light of evolution.
(Theodosius Dobzhansky, 1900–1975, geneticist and a central figure in the field of synthetic theory of evolution)

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Lyme Disease: Reservoir-Targeted Vaccines

16

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Contents

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Abstract

In many parts of the world, microbial diseases have been controlled by a combination of improved hygiene practices, surveillance, diagnosis, treatments, effective vaccines, as well as greater public education and awareness of risk factors. Control strategies are especially challenging for diseases caused by pathogens that persist in a mammalian wildlife reservoir and use vectors such as insects to cycle through that species. In this group, the most relevant illnesses that pose a direct human health risk are rabies, sylvatic plague, and Lyme disease [1].

Reservoir-targeted vaccines have been developed as vaccination strategies that target the host reservoir or the transmitting vector both for rabies and for Lyme disease. An example of a successful application is the oral vaccine (Raboral™) currently used by local governments in the United States to create barriers between infected wildlife and highly populated areas to prevent transmission of rabies.

In this chapter I will discuss the development of an oral reservoir-targeted vaccine to curb transmission of *Borrelia burgdorferi* within wildlife and its projected impact on reduction of the incidence of Lyme disease in humans.

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16.1 Lyme Disease and the Pathogen

Lyme borreliosis or Lyme disease was clinically characterized in the mid-1970s after an epidemic of arthritis affecting multiple joints in an asymmetrical pattern was observed in children around the town of Lyme, Connecticut, USA [2]. The disease is caused by a group of related spirochetes that are transmitted to natural and secondary reservoir hosts by specific *Ixodes* spp. ticks [3].

Lyme spirochetes are widely distributed throughout the temperate zones of the Northern Hemisphere, and their range continues to expand as lands that were previously denuded for agriculture become reforested, creating new habitats for deer, ticks, and susceptible vertebrate reservoir hosts [4] (Fig. 16.1).

Lyme borreliosis accounts for >90 % of all vector-borne diseases with nearly 30,000 confirmed cases reported each year in the United States (http://www.cdc.gov/lyme/). In Europe, as many as 60,000 cases occur each year within the range of *I. ricinus*, its primary vector in the region [5].

Borrelia spp. fall into two major phyletic groups, one containing the causative agent of Lyme disease and the other containing the spirochetes responsible for relapsing fever [4]. Phylogenetic analysis has led to the division of Lyme disease spirochetes into numerous species referred to *B. burgdorferi* sensu lato (s.l.). Among the more than 20 named and unnamed species in the *B. burgdorferi* s.l. complex, three genospecies predominate as human pathogens: *B. burgdorferi* sensu stricto in the United States and Western Europe and *B. garinii* and *B. afzelii* in Eurasia [4].

Spirochetes are deposited into the bite wound along with the tick saliva during tick feeding [6]. Infection becomes increasingly likely after the tick has been attached for 48 h [7]. Because the *B. burgdorferi* genome does not encode any known toxins nor the machinery that would be required to secrete them [8], tissue damage and disease are mediated by the inflammatory response that follows infection in the mammalian host [9]. *Erythema migrans* is the most common clinical manifestation of Borrelia infection

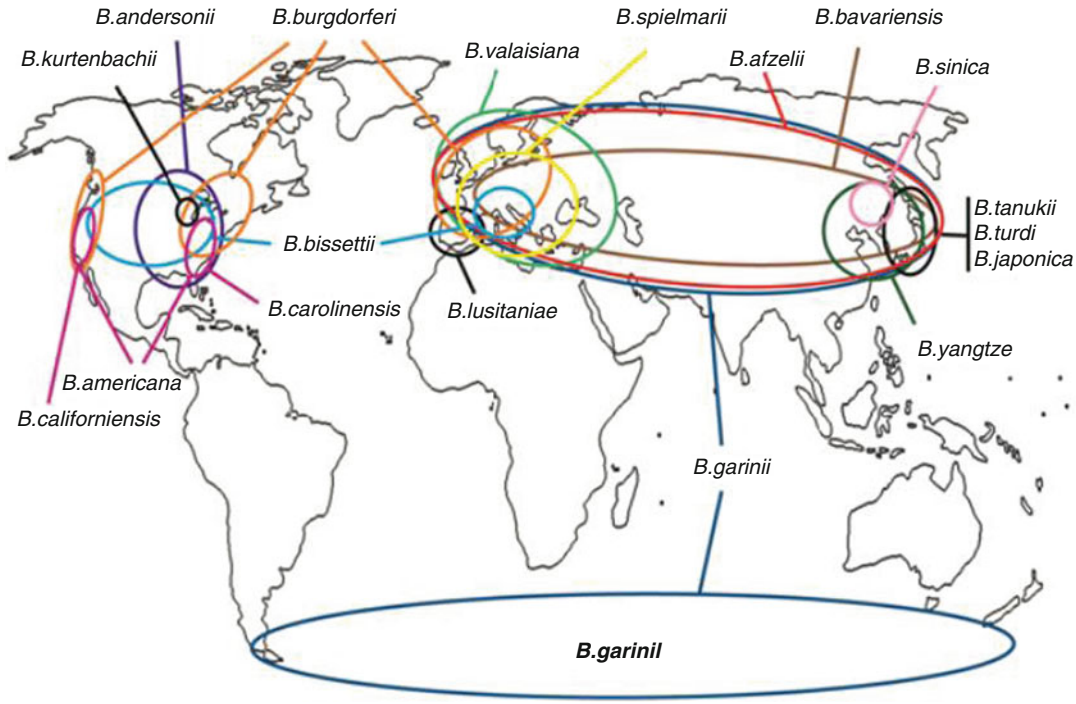

Fig. 16.1 Worldwide geographic distribution of the Lyme borreliosis species and its tick vectors (Printed with permission of Elsevier [SciVerse Science Direct] [Publisher of Margos and Fish, IGE, 2011])

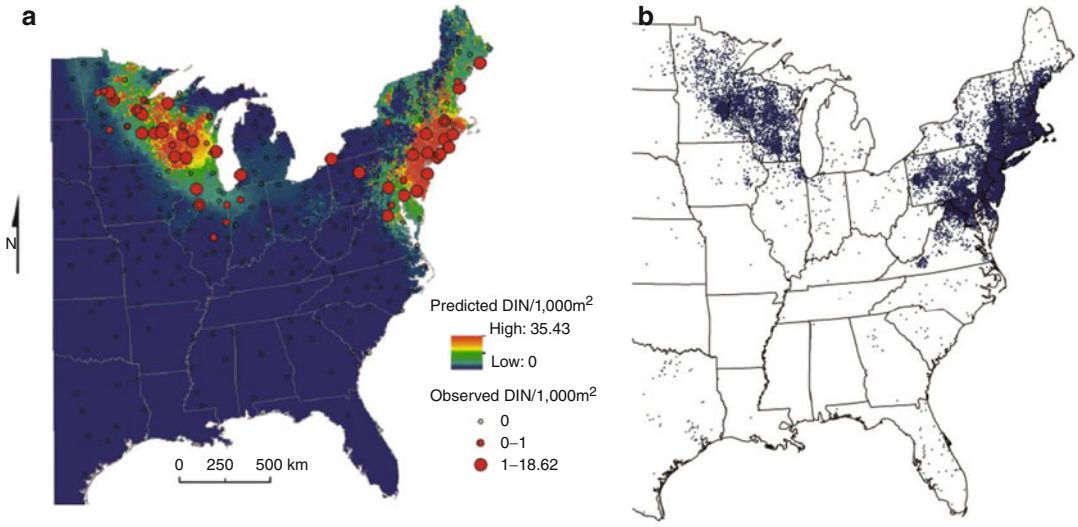

Fig. 16.2 Correlation between nymphal infection prevalence and Lyme disease incidence in the USA. (**a**) Predicted and observed density of infected host-seeking *Ixodes scapularis* nymphs, 2012. (**b**) Reported cases of Lyme disease to the CDC, 2010 (Printed with permission of The American Society of Tropical Medicine and Hygiene [Publisher of Diuk-Wasser, Fish, 2012; **b** is available for public use from the CDC website www.cdc.gov])

(60–80 % [10]) and develops after an incubation period of 2–32 days [11]. Most cases of *erythema migrans* occur between June and August. Like most tick-borne infections, slightly more men are infected than women. In the USA there is a bimodal age distribution with the highest incidences in children 5–9 years old and in adults 45–59 years old, but people of all ages are at risk [12]. Low-level spirochetemia probably occurs in the majority of untreated patients [13], occasionally affecting the peripheral or central nervous system, joints, or heart [11].

In the United States most infections are caused by *B. burgdorferi* sensu stricto (s.s.) which are characterized mostly by arthritis and occasionally carditis and neurological symptoms. In Europe most infections are caused by *B. afzelii* which cause dermatological manifestations such as acrodermatitis chronica atrophicans, *B. garinii* which cause neuroborreliosis, and *B. burgdorferi* s.s. which cause carditis and neuroborreliosis [14]. Late Lyme neuroborreliosis characterized by slowly progressive encephalomyelitis is uncommon [15–17].

Five other species of Borrelia have been isolated or detected by PCR in specimens from small numbers of patients in Europe (*B. bavarensis*, *B. spielmanii*, *B. lusitaniae*, *B. valaisiana*, and *B. bissettii*) [4, 18] (Fig. 16.2).

16.2 The Enzootic Cycle and Transmission

In endemic areas, transmission of Lyme borrelia occurs in suburban areas or rural areas used for forestry and recreational activities [19]. A typical habitat for the transmission of Lyme borrelia is much the same throughout the geographical range of this disease. It usually consists of deciduous or mixed woodland, occasionally coniferous, with a substantial understory and a layer of decaying vegetation on the ground, thus providing sufficient humidity for the development and survival of ticks and supporting a range of potential vertebrate reservoir hosts [3].

B. burgdorferi is transmitted mainly by four species of hard tick within the *I. ricinus* complex: *I. scapularis* and *I. pacificus* in eastern and western North America, respectively, *I. ricinus* in Europe, and *I. persulcatus* in Asia [2, 3, 20]. These ticks go through a four-stage life cycle (egg, larvae, nymph, and adult) feeding only once per active stage. Male ticks rarely feed and never engorge.

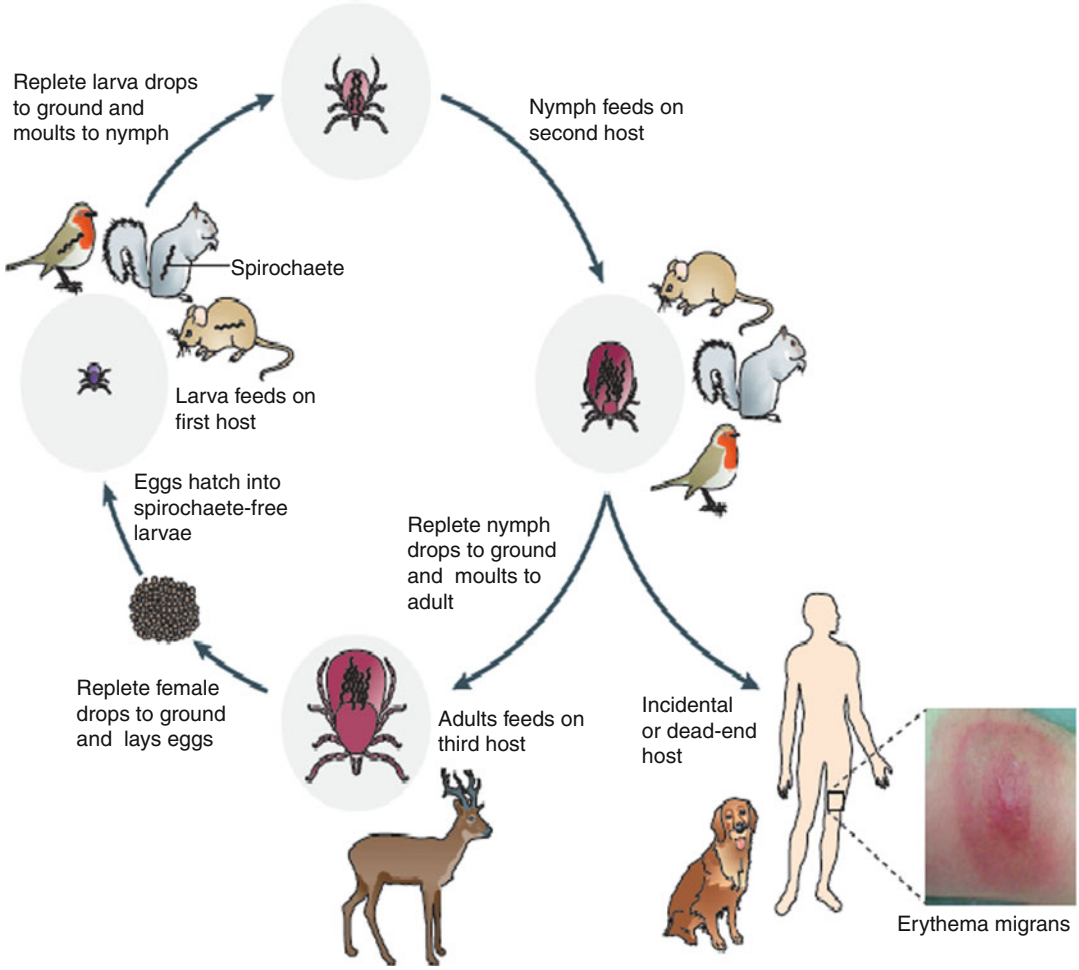

Fig. 16.3 The enzootic cycle of Borrelia burgdorferi (Printed with permission of Macmillan Publishers Limited [Publisher of Nature Reviews, Radolf, 2012])

Unfed (flat) ticks attach to the skin of a host as the animal passes through vegetation. After feeding for a few days (3 days for larvae, 5 days for nymphs, and 7 days for adult females), the ticks drop off their host and hibernate near the soil surface where they need a minimum relative humidity of 80 % for survival. The ticks take several months to develop into the next developmental stage [3]. The larvae are uninfected as they hatch—there is no transovarial transmission [4]—and *B. burgdorferi* is acquired upon feeding on an infected reservoir host. After molting to the nymphal stage, the tick transmits the pathogen to the animal or human that provides its next blood meal. Transmission of Lyme borrelia occurs through injection of tick saliva during feeding. A feeding period of at least 36 h is needed for transmission of *B. burgdorferi* by *I. scapularis* or *I. pacificus* ticks [21–23]. Transmission of *B. afzelii* by *I. ricinus* can be faster. Experiments in gerbils have put this number at 17 h although results need to be repeated by additional investigators and the minimum feeding time has not been determined [24]. Of note, transmission of Lyme borrelia to humans occurs through the nymphal stage for *I. scapularis*, *I. pacificus*, and *I. ricinus* but not for *I. persulcatus*. This species transmits Lyme borrelia to humans in its adult stage (Fig. 16.3).

The life cycle of all four species of ticks has distinct seasonality. *I. scapularis* nymphs are

Table 16.1 The reservoir competence of vertebrate species

	Species	Geographical area	References
Competent	Mouse	USA/Europe	[29–31]
	Voles	Europe	[31]
	Chipmunk	USA	[29, 30]
	Shrew	USA	[29, 30, 32]
	Squirrel	USA/Europe	[29, 30, 33]
	Ground birds	USA/Europe	[30, 33]
	Striped skunk	USA	[30]
	Lizards	USA/Europe	[23, 36]
Not competent	Deer	USA/Europe	[30, 35]
	Cattle	Europe	[25, 33, 36, 37]
	Opossum	USA	[30]
	Raccoon	USA	[30]
Not confirmed	Sheep	Europe	[20]
	Hare	Europe	[38]
	Hedgehog	Europe	[39]
	Badgers	Europe	[33]
	Red foxes	Europe	[33]

Reservoir competence is the probability that an infected host transmits *B. burgdorferi* to feeding ticks

active in early summer, and adults become active in autumn and remain so until winter and early spring. In the case of *I. ricinus* and *I. persulcatus*, nymphs and adults become active in early spring and continue to seek hosts until midsummer or until later in the year in sheltered humid environments. With *I. ricinus* a second peak of activity can occur in the autumn. Patterns of activity of *I. pacificus* seem more like those of *I. ricinus* than *I. scapularis*. In all species, peak activity usually occurs slightly later in larvae than in nymphs, especially for *I. scapularis* in eastern USA. The 3-month difference between one cohort of *I. scapularis* nymphal activity and the following cohort of larval activity allows plenty of time for transmission from infected reservoir hosts to larvae and could explain the high transmission rate in eastern USA [23]. Most transmission to humans, manifested by cases of erythema migrans, occurs from late May to late September, coinciding with the activity of nymphs and with the increasing recreational use of tick habitats by the public [3].

Field studies in North America and Eurasia have identified a variety of small mammal and avian reservoirs in enzootic transmission cycles [4] (Table 16.1). The white-footed mouse, *Peromyscus leucopus*, is considered to be the main reservoir in the northeastern United States, whereas rodents and migratory birds are the principal reservoirs in Europe for *B. afzelii* and *B. garinii*, respectively [2]. In most tick habitats, deer are essential for the maintenance of tick populations because they are one of the few wild hosts that can feed sufficient numbers of adult ticks and keep the cycle ongoing, but they are not competent reservoirs for spirochetes. Cattle are incompetent hosts, and sheep also appear likely to be incompetent reservoir hosts [20, 25, 26]. The different pathogenic genospecies of *B. burgdorferi* sensu lato show a slight predilection for some vertebrates as reservoir hosts, though this host specificity does not seem to be absolute. One factor thought to be relevant to reservoir competence is the susceptibility of the particular genospecies of Lyme borrelia to complement-mediated killing by the animal host [27]. Small populations of deer in a tick habitat can be regarded as a good indication of Lyme borreliosis risk because an array of other hosts, including reservoir-competent animals, are also likely to be present. However, if most animals in a habitat are those which do not act as reservoirs for Lyme borrelia, such as deer or cattle, Lyme borreliosis risk decreases because ticks will feed mostly on these animals and will therefore not become infected [3, 28].

16.3 Diagnostic and Classic Therapy

16.3.1 Clinical Presentation and Diagnosis

The clinical presentation of LB ranges from acute to chronic illness, with wide variation attributed to the different *Borrelia* genospecies and genotypes implicated in the infection [16, 18, 40].

Briefly, several days or weeks after a tick bite, if *Borrelia* infection occurs, in 60–80 % of cases, this will be characterized by erythema migrans (skin rash at the tick bite site, about 10 cm across that may expand peripherally and may or may not be itchy) [10, 16, 40]. Other early symptoms include influenza-like symptoms, fever, fatigue, headaches, and muscle or joint pain. However, early infection may be completely asymptomatic. Several weeks or months after infection (with or without a previous history of erythema migrans), neuroborreliosis (noted in 10–20 % of symptomatic patients) in the form of meningoradiculitis, meningitis or meningoencephalitis [17], Lyme arthritis, or Borrelia lymphocytoma may occur [16]. Less frequently, multiple erythemata or carditis is diagnosed [16, 41]. Months or even years after *Borrelia* infection, acrodermatitis chronica atrophicans (common in Europe), lymphocytoma, chronic arthritis (common in the USA), encephalomyelitis, or chronic neuroborreliosis (rare in Europe) may be observed [16, 18].

Microbial or serological confirmation of *Borrelia* infection is needed for all manifestations of the disease except for typical early skin lesions. The diagnosis of some chronic forms of LB is currently controversial [42], and it has also been suggested that the overdiagnosis and overtreatment of LB may be an important problem [18].

Thus, diagnosis is primarily clinical; *erythema migrans* is a pathognomonic sign and takes into account the risk of tick bite. Diagnosis of other forms of LB requires confirmation by means of a direct or indicted diagnostic test [15] (Table 16.2). A wide range of methods have been developed for the direct detection of *B. burgdorferi* s.l. in clinical tissue specimens, including microscopic examination, detection of *B. burgdorferi* specific proteins or nucleic acids, and cultivation. Future diagnostic methods may include PCR-based molecular techniques that can rapidly confirm clinical diagnosis of LB and identify *Borrelia* genospecies in tissue specimens or cultured isolates [43]. The most commonly used methods for indirect detection of *B. burgdorferi* are serological indirect immunofluorescent antibody assays (IFA) and enzyme-linked immunosorbent assays (ELISA) [44]. In more than 50 % of cases, diagnosis of LB can be made on the basis of an expanding erythema (confirmed after a 1-week follow-up). In the absence of erythema migrans, at least one other clinical manifestation must be noted and confirmed using serological diagnosis of *Borrelia* in blood or CSF. Nevertheless, specific antibodies are often not detectable in the early stage of infection with currently available tests. According to the most recent European and American guidelines [45, 46], serological diagnosis should follow a two-step procedure starting with a sensitive assay such as ELISA. Positive ELISA results should be confirmed by a specific assay such as immunoblot.

16.3.2 Treatment

In vitro studies have shown that Lyme borrelia are susceptible to tetracyclines, most penicillins, many second-generation and third-generation cephalosporins, and macrolides. Lyme borrelia are resistant to specific fluoroquinolones, rifampicin, and first-generation cephalosporins [15, 47, 48].

Although erythema migrans will eventually resolve without antibiotic treatment, oral antibiotic treatment is recommended to prevent dissemination and development of later sequelae (Table 16.3). Doxycycline, amoxicillin, phenoxymethylpenicillin, and cefuroxime axetil are highly effective and are the preferred agents for this manifestation. Doxycycline is the only drug for which both a prospective and a large retrospective clinical trial have shown that only 10 days of treatment is effective [49, 50]. Doxycycline, however, can cause photosensitivity and is contraindicated in children younger than 8 years and in women who are pregnant

Table 16.2 Diagnostic testing of Lyme borreliosis. Manifestations, brief clinical case definitions and recommended diagnostic approach for the diagnosis of Lyme borreliosis in routine clinical practice

	Primary diagnostic testing	Supporting diagnostic testing	Supporting clinical findings
Erythema migrans			
Expanding red or bluish-red patch (≥5 cm in diameter), with or without central clearing	Diagnosis on the basis of history and visual inspection of the skin lesion Laboratory testing not needed or recommended	Culture or PCR of a skin biopsy specimen useful in research studies, but not needed for routine clinical practice	Tick bite at site; regional lymphadenopathy in North American patients
Advancing edge is typically distinct, often intensely colored and not noticeably raised	If lesion is atypical, then acute-phase and convalescent-phase serological testing are recommended because of insensitivity of acute phase testing		
Borrelial lymphocytoma (*a rare manifestation*)			
Painless bluish-red nodule or plaque, usually on ear lobe, ear helix, nipple or scrotum; more frequent in children (especially on ear) than in adults	Serological testing usually positive at time of presentation; if negative, test convalescent phase sera (2–6 weeks later)	Lesion biopsy might be necessary to rule out neoplasm Culture or PCR of a skin biopsy specimen useful in research studies but not needed for clinical practice	Tick bite at site; recent or concomitant erythema migrans
Lyme neuroborreliosis			
In adults, mainly meningo-radiculitis, meningitis and peripheral facial palsy; rarely encephalitis, myelitis; very rarely cerebral vasculitis	Pleocytosis and demonstration of synthesis of intrathecal antibodies to *Borrelia burgdorferi* sensu lato	Detection of Lyme borrelia by culture or PCR of cerebrospinal fluid	Recent or concomitant erythema migrans
In children, mainly meningitis and peripheral facial palsy	Serological testing usually positive at time of presentation; if negative, test convalescent phase sera (2–6 weeks later)	Intrathecal synthesis of total IgM, IgG or IgA	
Cardiac Lyme borreliosis (*a rare manifestation*)			
Acute onset of atrioventricular (I–III) conduction disturbances, rhythm disturbances and sometimes myocarditis or pericarditis Alternative explanations should be excluded	Serological testing usually positive, but if negative and clinical suspicion strong, test convalescent phase sera (2–6 weeks later)	None recommended (Detection of Lyme borrelia by culture or PCR from endomyocardial biopsy restricted to research studies)	Recent or concomitant erythema migrans, neurological disorders or both
Ocular manifestations (*rare*)			
Conjunctivitis, uveitis, papillitis, episclerifs, keratitis	Serological testing	Detection of Borrelia burgdorferi sensu lato by culture or PCR from ocular fluid	Concomitant or previous other well-defined Lyme borreliosis manifestations
Lyme arthritis			
Recurrent attacks or persisting objective joint swelling in one or more large joints	Serological testing As a rule, high concentrations of specific serum IgG antibodies present	Synovial fluid analysis. Detection of *Borrelia burgdorferi* sensu lato by PCR of synovial fluid or tissue	Previous other well-defined Lyme borreliosis manifestations

(continued)

Table 16.2 (continued)

	Primary diagnostic testing	Supporting diagnostic testing	Supporting clinical findings
Acrodermatitis chronica atrophicans			
Long-standing red or bluish-red lesions, usually on the extensor surfaces of extremities	Serological testing	Histology	Previous other well-defined Lyme borreliosis manifestations
Initial doughy swelling Lesions eventually become atrophic Possible skin induration and fibroid nodules over bony prominences	As a rule, high concentrations of specific serum IgG antibodies present	Detection of *Borrelia burgdorferi* sensu lato by culture or PCR from skin biopsy useful in research studies, but not for routine clinical practice	

Table 16.3 Treatment of Lyme borreliosis

	Treatment regimen	Duration	Comment
Early-localised and early-disseminated Lyme borreliosis			
Erythema migrans	Oral	14 days	A 10-day course of treatment with doxycydine is effective in the USA, but the efficacy of 10-day courses of the other first-line oral antibiotics is less well substantiated No studies have been done in Europe Doxycydine is also active against *Anaplasma Phagocytophilum*
Meningitis or radiculopathy	Parenteral or doxycydine	14 days	Evidence from European studies shows that oral doxycydine treatment is as effective as parenteral treatment, although this finding has not been systematically tested in North America
Cranial nerve involvement	Oral	14 days	Although any of the first-line oral antibiotics seem to be effective in patients with cranial neuropathy, there is restricted evidence for its effectiveness in patients with a cranial neuropathy other than facial palsy or with drugs other than doxycydine
Cardiac disease	Oral or parenteral	14 days	Information on treatment is restricted A parenteral regimen is preferential in patients who are being monitored in hospital or are in hospital for placement of a temporary pacemaker When heart block has improved and the patient is ready to be discharged, an oral antibiotic treatment regimen can be given Those who are managed as outpatients can be treated with an oral antibiotic
Borrelial lymphocytoma	Oral	14 days	Little information is available on treatment The same approaches are used as for the treatment of erythema migrans Not recorded in North America
Late Lyme borreliosis			
Arthritis without neurological disease	Oral	28 days	Patients are usually concomitantly treated with NSAIDs

Table 16.3 (continued)

	Treatment regimen	Duration	Comment
Recurrent arthritis after one course of oral treatment	Oral Parenteral	28 days 14–28 days	Parenteral treatment is usually reserved for patients without even a partial response to oral treatment.
Antibiotic-refractory arthritis	Symptomatic treatment	As required	Antibiotic refractory Lyme arthritis is defined as persistent synovitis for at least 2 months after completion of a course of intravenous ceftriaxone (or 1 month after completion of two 4-week courses of an oral antibiotic regimen); additionally, PCR on synovial fluid (and synovial tissue if available) is negative for borrelial nucleic acids
Central or peripheral nervous system disease	Parenteral	14–28 days	No studies have compared 14-day treatment with 28-day treatment, partly because of the rarity of cases
Acrodermatitis chronica atrophicans	Oral	21–28 days	No studies have compared 21-day treatment with 28-day treatment, nor treatment with different antibiotics
Post-Lyme borreliosis syndrome			
Post-Lyme borreliosis syndrome	Symptomatic treatment	As required	Consider and assess other potential causes of symptoms

or breastfeeding [15]. The preferred parenteral drug for Lyme borreliosis is ceftriaxone because it is highly active against Lyme borrelia in vitro, crosses the blood–brain barrier well, and has a long serum half-life, which means it can be taken only once a day. Alternative choices for parenterally given antibiotics are cefotaxime and intravenous penicillin. Parenteral antibiotic treatment is recommended for treatment of patients with late Lyme neuroborreliosis and as an initial treatment for those with cardiac Lyme borreliosis who are admitted to hospital for monitoring [3].

16.4 Vaccine

At present, there are multiple proven strategies for prevention of Lyme disease transmission. Avoidance of tick-infested environs or covering bare skin, and using tick repellents when in such environments, is a very effective method. Removal of wood chips where lawns are adjacent to forests, application of acaricides, and the construction of fences to keep out deer [49] are also effective as these disturb the habitat where density of host-seeking ticks is high. Bathing within 2 h of tick exposure decreases the risk of Lyme borreliosis [50]. Daily inspections of the entire skin surface (including scalp) to remove attached ticks are recommended because of the delay between the time of tick attachment and transmission of Lyme borrelia. Clinical studies have shown that more than 96 % of patients who find and remove an attached *I. scapularis* tick will not contract Lyme borreliosis, without any other intervention, even in highly endemic geographical regions [51]. If the tick is not found or removed, the probability of infection approaches the infection rate in the regional tick population (typically 25 % of nymphal stage *I. scapularis* ticks are infected in highly endemic areas of the northeastern and midwestern USA and 10 % of nymphal *I. ricinus* ticks in Europe) [52, 53]. Chemoprophylaxis can reduce the chance of developing Lyme borreliosis after removal of *I. scapularis* or *I. persulcatus* tick from the skin [3, 51, 54].

No vaccine is available to prevent human Lyme borreliosis [3]. However, new strategies for the prevention of Lyme disease including novel human vaccines, anti-tick vaccines, and reservoir-targeted vaccines and interventions are currently under development. The latter reservoir-targeted vaccines and interventions are further discussed in this chapter.

I. scapularis are widely distributed across the United States, in many areas that Lyme borreliosis is not found to be endemic. One reason for the lack of Lyme borreliosis in these areas is that lizards are the natural host for *Ixodes* ticks, and *B. burgdorferi* is killed when exposed to lizard blood. Thus, eliminating the hosts or reducing vector competence for an organism (i.e., preventing the vector from acquiring or transmitting an organism) may be an effective strategy for preventing Lyme disease in humans [55]. Although elimination of Lyme borrelia from nature is unrealistic, diminishing their threat to humans is an achievable goal [2].

The major reservoir host that is competent for transmission of *B. burgdorferi* in the USA is the white-footed mouse (*Peromyscus leucopus*) [56, 57]. However, chipmunks (*Tamias striatus*), squirrels, shrews, and other small vertebrates are becoming increasingly recognized as important hosts. Further, birds may also play a major role in dispersing *B. burgdorferi* reach [58].

Researchers have been investigating the vaccination of reservoirs of *B. burgdorferi* to reduce carriage of the organism. Tsao et al. performed an ambitious study where *Peromyscus* mice were captured and vaccinated subcutaneously with either the outer surface protein A of *B. burgdorferi* (OspA) or a control vaccine. Vaccination significantly reduced the prevalence of *B. burgdorferi* the following year in ticks collected from sites where OspA was given compared with control [59]. Currently, several groups are developing methods for oral distribution of an OspA vaccine to mice and other reservoir hosts as bait with the ultimate goal of disrupting the enzootic cycle of this spirochete [60, 61].

There is a precedent for the development of bait vaccines to be used as a strategy to reduce transmission of pathogens [62]. Baits and baiting systems for delivery of rabies [63–65] and plague vaccines [66] have proven successful. Other systems of tick control have been explored to reduce the risk of Lyme borreliosis. In one study, acaricide self-treatment of white-tailed deer resulted in reduction of tick density [67–70]. In another study, a rodent-targeted acaricide (fipronil) delivered to white-footed mice (*P. leucopus*) in modified commercial bait boxes was also effective in reducing nymphal and larval tick infestations (tick density) [71]. In an alternative approach, a doxycycline rodent bait formulation prevented tick transmission of *B. burgdorferi* to vertebrate hosts as well as cured established infections in mice [72, 73]. A decrease in tick density or tick infection, as well as the decrease of vertebrate host infection with *B. burgdorferi*, is expected to result in the overall decrease in human risk to Lyme disease.

16.4.1 Animal Models of Lyme Disease

As mice and other rodents are natural reservoirs for *Borrelia burgdorferi* sensu *lato*, it is not surprising that wild *Peromyscus* spp. mice show no observable changes during infection with *B. burgdorferi*, as symptomatic disease could leave the infected animal with a survival disadvantage and/or limit the opportunities for transmission of spirochetes to new cohorts of vectors [74]. However, when infected with *B. burgdorferi*, specific inbred strains of the laboratory mouse *Mus musculus* have been found to exhibit features similar to those of human Lyme disease [9, 75]. For example, C3H and BALB/c mice develop both ankle joint arthritis and carditis on infection with *B. burgdorferi*, whereas C57BL/6 and DBA mice are more resistant to developing signs of infection and typically have only minimal inflammation in the heart and joints. No strain of inbred mouse develops erythema migrans, meningitis, or encephalitis, and thus all are imperfect models for human Lyme disease. Rhesus monkeys can also be infected with *B. burgdorferi* and are used as a model of neuroborreliosis because of their propensity for central nervous system infection, particularly when immunosuppressed with corticosteroids. Rhesus monkeys also develop erythema migrans, mononeuritis multiplex, and arthritis, making them the animal model that is most similar to humans for this disease [75]. However, for reasons of cost and ease of genetic manipulation, animal models other than mice have been studied sparingly. Although *P. leucopus* do not develop evident signs of

disease, spirochete dissemination can be determined by culture of *B. burgdorferi* from blood, heart, and bladder tissues further confirmed by PCR amplification of *B. burgdorferi* genes such as FlaB, OspA, or OspC [76].

16.4.2 The OspA Vaccine

Previous studies indicated that immunization with OspA induced a long-term protective immune response in mice [77]. Spirochetes were killed inside the midgut of engorging ticks in the presence of anti-OspA antibodies [78]. The OspA vaccine successfully completed Phase I, II, and III trials and was approved by the Federal Drug Administration in the United States in 1998. Vaccinated individuals showed approximately 80 % protection against *B. burgdorferi* infection after receiving three vaccine doses with OspA using aluminum as an adjuvant [79]. One drawback of the OspA vaccine in humans was that protective immunity correlated with high titers of OspA antibodies after immunization, and it was shown that 5 % of the vaccine recipients developed insufficient antibody responses against OspA. Vaccine failure was associated with decreased cell surface expression of Toll-like receptor (TLR)-1 [80]. Thus, high antibody titers did not persist long after vaccination, and additional boosters would be necessary to maintain protective titers [79, 81]. Despite some weaknesses, OspA remains the best immunogen to develop a vaccine against Lyme disease.

Two groups used OspA to develop oral delivery systems to vaccinate reservoir hosts, hence developing reservoir-targeted bait vaccines. One such delivery system is based in vaccinia virus (VV) expressing OspA [61]. Another delivery system is based in *E. coli* expressing OspA [60]. Both systems are equally effective in eliciting production of protective levels of anti-OspA antibodies in inbred *Mus musculus* and in *Peromyscus leucopus* mice that receive the vaccine orally [60, 61, 76, 82]. Further, both vaccines are equally effective in clearing *B. burgdorferi* from infected ticks that feed on vaccinated mice. However, vaccinia virus-based vaccines are infectious to people who are immunocompromised or suffer from eczema [83]. Given that a reservoir-targeted vaccine (RTV) against Lyme borreliosis would be deployed near suburban areas, the fact that Vaccinia is an infectious agent will seriously complicate regulatory approval. Thus, a bacterial-based delivery system is seen as the safest approach. Therefore, all of Centers for Disease Control and Prevention efforts to test an RTV against Lyme borreliosis in the field have been placed on the bacterial delivery system.

The bacterial delivery system is based in *Escherichia coli* transformed with a plasmid encoding the full-length sequence of OspA from *B. burgdorferi* [60, 76]. The bacteria are induced to express the protein, the viability of the cells analyzed, and the live culture is lyophilized creating a delivery system without the need to purify the protein followed by repackaging in deliverable microspheres. In the studies reported, the bait vaccine was made daily before immunization by mixing 200 mg of lyophilized bacteria with rolled oats (containing approximately 2 mg/ml of OspA) and offered to *Peromyscus leucopus* ad libitum for ingestion. The OspA-RTV was tested for induction of a protective immune response in white-footed mice, the natural reservoir host of the spirochete. In studies to optimize the OspA-RTV for field deployment, several immunization schedules (daily delivery of bait for 4–16 weeks) and parameters mimicking exposure of the bait in the field (high temperature and humidity) were tested.

These studies showed that mice immunized with the OspA-RTV developed high titers of antibody to OspA that lasted throughout the full year in which the immunization was carried out and that high titers of antibody to OspA correlate with protection. In addition, the researchers determined that the minimum number of units required for white-footed mice to develop a protective immune response is five. Considering that nymphal *I. scapularis* ticks that infect humans with *B. burgdorferi* are active in the spring and that the larval ticks that carry *B. burgdorferi* infection to the next tick cohort (which will become nymphs the following year) are active in the summer, possible plans to deploy the OspA-RTV to wildlife could consist of delivery of bait for 5 days a week for 4

weeks. Or, an alternative plan could be implemented by deploying OspA-RTV two or three times per week for a period of 4 months, ranging from mid-April until mid-August. The rationale for a 4-month deployment rather than a less labor-intensive 1 month potentially equally effective deployment would be to vaccinate the young hosts that are born throughout both seasons.

16.4.3 Preclinical Development/Field Application

Field trials using *E. coli*-based OspA-RTV were recently performed in New York State and Pennsylvania in collaboration with the Centers for Disease Control and Prevention (CDC). These studies have been projected to assess RTV efficacy in reducing the infection prevalence of wild reservoir host species (white-footed mouse, *P. leucopus*) and the tick vector. Vaccine efficacy will be determined by calculating the amount of white-footed mice that have developed antibodies to OspA after OspA-RTV intake and by reduction of the proportion of *I. scapularis* ticks infected with *B. burgdorferi* in vaccinated plots compared to paired control plots in which the RTV is not deployed.

The OspA-RTV vaccine falls under USDA jurisdiction. The USDA process for licensing biological products is not nearly as long as the Federal Drug Administration (FDA) process, and the timeline ranges from 2 to 3 years depending on the complexity of the technology and how organized the applicant is. The *E. coli* itself is a derivative of generally recognized as safe *E. coli* B2 which is approved for use in biologics in the USA.

16.5 Strengths and Weaknesses

16.5.1 Strengths

A major strength of the *E. coli*-based RTV technology is that it combines excellent efficacy with safety after deployment in target areas in the field. The *E. coli* strain used is a B2 derivative that is generally recognized as safe (GRAS status).

A major strength of the vaccinia virus-based RTV technology is that it potentially requires one application in the field to induce effective immune responses to OspA.

16.5.2 Weaknesses

A weakness of the *E. coli*-based RTV technology is that efficacy of this vaccine is dependent on a lengthy immunization schedule; multiple deployments of RTV are necessary over several weeks given that a minimum of five vaccine doses must be ingested before mice can mount a significant immune response to OspA.

A weakness of the vaccinia virus RTV technology is that it is not safe for wide distribution near suburban areas where Lyme disease is endemic and humans will certainly encounter and handle baits.

Given that *I. scapularis* has a 2-year life cycle, it might be seen as a weakness of the concept in general, the fact that it should take several years of distribution of OspA-RTV in the field for an impact in reduction in nymphal infection prevalence. Thus, it would take several years of RTV application in the field for a reduction of Lyme disease incidence to occur.

16.5.3 Manufacturing and Market

Manufacturing and distribution of weatherproof *E. coli*-based OspA-RTV could be a cumbersome process as we envision that several tones of material would have to be produced to deploy in target areas of endemic regions.

The current market for a bait vaccine to indirectly prevent Lyme disease in the human population would be targeted to public health organizations both in the United States and internationally. Presently endemic areas of Lyme disease occur throughout the world in Asia, Europe, and North America with infections increasing every year. To our knowledge there is no bait vaccine against *B. burgdorferi* in the market. State and city health departments should form the primary domestic market.

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Anti-tick Vaccines for the Control of Ticks Affecting Livestock

17

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Abstract

Ticks are obligate hematophagous arthropod parasites affecting most terrestrial vertebrate species. Their importance as disease vectors is due to the abundance and diversity of organisms they transmit to their vertebrate host. In addition, secondary infections of the attachment site, direct toxicosis, and paralysis can occur due to tick feeding. The impact of tick-borne diseases is most heavily felt in the livestock sector, where production is limited in many areas due to high tick infestations and tick-borne disease prevalence.

Currently, large-scale tick control is achieved through acaricide application, but continued use has resulted in resistance to several active ingredients. As an alternative, the immunological control of ticks through vaccination has been proposed. Proof of concept has been shown culminating in commercial vaccines for the control of the cattle tick *Rhipicephalus microplus*. Despite this initial success, the development of anti-tick vaccines faces a number of unique obstacles due to complex interactions between tick and vertebrate hosts.

This complexity does however allow novel areas of vaccine development to be followed that are unavailable to other vector-borne dis-

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eases. Tick antigens localized in the tick gut are concealed from the host immune response during normal tick feeding. With the uptake of the blood meal from a vaccinated host, damage to the gut wall occurs which may result in death or decreased reproductive capacity. The use of "concealed antigen" candidates has been successful in effectively reducing tick populations with successive generations. Furthermore, a number of "exposed antigen" candidates, present in the saliva or cement cone, have also been investigated for their ability to interfere with tick feeding and block parasite transmission. With the completion of tick genome sequencing projects the number of candidate antigens for evaluation as vaccine candidates increasing. In this chapter, the history and potential future of anti-tick vaccine development are outlined with particular reference to control of ticks and tick-borne diseases affecting livestock. Anti-tick vaccine advances for the major tick species affecting livestock, *Rhipicephalus*, *Amblyomma*, and *Hyalomma*, will be discussed.

17.1 Introduction

Ticks are widely distributed throughout the world affecting 80 % of the world's cattle population [1]. The economic importance of ticks and tick-borne diseases (TBD) has been estimated by a number of studies; however, they most likely represent an underestimation of the real impact of these arthropod vectors and their transmitted diseases. This is partly due to studies having a single focus such as direct losses and cost of control and partly because they often focus only on a single disease or tick species [2–4]. To further complicate estimations, losses are measured differently for the livestock sector compared to human/companion animals.

Tick feeding has devastating effects including disease transmission, paralysis, toxicosis, and secondary infections of the tick-feeding site [4, 5]. The effect of ticks and tick-borne diseases is particularly pronounced in the livestock sector where it is repeatedly rated highly for its impact on the livelihood of farmers, particularly in countries of the South which are heavily dependent on agricultural production [3, 4]. Heavy tick infestations in livestock can result in anemia, reduction of body weight, loss of body condition, and hide damage, an important factor in leather production industries [2, 6].

Although argasid ticks of veterinary importance exist, this review will focus solely on Ixodidae ticks. There are six genera of ixodid ticks of importance, namely, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Rhipicephalus*, and *Ixodes* (summarized in Table 17.1).

Ixodes genus ticks are most well known for their transmission of *Borrelia*, the agents of Lyme disease common in Europe, Asia, and North America. As this species is not of concern in the livestock sector, it will not be discussed further.

Ticks can be tolerated on livestock if numbers remain low and the distribution does not overlap with its associated tick-borne disease. In some instances, near-complete eradication of ticks and tick-borne disease has been achieved, and complete elimination of tick species from a geographic area is often not feasible [7, 8]. Historically, tick and tick-borne disease control has focused on the control of ticks at tolerable levels through acaricide use and treatment of disease with appropriate drugs. In some cases, acaricide-based tick control is often the only method of reducing tick populations without sacrificing productivity [8, 9].

Acaricides are commercially available in a number of formulations that are applied directly either onto livestock or in dipping vats where multiple animals can be passed through at regular time intervals. Acaricide application relies heavily on correct formulation and administration to be effective [9]. A large number of chemical compounds have been found to be effective against ticks including arsenic (introduced ≈ 1983), DDT (≈1946), cyclodienes and toxaphene (≈1947), organophosphates-carbamate group (≈1955), formamides (≈1975), and macrocyclic lactones (≈1981). The potency and usefulness of many of the abovementioned compounds is gradually eroding with resistance developing in many tick species of *Rhipicephalus*, *Amblyomma*, and *Hyalomma*.

Multiple acaricide-resistant tick stocks have been identified, limiting or entirely excluding the

Table 17.1 A summary of important tick and tick-borne diseases of livestock [4]

Genus	Distribution	Tick-borne diseases
Amblyomma	Widely distributed through Africa (*A. variegatum*), Southeast Africa (*A. hebraeum*)	Cowdriosis of ruminants [*Ehrlichia* (*Cowdria*) *ruminantium* (Rickettsia)] Benign theileriosis of cattle [*Theileria mutans* (Protozoa)] Severe secondary infections of tick-feeding site in cattle
Dermacentor	Europe, Asia, North America, Africa[a]	Bovine anaplasmosis [*Anaplasma marginale* (Rickettsia)] Equine babesiosis [*Babesia caballi* (Protozoa)]
Haemaphysalis	Asia, Europe, and lesser extent in Australia	Bovine babesiosis [*Babesia ovata* (Protozoa)] East Asian bovine theileriosis [*Theileria buffeli* (Protozoa)] Babesiosis of small ruminants [*Babesia motasi* (Protozoa)] Bovine babesiosis [*Babesia major* (Protozoa)]
Hyalomma	Asia, Europe, Africa	Bovine tropical theileriosis [*Theileria annulata* (Protozoa)] Theileriosis of small ruminants [*Theileria lestoquardi* (Protozoa)]
Rhipicephalus[b]	America (North and South), Africa, Asia, Australia, Europe	Bovine babesiosis [*Babesia bovis* and *Babesia bigemina* (Protozoa)] Theileriosis of cattle [*Theileria parva* (Protozoa)] Benign bovine theileriosis [*Theileria taurotragi* (Protozoa)] Equine piroplasmosis [*Theileria equi* and *Babesia caballi* (Protozoa)] Bovine anaplasmosis [*Anaplasma marginale*, *Anaplasma centrale* (Protozoa)] Babesiosis of small ruminants [*Babesia ovis* (Protozoa)] Anaplasmosis of small ruminants [*Anaplasma ovis* (Protozoa)]

[a]Although present in Africa, not considered important
[b]The Rhipicephalus genus now includes tick species formerly known as *Boophilus*. Most important tick genus and includes cattle tick *Rhipicephalus microplus*, the main vector for bovine babesiosis, and *Rhipicephalus appendiculatus*, the vector for East Coast fever (bovine theileriosis) in cattle

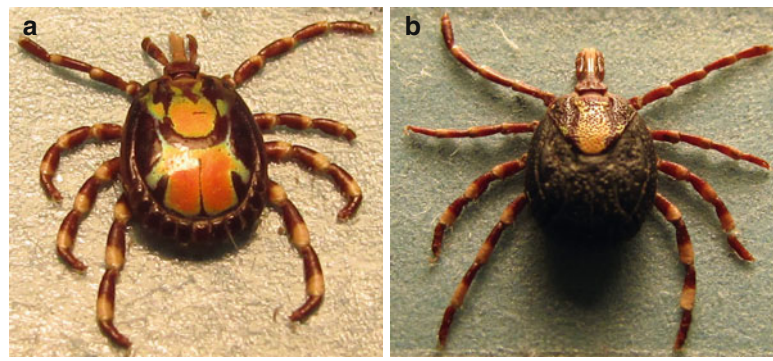

Fig. 17.1 Due to their large mouthparts, feeding by Amblyomma ticks may result in severe secondary infections of the tick-feeding site (**a**): *Amblyomma variegatum* adult male tick, (**b**): *Amblyomma variegatum* adult female tick

use of many acaricides [9–11]. In addition to resistance, chemical control through acaricide application results in environmental pollution and residue tainting of meat and milk products. In economically weak countries, use of acaricides for tick control may be even less feasible due to the high cost involved in infrastructure maintenance and product purchase [2]. For small-scale farmers, the formation of cooperatives may be an alternative to allow acaricide-based tick control [12]. Where ticks have been removed by systematic acaricide application, collapse of protocols and infrastructure due to political unrest has resulted in the reestablishment of tick populations and high livestock mortality associated with disease outbreaks [6].

Figures 17.1, 17.2, 17.3, 17.4, and 17.5 give an overview on ticks affecting livestock in Africa.

17.2 Vaccination Against Ticks

The guiding principle for anti-tick vaccination stems from early studies conducted on acquired host resistance to tick infestations. Repeated exposure of hosts to ticks or tick organ homogenates induced resistance to tick reinfestation. While the degree of resistance may vary between different tick and host species, evidence strongly suggests that natural resistance against tick infestation develops based on adaptive immune response mechanisms [13–15]. Ticks feeding from hosts vaccinated with tick components take up effector molecules during feeding that mediate deleterious effects on the ticks. This effect manifests as reduction of feeding time, tick mortality (during or after feeding), reduced engorgement weights, and reduced reproductive capacity of adult females. Eggs laid from ticks fed on vaccinated hosts may also show reduced hatching rates. The overall result culminates in reduction of tick populations and tick-borne diseases.

Fig. 17.2 Adult *Rhipicephalus appendiculatus* tick. Most known for the transmission of *Theileria parva* to cattle (East Coast fever), the tick species also transmits *Theileria taurotragi* (benign bovine theileriosis), *Anaplasma bovis* (bovine ehrlichiosis), *Rickettsia conorii* (tick typhus), and Nairobi sheep disease virus

There are several advantages of anti-tick vaccination over acaricide use for tick control. These include reduced concerns regarding long-term contamination of animal products and environment with harmful residues and increased sustainability in terms of required infrastructure. Additionally, the resistance development to anti-tick vaccines is likely to be slower than that against acaricides, and the costs of anti-tick vaccine development, production, and registration are thought to lower than

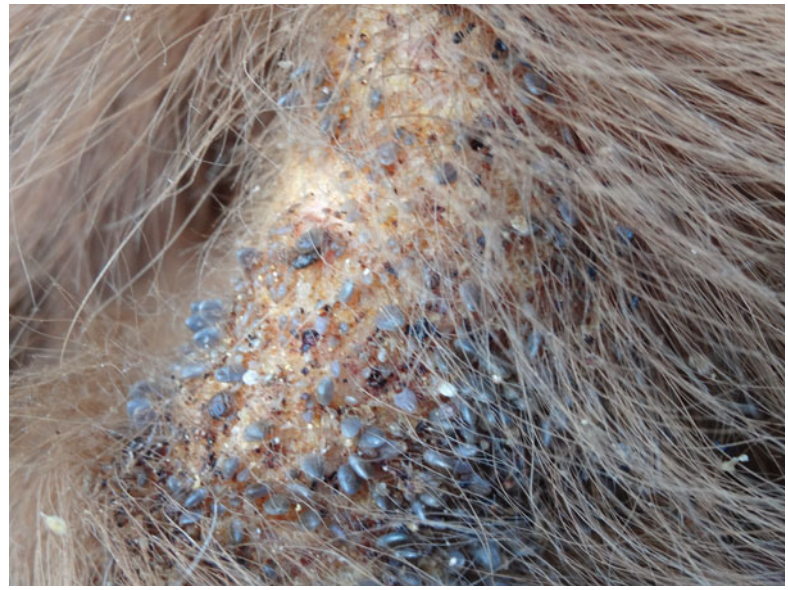

Fig. 17.3 Immature *Rhipicephalus* ticks feeding on the ear of cattle (Courtesy of S. Mwaura)

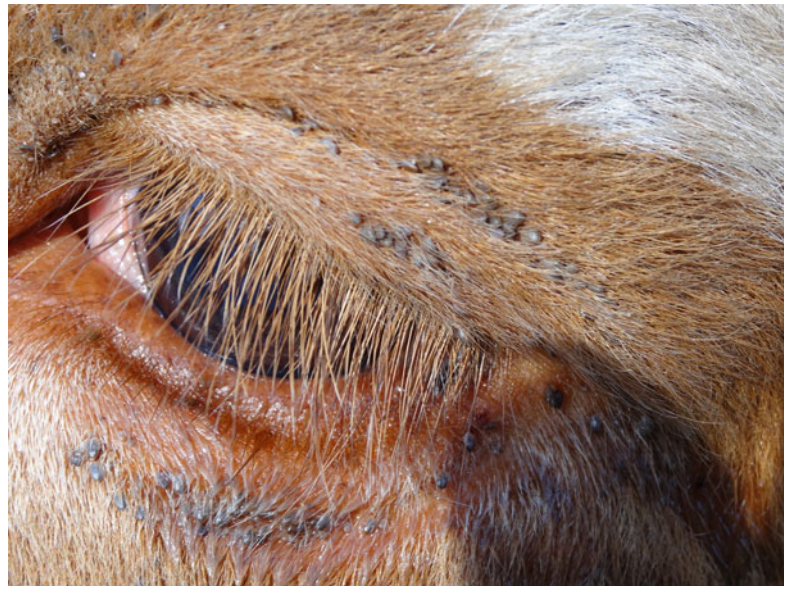

Fig. 17.4 Immature *Rhipicephalus* ticks feeding around the eye of cattle (Courtesy of S. Mwaura)

Fig. 17.5 Collection of acaricide-resistant adult *Rhipicephalus* ticks in Kenya (Courtesy of S. Mwaura)

for a new acaricidal compound (estimated at US $100 million) [5, 16, 17]. Although anti-tick vaccination is often viewed as an alternative to acaricide usage, it can also be seen as a complementary approach within the framework of integrated pest management approaches (IPM). Where used appropriately, anti-tick vaccination results in reduction of acaricide use and potentially decreases acaricide resistance development [5, 17–20].

17.3 Anti-tick Vaccine Candidates

Many of the anti-tick vaccine targets have been identified using conventional immune-screening techniques. Immunization of vertebrate hosts with tick homogenates or purified tick extracts generates immune sera. These sera are used to screen for tick antigens detected by the host.

The identification of tick proteins essential for tick survival is a useful method for more

targeted antigen discovery, which is made increasingly possible as information is gathered on tick biology. With the availability of genome sequences for a number of tick species, the number of candidates for discovery is expanding through reverse vaccinology. A repository of the *R. microplus* genome data is a valuable resource for candidate mining [21], and hopefully, such repositories for other important tick species will follow. The use of other techniques such as RNA interference (RNAi) has been useful in confirming the importance of anti-tick vaccine candidates and is likely to play a role in future anti-tick vaccine antigen discovery [22].

*A*nti-tick vaccine candidates have been classified into two categories: exposed or concealed antigens. Exposed antigens are secreted in tick saliva during attachment and feeding on a host, while concealed antigens are normally hidden from the host immune response. While Willadsen proposed that antigens should rather be classified according to structure or function [5], the main advantage of using the exposed vs. concealed distinction is that it has obvious important implications for potential immune evasion mechanisms by the tick. Molecular mimicry by ticks of host components has been observed, and vaccination may induce host sensitivity and autoimmune reactions when exposed antigens are used [23].

One advantage of using exposed antigens is that natural boosting occurs through tick feeding. Mechanistically, vaccination with exposed antigens is thought to induce a focal hostile environment unsupportive for tick attachment and feeding. Concealed antigens do not come into contact with the host immune response during natural tick feeding. Although often contained within the thoracic cavity of the tick, some salivary gland proteins can be characterized as concealed if they are not secreted into the tick-feeding site.

One difficulty in the development of concealed anti-tick vaccines is that the antigen must be accessible to the induced humoral vaccine response. This often limits the number of candidates to those coming into prolonged and direct contact with the blood meal or where the humoral response can be transported over the gut barrier into the haemolymph [24–26]. The second limitation of concealed antigens relates to natural boosting of the immune response. As the antigens do not come into contact with the immune response within the host, sufficiently high antibody levels must be induced through repeated vaccination. On the positive side, there is low natural selection pressure on these antigens, and emergence of resistance is not of major concern.

As the blood meal acts as the carrier for the effector immune responses, the anti-tick effect can take place over a longer period of time compared to exposed antigens. This effect may even extend beyond the mere feeding period into the inactive stages where digestion and molting/egg laying takes place.

17.3.1 Review of Successful Anti-tick Vaccine Candidates

The Bm86-based anti-tick vaccine remains the only anti-tick vaccine commercially produced and has become the benchmark for future anti-tick vaccine development and evaluation. The gut-associated Bm86 glycoprotein was first identified in *R. microplus* although homologues in other tick species have since been identified [27–33]. The biological function of Bm86 remains unknown although it is thought to play a role in the digestion of the blood meal [34]. In *R. microplus*, expression of Bm86 is increased during embryogenesis reaching the highest level in unfed larvae. Expression decreases during feeding and molting with lowest levels of expression detected during the resting stages of the tick [35]. Bm86 has a translated coding sequence of 650 amino acids and a size of 71.7 kDa. The protein contains four potential N-linked glycosylation sites and a leader peptide suggesting transport to the cell surface [28]. Localization studies have shown that the molecule is located predominantly on the microvilli of gut epithelial cells [29, 36]. A single C-terminal transmembrane sequence is present in the unprocessed protein which is replaced by a glycosylphosphatidylinositol anchor in the mature protein. The protein also contains multiple predicted EGF repeats rich in cysteine residues [28, 34].

Vaccination has been performed mostly with the whole molecule, and protective epitopes for Bm86 have not been well determined. The site of a protective B-cell epitope was defined and additional epitopes are likely to exist [37]. Overlapping cross-reactive immune-reactive epitopes have been found between Bm86 and the *R. decoloratus* homologue, Bd86 [36, 38]. Vaccine efficacy is directly related to anti-Bm86 antibody titer, and ability to control tick populations is directly related to achieving a strong antibody response [39–42].

Substantial animal-to-animal variation has been observed in the ability to generate anti-Bm86 antibody titers which is likely related to the MHC Class II haplotypes expressed [42, 43]. Antibodies to Bm86 and cattle complement system are taken up during the blood meal. Antibody binding results in lysis of the gut epithelial cells culminating in impaired blood meal digestion. Strong antibody responses may induce tick mortality due to blood leakage from the gut into the hemolymph, and ticks may turn reddish instead of grey [34].

Recombinant expression of Bm86 has been attempted in several expression systems including *Escherichia coli* [34], *Aspergillus nidulans Aspergillus niger* [44], and *Pichia pastoris* [45–48]. Vaccine trials showed that Bm86 vaccination targeted mainly the adult stage of *R. microplus*, particularly the number of adult females fully engorging and post-engorgement mortality. Reproductive capacity of adult *R. microplus* females was affected in terms of egg laying capacity and hatching of eggs [5, 49].

Under field situations, vaccination of cattle reduced tick numbers by 56 % within a single generation and reduced the reproductive capacity by 72 % [50]. Reversal of negative effects of tick feeding on live weight of vaccinated animals by an average increase in live weight of 18.6 kg over a 6-month period was observed [40, 41]. Extensive field trials in Cuba, Brazil, Argentina, and Mexico showed between 55 and 100 % control of *R. microplus* ticks within a 36-week period [42, 51].

Importantly, complete control of acaricide-resistant ticks could be accomplished by integrating Bm86 vaccination with acaricide use [52] showing that integrated control systems are effective in controlling tick populations. Vaccination also decreased the amount of acaricides required to control tick populations and prolonged the time interval between cattle dippings [5]. Bm86 vaccination has been extensively evaluated for its ability to control other tick species. Almost complete cross-protection against *Rhipicephalus annulatus* has been reported [53, 54]. Significant protection against *Hyalomma anatolicum*, *H. dromedarii*, and *R. decoloratus* has been observed; however, no cross-protection however was seen against *R. appendiculatus* or *Amblyomma variegatum* [38, 55].

Amino acid sequence variations in Bm86 exist between different stocks of *R. microplus*, and this was thought to contribute to differences in vaccine effectiveness over different geographic locations. Thus far, no definite correlation between vaccine effectiveness and Bm86 sequence homology has been shown [5]. A laboratory *R. microplus* tick strain from Argentina was shown to be resistant to Bm86 vaccination [47, 51], and the nucleotide sequence of the Bm86 gene showed substantial differences including 21 amino acid substitutions (out of 610 amino acids in the mature protein) [56]. The protein was subsequently renamed to Bm95 although whether it should be separately classed remains debatable.

Bm95 has been successfully produced in *P. pastoris* [48] and incorporated in a number of vaccine trials. Effects of Bm95 vaccination are similar to Bm86 vaccination with tick rejection, damage, and death. Decreases in engorgement weights, oviposition, and egg hatching are also demonstrated [57]. A high level of cross-protective vaccine efficacy against Indian *Rhipicephalus haemaphysaloides* with Bm95 vaccination has been illustrated in cattle [58]. Using a novel production technique, fusion of immunogenic Bm95 peptides and the *A. marginale* MSP1 N-terminal region was protective against *R. microplus* infestations in rabbits. The reduction of tick oviposition and fertility was comparable to the commercial Bm86 vaccine [59, 60].

17.3.2 Commercialization of Bm86-Derived Vaccines

Commercialization of the Bm86 anti-tick vaccine in Australia under the name TickGARD™ was undertaken by Commonwealth Scientific and Industrial Research Organization (CSIRO) in collaboration with Biotech Australia Pty. Ltd. and released by Hoechst Animal Health in 1994. Within 4 years, the commercial success of TickGARD™ grew till it became the highest value tick treatment product in Australia. Unfortunately, a series of changes within the commercial partners involved in TickGARD™ production and distribution resulted in discontinuation of production.

The vaccine was briefly reintroduced by Intervet Australia Pty., Ltd. (Bendigo, Australia), but it is currently not available for sale [20]. The Gavac Bm86-based vaccine has been far more successful in areas of Latin America. It was developed by the Center for Genetic Engineering and Biotechnology (Havana, Cuba) and released by HeberBiotec, SA (Havana, Cuba) in 1993. The vaccine has been extensively used in Cuba where it has resulted in reduction of babesiosis and anaplasmosis as well as a dramatic reduction in required acaricide use [20, 42, 61]. Due to state-mandated control programs, Cuba illustrates a best-case scenario outcome for anti-tick vaccine deployment [20]. The vaccine was registered in 1994, 1995, and 1997 for Colombia, Brazil, and Mexico, respectively, with these countries reporting on similar control levels achieved [20].

17.3.3 Bm86 Homologues of Other Tick Species and Their Vaccine Potential

Homologues of Bm86 from a range of other tick species have shown promise as tools for tick control. Bm86 vaccination of cattle induces cross-reacting antibodies binding to gut sections of *R. decoloratus* although its anti-tick effect has not been determined. Mouse monoclonal antibodies induced against a synthetic Bd86-derived peptide recognized Bm86 homologues in *R. microplus*, *R. decoloratus*, *H. anatolicum anatolicum*, and *R. appendiculatus* [35]. Identification and RNAi silencing of the *Haemaphysalis longicornis* Bm86 homologue showed a significant reduction in tick engorgement weight [62]. The *R. annulatus* homologue of Bm86, Ba86, has been successfully expressed in *P. pastoris* and evaluated in cattle.

Vaccination significantly affected tick infestations, oviposition, and egg fertility. Additionally, cross-protection occurred between Ba86 and *R. appendiculatus* ticks. Interestingly, the efficacy of both Ba86 and Bm86 was higher for *R. annulatus* than for *R. microplus* indicating that Bm86-like effects may be tick species dependent rather than sequence homology dependent [60]. Vaccination of cattle with *H. anatolicum anatolicum* homologue, Haa86, resulted in significant increase in tick rejection and reduction of tick engorgement weights and egg mass [32]. The effect of Bm86 homologue vaccination on the transmission of tick-borne diseases has been rarely studied. In one study, Haa86 vaccination reduced transmission of *Theileria annulata* parasites to susceptible calves [63]. The expression pattern of the *R. appendiculatus* Bm86 homologue, Ra86, is notably different to the Bm86 expression [35].

In a laboratory tick stock of *R. appendiculatus*, Ra86 homologues exist as two highly divergent allelic variants. Nucleotide sequence similarity between Ra86 and Bm86 is 73 and 74 % for the two variants Ra85A and Ra92A, respectively [33]. Vaccination of rabbits with baculovirus-expressed Ra86 variants significantly affected engorgement weight and egg weight [64]. None of these effects could be replicated in the bovine host using the same baculovirus construct for expression of Ra86 homologues. However, a previously unreported significant effect on the molting of nymph ticks to the adult stage occurred after Ra86 vaccination of cattle [65].

17.3.4 Other Anti-tick Vaccine Candidates

A number of other anti-tick vaccine candidates have shown promise for control of both homologous and heterologous tick species, although none have been taken beyond initial proof-of-concept studies. These antigens are summarized in Table 17.2.

Table 17.2 List of anti-tick vaccine candidates evaluated for the control of ticks on livestock

Antigen	Location and function	Effect of vaccination	References
BMA7	63-kDa membrane-bound glycoprotein widely distributed in tick tissues Biological function unknown	Vaccination with *R. microplus* BMA7 showed significant protection against tick infestation Effect was less than the Bm86-mediated vaccination	[95]
SBm7462	The synthetic peptide SBm7462 derived from Bm86	Cattle vaccination resulted in a efficacy above 80 %	[37]
Bm91	Membrane-bound carboxydipeptidase Identified in *R. microplus* Located in the tick salivary gland	Increases the effect of Bm86 vaccination	[96, 97]
Serine proteinase inhibitors	The serine proteinase inhibitors (serpins) have been the focus of a number of promising anti-tick vaccine studies	Vaccination of rabbits with recombinant HLS1 resulted in 44 and 11 % mortality in nymph and adult ticks, respectively Vaccination of rabbits with H. longicornis serpin-2 resulted in a mortality rate of 45 and 43 % in nymph and adult ticks, respectively Four serpins have been identified in *R. appendiculatus*, named RAS1-4. Vaccination of cattle with recombinant RAS1 and RAS2 conferred protective immunity with 61 % reduction in nymphal engorgement weight, 28 and 43 % adult female and male mortality rate, respectively	[98–101]
Troponin I-like protein	Isolated from *H. longicornis* and induced specific antibody responses in both rabbits and mice. Located in muscle, cuticle, gut, and salivary glands	Vaccination of mice and rabbits resulted in significant extension of feeding times for larvae and adult ticks, low larval engorgement rates, and reduction in egg weights	[102–104]
Vitellin	Most abundant yolk protein of *R. microplus* eggs. Antibodies raised to vitellin also recognized a 200-kDa polypeptide in the hemolymph of adult tick	In its native form, induced an immune response protecting sheep against infestation with *B. microplus*	[105]
Voraxin	Engorgement factor	Rabbits immunized against the *A. hebraeum* voraxin, engorgement was seen to be reduced by 74 %, while all ticks fed on control rabbits fully engorged	[106]
Glutathione S-transferases	Glutathione S-transferases (GST) are a family of enzymes involved in detoxification of xenobiotics and endogenous compounds, and they have been identified in a number of tick species	Recombinant *H. longicornis* GST-Hl as an anti-tick vaccine candidate has been evaluated in cattle for cross-protection against *R. microplus* where an overall vaccine efficacy of 57 % was shown against *R. microplus*	[107–111]
RIM36	Isolated from *R. appendiculatus*, and it is present in salivary glands (type III salivary gland acini) and the cement cone	Immunodominant, under natural infestation vaccination of cattle, did not induce a clear protective effect although strong antibody responses were induced	[112–114]

(continued)

Table 17.2 (continued)

Antigen	Location and function	Effect of vaccination	References
HL34	Saliva protein identified in *H. longicornis* through tick saliva immune rabbit serum immunoscreening of an adult tick cDNA library	Vaccination of rabbits resulted in increased morbidity and mortality in nymphal and adult stages of *H. longicornis* although the effect did not reach statistical significance	[115]
p29	29-kDa extracellular matrix-like protein from *H. longicornis* is thought to be a component of the cement cone	Recombinant p29 immunization of rabbits resulted in mortality rates of 40 and 50 % of *H. longicornis* larvae and nymphs, respectively. Reduction in engorgement weight of adult female ticks of 17 % was noted	[116]
Histamine-binding protein	*Identified in R. appendiculatus and has a role in itch response*	No anti-tick effect in multivalent vaccination. Mild transmission blocking effect	[117]
Male immunoglobulin-binding protein	*Identified in R. appendiculatus*	Vaccination of guinea pigs resulted in slight female feeding impairment	[24]
β-*N*-Acetylhexosaminidase	Identified in *R. microplus*	Direct injection of rabbit polyclonal antibodies into replete females resulted in a 26 % reduction in oviposition. No evaluation as a recombinant vaccine	[118, 122]
5′-Nucleotidase	Identified in *R. microplus*	Inactive, truncated form of the protein produced a significant effect in sheep only where high antibody titers were induced. No effect in cattle	[119, 120]
Calreticulin	Identified in *A. americanum*, *R. microplus*	Poorly Immunogenic in cattle, no effect	[121]
Yolk pro-Cathepsin	Identified in *R. microplus*	Antigenic in cattle, vaccination resulted in a protection rate of 25 %	[123–126]

17.3.5 Dual-Action Anti-tick Vaccines

A dual-action anti-tick vaccine candidate represents a novel concept in that exposed antigen shares antibody binding epitopes with a concealed antigen. The advantage of this group of antigens lies in the fact that natural boosting occurs through exposed epitopes while targeting concealed antigens. The best studied of these dual-action candidates is the 64P protein also known as TRP64 and 64TRP. The protein identified in *R. appendiculatus* is 15 kDa and appears to form part of the cement cone [66]. Cross-reactive epitopes were found within the salivary glands, hemolymph, midgut of adult females, and whole body extracts for larvae and nymph *R. appendiculatus* [66]. One of the striking features of this antigen is that vaccination of truncated versions affected different stages of ticks [66]. Vaccination-mediated effects manifested as engorgement weight and egg mass reduction and direct tick mortality [66]. Inflammatory and adaptive immune responses induced by vaccination with TRP64 disrupt tick feeding. Where feeding does occur, antibodies taken up bind to cross-reactive midgut epitopes and rupture of the tick midgut and death of engorged ticks occur [66]. A broad, cross-protective effect was seen against *I. ricinus*, *R. sanguineus*, *A. variegatum*, and *R. microplus*. This cross-reaction may be based on strong epitope conservation in the different tick species to maintain molecular mimicry of host collagen and keratin [66–68], and natural boosting of antibody titers clearly occurs after tick infestation [67].

While anti-tick effects have been demonstrated in mice and guinea pigs [66, 67], it failed to show an effect in rabbits using a baculovirus-prepared antigen [64]. Furthermore, no tick effect could be established using a bacterial-expressed TRP64 as part of a multivalent vaccine in cattle. The potential of TRP 64 as anti-tick vaccine candidate was substantiated after transmission blocking of tick-borne encephalitis virus to susceptible mice by *I. ricinus* ticks. The protective effect of the 64TRP vaccine was comparable to that of a single dose of a commercial tick-borne encephalitis vaccine [69].

During the uptake of the blood meal, the amount of iron increases dramatically in the tick gut where tick ferritins act as iron-storage proteins. Ferritin 2 (Fer2) is a gut-specific protein secreted into the tick hemolymph where it acts as an iron transporter. It is expressed in all tick developmental stages, and silencing by RNAi of Fer2 has an adverse impact on tick feeding, oviposition, and larvae hatching [70].

Vaccination of cattle with recombinant homologues of Fer2 in *Ixodes ricinus* and *R. microplus* showed excellent control of *R. ricinus* and *R. microplus* and *R. annulatus* for the *R. microplus* homologues [71]. Notably, the vaccine efficacy of recombinant Fer2 was comparable to the benchmark obtained with Bm86 vaccination against *R. microplus* [71].

An antigen that received much attention is subolesin. Initially termed 4D8, the antigen was first discovered in *Ixodes scapularis* [72], but it has been characterized in several other tick species [73–75]. Targeted reduction of subolesin mRNA using RNAi resulted in degeneration of tick guts, salivary glands, reproductive tissues, and embryos [74–78]. Immunization of cattle with recombinant subolesin resulted in reduced *R. microplus* survival and reproductive capacity [79, 81]. Additionally, subolesin was found to control tick gene expression and impact the tick innate immune response to pathogens reducing tick infection by tick-borne diseases *Anaplasma marginale*, *Anaplasma phagocytophilum*, and *Babesia bigemina* [77, 80–86]. Most recently, immunogenic peptides of subolesin were fused to the *A. marginale* MSP1a N-terminal region, named SUB-MSP1a [30, 59, 77, 87]. Vaccination of cattle showed high efficacy for control of *R. microplus* and *R. annulatus* through negative impact on tick reproduction and egg fertility [87].

17.4 Transmission Blocking Anti-tick Vaccines

An anti-tick vaccine able to reduce or block transmission of pathogens from the tick vector to the vertebrate host would aid in TBD control. Transmission blocking vaccines would be ideal in allowing endemic stability development by reducing clinical disease incidence in vaccinated herds. It is well known that saliva aids the transmission of many tick-borne diseases. Saliva-activated transmission (SAT) is termed as "the indirect promotion of tick-borne pathogen transmission via the actions of bioactive tick saliva molecules on the vertebrate host" [88]. *Ixodes scapularis* salivary gland protein P11 facilitates migration of *Anaplasma phagocytophilum* from the tick gut to salivary glands [89]. The rate of in vitro infection of lymphocytes by *Theileria parva* is increased in the presence of *R. appendiculatus* salivary gland extracts [90]. Targeting these SAT components seems to be ideal for transmission blocking; however, more research is required to identify suitable nonredundant candidates.

17.4.1 The Future of Anti-tick Vaccine Development

The ongoing genome projects of several tick species will provide the community with a wealth of potential novel candidates to be tested as anti-tick vaccines [17, 21]. The use of RNAi experiments can help to functionally prioritize candidates that will be taken further for the study through genetic manipulation of ticks [22]. Ideally, future anti-tick vaccines should simultaneously target a range of tick species since several tick species may co-feed on livestock. A broad action anti-tick vaccine may be developed by identification of antigens with common, conserved, and essential functions

across several tick species or by incorporation of multiple antigens into a cocktail.

After the identification of novel candidates, evaluation in the suitable animal model is of paramount importance. Testing of candidates in small animal models is accessible to many research laboratories around the world. Large animal facilities handling livestock experiments however are expensive and rare. Additionally, for many ticks and TBD, appropriate in vivo challenge models have not been developed yet. However, it has been shown several times that sera generated from tick immune animals through repeated tick infestation detect different spectra of tick antigens depending on the animal species used. This difference in immune-dominance of tick components is most likely a consequence of long-term host-pathogen coevolution [23].

One clear example is seen when *Rhipicephalus sanguineus* ticks are fed on nonnatural hosts like guinea pigs. Resistance to tick infestation is developed even after a low number of tick bites manifested as high tick mortality, reduced female tick weight, and reproductive capacity [91]. In contrast, when *R. sanguineus* ticks are fed on dogs (their natural host), the reaction is significantly reduced and mainly restricted to immediate inflammatory responses in the skin with a reduced delayed hypersensitivity response [91]. Molecules present in tick saliva are thought to inhibit the host immune response, and in nonhost species, repeated tick exposure results in immune responses not present in the natural host systems [23]. When testing the identical vaccine candidates for efficacy in different host-pathogen interaction, systems conflicting results are often observed. Therefore, evaluation of novel candidates should be carried out at an early developmental step in the relevant natural host.

The commercialization of novel anti-tick vaccines should also take into account the lessons learned from Bm86 development [20]. Despite being commercially available, the vaccine would not be deemed a commercial success. The only two commercial products based on Bm86, Gavac and TickGARD are either limited to used in Latin America or removed from the market, respectively. This is striking for a product that targets a highly economically important tick species with a global distribution. Expectations regarding the performance of an anti-tick vaccine should be explained thoroughly to consumers. Since the eradication of distinct tick species from the globe is unrealistic, anti-tick vaccines will be most likely play a part in integrated pest control strategies. These strategies have shown to reduce both tick load and TBD transmission and have the potential to fulfill farmer and government requirement for disease control [20, 92, 93]. Early integration of testing anti-tick vaccines together with acaricide treatment schedules will probably pave the way for efficient commercialization of novel products in conjunction with better farmer product information.

In conclusion, the need for novel methods of tick and TBD control is growing, and there seems to be acceptance of farmers willing to use anti-tick vaccination [94]. Development of novel commercial anti-tick vaccine products is beyond the possibilities of most academic institutions, and novel public-private partnerships could speed up substantially the establishment of sustainable, cheap, and environmentally friendly tick and TBD control strategies globally [17].

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Development of Safe and Efficacious Bluetongue Virus Vaccines

18

Polly Roy and Meredith Stewart

Contents

Abstract

Bluetongue virus (BTV) infection results in an agriculturally important disease of livestock. Traditionally BTV outbreaks were controlled by the use of attenuated live virus vaccines. However recent BTV outbreaks in Europe had been controlled by killed virus vaccine. These vaccines are not DIVA-compliant (unable to differentiate between infected and vaccinated animals). This review summarises the current efforts in development of DIVA-compliant subunit vaccines, with emphasis of virus-like particles as improved bluetongue vaccines.

Although current commercial vaccines that have been used to control virus spread in Europe are mainly based on inactivated viruses, these are not DIVA compliant. Thus, this has lead to many new approaches in order to develop a DIVA-compliant vaccine with accompanying tests. This review describes the different technologies, with emphasis of virus-like particles as improved bluetongue vaccines.

18.1 Introduction

Bluetongue (BT) has been recognized as a viral disease of agriculturally important livestock for more than 100 years. In the eighteenth century, BT disease was observed in domestic ruminants, mainly sheep and less frequently cattle, goats,

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and buffalo, as well as in wild ruminants (e.g., blesbuck, deer, elk, and antelope) in the southern regions of Africa. BT disease was confined to Africa for many decades with the first confirmed outbreak outside of Africa occurring in Cyprus in 1924. This was followed by further outbreaks in Cyprus between 1943 and 1944 with ~2,500 sheep dead and with a mortality rate reaching 70 % [1]. There was then no reported incidence of BT in Europe until 1956 with a major epizootic beginning in Portugal and extending along the Iberian peninsula into Spain resulting in the death of >180,000 sheep [2]. Outbreaks of BT disease in the Middle East, south east Asia, southern Europe, and the United States in the early 1940s and 1950s led to its description as an emerging disease [3].

An important factor in the spread and emergence of bluetongue virus (BTV), the causative agent of BT, worldwide, is the availability of suitable vectors, usually biting midges of the *Culicoides* species. Virus isolates, from 26 different serotypes (BTV-1, -2, -3, etc.), have been made in tropical, semitropical, and temperate zones of the world, including North and South America, Australia, southern Europe, Israel, Africa, and Southeast Asia. In 1998, significant outbreaks of BT occurred in Europe, initially in Greece and then in many other Mediterranean countries causing the death of over a million sheep by 2005 [4]. In 2006 and 2007, BTV-8 emerged in north western Europe eventually expanding into the UK, Denmark, and the Czech Republic. This outbreak was extensive, >1.8 million animals were infected [5], due to the potential ability of this BTV isolate to overwintering.

The virulence of the virus was such that even infected cattle, which generally do not show any visual disease symptoms, displayed classic BT disease resulting in animal fatality [6, 7]. The factors associated with the virulence between strains of BTV in different animal hosts have yet to be deduced. The spread of BTV in Europe was controlled by vaccination using an inactivated virus preparation but not the attenuated live virus vaccines which are commonly used in Africa and many other countries (see Sect. 18.6). Due to these recent outbreaks of various BTV serotypes in worldwide, there has been a number of new approaches toward the development of a bluetongue virus vaccines including recombinant protein technology. These vaccines are being developed to improve the safety and breadth of protection afforded. This review briefly examines the disease, virus, and transmission and describes some of the technologies involved in improved bluetongue vaccines focusing on the use and development of virus-like particles (VLP).

18.2 Disease and Pathogenesis

BT is noncontagious disease of livestock. Traditionally the disease was observed in sheep with clinical manifestation ranging from subclinical infection to high morbidity and mortality. The clinical outcome of BTV infection is dependent on virus strain and host species. In particular, European fine wool and mutton breeds are highly susceptible to BTV infection and disease [8]. Other ruminants including cattle, goats, deer, and camelids (camels, llamas, alpacas, guanaco, and vicuña) are generally asymptomatic but may display milder clinical signs. Generally 5–10 % of BTV-infected cattle develop mild to severe disease [9], which was also observed in the recent BTV-8 outbreak in Europe [10]. Due to the asymptomatic or mild response to infection, these animals can potentially act as reservoirs of infectious virus. This is due, in part, to the prolonged viremia observed in these animals that enables the insect vectors to circulate and transmit the infectious virus from animal to animal. In particular, cattle and calves born can be viremic for up to 3 years postinfection [9].

In 1905, the first detailed description of the BT was published [11] with the disease referred to as malarial catarrhal fever [12]. Distinctive lesions in the mouth of the infected animals with a dark blue tongue were the characteristic symptoms; however, the swollen cyanotic tongue, which gave rise to the name bluetongue, is a quite rare clinical outcome. Other commonly observed clinical signs include pyrexia, tachypnea, and

lethargy. The pathophysiologic features associated with BT resemble many other viral hemorrhagic fevers including those observed in African horse sickness virus (AHSV) and epizootic hemorrhagic disease virus (EHDV), the two related orbiviruses [13–15]. Viremia is usually detectable around 3–5 days postinfection of BTV in sheep and results in widespread edema and hemorrhages of lymph nodes, lungs, heart, and skeletal muscles [16]. In addition, necrosis of the mucosal surfaces in the oral-nasal and alimentary systems may also be observed during infection. The lungs are especially susceptible to permeability disorders of the vasculature induced by BTV [17, 18]. Both BTV and the related EHDV can cross the placenta [19, 20], and infection of pregnant cattle and sheep can result in fetal infection, abortion, or congenital anomalies [21–23].

The cellular tropism of BTV infection in natural infection of ruminants includes mononuclear phagocytic, dendritic cells (DC) and endothelial cells (EC) [17, 18, 24]. Conventional dendritic cells are the primarily targets of BTV infection and are responsible for dissemination of the virus from the skin to the draining lymph nodes and are critical to the pathogenesis of BT [18, 24–26]. BTV infection causes hemorrhagic disease in ruminants and induces cell death, predominantly due to apoptosis rather than necrosis, and a correlation may exist between cytopathic effects (CPE), apoptosis of host cells, and pathogenesis [27]. In BTV-infected mammalian cells, both the biochemical and morphologic hallmarks of apoptosis are observed within 24 h of infection, including activation of NF-κB, caspase-3, DNA fragmentation, membrane blebbing, and cellular shrinkage. Those changes are not observed in insect (*Culicoides* species) cells although these cells are efficient for BTV replication [28].

18.3 Bluetongue Virus

BTV belongs to the *Orbivirus* genus of the *Reoviridae* family. BTV virions are architecturally complex structures composed of seven discrete proteins (Fig. 18.1). The particle is non-enveloped icosahedral capsid that is organized into three concentric protein shells, subcore, core, and outer capsid layers [29]. The genome consisting of ten linear dsRNA molecules is encapsidated within the virion [30, 31] (Fig. 18.1). The segments are classified in three size classes: large (S1-3), medium (S4-6), and small (S7-10), and all have a conserved pentanucleotide sequence at the 5′ ends and a different conserved pentanucleotide sequence at the 3′ ends [30]. The segments encode 12 proteins and seven structural (VP1-VP7; Fig. 18.1) and four nonstructural (NS1, NS2, NS3/NS3A, and NS4) proteins [30, 32, 33].

BTV enters into the susceptible cells through endocytic pathway [34–36] (Fig. 18.2). The outer capsid composed of VP2 and VP5, both of which attach to the underlying core surface layer, VP7 [37, 38]. VP2 and VP5 are involved in cell

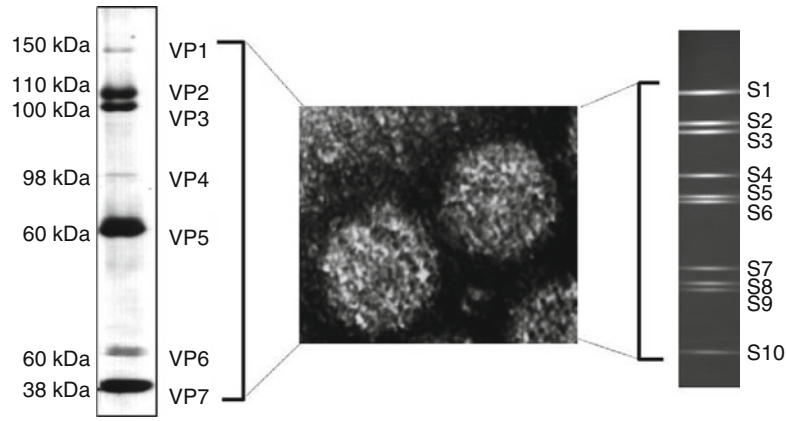

Fig. 18.1 BTV particle consists of seven proteins and a genome of ten discrete double-stranded RNA molecules. SDS-PAGE of purified virus particles (*left*), electron micrograph of BTV particles (*middle*), and non-denaturing PAGE of BTV dsRNA genome (*right*)

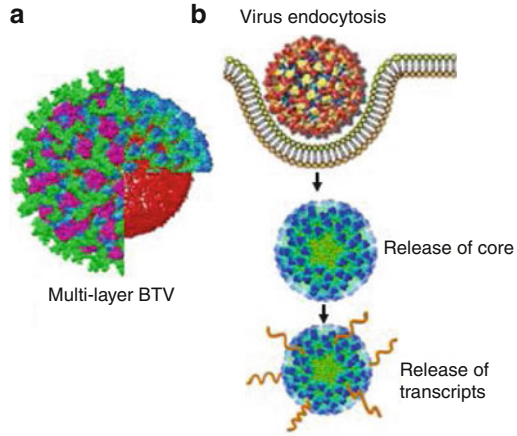

Fig. 18.2 (**a**) Schematic of BTV virus particle. The outer capsid consists of VP2 (*green*) and VP5 (*magenta*), the inner core of VP7 (*blue* and *green*), and the VP3 subcore (*red*). (**b**) Entry of the virus particle via endocytosis to release the core in to cytoplasm. The core becomes transcriptionally active upon entry into the cytoplasm to release virus transcripts

attachment and membrane penetration during the initial stages of infection [34, 36]. The attachment protein, VP2, is the most variable viral protein of BTV; it confers serotype specificity and possesses the neutralization epitopes [39–46]. Three-dimensional structural analysis by high-resolution amend to cryo-electron microscopy has revealed that VP2 has an exposed receptor-binding tip domain and an internal sialic acid binding site; both are necessary for cell surface binding activity [38]. VP5 acts as a fusion-like protein at low pH of endosome enabling the virus to penetrate the endosomal membrane [35, 47].

Shortly after entry, the outer capsid is removed to release a transcriptionally active core particle into the cytosol (Fig. 18.2). The core is made up of two major proteins, VP3 and VP7, and three minor enzymatic proteins, VP1, VP4, and VP6, in addition to ten segments of dsRNA genome. VP3 forms the icosahedral subcore structure of the particle with the three minor proteins located at the fivefold axis [48, 49]. VP7, the major core protein, is the group-specific antigen [39] and has integrin receptors binding sites for entry into insect vector cells [50]. The transcriptionally active core particle upon release into the cytosol continuously synthesizes and extrudes multiple copies of 10-capped transcripts into the host cell cytoplasm. These transcripts (mRNAs) are not only responsible for viral protein synthesis but also function as templates for complementary negative-strand RNA synthesis. However, ssRNA segments, not dsRNAs, are packaged by the assembling nascent progeny core particle, prior to serving as templates for genomic dsRNA synthesis [51]. In the virus-infected cells, the core assembly takes place within virus inclusion bodies (VIBs) formed by the nonstructural NS2 [30, 52–54]. All three minor enzymatic proteins, viral transcripts, and the two major structural proteins, VP3 and VP7, are recruited by NS2 allowing their assembly. These newly generated cores then egress from the VIBs, and the outer capsid proteins VP2 and VP5 are assembled onto the core particles as they are transported, along the lipid rafts in association with NS3, to the cellular membrane [55, 56]. NS3/NS3A are involved both in trafficking and release of the assembled particle [57]. The nonstructural protein, NS1, forms tubular structures in infected cells and is one of the most abundantly expressed proteins [58]. Although the precise function of tubules is not known, recently it has been shown to upregulate the expression of viral proteins [59].

18.4 Transmission and Distribution

Bluetongue disease is endemic in many tropical and subtropical regions including North (USA) and South America, Australia, Southeast Asia, and the African continent. However, in the last 15 years both the distribution and emergence of new serotypes have changed. This change is typified in mainland Europe with increased frequency of outbreaks of different serotypes in countries along the Mediterranean basin and the first outbreak of BTV in northern Europe, which reached as far north as Norway. Molecular epidemiology studies showed that of the 24 serotypes, eight different serotypes (BTV-1, -2, -4, -6, -8, -9, -11, and -16) have been introduced into mainland Europe since 1998 [60]. Of these, BTV-8 caused the most severe disease in northern European

sheep and cattle. Furthermore, a new serotype, BTV-25, was identified in Europe [61] and another, BTV-26, in Kuwait [62].

BTV is transmitted from animal to animal via blood feeding midges (*Culicoides* species) [30, 63]. Climate changes (temperature, rainfall) may be responsible for increased distribution and size of insect populations of *Culicoides imicola*, the vector responsible for facilitating spread of the virus in Africa and Asia. This has coincided with the emergence of BTV in Europe and of new serotypes in the USA. Furthermore, novel *Culicoides* spp. (*Culicoides chiopterus*, *C. dewulffi*, *C. obsoletus*, *C. scotius*, and *C. pulicaris*) have been identified in Europe that are able to transmit the virus [60, 64]. The enlarged distribution of the vector populations and virus transmission by novel species of *Culicoides*, which are abundant in central and northern Europe, have allowed the geographic range of BTV to remain extended into Europe [60, 64].

Thus, BTV now represents an ongoing threat to livestock in all European countries. In the Americas, the endemic serotypes BTV-10, -11, -13 are transmitted by *C. sonorensis*, while BTV-2 transmission is restricted to *C. insignis* [65–67]. Since 1998, ten serotypes -1, -3, -5, -6, -9, -12, -14, -19, -22, and -24 have also been isolated in the southeastern USA, generally restricted to the Caribbean/Central American regions [67–70]. The transmission of these serotypes has also highlighted the possibility that other *Culicoides* spp. (*C. pusillus*, *C. furens*, *C. filarifer*, and *C. trilineatus*) are involved in changing the distribution and emergence of new serotypes [67, 69].

18.5 BTV Diagnostic

Bluetongue virus and infection of ruminants is a significant issue associated with international trade restriction and animal movement. The 2007 outbreak of BTV-8 was estimated to result in economic losses up to $1.4 billion to the French economy, mainly due to the infection of and restricted movement of cattle [71]. This was due partly in the virus infection resulting in morbidity which ranged between 0 and 100 % with an average of 20 % in sheep [72] and 10 % in cattle [72] and also due to the prolonged viremia in cattle, which are capable of infecting *Culicoides* spp. and spreading the virus to other ruminants including sheep and goats. In the USA alone, the losses associated with testing and restricted movement of cattle costs $130 million annually [71]. Therefore, the OIE has a number of internationally prescribed tests to isolate, identify, and serologically test animals for BTV infection.

In order to identify BTV infection, a number of virus isolation procedures are utilized, including inoculation of sheep, isolation of *in vitro* cell culture using insect cell lines, e.g., C6/36, and the most sensitive method the inoculation of embryonated chicken eggs (ECE; [73]). The serotype of the virus is determined by virus neutralization. Plaque reduction assays, which result in a fixed reduction of up to 90 %, are the most reliable method in determining serotype. Other methods including microtiter neutralization (50 % of 50–100 $TCID_{50}$), immunofluorescence inhibition, and reverse transcription polymerase chain reaction (RT-PCR) with specific primers and sequence analysis are becoming common practice due to ease of use. RT-PCR is now recognized as a prescribed international procedure to test for BTV infection [7].

These methods are used alongside serological tests to determine the BTV status of a region. Serological methods that determine the serotype specificity of antibodies in sera are generally more complex, expensive, and time consuming, because they assess whether the sera inhibit the infectivity of panels of known virus serotypes in neutralization test. Therefore competitive enzyme-linked immunosorbent assay (ELISA) designed to specifically detect BTV group-specific antigen, VP7, is recommended [7]. This utilizes a specific monoclonal antibody to the highly conserved core protein VP7 of BTV to prevent cross-reactivity with VP7 of the closely related epizootic hemorrhage disease virus (EHDV). An alternative test for international trade is the agar gel immunodiffusion (AGID; [74]) test, which replaced the complement fixation (CF) assay used to detect

BTV antibodies, although the CF test is still used in some countries [7].

18.6 Bluetongue Virus Vaccines

18.6.1 Attenuated Virus Vaccines

Vaccination strategies in different countries were developed according to their individual policies, the geographic distribution of the incurring serotypes of BTV, and the availability of appropriate vaccines. In South Africa, the country of BTV origin, the live attenuated virus vaccines have been used for over 40 years and are known to induce an effective and lasting immunity [75–77]. These live attenuated vaccines have also been used in Italy as part of the Sardinian BT vaccination campaign with a combination of monovalent BTV-2, BTV-4, and BTV-16 [78]. Due to the high number of circulating serotypes, live modified vaccines are often administered as a multivalent vaccine particularly in South Africa. This is due to the serotype-specific protection afforded by the neutralizing epitopes on the outer capsid protein, VP2.

Live attenuated BTV vaccines were developed by serial passage in embryonated chicken eggs. Despite their success in endemic areas, the use of attenuated virus vaccines has some drawbacks. For example, teratological effects as a result of vaccination with attenuated BTV are well documented [79, 80]. Adverse reactions to attenuated virus include depressed milk production in lactating sheep and abortion/embryonic death and teratogenesis in offspring from pregnant females that are vaccinated during the first half of gestation [81]. Furthermore the stimulation of a strong protective antibody response by these attenuated vaccines is directly correlated with their ability to replicate in the vaccinated host [7].

The viremia observed following vaccination in both laboratory experiments and in the field has been sufficient for the vaccine strain to be transmitted to biting midges [80, 82]. This leads to a greater potential risk for spread by vectors, with eventual reversion to virulence and/or reassortment of vaccine virus genes with those of wild-type virus strains. The segmented nature of BTV genome allows for reassortment of genes between strains that coinfect the same animal to be a possibility [83]. This has been observed in the field in Italy in 2002 with BTV-16 strain circulating found to be a reassortment between BTV-2 and BTV-16 live attenuated virus vaccine [84]. Attenuated virus for BTV may offer a route to control disease with protective efficacy afforded, but they are not suitable for a program designed to eradicate the disease in an area.

18.6.2 Inactivate Virus Vaccines

Recently, chemically inactivated BTV vaccines were used successfully for mass vaccination of sheep and cattle in many northern European countries during the BTV-8 and BTV-1 outbreaks. Inactivated vaccines are a significant advancement on the attenuated virus vaccines provided quality control is rigorously implemented and complete attenuation is achieved. These inactivated virus vaccines have been demonstrated to be safe and immunogenic in animals but are currently only available for a limited number of serotypes [85]. However, adverse reaction upon vaccination of field animals has been associated with some of these inactivated virus preparations, which had included mild BT symptoms [85–87].

It is noteworthy that during the BTV-8 outbreak in 2007, vaccination of sheep and cattle in Germany with the inactivated vaccine (BLUEVAC 8) was often associated with severe side effects including abortions, lameness, and bloody feces as well an increase in body temperature (Giese M, 2012, personal communication). This may be due to the agent used to inactivate the vaccines which include formalin, beta-propiolactone, binary ethylenimine and gamma radiation, or the different adjuvants [88–91] or viral proteins in the preparation causing a pathogenic effect. Other potential disadvantages of this type of vaccine is that production is highly costly due to large amounts of antigen required for each vaccination and the need for booster immunizations, as inactivated vaccines generally induce a

relatively transient immunity in comparison to live attenuated vaccines.

Importantly, the biggest limitation in the use of both the live attenuated and inactivated vaccines is the significant economic impact they have on agricultural trade. These vaccines do not allow the differentiation of naturally infected versus vaccinated animals (DIVA) and therefore are not DIVA compliant, and restriction in animal trades and movements are imposed. Furthermore, the DIVA compliance of these vaccines is hampered by a lack of commercially available tests to allow the differentiation between vaccinated and naturally infected animals. This is because inactivated and live virus vaccine preparations are not free of all viral components and thus do not allow a serological differentiation between infected versus vaccinated animals. Further, due to the presence of genomic RNA after vaccination [92] and short-term BTV viremia in sheep and long-term viremia in cattle, a genetic DIVA is not always feasible [7, 23, 93, 94].

18.6.3 Recombinant Protein-Based Vaccines

A number of recombinant protein-based technologies have been investigated including baculovirus-based [43, 77, 95, 96], poxvirus-based [97–99], and herpesvirus-based [100] expression systems in order to be DIVA compliant and to reduce the potential adverse effects associated with inactivated and attenuated virus vaccines. For the most part, recombinant proteins have been assessed in sheep in experimental vaccination challenge trial and shown to afford protection efficacy but have yet to be commercialized [75, 77, 95]. The recombinant protein-based vaccines for BTV are based on the serotype determining outer capsid, VP2, ability to elicit protective immunity in vaccinated sheep [40, 41, 43]. This is due to VP2 alone possessing the multiple neutralizing epitopes [42, 46, 101]. These results clearly demonstrated that the neutralizing antibody response to the virus was directed at this protein [40, 41, 43, 95]. Later studies that combined both outer capsid proteins (VP2 + VP5) resulted in better protection than VP2 alone [43] in vaccinated sheep (Fig. 18.3). In addition, VP2-based vaccines have been developed using other live virus (i.e., poxvirus) vector systems, which have been designed to express the either a single or multiple proteins in animals upon vaccinations [98, 102].

18.6.4 Bluetongue Virus-like Particles (VLPs)

Baculovirus-expressed recombinant VP2 was shown to elicit serotype specific in vaccinated

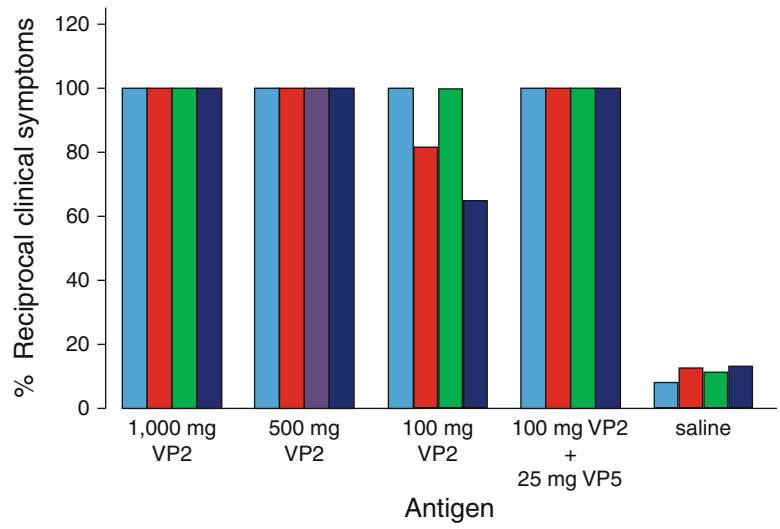

Fig. 18.3 Effect of BTV antigen dose and composition on protection of sheep from clinical signs of BTV post-challenge with virulent virus. Animals were vaccinated with different concentration of VP2 and VP2 + VP5 combination, and clinical response to a virulent virus challenge was determined

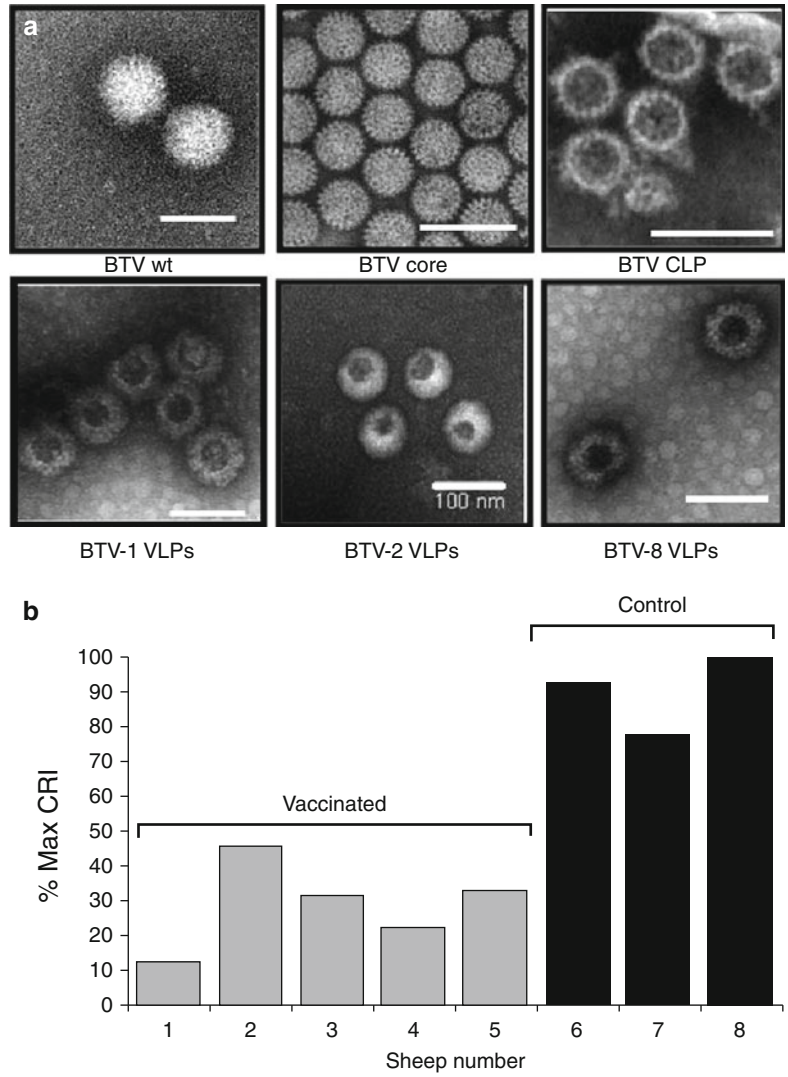

Fig. 18.4 (**a**) Electron micrograph of BTV virions, cores, core-like particles, and viruslike particles. (**b**) Vaccination of sheep with CLPs decreases clinical scores of BT disease in comparison to control animals

sheep and was afforded complete protection against a homologous virulent virus challenge [103–105]. Vaccination of recombinant VP2 along with VP5 reduced the amount of protein required to afford protection against a virus challenge [43]. This leads to the observation that presenting VP2 in the appropriate conformation would reduce the amount of protein required for vaccination [96]. This was taken to a logical conclusion and the two BTV outer capsid proteins together with scaffolding core proteins, VP3 and VP7, were expressed by recombinant baculovirus expression system to generate double-capsid virus-like particles (VLPs) that mimicked the virus structure but did not contain any of the viral genome or enzymatic proteins [106] (Fig. 18.4a). This was the first example that the assembly of BTV particles did not require the presence of NS1 or NS2.

3D reconstruction of the BTV VLP demonstrated that it was structurally identical to the virion [49]. The VLPs had the characteristic icosahedral structure (~90 nm) with the four recombinant proteins in the same position and ratios as the native protein. Further research demonstrated that it was possible to co-express the outer capsid proteins from different serotypes onto a highly conserved inner core [107,

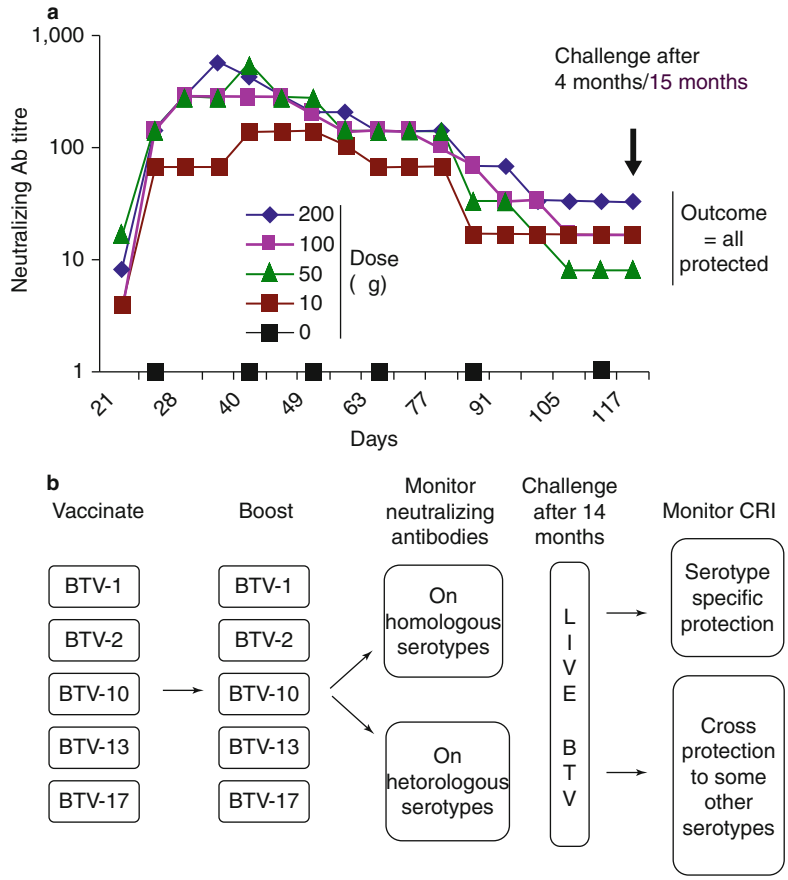

Fig. 18.5 (**a**) Neutralizing antibodies titers of sheep with different concentrations of BTV VLP. Long-term clinical and virological protection against BTV challenge at 4 and 15 months postvaccination even after neutralizing antibodies had reduced (Adapted from Roy et al. [103]). (**b**) Schematic representation of VLP vaccination challenge trial in sheep. Sheep were vaccinated with VLP from different serotypes and results of challenge with a virulent homologous and heterologous virus (Adapted from Roy et al. [103])

108]. Vaccination of sheep with preparations that with core-like particles (CLPs) in the absence of VP2 and VP5 showed low level of protection against virulent virus challenges with slight fever and some pathological changes associated with BT [76, 105] (Fig. 18.4b). The partial protection conferred by the CLPs may be, in part, due to cell-mediated immune (CMI) response directed against antigenic sites on the inner core proteins. Indeed, each of the structural and nonstructural BTV proteins has been shown to induce a CTL response [99]. Vaccination of animals with VP7 alone or in combination with other BTV proteins had been demonstrated to elicit a cell-mediated immune response, and it is postulated that CMI response may reduce BTV viremia [99, 109, 110], although the reduction in BTV viremia is unlikely to stop virus transmission [105].

More recently, both CD8 and CD4 epitopes have been mapped within VP7 sequence [111]. Animals showed strong group-specific VP7 antibody responses but no neutralizing antibodies.

Among all of these potential subunit vaccines, BTV VLPs that are formed by co-expression of four BTV structural proteins, displaying each protein in correct configurations, have been tested in animals most extensively and shown to be most promising. In each study, VLPs were proven to be excellent immunogens both for preventing disease in sheep and preventing detectable virus replication in small-scale experiment trials (Fig. 18.5b) [77, 96, 103–105, 112, 113].

The success of BTV VLP is due to its ability to elicit a strong neutralizing antibody response in conjugation with the CMI, which also plays role in recovery from infection and protection against reinfection [109, 110]. Early studies in South Africa demonstrated animals were protected against a BTV challenge when vaccinated with as little as ~10 μg of VLP (of which VP2 is

Table 18.1 Summary of the VLP vaccination trial in European sheep breeds

Vaccine		NAb titer (pre-challenge)	Challenge virus	Ct Day 7
Single	BTV-1	>128	BTV-1	40
		128	BTV-1	40
Single	BTV-2	32–64 (10 μg)	BTV-2	40
		128 (20 μg)	BTV-2	40
Single	BTV-8	>128	BTV-2	40
Cocktail	BTV-1	64–128	BTV-1	40
	BTV-4	>16	BTV-4	40
Cocktail	BTV-1	>128		
	BTV-2	32–128		
	BTV-8	64–128	BTV-8	40

Sheep were vaccinated with either BTV VLP vaccine consisting of either a single serotype or multiple serotypes. Neutralization antibody titers for each serotype was determined pre-challenge for each serotype in the vaccine preparation. Animals were then challenged with a virulent virus challenge, and Ct values detecting replicating dsRNA are given for 7 days postinfection. Ct values of 40 are considered to be negative for virus replication

only ~1–2 μg; Fig. 18.5a) [96]. Extensive clinical trials in Europe using susceptible sheep breeds (i.e., Merino, pre-Alps, Karagouniko crossbred) have been carried out in recent years with BTV VLPs representing different European serotypes (Table 18.1) [96, 103–105, 113]. These trials have also assessed VLPs delivered singly or in cocktail as well as the evolutionary lineage of the virus strains in order to assess their potential for commercialization.

The influence of evolutionary divergence (eastern and western topotypes [114–117]) on vaccine development clearly highlighted that the development of serotype-specific neutralizing antibodies was more critical than the topotype of the circulating strain [105]. This suggests that isolate variation or evolutionary distance of the strain was not a critical factor for BTV vaccines [105]. Furthermore, delivery of VLPs as a cocktail of different serotypes does not influence or affect the ability of each serotype to raise a serotype-specific neutralizing antibody response [43, 96, 112, 113] or interfere with the protective efficacy of the vaccine when animals were challenged (Table 18.1) [43, 113]. These findings highlighted that multiple serotype vaccines could be developed using known serotypes rather than generating new vaccines for each BTV outbreak.

VLPs have been highly effective in sheep trials, and since they do not contain any of the virus nonstructural proteins, it is possible to distinguish between vaccinated animals and those that are infected with the virus, thus addressing one of the major problems with current vaccines. A further advantage of the VLP approach is that, since the immunogen is entirely protein based, there is no genetically modified virus component that requires expression of foreign genes in the vaccinated animal.

18.6.5 Designing Replication-Deficient BTV Strains as Vaccine Candidates

The reverse genetic (RG) system developed for BTV [118] has allowed direct introduction of mutations/deletions in the viral genome thereby the recovery of replication-defective virus strain using specific complementary cell lines [119, 120]. The replication-defective viruses lack the essential catalytic genes and are noninfectious in normal noncomplementary cells although can enter the susceptible cells where these particles are only able to undergo a single cycle of replication; this is due to the inability of the progeny virus to package dsRNA (Fig. 18.6). This means that these defective viruses should have all the benefits associated with robust immune response similar to live virus vaccines but no risk of infection. Further, the "core" elements, similar to VLPs, can be used for generating vaccines for new serotypes simply by substituting the outer capsid protein genes. For example, using the RG system, reassortants can be generated by transfecting the cells with the two RNA segments encoding VP2 and VP5 (e.g., BTV-8) together with the remaining eight segments that from the parental replication-defective virus strain of BTV-1 [120]. These defective viruses have been shown to be highly protective in sheep and cattle [120]. The ability to use the same backbone and

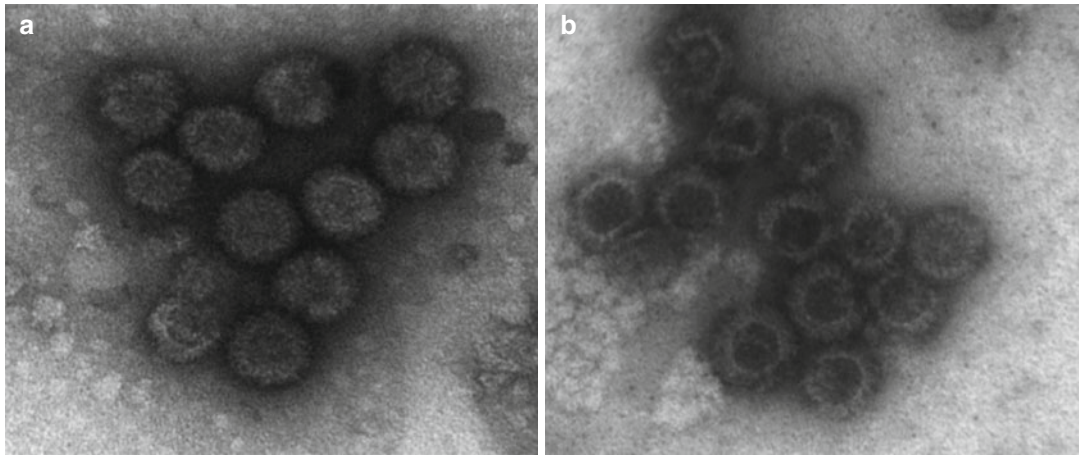

Fig. 18.6 BTV-1 VP6-deficient viruses lack viral genome. BTV-1 replication-deficient purified core particles. (**a**) Cores from BSR cells expressing VP6 *in trans* and (**b**) cores from wild-type BSR cells

to reassort only the VP2 and VP5 will enable the licensing of new vaccines to be undertaken rapidly. The RG system will also allow inclusion of marker sequence into the virus genome to differentiate the vaccine strains from wild-type viruses.

Conclusion

BT is an important agricultural disease that has significant economical impact. Although there are effective vaccines based on either killed or attenuated virus, vaccinated animals are subject to trade restrictions and potential health side effects. The development of the replication-deficient virus strains and VLPs offer two new potential DIVA-compliant vaccines that can be adopted to be used within the existing manufacturing technologies. The uptake of the technologies would be dependent on the specific trade and agricultural requirements and BTV status of the country. Thus vaccine adoption can be tailored to the needs of a country. The development of recombinant protein-based vaccines for BTV including the highly successfully baculovirus produced VLP immunogens are a step forward. These vaccines are easily amenable to the development and commercialization of DIVA tests that can be directed at any of the minor structural as well as the nonstructural proteins. This can be achieved simply and inexpensively as recombinant proteins for these proteins have been expressed and used in ELISA-based systems [121]. These immunological DIVA tests would be viable as VLP immunogen elicits a long-lasting immune detectable response (>24 months; [96]) in comparison to inactivated or killed vaccines (~12 months). For the killed and attenuated vaccines to be DIVA compliant, a number of different approaches would have to be undertaken including the removal of the nonstructural proteins, incorporation of specific immunological tags, or removal of epitopes, all of which would add to the cost of production of vaccine and of the DIVA test.

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Non-typhoidal Salmonellosis

19

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and María-Jesús Grilló

Contents

Abstract

Salmonella is a ubiquitous Gram-negative bacterium belonging to the *Enterobacteriaceae* family that can infect animals and humans, with gastroenteric and systemic symptomatology from moderate to severe. Animals can act as asymptomatic carriers that excrete *Salmonella* spp. intermittently in faeces and contaminate carcasses. At present, poultry and swine are recognised as the main sources of infection for humans. Control of human salmonellosis is based on sustainable biosafety and hygienic measures "*from farm to folk*" but efficient vaccines would contribute to avoid animal infections. Since no commercial vaccines are available, a wide variety of experimental work is carried out to test both non-living and live attenuated vaccines in animal models, using either subcellular components of *Salmonella* administered with adjuvants or live genetically modified bacteria lacking structural elements, essential metabolites or virulence genes. A special effort should be conducted to design effective vaccines antigenically tagged to allow distinguishing between infected and vaccinated animals.

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19.1 Pathogen

Salmonella is a Gram-negative, facultative anaerobic, motile, non-lactose fermenting bacterium that belongs to the *Enterobacteriaceae* family. This microorganism is frequently excreted in

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animal and human faeces, being thus ubiquitous and frequently found in sewage, farm effluents and any material subjected to faecal contamination.

Taxonomic classification of *Salmonella* has been complex and controversial due to the constantly increasing number of serotypes. Nowadays, *Salmonella* is classified in two species: *S. enterica* and *S. bongori* [1], and, in turn, *S. enterica* has been subdivided into six subspecies, according to their biochemical characteristics and susceptibility to bacteriophages [2]. These six subspecies were formerly named by roman numbers and currently substituted as follows:
Subspecies I: Subspecies *enterica*
Subspecies II: Subspecies *salamae*
Subspecies IIIa: Subspecies *arizonae*
Subspecies IIIb: Subspecies *diarizonae*
Subspecies IV: Subspecies *houtenae*
Subspecies VI: Subspecies *indica*

Subspecies I strains are usually isolated from humans and warm-blooded animals, while subspecies II, IIIa, IIIb, IV and VI strains and *S. bongori* are usually isolated from cold-blooded animals and the environment (rarely from humans). These six subspecies are subdivided in 51 serogroups (named either by alphabetic and/or numeric order) and more than 2,600 serotypes [3] (Table 19.1).

Classification into serotypes is carried out applying the Kauffmann-White scheme, according to characteristics of antigens: (1) somatic (O antigens) expressed by the lipopolysaccharide (LPS) O-chain; (2) flagellar (H antigens) expressed by flagellar proteins, motility, and first and second phase antigens expression; (3) capsular (Vi antigens) [2]. Accordingly, *Salmonella* strains are named orderly by the species, subspecies and the name of the serotype (if exists) or the complete antigenic *formulae*. For instance, *Salmonella enterica* subspecies *enterica* serotype Typhimurium can be also named as *Salmonella enterica* subspecies *enterica* serotype 1,4,[5],12:i:1,2. In practice (and herein), abbreviations can be "*S.*" (italics and capitals) followed by the name of the serotype in non-italics and with the first letter in capitals, e.g. *S.* Typhimurium.

Table 19.1 Classification of *Salmonella* spp. and number of serotypes identified

Species	Subspecies	Serogroup[a]	No. of serotypes
S. enterica			2,587
	enterica (I)	A-C4, D1, D2, E1-E4, F-Z, 51–54, 57, 67	1,547
	salamae (II)	B-C2, C4, D1-D3, E1, E2, F-Z, 51–53, 55–60, 65	513
	arizonae (IIIa)	F, G, I-L, O, P, R-Z, 51, 53, 56, 59, 62, 63	100
	diarizonae (IIIb)	C1, C3, C4, F-M, O, P, R-V, X-Z, 51–53, 57–61, 63, 65	341
	houtenae (IV)	C1, F, H-L, P, R-Z, 51, 53, 57	73
	indica (VI)	C1, F, H, K, S, W, Y, Z, 59	13
S. bongori (*V*)		D1, G, H, R, V, Y, 60, 61, 66	23

[a]Nomenclature by alphabetic (A–Z) or numeric (from 51 to 67) order. Serogroups C, D, Y and E are subdivided into numbers from 1 to 4

The strains most frequently isolated in clinical cases of human salmonellosis belong to serogroups A, B, C1, C2, D and E and to serotypes *S.* Enteritidis and *S.* Typhimurium [4]. Further typing, such as phage-typing and Pulsed Field Gel Electrophoresis (PFGE), can be assessed in order to classify field strains, particularly in case of outbreaks. Other characteristics of circulating *Salmonella* strains, as the antimicrobial resistance pattern, are of clinical importance to apply effective antimicrobial therapies in patients at risk and/or with complicated disease (see below).

19.2 Disease

Salmonellosis is one of the main zoonosis worldwide distributed, being an important foodborne disease in developed countries. In fact, in the USA, non-typhoid *Salmonella enterica* infections are the first cause of acute gastroenteritis,

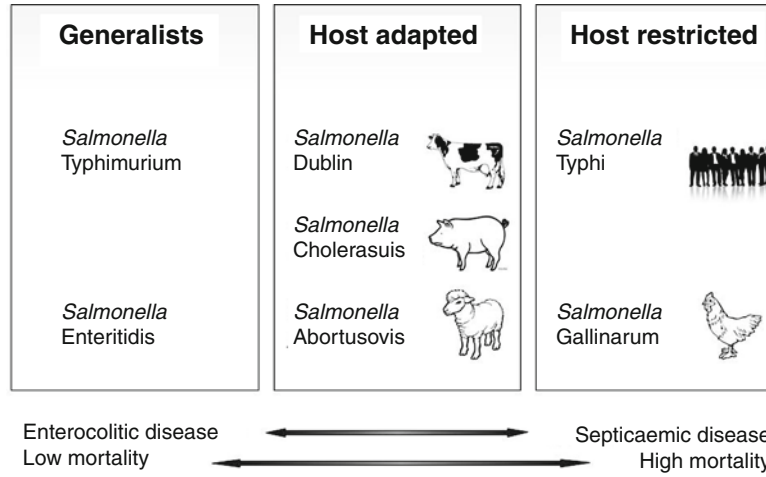

Fig. 19.1 Host adaptation in *Salmonella* infection and effects on clinical forms in the host

registering 1,027,561 human cases in 2011, from which 19,336 (1.9 %) cases required hospitalization and 378 (1.95 %) cases were lethal [5]. In the European Union (EU), salmonellosis is the second most frequent zoonosis, after campylobacteriosis, registering 99,020 human cases in 2010 [4]. During the last decade, a decreasing incidence of salmonellosis has been reported [4], probably as result of both control measures implemented in the food chain *from farm to fork* and public awareness on the importance of hygienic control measures against foodborne infections.

Among the over 2,600 serotypes of *Salmonella* species described, clinical manifestations and mortality differ according to both bacterium and host characteristics. All serotypes are considered potentially pathogenic to humans, with different degree of adaptation to the host [6] (Fig. 19.1). Thus, some serotypes such as *S*. Typhi, *S*. Paratyphi and *S*. Sendai cause severe systemic disease to humans. However, other serotypes are specifically adapted to animals, such as *S*. Choleraesuis to pigs, *S*. Dublin to cattle, *S*. Abortusovis to sheep and *S*. Gallinarum to poultry, and only occasionally affect to humans and cause only mild symptoms [7, 8]. Ubiquitous serotypes such as *S*. Typhimurium, *S*. Enteritidis and *S*. Infantis are the most recognized as zoonotic agents, affecting a wide range of animal species [9–11].

Poultry and swine asymptomatically infected, mainly by *S*. Enteritidis and *S*. Typhimurium, respectively, are considered the main sources of human *Salmonella* infections [4, 9], representing 75.6 % of total serotypes reported in human salmonellosis in the UE [6]. Other animals, such as wild birds, rodents, lizards or domestic turtles, asymptomatically infected by *Salmonella* may contribute to spread and be source of human infections [5, 12, 13].

Clinically, swine and poultry salmonellosis can produce septicaemic or enterocolitic forms (Fig. 19.1). The former is characterized by profuse diarrhoea, symptoms of systemic infection (fever, prostration, etc.) and, in absence of antimicrobial treatment, conclude with a high mortality rate. The enterocolitic form is characterized by symptoms of acute or chronic gastroenteritis, being diarrhoea the most common symptom. Animals submitted to treatment could recover from infection, eliminating the microorganism during months by faeces, for long-lasting periods, and acting as asymptomatic carriers [14]. Subclinical asymptomatic salmonellosis are the most frequent presentations in poultry and swine (the main sources of human infections), produced by a wide range of serotypes that infect tonsils, intestinal tract and mesenteric lymph nodes, and are excreted intermittently [14, 15]. While clinical forms are easily identifiable, asymptomatic carriers cannot be detected in routinely inspections, representing a major risk for humans [16].

Human salmonellosis is mainly acquired by ingestion of raw or undercooked contaminated food from animal origin, mainly from poultry

(43.8 %; eggs and meat) and pigs (26.9 %; meat), and also by ingestion of unpasteurized cow milk [11]. In fact, the decreasing incidence of human salmonellosis observed last years has been associated to the implementation of *Salmonella* control campaigns in eggs and poultry products intended for human consumption [6]. Accordingly, infections of swine origin are becoming more relevant, being *S*. Typhimurium and *S*. Derby the serotypes most common isolated in the EU [17, 18].

The human infection courses with an acute gastroenteritis that appears within 12–72 h after ingestion of contaminated food and includes symptoms as diarrhoea, vomiting, abdominal pain and fever. Treatment is based on rehydration and occasionally requires antibiotic administration, only when bacteria reach the bloodstream causing bacteraemia. This complication is particularly dangerous, even fatal, in immune-compromised patients, such as those with HIV infection, cancer or under immunosuppressive treatment, or with altering endogenous intestinal flora and, particularly, in children and aged people. In these cases, the establishment of an effective antibiotic treatment is essential to control the infection, and, thus, the antimicrobial resistance profile of the pathogen should be assessed during the earlier phase of infection. An additional problem associated to the occurrence of human salmonellosis is the emergence of *Salmonella* strains carrying resistance to multiple antimicrobial agents, frequently associated to antibiotic administration to animals.

19.3 Pathogenesis

After oral ingestion, the acidic environment of the stomach destroys a high proportion of *Salmonella*. The surviving *Salmonella* reach the distal ileum and caecum, replicate in enterocytes and go through the intestinal barrier, captured by the M cells overlying Peyer's patches, phagocytes expressing CD18 molecules and/or active entrance in non-phagocytic enterocytes (Fig. 19.2) [19]. Once in the lamina media, the invasion of the macrophages (target cells of the pathogen) requires two different type III secretion systems (T3SSs) encoded on separate *Salmonella* pathogenicity islands (SPI-1 and SPI-2) [20]. Both SPI-1 and SPI-2 provide a variety of proteins required for delivering the bacterial effectors into the host cells, modulating the host cell functions (SPI-1), such as cytoskeletal reorganization and cytokine gene expression, and the transformation of *Salmonella*-containing vacuole (SCV) into an intracellular replicative niche (SPI-2) [21]. Following the local infection of *Salmonella*, the interaction of the bacterial LPS with Toll-like

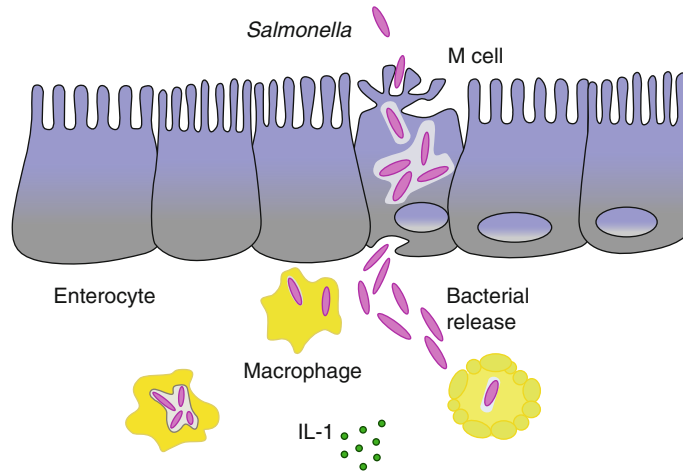

Fig. 19.2 Graphical representation of *Salmonella* pathogenesis in the intestine

receptor 4 (TLR-4) activates dendritic cells and macrophages and, subsequently, triggers the host immune response [22]. The pathogen reaches the mesenteric lymph nodes and the general lymphatic system, being disseminated through the bloodstream to spleen, liver and other organs, causing bacteraemia.

During enteric acute infection, the pathogen is massively excreted through faeces and, eventually, could induce persistent asymptomatic infections, with intermittent excretion through faeces for long-lasting periods. Asymptomatically infected hosts act as source of infection to healthy hosts, either by direct contact or indirect food contamination. Establishing persistent infections is a complex process involving both host and pathogen components.

On one hand, the host displays the innate immune response to eliminate the infection from the organisms (see below), and, on the other hand, *Salmonella* compete with host microbiota to reach its target cell and, thus, develop or activate strategies to survive in extreme environments [23]. In fact, it has been described that fimbria and adhesins are displayed as an attempt to be maintained inside the intestinal tract [24]. Similarly, resistance to stomach acid pH and ability to use inflammation related-metabolites provide a growth advantage against host microbiota [25]. Also, external factors, such as recent antibiotic treatment, could favour the establishment of *Salmonella* infection by competitive microbiota inhibition and prolong excretion of the pathogen [26]. For this reason, antibiotic therapy is only recommended for septic clinical form of *Salmonella* infections, but not for mild to moderate healthy patients (see below).

First recognition of *Salmonella* by the innate immune system is mediated by TLR-4 via MD2, as LPS specific receptor, activating the transcriptional responses to extracellular and SCV vacuolar pathogen [27]. Stimulation of this receptor triggers the expression of cytokines (such as IL-1β and TNF-α) and proteins (such as proteolytic enzymes and antimicrobial cationic peptides) from the macrophages. Thereafter, a variety of other mechanisms contribute to control *Salmonella* by the immune system, as the acidification of the SCV, defensins and reactive oxygen intermediates secretion [28]. Following primary infection, both antibodies (mainly directed to *Salmonella* LPS and proteins) and specific T-cell responses can be detected in domestic animals and humans. Humoral response is not limited to live bacteria, since non-live or subunit vaccines are able to elicit specific antibodies production, but cellular immune response is only detected after active infection. It has been widely stated elsewhere that humoral and cellular immune response levels are not always correlated with the innate immune system status to prevent or eliminate the pathogen [29].

19.4 Isolation and Typing of *Salmonella*

In order to preserve the consumer's health, EU authorities have established regulations to be accomplished compulsory in all Member States, for controlling *Salmonella* spp. in both foodstuffs and animal infections. For foodstuffs, the microbiological and hygiene criteria is established in Annex I of the Commission Regulation (EC) 2073/2005 of 15 November 2005 [30], requiring absence of *Salmonella* spp. in 5 samples of 10 or 25 g/sample, depending on the type of product, all along the product shelf life or in animal carcasses after slaughtering and before refrigeration. To control animal salmonellosis, exhaustive controls should be performed along the productive chain *from farm to fork* [31, 32]. Currently, swine salmonellosis control is being implemented in all the EU territory, after performing well-standardized reference studies in the 27 Member States [31].

The ISO 6579:2002/Am 1:2007 (ISO ahead) [33] is the standardized method internationally recommended for *Salmonella* isolation, using slight modifications depending on the type of sample (stool, lymph nodes, food, water). In general, samples are taken individually, although both animal and environmental samples could be taken in pool for epidemiological purposes. In subclinical infections, *Salmonella* might be excreted intermittently through faeces; thus, follow-up sampling should be taken in hosts nontreated with antibiotics.

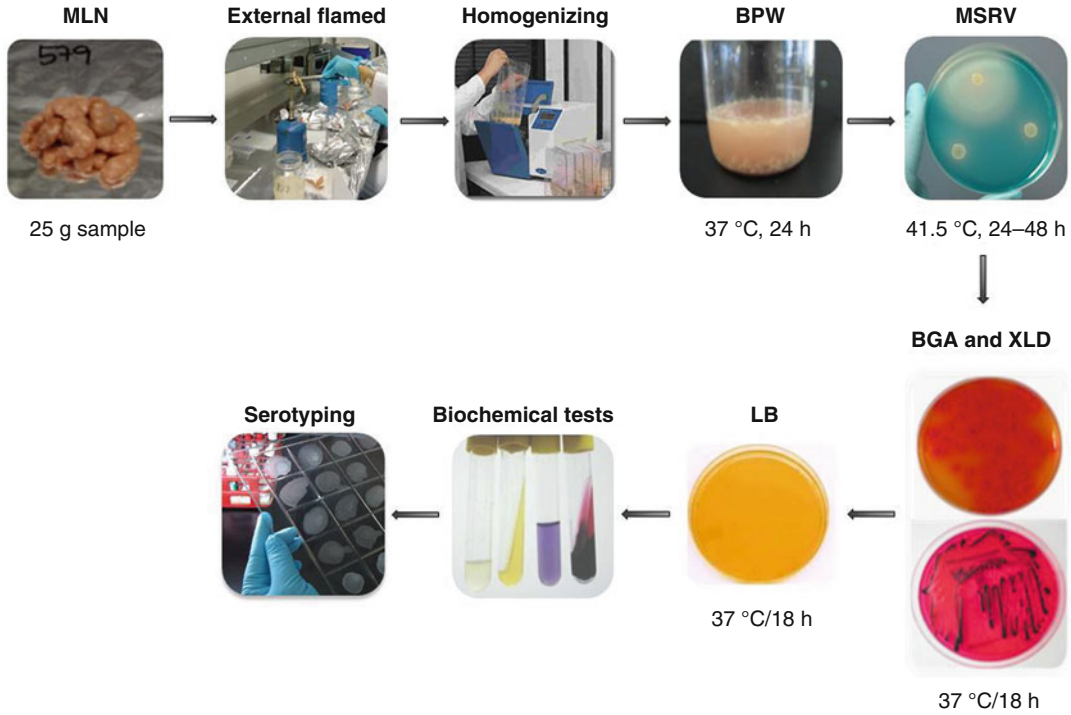

Fig. 19.3 Isolation of *Salmonella* spp. from mesenteric lymph nodes (*MLN*) following the ISO 6579:2002/Am 1:2007. *BPW* buffered peptone water, *MSRV* Modified Semisolid Rappaport-Vassiliadis medium, *BGA* Brilliant Green Agar, *XLD* Xylose Lysine Deoxycholate, *LB* Luria Bertani Agar

The ISO method comprises a stepped use of culture media, from none to highly selective, to achieve a successful isolation of *Salmonella* (Fig. 19.3). Thus, suspected colonies should be isolated after a non-selective pre-enrichment in buffered peptone water (BPW), followed by a semi-selective enrichment in Modified Semisolid Rappaport-Vassiliadis medium (MSRV) and a final selective culture in two solid selective media, such as Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA). After purification of single colonies in agar plates, biochemical tests, such as Urea agar, Lysine, Indole and Triple Sugar Iron agar (TSI) or a commercial Analytical Profile Index (API) should be applied to confirm the identity of suspected colonies. Finally, bacteria should be submitted to confirmatory serotyping by slide agglutination with specific monoclonal mouse sera directed to identify variants of the O, H and Vi antigens (see above). This technique requires the use of over 150 specific sera and carefully trained personnel, thus should be performed in *Salmonella* Reference Centres.

Additional techniques, such as phage-typing and molecular typing, can be used for a better characterization of the *Salmonella* strains, particularly useful in both global surveillance and outbreaks investigations. Phage-typing is determined by lytic or lysogenic activity of specific collections of phages, such as the 17 *S*. Enteritidis typing phages (SETP) [34] or the 34 *S*. Typhimurium typing phages [35], as described by the Health Protection Agency (HPA; Colindale, United Kingdom). Molecular typing techniques most widely used are Multiple-Locus Variable-Number Analysis (MLVA) and PFGE, the latter being the technique most widely accepted for fingerprinting strains in outbreak situations and phylogenetic studies. PFGE is relatively inexpensive but is time consuming, laborious and requires well standardization, displaying different sensitivities for different serotypes. The evolution of molecular biology has led to the emergence of novel

diagnostic techniques, such as genotyping by analysis of genes encoding O and H antigens, using multiplex PCR [36] or bead arrays [37] or ORFeome comparisons [38, 39].

19.5 Serological Diagnosis

ELISA and other serological tests can be useful tools in certain epidemiological situations [40]. However, serology is not an indicative of *Salmonella* infection at the time of sampling, since humoral response persists in the organism long-lasting periods than bacteria, and, conversely, *Salmonella* infections occur quickly, while seroconversion requires long-lasting periods.

For human diagnosis, four ELISA tests have been developed for detecting *S*. Typhi whereas are scarce for non-typhoid *Salmonella*. In fact, most laboratories currently use their own in-house tests with acceptable success, and the need for a standardized ELISA has been sidelined or, in some cases, discarded in favour of PCR or other molecular techniques. In this context, several authors point out towards the combination of LPS belonging to different serogroups, in order to improve the detection of a high number of serotypes [41].

In veterinary, a wide variety of commercial ELISA tests are available for monitoring the infection in pigs, poultry as well as food. These tests allow a quick diagnosis, using either sera or meat juice collected for animal health surveillance studies, but seroprevalence is not always in agreement with the actual infectious status, limiting the use of ELISA tests to areas with low expected *Salmonella* prevalence but not as the only infection control tool [42].

19.6 Therapy Against *Salmonella* Infection

General recommendations for enterobacterial infections treatment are hydration and soft diet, accompanied by meticulous personal hygiene. Only exceptionally, severe ill or at risk patients (immunosuppressed, infants, etc.) should be treated with antimicrobial agents. Drugs usually applied against human salmonellosis are fluoroquinolones (ciprofloxacin), trimethoprim-sulfamethoxazole or amoxicillin-clavulanic acid in adults and third-generation cephalosporin in children [43, 44]. Indiscriminate antibiotic treatment could lead to the emergence of multidrug-resistant strains, as happened after the 1980s massive treatments with ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole. In these situations, antimicrobial agents are not only noneffective in further treatments but also induce increasing illness severity and mortality rate. Moreover, it has been proven that children treated with ampicillin or amoxicillin frequently have prolonged *Salmonella* excretion periods and clinical relapses, due to the elimination of the endogenous microbiota and the strengthening of *Salmonella* throughout the gastrointestinal tract [45].

Animals are considered as the main source of multi-resistant *Salmonella* strains, as result of selective pressure derived from the systematic use of antibiotics in the diet as both growth promoters and treatment of multiple infectious processes during its productive life [46]. In 2008, in the EU, swine and cattle showed the highest number of *Salmonella* strains resistant to tetracycline, ampicillin and sulphonamides [43]. It should also be considered that the use of antimicrobials for therapy or growth promoting also disrupts the gut flora which often increases the susceptibility of pigs for *Salmonella* infection [47]. The use of antibiotics may thus act as a trigger for the spread of a *Salmonella* infection within a herd, which would not have occurred if the animals were untreated. Besides selective pressure, acquisition of antimicrobial resistance could be favoured by other factors, such as a genetic tendency of some *Salmonella* serotypes to acquire and fix genetic elements.

It can be concluded, as early also was recommended by WHO [48], that control of *Salmonella* infections should not be based on the use of antimicrobials and that the emergence of antimicrobial resistance is an additional serious reason why they should be used with great care, as exemplified by the emergence of the multi-resistant *S*. Typhimurium DT104 [49]. The

appearance of the classical profile of penta-resistance ACSSuT (aminopenicillins, chloramphenicol, streptomycin, sulphonamides and tetracyclines) in this DT104 clone has been associated to both the use of antimicrobials in animals and the international trade of infected animals [50, 51]. Thus, the emergence of *Salmonella* strains resistant to multiple antibiotics, including fluoroquinolones and third-generation cephalosporins, represents an important public health concern, recently associated to swine origin [43, 52]. In consequence, current European regulations prohibited the use of antimicrobials as growth promoters since 1 of January 2006 and recommend a limited usage of antibiotics in animals and the antimicrobial resistance surveillance of all *Salmonella* spp. isolates [53].

Otherwise, alternative novel therapies based on diets that modify the composition of endogenous intestinal bacterial flora are being implemented to favour microorganisms that contribute to eliminate *Salmonella* from the gastrointestinal tract [54].

19.7 Vaccine Working Mechanisms

Seroconversion, based on IgM followed by IgG antibodies, is induced at the first stage after vaccination. Moreover, IgA production can be detected in case of mucosal vaccination. LPS, flagellin, fimbriae and other proteins (lipoproteins, outer membrane proteins, heat shock proteins) are the main responsible elements for the stimulation of the immune system. The elicitation of the humoral immune response has been cited in a wide range of hosts, from laboratory experimental models (mice) to livestock (i.e. calves) and humans, receiving subunit, killed or live attenuated vaccines. Taking into account all the results described in the literature, a correlation between the presence of antibodies and resistance to infection cannot always be established. Moreover, protective effects based on the humoral immune response have only been described in some experimental conditions such as low dosage or moderately virulent strains challenge.

Live attenuated, but not subunit and killed, vaccines have been described as capable of inducing Th1 cellular immune responses (CD4 and CD8 lymphocytes), accompanied by the presence of cytokines (IL-12, IFN-γ) in different hosts. In fact, some subunit and killed vaccines have been reported to be Th2 immune response inductors. As it has been stated above regarding the humoral response, cellular immune response does not always correlate with protection after administration of *Salmonella* vaccines. Therefore, further investigations are needed to the elucidation of the mechanisms of protective immunity against *Salmonella*.

19.8 Vaccine Control and Animal Models

The approach to ensuring the purity, safety, potency and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high-quality products for use in animal health programmes [55]. The designed vaccine should be tested in terms of purity (Gram staining, homogeneity of growth in culture media and sero- and phage-typing), innocuousness (lethality in mice and the final host, side effects, stability and absence of reversion in case of live attenuated vaccines, transmission to milk or eggs), efficacy (level of protection in both mice and final host) and environmental behaviour (persistence of the vaccine in stools and litter, capability of infecting surrounding animals) [55]. Besides, all these final control measurements should be applied during the production of different batches, to ensure homogeneity in the method of manufacturing.

Primate salmonellosis closely resembles human symptomatology but the use of these animals is very limited due to practical, economic and ethical reasons. Otherwise, mice and calf models have been successfully used for elucidating both virulence and immunological mechanisms [56, 57]. Mice experimental infection with *Salmonella* causes rapid systemic infection, evidenced by symptoms as fever,

piloerection, prostration and starvation, followed by liver and spleen colonization and finally death [58]. Moreover, mice have been used to evaluate the efficacy of *Salmonella* vaccines either against lethal [59] or sublethal [60, 61] challenges.

Thus, human enteritis is characterized by diarrhoea, the bovine model has been considered suitable, since calves infected by *S*. Dublin displayed bacteraemia, abortions and became as chronic carriers, whereas calves receiving *S*. Typhimurium were asymptomatic carriers [58]. The model of ligation iliac loop, in calves, pigs, rabbits and mice, has been widely used due to its potential to study bacterial virulence factors and early gastrointestinal steps of infection, although this model does not predict the degree of protection of a vaccine [58].

19.9 Vaccines and Rationale for Vaccination

As zoonotic agent, prevalence of *Salmonella* infections in humans is directly related to prevalence in animals. Since there are no anti-*Salmonella* vaccines safe enough to be applied in humans, the control of this infection should be based on animal prophylaxis and hygienic measures directed to avoid dissemination of the pathogen to both other animals and humans, the latter mainly through foodborne of poultry and swine origin. In order to prevent the consumer's health, current legislations involve a complete control of *Salmonella* "*from farm to fork*" [31, 32]. Critical points throughout primary production, e.g. feed testing, health and hygienic controls at slaughter and hygienic measures during handling and consumption of poultry and swine meat and derivatives, are considered essential to control *Salmonella* dissemination and infections.

19.9.1 Nonliving Vaccines

Inactivated and subunit vaccines have been used widely in the past, in both humans and animals, with variable success [62, 63]. Different simple or combined bacterial fractions, such as Vi-polysaccharide, LPS, O-Chain, fimbriae or porins, have been used as non-live vaccines. For instance, combination of LPS to protein carriers has demonstrated to induce antibody responses in rabbits and mice but limited efficacy against a challenge infection [64]. In order to improve the efficacy, non-live vaccines require to be administered in combination with classical (e.g. aluminium hydroxide, complete (CFA) and incomplete (IFA) Freund's adjuvants) or novel (e.g. extra domain A of fibronectin (EDA) [61]) adjuvants. In general, non-live vaccines are safe but induce strong humoral responses and poor Th1 cell-mediated immunity, leading to low efficacy [29, 65] and undesirable serological interference with the diagnosis of the infection, being LPS and fimbriae the immunodominant antigens of *Salmonella* used in diagnostic tests. In fact, differentiation of infected and vaccinated animals (DIVA) is a priority in the design of vaccines against animal salmonellosis (see below).

Current investigations in animal salmonellosis vaccines are mainly directed towards the design of live vaccines allowing an attenuated, safe and efficient *Salmonella* strain that, in turn, induces a serological response allowing DIVA. However, the concept of attenuation varies depending on both the *Salmonella* serotype and the animal species involved since, in fact, most of *Salmonella* serotypes affecting humans are "attenuated" for animals, without inducing illness (see above). Accordingly, a "safe" *Salmonella* vaccine should be understood as unable to be excreted and contaminate both environment and food chain.

19.9.2 Live Attenuated Vaccines

In general, live vaccines are considered better than inactivated vaccines, since the former could (1) induce both cell-mediated and humoral immune responses, (2) be effective after one single-dose administration, (3) induce mucosal immune response, after oral administration, (4) be used as carrier for delivery of other recombinant antigens and become a multivalent vaccine and (5) have low cost of production and easy storage [29].

Early developed live *Salmonella* vaccines were spontaneous mutants obtained after *in vitro* culture, as *S*. Gallinarum 9R [66]; temperature treatment, as TS *S*. Typhi [67]; chemical selective pressure, as nitrosoguanidine in *S*. Typhimurium-NTG [68] or streptomycin in *S*. Abortusovis Rv6 [69]; or ultraviolet radiation [70]. These vaccines have proven to be effective in mice, poultry and cattle, and some of them have been licensed for their use. However, severe side effects, such as septic arthritis or hepatitis, have been described [71]. Advances in both *Salmonella* pathogenomic knowledge and molecular biology technology have open the possibility to design new live *Salmonella* attenuated strains with well-defined and non-reverting mutations in genes related to virulence and/or immunogenicity. The functional identification of *Salmonella* genes has led the possibility to select and mutate those involved in the *in vivo* bacterium survival and infection processes, including those encoding bacterial structural components and essential metabolite biosynthesis and virulence genes. All of them are described below.

19.9.2.1 Mutants in Bacterial Structural Components

Since LPS is both a major virulence factor and the immunodominant antigen in serological diagnostic tests, development of rough LPS mutants has been an interesting approach to build vaccines allowing DIVA. In general, complete LPS core *Salmonella* mutants are considered more effective than deep rough mutants against a virulent infection. In fact, several *Salmonella* mutants lacking different LPS portions, such as Δ*waaH* and Δ*waaL*, have been proposed as live vaccine candidates [61, 72, 73], but other rough mutants have been considered too attenuated (e.g. *S*. Typhimurium Δ*waaG*) to confer protection enough to prevent virulent infections or too virulent to be safe vaccines, being discarded as vaccine candidates [74].

Since galactose is a component of the LPS core, galactose epimeraseless or *galE* mutants are unable to synthetize the enzyme uridine diphosphate galactose (UDP-Gal) epimerase and, therefore, do not convert the uridine diphosphate glucose (UDP-Glu) to UDP-Gal and vice versa. This type of Δ*galE* mutants was developed in eighties, showing an incomplete LPS (deep rough phenotype) in absence of this sugar *in vitro*. However, if galactose is exogenously provided, like *in vivo*, Δ*galE* mutants could revert to smooth phenotype and, therefore, revert to virulent form. This phenomenon, which has been described in calves vaccinated with *S*. Typhimurium Δ*galE* mutant, could generate not only non-protection against a virulent challenge but also induce diarrhoea, fever and even death in calves [75, 76]. Similar results were obtained with Δ*galE* mutants in *S*. Typhi [77] and *S*. Enteritidis [78] genetic backgrounds. In an attempt to avoid rough-to-smooth phenotype reversion, a *S*. Enteritidis Δ*gal* operon (including *galM*, *galK*, *galT* and *galE* genes) mutant has been described [61]. Despite absence of side effects, no protection was observed in mice vaccinated with *S*. Enteritidis Δ*gal* operon and challenged by intraperitoneal route, indicating that deep rough mutants are not effective vaccines against smooth *Salmonella* infection [61].

Synthesis of outer membrane proteins (Omp) OmpC and OmpF is regulated by *ompR* gene. *S*. Enteritidis Δ*ompR* gene was highly attenuated and able to induce a moderate protection after oral challenge [79]. Besides, individual Δ*ompC* and Δ*ompF* attenuated mutants have been described. In the DIVA context, Omp mutants arise as an alternative to LPS mutants leading to absence of anti-Omp antibodies in vaccinated but not infected animals.

19.9.2.2 Mutants in Bacterial Essential Metabolites

Mutations in genes encoding the aromatic (*aro*) synthetic pathway have been described as attenuated and effective vaccines in different animal models [29]. Genes *aroA*, *aroC* and *aroD* have been widely used to design single and double mutant vaccines, in *S*. Dublin and *S*. Typhimurium for calves [80] and *S*. Enteritidis and *S*. Gallinarum for chickens [81]. Moreover, Δ*aroC*Δ*aroD* and Δ*aroA*Δ*aroC* double mutants maintain the immunogenicity with minimal chance to revert to virulent phenotype, being thus proposed as candidate vaccines [82].

Genes blocking the synthesis of adenosine monophosphate (*pur* mutants) require an external input for adenine, leading to a drastic *in vivo* attenuation. *S*. Typhimurium and *S*. Dublin Δ*purA* mutants demonstrated a reduced ability to colonise and persist in mice, stimulating an insufficient immune response and leading to low protection level [83, 84].

Finally, adenylate cyclase (*cya*) and cyclic AMP receptor (*crp*) genes regulate the expression of other genes involved in the utilization of carbohydrates, amino acids and cell surface structures, such as Omps, fimbriae and flagella. Despite Δ*cya* and Δ*crp* mutants are highly attenuated and their survival in the spleen is very limited, oral immunization has led to protection in mice against an oral challenge. The *S*. Typhimurium and *S*. Cholerasuis Δ*cya*Δ*crpA* double mutants were effective against parental or oral challenges, in chickens and swine, respectively [85, 86].

19.9.2.3 Mutants in Bacterial Virulence Genes

Salmonella virulence genes have been studied in order to reduce its capability of growing in the host but maintaining the stimulation of the host immune system to fight against virulent infections. In this context, several genes, both chromosomic (*invA*, *hilA*, *PhoP*/*PhoQ* two-component regulatory system) and plasmidic (*spvB*, *spvC*), have displayed different degrees of virulence. For instance, whereas *S*. Enteritidis Δ*hilA*, Δ*spvB* and Δ*spvC* mutants showed moderate to high virulence, Δ*invA* and Δ*phoP* mutants showed low virulence but also low protection against a virulent infection [78, 87].

Conclusions

Salmonellosis is a major zoonosis that is mainly acquired through food. Incidence of human salmonellosis is directly related to incidence of infection in poultry and swine, frequently asymptomatic. Since there are no vaccines safe enough to be administered to humans, health authorities advise a control of the infection at the animal stage "from farm to fork". Since antimicrobial treatments should be avoided at farm level in order to avoid the emergence of *Salmonella* strains with multidrug resistance, the animal vaccination could successfully reinforce (but not to substitute) the control programmes based on hygienic and sanitary measures. Live attenuated vaccines are generally more effective than subcellular vaccines. However, live vaccines retain some residual virulence, and its large-scale application may involve a risk of introducing new pathogens genetically modified into the food chain. Since a practical point of view, future directions in developing animal vaccines should be focused on new *Salmonella* live vaccines able to allow discriminating between infected and vaccinated animals (DIVA).

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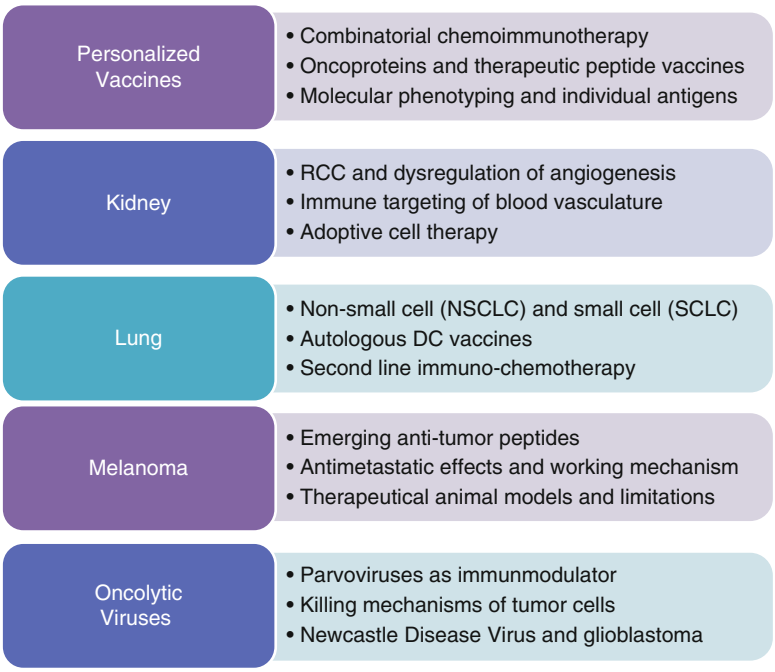
Part IV

Cancer Vaccines

Overview of Part IV



The first-personalized cancer vaccine that reached the market was the renal cell carcinoma vaccine Oncophage in Russia in 2009. Both the US and EU regulators rejected Oncophage after unsuccessful late-stage studies. The base for each P-vaccine is the individual genetic profiling. This new approach is scientifically approved and without question a new promising therapeutical strategy. But there is no one-to-one relationship between the analyzed DNA and the transcribed mRNA and the translated protein. The functional response to a P-vaccine depends mainly on the efficiency of the immune system.

An overload of tumor cells and a stringent chemotherapy will lead to the loss of immune competence. Therefore, for the success of a P-vaccine, a therapeutical window (see Chap. 1) based on a set of various biomarkers is essential.

Combination Therapy. The use of chemotherapy in combination with immunotherapy has been controversial due to the immunosuppressive effects of the chemotherapeutic agents. During the last decade, data have accumulated that point to possible advantages of combining these two treatments. To this end, some chemotherapeutic drugs lead to an immunogenic death of cancer cells and selective depletion of immunoregulatory cell subsets.

Personalized Peptide Vaccine. In addition to immunogenic antigens derived from oncogenic infectious agents that are recognized as foreign by the host immune system, vaccination with "self" antigen peptides with less immunogenicity has also shown substantial progress. In particular, improved strategies for vaccination, such as prescreening of antigens for "personalization," the combined use of multiple peptides, and combination therapy with other treatment modalities have increased the clinical benefits.

Renal Cell Carcinoma and Its Vasculature. Instability in the tumor vasculature increases interstitial pressure within the tumor, preventing the delivery of tumoricidal drugs and immune effector cells into the tumor microenvironment (TME). Tumor vascular endothelial cells (VEC) exhibit differential gene expression when compared to VEC isolated from healthy tissue, with such transcripts believed to underlie tumor blood vessel destabilization. Silencing these genes using siRNA approaches may block VEC migration and vascular tube formation, thus supporting tumor VEC as a highly relevant therapeutic target.

Lung Cancer Immunotherapy. There is no ideal source of antigen. Distinctions between autologous vs allogeneic, monovalent vs multivalent, and whole cell vs recombinant preparations are part theoretical and part pragmatic. Autologous tumor antigen would offer the advantage of a patient-specific vaccine that circumvents the need to know the antigens patient's cancer expresses, but the volume of tumor tissue generally required to produce autologous tumor vaccines restricts that approach to individuals with a surgically resectable tumor.

Melanoma. Natural peptides with antitumor activity have been described from a variety of sources. Some of them are free molecules and others are internal sequences from proteins that are liberated by proteolysis or are chemically synthesized. Their antimicrobial and antitumor activities resemble ancient molecules of innate immunity that have been effective in the protection against threatening conditions, infective or others, before the emergence of antibodies and adaptive T cell immunity.

Parvoviruses. A number of experimental observations support the notion that PV-mediated oncosuppression is immunogenic. As mentioned, the oncotropism of PVs is not due to a better virus uptake by transformed cells but rather to the dependence of intracellular steps of the viral life cycle on the presence, absence, or activation of factors that are regulated as function of cell cycling and oncogenic transformation.

Newcastle Disease Virus. The therapy with the NDV-DC vaccine consists in the intradermal application of DCs, which are generated ex vivo from blood cells of a cancer patient and which are activated via incubation with a viral oncolysate of the patient's tumor. The induction of danger signals in tumor cells upon NDV infection is key to the stimulation of DCs for the generation of strong antitumor effects.

Cancer Vaccines and the Potential Benefit of Combination with Standard Cancer Therapies

20

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Contents

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Abstract

Numerous different kinds of cancer vaccines are in development, comprising a broad spectrum of different antigenic targets and formulations. Although many cancer vaccination trials have been conducted through the last decades, clinical benefit for the majority of patients still needs to be confirmed. An obstacle to successive immunotherapy might be immunosuppressive mechanisms such as regulatory T cells and myeloid-derived suppressor cells.

The use of chemotherapy in combination with immunotherapy has been controversial due to the immunosuppressive effects of the chemotherapeutic agents. During the last decade, data have accumulated that point to possible advantages of combining these two treatments. To this end, some chemotherapeutic drugs lead to an immunogenic death of cancer cells and selective depletion of immunoregulatory cell subsets.

The combination of immunotherapy and standard chemotherapy regimens or low-dose chemotherapy aiming at improved response to immunotherapy is under investigation in numerous laboratories and clinics, and the results look promising so far.

The cancer vaccine approach might also benefit from combination with other kinds of cancer therapeutics such as targeted therapies and immune-modifying antibodies.

Selected studies are reviewed to address present knowledge on combining cancer vaccines and existing cancer therapies. Also future perspectives are discussed.

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20.1 Introduction/Background

20.1.1 Cancer Vaccines

The use of cancer vaccines to induce a therapeutic host anti-tumour immune response has huge potential to complement traditional cancer therapies in a nonoverlapping way. In 2010 the first therapeutic cancer vaccine was approved by the FDA (sipuleucel-t, Provenge®) [1]. This was the culmination of decades of research and clinical trials investigating the potential anticancer effects of cancer vaccinations. Still, clinical outcomes from vaccination trials have with a few exceptions been disappointing, and new approaches and strategies are under intense investigation. This includes the vaccines themselves but also the timing of vaccinations and recently the combination of vaccines with other antineoplastic therapies.

In general, a successful vaccination requires two components: An antigen and an adjuvant. The "antigen" represents the embodiment of the target that should be cleared from the body. The second requirement – the adjuvant – is an amplifier of the response induced by the antigen itself. Cancer vaccines are therapeutic vaccines different from prophylactic vaccines since they are given with the purpose of overcoming an already existing disease in the patient. The principle of cancer vaccination builds on the immunisation against so-called tumour-associated antigens (TAA). Nucleated cells process intracellular proteins in the nucleus and present small fractions of these proteins on the cell surface in context with HLA molecules.

On the surface the peptides can be recognised by immune cells, i.e. T cells, which can then initiate an immune reaction against the cell. Some antigens expressed on the surface of tumour cells (TAA) are different from those seen on the surface of normal cells. TAA can be categorised into different subtypes: overexpressed antigens including differentiation antigens which are antigens with low expression on normal cells but highly expressed if the cells undergo malignant transformation, cancer testes antigens which are expressed solely on the immune-privileged testicular cells and on tumour cells and mutated antigens among others (Table 20.1). The application of peptides represent one of the simplest ways of targeting single or a few antigens, but other ways of achieving this is also possible, e.g. by using whole protein, RNA or DNA. Thus, cancer vaccinations target one or more of these TAA and many different vaccine approaches exist. In general, the adjuvant may induce an antibody response or a cellular response due to the fact that the response to some extent depends on the adjuvant.

Since most efforts in the field have addressed induction of cellular (T cell) responses, adjuvants with this focus are most intensively studied in cancer vaccinations. In this respect, activation of dendritic cells has attracted much attention, as they are regarded key mediators of crosstalk between the

Table 20.1 Categorisation of tumour-associated antigens

Tumour-associated antigens (TAA)	Examples of TAA	Cancers where the TAA typically is expressed	Expression on normal cells	Expression on malignant cells
Differentiation antigens	MART-1/Melan-A, gp100, tyrosinase	Malignant melanoma	Low	High
Overexpressed antigens	Survivin, hTERT	Almost all cancers		
	HER2/neu oncogene	Breast and ovarian cancer		
	CEA	Gastrointestinal and lung cancers	Especially during embryonic development	Present
	AFP	Germ cell tumours, hepatocellular carcinoma		
Cancer testes antigens	NY-ESO-1	Various cancers	Solely on testes cells	Present
	MAGE, GAGE	Malignant melanoma		
Mutation antigens	p53, BRAF, KRAS	Expressed in various degrees in several tumours	Present – but not mutated	Present and mutated
Viral antigens	HPV	Cervical cancer, head and neck cancer	Not present	Present

innate and adaptive immune responses, and has been described as nature's own adjuvant. However, other approaches including synthetic adjuvants (e.g. bacterial extracts in oil emulsions), DNA or viral vectors are being investigated. Adjuvants can increase the immunogenicity of the vaccine (e.g. Bacillus Calmette-Guérin (BCG), tetanus toxin, interleukin (IL)-2, interferon (IFN), thymalfasin, granulocyte-macrophage colony-stimulating factor (GM-CSF)) or decrease immunoregulatory mechanisms (e.g. CD25 antibody, chemotherapy).

20.2 Immunosuppressive Mechanisms

Many different and promising vaccine strategies have been investigated but so far convincing clinical efficacy has been lacking. Immunosuppressive mechanisms might be a crucial factor for this. In order to survive, tumours have developed several defence mechanisms to avoid attacks from the immune system (Fact Box 20.1). These mechanisms include the downregulation of major histocompatibility complex (MHC) molecules, the loss of antigens recognised by the immune system and also the secretion of molecules that attract and upregulate regulatory or inhibitory immune cells [2]. Especially two types of regulatory immune cells have been described: regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC).

Treg are a subset of CD4 T cells that are defined by the expression of CD4, CD25 and the transcription factor forkhead box P3 (FOXP3). Treg work through multiple different suppressive mechanisms; they kill T cells and antigen-presenting cells (APCs) through perforin- and granzyme B-dependent pathways, they release IL-10 and TGF-β, thereby inhibiting T cell activation and APC function, and they induce the enzyme indoleamine 2,3-dioxygenase (IDO) on APCs which lead to reduced T cell activation and tolerance in the microenvironment [3].

Another immunosuppressive cell subset is the MDSC which are a heterogeneous population of myeloid precursor cells. They are defined as HLA-DR negative and CD33CD11b positive and can be divided into a monocytic (CD14+) and a granulocytic subtype (CD15+). They inhibit CD4 and CD8 T cells directly by the depletion of cysteine and arginase or secrete reactive oxygen species also inhibiting T cell function [4]. Furthermore, both Treg and MDSC have the ability to upregulate and attract immunosuppressive mechanisms in the tumour environment.

Fact Box 20.1
Tumour defence mechanisms against immune attacks

- Reduced immune recognition/loss of antigen presentation
  - Downregulation of MHC molecules
  - Loss of antigen processing within the tumour
- Resistance to immune attacks
  - Induction of anti-apoptotic mechanisms
- Production of immunosuppressive cytokines within the tumour environment, e.g. TGF-β, IDO and VEGF
- Attraction or induction of immunosuppressive cell subsets, Treg and MDSC

The enzyme IDO is a tryptophan catabolic enzyme which has recently been recognised as an important factor in immune regulation and development of immune tolerance in the microenvironment of cancer cells. IDO creates a tolerogenic milieu in the tumour environment and in the tumour-draining lymph nodes, either by inhibiting T cells directly or by inducing immune suppression via Treg [5] In cancer patients IDO expression is seen in both cancer cells and antigen-presenting cells. Therefore, the induction of IDO-specific T cells through vaccination against IDO has the dual potential to block the development of tumour immune tolerance through elimination of immune suppressive IDO positive immune cells and elimination of IDO positive tumour cells [6]. A phase I trial with NSCLC patients is being conducted at our institute evaluating the safety and efficiency of vaccination with an IDO-derived peptide (www.clinicaltrials.gov ID: NCT01219348).

20.3 Chemotherapy

20.3.1 The Immunological Advantages of Chemotherapy

Even though the use of chemotherapy in combination with immunotherapy may seem controversial due to the immune-inhibiting potential of chemotherapeutics, evidence arises that this combination may have more beneficial than negative consequences. Several groups have in preclinical models examined the efficacy of different chemotherapeutic agents on the immune cells and the immune defence mechanisms [7–9].

Chemotherapeutics exert their immunomodulating function through multiple mechanisms (Fact Box 20.2). First, when tumour cells undergo apoptosis after the treatment with certain chemotherapeutics, several antigens are released. These antigens can be taken up by APC, presented for T cells, and an immune reaction against other cancer cells expressing these antigens on the surface can be initiated. This is called immunogenic apoptosis and is characterised by the expression of calreticulin on the cell surface [9] and the release of adenosine triphosphate [10]. Secondly, tumour cells can be more sensitive to T cell-mediated lysis after treatment with certain chemotherapeutics due to a cell membrane more permeable for granzyme B [11]. This can result in increased tumour cell killing. Thirdly, it has been observed that chemotherapy-induced lymphopenia is often followed by a secretion of cytokines, i.e. IL-7, IL-15 and IL-21. This can lead to a period of T cell proliferation where new antigens are presented to the proliferating T cell clones, and this reaction can be exploited to redirect immunity against a tumour hostile reaction [7]. Finally, as mentioned above, Treg play an important role in the regulation of immune reactions induced naturally as well as with immunotherapeutics. It has been shown that chemotherapeutic agents target Treg by direct killing and also by reducing the inhibitory function of these cells [12, 13].

Fact Box 20.2
The immunomodulating functions of chemotherapeutics

- Immunogenic apoptosis – the release of tumour-associated antigens after tumour cell apoptosis is induced by chemotherapy, characterised by
  - Expression of calreticulin on the surface of cancer cells
  - Release of adenosine triphosphate
- Fragile tumour cells – chemotherapy can induce cell membranes permeable for granzyme B → increased sensitisation of T cell-mediated killing
- Increased T cell proliferation after lymphodepletion → de novo presentation of antigens to the proliferating T cell clones → increased T cell reactivity towards tumour cells
- Reduced number and function of regulatory T cells

20.4 Combinatorial Chemoimmunotherapy (Table 20.2)

20.4.1 Combinatorial Chemoimmunotherapy with Low-Dose Immunomodulatory Chemotherapy

Cyclophosphamide is one of the chemotherapeutics most investigated for its potential immunomodulatory effects [8]. It has been shown that low/metronomic doses of cyclophosphamide might be able to reduce the number of regulatory T cells and influence the function of dendritic cells with increased expression of maturation markers and enhanced antigen presentation [7].

In a pilot study, a metronomic dose of cyclophosphamide, 50 mg p.o. daily for 3 months, was tested in 12 metastatic breast cancer patients [14]. The level of Treg was transiently reduced but recovered completely during treatment. The anti-tumour T cell response on the contrary was

Table 20.2 Examples of combinatorial chemoimmunotherapy

	Trial examples	Drug/dose	Vaccine	Cancer	No. of patients	Response/efficacy
Low-dose chemotherapy, cyclophosphamide (Cy)	Ge et al. [14]	Cy 50 mg/day	None	Breast cancer	12	Transient reduction in Treg, increased anti-tumour T cell response
	Emens et al. [15]	Cy 200 mg/m^2 + doxorubicin 35 mg/m^2	HER2+ allogeneic GM-CSF-secreting vaccine	Breast cancer	28	Increased HER2-specific humoral immunity
	Holtl et al. [16]	Cy 300 mg/m^2 day 3–4 prior to vaccination vs. no Cy	Allogeneic DC vaccine	Renal cell carcinoma	10 (+Cy) vs. 12 (−Cy)	Two patients with mixed response – also the ones with highest immune response; both received Cy
	Ghiringhelli et al. [17]	Cy 50 mg × 2/day every second week	None	Late-stage cancer	28	Selective reduction in Treg
	Ellebaek et al. [18]	Cy 50 mg × 2/day every second week	Autologous DC vaccine + IL-2	Metastatic melanoma	28	No reduction in Treg – disease stabilisation in 58 % of patients
Standard-dose chemotherapy	Kyte et al. [20]	Temozolomide	Telomerase peptide vaccine	Metastatic melanoma	25	Increased number of immune responders and long-lasting clinical responses
	Harrop et al. [23]	5-FU, leucovorin, irinotecan	TroVax (5 T4-encoding virus vaccine)	Metastatic colorectal cancer	12	Increased immune responses and the correlation of clinical activity and immune reactivity
	Nistico et al. [24]	Dacarbazine ±	Melan-A/MART-1 peptide vaccine	Stage II-IV disease-free melanoma	5 (+ dacarbazine), 5 (− dacarbazine)	Increased number of anti-tumour T cells in 3 patients treated with the combination who stayed disease-free
	Quoix et al. [25]	Cisplatin and gemcitabine	± TG4010 (MUC-1- and IL-2-encoding pox virus)	Non-small cell lung cancer	74 patients in each group (± vaccine)	Improved ORR and prolonged survival among patients treated with the combination

increased along with the reduction in Treg and maintained at high levels throughout the treatment. This was found to be due to a reduced suppression of pre-existing tumour-specific T cells as well as to a de novo generation of tumour-specific cells. Furthermore, the number of tumour-reactive T cells correlated with clinical benefit. Thus, even though this dose of cyclophosphamide was not able to constitutively reduce the level and function of Treg, it still had an impact on the anti-tumour response.

A phase I study of Emens et al. [15] tested the most optimal doses of cyclophosphamide and doxorubicin when combined with a HER2-positive allogeneic GM-CSF-secreting tumour vaccine. Different doses of each drug were tested to determine the chemotherapy doses most optimal for induction of HER2-specific immunity. Results showed that 200 mg/m^2 of cyclophosphamide and 35 mg/m^2 of doxorubicin could improve the magnitude of HER2-specific humoral immunity and preserve DTH development. It also showed that this has a narrow therapeutic window since larger doses of cyclophosphamide would interfere with immunity and DTH development, emphasising that dosing of chemotherapy is important when immunomodulating effects want to be preserved.

In another study, metastatic renal cell carcinoma patients were treated with an allogeneic dendritic cell vaccination with (10 patients) or without (12 patients) cyclophosphamide in a phase I/II trial [16]. Out of 22 patients treated, 2 had a mixed response and 1 patient had stable disease – all of whom were treated with cyclophosphamide. Very weak or absent immune responses were found, but the 2 patients with mixed response had the strongest KLH-specific and antigen-independent proliferative response observed which might indicate that cyclophosphamide was able to augment immune and clinical responses.

Finally, Ghiringhelli et al. [17] treated 28 patients with late-stage cancer with metronomic doses of cyclophosphamide, 50 mg twice a day in 1 week, altering with 1 week off treatment. This regimen resulted in a selective reduction of Treg. However, in a clinical trial [18, 19], this cyclophosphamide regimen was used in combination with a dendritic cell vaccine and IL-2, but in this trial, the number of Treg was not reduced. Whether this was due to the co-treatment with IL-2 will be assessed in a new trial with metronomic doses of cyclophosphamide in combination with DC vaccination but without IL-2 (clinicaltrial.gov Identifier NCT00978913).

20.4.2 Combinatorial Chemoimmunotherapy with Standard-Dose Chemotherapy

Several clinical studies have focused on the feasibility of adding cancer vaccination strategies to standard chemotherapy. In a Norwegian study from 2011 [20], Kyte et al. treated 25 metastatic melanoma patients with standard chemotherapy, temozolomide, in combination with a 16-amino acid-long telomerase peptide vaccine. Temozolomide was given in a dose of 200 mg/m^2 in 5 consecutive days every 4 weeks, and vaccine injections were given with increasing intervals with the first 5 injections given during week 2 and 3 after chemotherapy was initiated. Clinical results revealed that 5 patients obtained a partial response and a further 6 patients had stable disease. All 5 clinical responders developed vaccine-specific T cell responses during vaccination and this lasted throughout the period of tumour regressions. Survival analyses showed that OS was extended compared to matched controls and responders survived up to more than 5 years.

Two interesting observations were done: First, this group has previously published results from a clinical trial treating melanoma patients with vaccine only, but in the trial combining the vaccine with chemotherapy, an increased number of patients with an immune response towards the vaccine were observed. Secondly, the clinical responses observed were long lasting (up to 5 years) and developed gradually (up to almost a year) and not shortly after treatment induction as would have been expected with chemotherapy. These facts indicate that the combination of standard doses of temozolomide and vaccination can have a positive influence on the induction of

immune responses as well as the durability of clinical responses.

To this end, in three consecutive studies [21–23] Harrop et al. have treated metastatic colorectal cancer patients with the vaccine TroVax; a tumour-associated antigen 5T4 engineered into the virus vector modified vaccinia Ankara. As in the above-described study, immune responses induced during the vaccination course were of greater magnitude and longevity when administered in combination with standard chemotherapy (5-fluorouracil, leucovorin and irinotecan) [23] than when given as monotherapy [21]. Also, signs of clinical activity with stable CEA levels after completion of chemotherapy seemed to correlate with the induction of 5T4-specific immune responses.

Ten patients with disease-free malignant melanoma stages II–IV were randomised to receive dacarbazine plus a Melan-A/MART-1 peptide vaccine or the vaccine alone in a phase I/II pilot study [24]. Immunological analyses revealed that an expansion of peptide-specific effector memory CD8+ T cells capable of killing Melan-A$^+$ tumour cell lines were observed among patients treated with dacarbazine and vaccination but not in the vaccine-only group. Also, an upregulation of immunoregulatory factors in the gene expression profile was seen the day after administration of chemotherapy which could potentially enhance immune responses induced by vaccination. Only 1 patient with stage II disease in the vaccine-alone group remained disease-free after a follow-up of 29 months, whereas in the combination arm, 3 patients with stage III/IV disease were without evidence of disease. Interestingly, those 3 patients showed an impressive expansion of CD8+ anti-tumour T cells with an effector memory phenotype. Still, conclusions cannot be drawn from this study including only 5 patients in each arm.

In another interesting phase IIB trial [25], patients with non-small cell lung cancer (NSCLC) who were to receive first line chemotherapy were randomised to receive either chemotherapy alone or chemotherapy in combination with TG4010, a pox virus coding for MUC-1 tumour-associated antigen and IL-2. The primary endpoint of this study was 6-months PFS of 40 % or more in the combination arm, which was reached. Clinically, they found that the objective response rate was better in the combination arm and that objective responses were associated with a prolonged overall survival in this group. Also, analyses of immunophenotypes showed that patients with an increased number of NK cells (CD16+CD56+CD69+) had a worse clinical outcome indicating that response to treatment was conditioned by the patients' immune status.

In conclusion, these studies show that chemotherapy possesses the ability to modulate the immune system and improve the circumstances under which vaccines are given in order to exert their immune-stimulating potential (Fig. 20.1). Still, it is also clear that the optimal dosing and scheduling of chemotherapy as well as the best chemotherapeutic drug remain unknown and research in this area is therefore highly warranted.

20.5 Other Cancer Therapies

20.5.1 Cancer Vaccines in Combination with Other Cancer Therapies (Table 20.3)

20.5.1.1 Radiotherapy

Not only can chemotherapy induce changes in the immune system that might be beneficial for combination with cancer vaccines. Radiotherapy (RT) can induce some of the same modulations as do chemotherapy, including the induction of immunogenic apoptosis resulting in increased antigenic presentation by DCs [26]. Also, if cell death is not induced by irradiation, it can lead to increased expression of MHC I complexes and adhesion molecules by the surviving cancer cells which can increase T cell recognition and killing [27]. This might again result in an increased number of circulating tumour-specific T cells with the potential to mediate tumour regression in other parts of the body than on the irradiated sites.

Tumour regression at sites distant from the locally irradiated site is called an abscopal effect, a phenomenon described only in few cases [28].

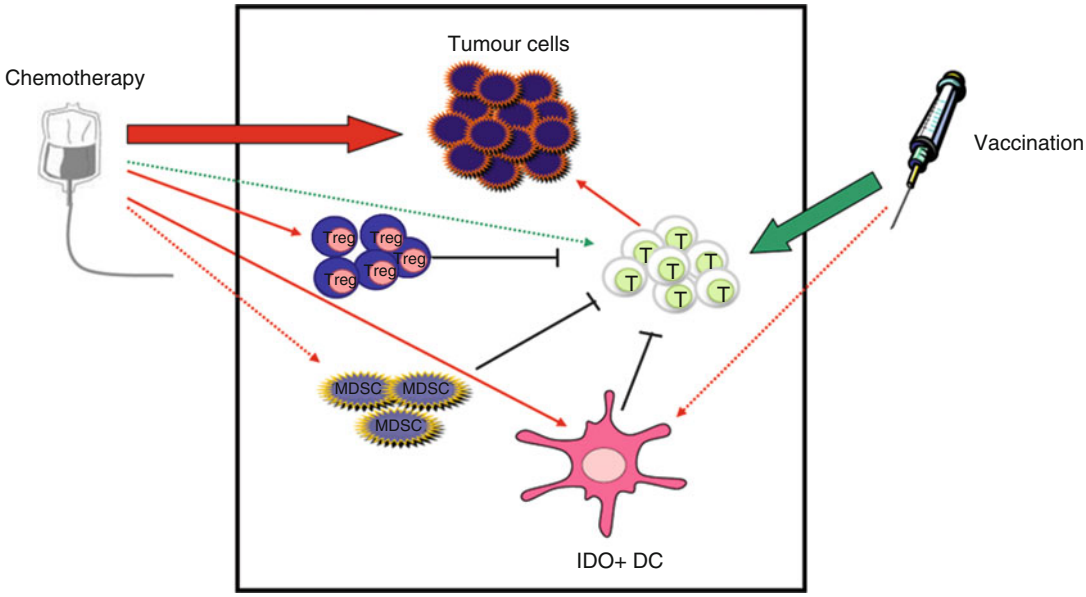

Fig. 20.1 The interaction between tumour cells, effector T cells and immunoregulatory cell subsets. The effector T cells (*T*) have little cytolytic activity towards the tumour cells in the immunosuppressive tumour milieu. There function is inhibited by regulatory T cells (Treg), myeloid-derived suppressor cells (*MDSC*) and IDO+ dendritic cells (*DC*) as well as the tumour cells' ability to escape from T cell recognition by, e.g. downregulation of MHC molecules. Chemotherapy and cancer vaccinations can work in synergy; the vaccines giving rise to a number of tumour-specific T cells and the chemotherapy killing tumour cells directly and priming the tumour for T cell killing, decreasing the number and functionality of regulatory cell subsets, and inducing cytokine release leading to T cell proliferation. Also, selective inhibition of, e.g. IDO+, DC by specific immunisation against the IDO peptide can further improve T cell activity. Inhibitory activity by chemotherapy/vaccination is indicated by *red arrows* whereas increasing activity is indicated by *green arrows*

Patients with NSCLC in stable disease or response after chemotherapy or chemoradiation were included in a phase IIB study [29] and randomised to vaccination with BLP25 liposome vaccine plus best supportive care (BSC) or BSC alone. A remarkable prolonged survival time for patients in the vaccination arm was shown, most pronounced in the subgroup of patients with stage IIIB locoregional disease (30.6 vs. 13.3 months). It was hypothesised that, besides the fact that patients with less immunosuppression and less aggressive disease have more time for development of an immune response, these patients were more often pre-treated with RT maybe contributing to the effects of immunotherapy. A new trial has been initiated including only patients with stage IIIB disease pretreated with chemoradiotherapy.

Only few clinical studies have been conducted, examining the efficacy of RT in combination with cancer vaccines. Gulley and colleagues [30] conducted a clinical phase II trial with prostate cancer patients undergoing local RT and randomised patients to RT alone or RT in combination with a recombinant vaccine encoding PSA. They found a 3-fold increase in PSA-specific T cells in the combination arm, whereas no increase was observed in the RT alone arm.

Thus, RT seems to have the immunomodulating potential for being combined with immunotherapy, and future clinical trials are being conducted or are in the process of being planned [31], among these two studies is combining RT with ipilimumab (ClinicalTrials.gov identifier: NCT00861614 and NCT01557114).

20.5.1.2 Targeted Therapies

Another well-established antineoplastic treatment modality is the targeted therapies of which more and more are being approved for treatment.

Table 20.3 Examples of cancer vaccines in combination with other cancer therapies

	Trial examples	Drug	Vaccine	Cancer	No. of patients	Response/Efficacy
Radiotherapy (RT)	Butts et al. [29]	Chemotherapy/ chemoradiotherapy	± Stimuvax (BLP25 liposome vaccine)	Non-small cell lung cancer	88 (+ vaccine) and 83 (– vaccine)	Prolonged survival for patients treated in the vaccination arm – especially those pretreated with chemoRT
	Gulley et al. [30]	Local RT	± PSA-encoding vaccine	Prostate cancer	19 (+ vaccine) vs. 11 (– vaccine)	Threefold increase in PSA-specific T cells in the combination arm
Targeted therapy	Comin-Anduix et al. [36]	BRAF inhibitor (PLX4032)	None	Malignant melanoma cells BRAF V600E+ (in vitro study)		No negative effects on cytotoxicity of T cells
	Boni et al. [38]	BRAF inhibitor	None	Malignant melanoma cells BRAF V600E+ (in vitro study)		Increased antigen expression
Immunotherapy	Schwartzentruber et al. [40]	High-dose IL-2	± gp100 peptide vaccine	Metastatic melanoma patients	94 (IL-2 alone) vs. 91 (IL-2 + vaccine)	Improved clinical response and progression-free survival in the combination arm
	Hodi et al. [42]	± Ipilimumab (Ipi)	± gp100 peptide vaccine	Metastatic melanoma patients	403 (Ipi + vaccine), 137 (Ipi alone), 136 (vaccine alone)	No clinical benefit was seen in the combination arm

Targeted therapies have been approved in first line treatment for chemotherapy-resistant cancers such as renal cell carcinoma [32, 33] and in some cases also for melanoma patients [34]. These therapies might very well also have a positive impact on the immune system by the induction of immunogenic cell death and the release of tumour antigens. Furthermore, the fast and impressive but often short-lasting objective responses observed with some of these therapies might benefit from being combined with the slower but more sustained responses seen with immunotherapy.

A prolonged time to disease progression effectuated by the targeted therapies might also expand the time from where a clinically significant immune response can be induced by, e.g. cancer vaccinations [35]. Recent research have found that BRAF inhibitors do not negatively influence the function of cytotoxic T cells [36, 37] and that these agents might increase antigen expression on the surface of melanoma cells, making them more prone to T cell killing [38]. This suggests that a combination of BRAF inhibitors and immunotherapy could be possible and discussions on the combination of BRAF inhibitors and anti-CTLA-4 or MEK inhibitors are ongoing.

Recently we have shown that blockade with the BRAF inhibitor, vemurafenib, could increase the anti-tumour reactivity of tumour-infiltrating lymphocytes when cocultured with $BRAF^{V600}$ mutant autologous melanoma cell lines pretreated with vemurafenib. Improved tumour recognition was associated with upregulation of MHC I molecules and heat shock protein on the tumour cells [39]. These data suggest that a combination of vemurafenib and adoptive cell transfer could be beneficial for patients with $BRAF^{V600}$ mutant malignant melanoma, but increased tumour recognition by tumour-specific T cells could also be exploited in a cancer vaccination setup.

20.6 Other Immunotherapies

The induction of tumour-specific cytotoxic T cells by cancer vaccination could in theory be augmented by the synergistic effects of other immunotherapeutic strategies such as treatment with cytokines or the blockade of inhibitory T cell receptors, e.g. CTLA-4 or PD-1 antibodies.

In a study from 2011 [40], Schwartzentruber and colleagues reported of the findings from a phase III trial randomising 185 metastatic melanoma patients between treatment with high-dose IL-2 alone or in combination with a gp100 peptide vaccine. Results showed a significant improvement in clinical response with an objective response rate of 16 % in the combination group compared to 6 % in the IL-2-alone group. Also, progression-free survival was significantly improved and overall survival was in favour of the combination (17.8 months vs. 11.1 months, $p=0.06$). Only 7 out of 37 immune evaluable patients from the combination arm developed measurable peptide-specific T cells, and immune analyses did not show any correlation between the anti-tumour response and clinical response. Still, results from this randomised study underline the possible advantages of combining cytokine therapy with cancer vaccination.

The impact on the immune system of the CTLA-4 antibody ipilimumab (Yervoy, BMS) has been investigated by Weber et al. [41] who found that the percentage of activated CD4+ and CD8+ T cells and the percentage of effector memory T cells were increased during treatment. Concomitant with this, a decrease in the level of naïve T cells was seen. These data suggest that ipilimumab increases T cell activity, making it an obvious agent for combination with cancer vaccines. In a large randomised phase III trial [42], ipilimumab was tested for its efficacy using a 3-arm randomisation strategy with ipilimumab alone, together with a gp100 peptide vaccine, or the gp100 vaccine alone. This study showed that ipilimumab alone or in combination with the gp100 vaccine was superior to the gp100 vaccine alone, but surprisingly, no benefit was seen from combining ipilimumab with the vaccine. This has also been observed in other studies combining ipilimumab with gp100 vaccination [43].

Why the IL-2 and a gp100 vaccine seem to have synergistic clinical effects while the combination of CTLA-4 antibody with the same vaccine does not improve clinical response to treatment is unknown but most likely due to dif-

ferent mechanisms of action. This underlines that the outcome of combining a cancer vaccine with an immune-activating agent is quite unpredictable and might not lead to neither synergistic nor additive clinical efficacy.

20.7 Perspectives

Drug resistance is the major problem that limits the effectiveness of chemotherapies used in the treatment of cancer. A frustrating property of such acquired resistance is that the tumour not only become resistant to the specific drug in use but may also acquire cross-resistance to other drugs with different mechanisms of action [44]. Cancer-associated defects in apoptosis play a vital role in resistance to chemotherapy and radiotherapy. An important reason for this impaired apoptosis is an overexpression of the anti-apoptotic regulators of apoptosis proteins, e.g. the tumour antigen survivin [45].

Additionally, drug inactivation by the tumour antigen CYP1B1 may represent a mechanism of resistance, influencing the clinical outcome of chemotherapy [46]. Consequently, immunotherapy targeting these antigens in combination with conventional chemotherapy appears to be particularly appealing. In such a setting, conventional therapy would kill the majority of the cancer cells, leaving only cells that express high levels of antigens, which would be particularly vulnerable to killing by vaccination-induced T cells. The synergy of these measures could potentially give a more effective treatment than the added effect of either regime alone, thereby strengthening the already-described synergistic effect of anticancer vaccines and chemotherapy. Thus, the synergistic effects of conventional and immunological therapies necessitate rethinking of the clinical strategies not only with respect to the chosen chemotherapeutics but also considering design of the selected immunotherapy.

Undoubtedly, the combination of cancer vaccines with other antineoplastic treatments has high potential and the possibilities are numerous. However, interactions between the individual therapies are in general very difficult to predict and thorough clinical testing is therefore essential. The diversity from immunogenic cell death induced by some chemotherapeutics and radiotherapy regimens to the synergistic effects of cytokines and vaccines points towards a number of potential new clinical trials which should be based on reasonable scientific arguments and solid preclinical work.

At our institute we have initiated 2 new trials combining standard chemotherapy regimens with cancer vaccines (www.clinicaltrial.gov Identifier: NCT01446731 and NCT01543464). Furthermore, a trial combining adoptive cell therapy with a peptide vaccine is planned.

Cancer immunotherapy is a field in rapid growth; more than 50 phase III trials testing immune strategies for cancer treatment are ongoing (www.clinicaltrial.gov); several of which are combining cancer vaccines with immune-modulating agents in a search for ways to increase efficacy. It is very likely that some of these trials will reach clinical significance and thereby pave the way for a new era of combinatorial cancer immune therapy.

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Personalized Peptide Vaccine as a Novel Immunotherapy Against Advanced Cancer

21

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Contents

Abstract

Along with recent advancements in the field of basic and clinical immunology over the past several decades, cancer immunotherapy has shown progress as a novel treatment modality against cancer. We have developed a novel immunotherapeutic approach, the personalized peptide vaccination (PPV), in which, 2–4 suitable peptides are selected based on the patients' preexisting immune responses against vaccine antigen candidates. Recent clinical trials of PPV have demonstrated the feasibility of this new therapeutic approach in various types of advanced cancers. Inflammatory factors, such as IL-6 and CRP, and immune suppressive cells, such as myeloid-derived suppressor cells (MDSC), have been identified as predictive biomarkers in the vaccinated cancer patients. In the near future, other therapeutic approaches to inhibit the inflammatory factors and MDSC could be combined to increase the clinical efficacy of PPV for advanced cancer patients.

21.1 Introduction

Along with recent advancements in the field of basic and clinical immunology over the past several decades, cancer immunotherapy has shown progress as a novel treatment modality against cancer [1, 2]. Indeed, 17 immunotherapy products have so far received approval of the US

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Food and Drug Administration (FDA) in the past quarter century [1]. These include nonspecific immune stimulators, cytokines, monoclonal antibodies, radiolabeled antibodies, immunotoxins, and cell-based therapies.

Notably, two novel immunotherapeutic agents have recently been approved by the US FDA for patients with advanced cancer [3, 4]. In 2010, an autologous dendritic-cell-based vaccine, sipuleucel-T (Provenge; Dendreon Corporation, Seattle, WA), which is designed to stimulate T cell immune responses against human prostatic acid phosphatase (PAP), was approved for treatment of patients with castration-resistant prostate cancer (CRPC), since this vaccine showed an improvement in overall survival by 4.1 months in the IMPACT study, the largest phase 3 randomized controlled trial of sipuleucel-T [3].

In 2011, another drug, ipilimumab, which is a blocking antibody against one of the immune checkpoint molecules, cytotoxic T-lymphocyte antigen 4 (CTLA-4), has been approved by the FDA for melanoma patients. In the pivotal phase 3 trial, this drug led to a 3-month improvement in overall survival with a disease control rate of 28.5 %, where 60 % of the responding patients maintained disease control for more than 2 years [4]. More recently, antibodies against programmed death 1 (PD–1) molecule, a T cell co-inhibitory receptor, and one of its ligands, PD-L1, which plays a pivotal role in the ability of tumor cells to evade the host's immune system, have shown promising results in the treatment of various types of cancers, although they have not yet been officially approved [5, 6]. Topalian et al. demonstrated that anti-PD-1 antibody produced objective responses in approximately one in four to one in five patients with non-small-cell lung cancer, melanoma, or renal-cell cancer [5]. Brahmer et al. showed that antibody-mediated blockade of PD-L1 induced durable tumor regression (an objective response rate of 6–17 %) and prolonged stabilization of disease (rates of 12–41 % at 24 weeks) in patients with advanced cancers, including non-small-cell lung cancer, melanoma, and renal-cell cancer [6]. Currently, these promising advancements are generating great optimism and enthusiasm for the further development of cancer vaccines.

21.2 Peptide-Based Immunotherapy

A rapidly increasing number of tumor-associated antigens (TAAs) and HLA-restricted peptide epitopes derived from them have been identified by several different approaches, such as complementary DNA (cDNA) expression cloning, serologic analysis of recombinant cDNA expression libraries (SEREX), and reverse immunological methods [7, 8]. The epitopes derived from TAAs that are expressed preferentially in tumor cells, but expressed restrictively in normal tissues, have been utilized as therapeutic peptide vaccines against cancers during the past two decades, but most of the clinical trials performed so far have showed only limited success [9, 10].

Nevertheless, recent clinical trials have demonstrated some significant advances in therapeutic peptide vaccines. For example, therapeutic HPV vaccines have been reported to be effective for people at high risk of developing HPV-related cancers. Melief and his colleagues showed that a vaccine composed of a synthetic long peptide pool derived from HPV-16 E6/E7 oncoproteins successfully induced HPV-specific immune responses and caused measurable regression of HPV-infected precancerous genital lesions in a majority (79 %) of patients [11]. In addition to these immunogenic antigens derived from oncogenic infectious agents that are recognized as foreign by the host immune system, vaccination with "self" antigen peptides with less immunogenicity has also shown substantial progress. In particular, improved strategies for vaccination, such as prescreening of antigens for "personalization," the combined use of multiple peptides, and combination therapy with other treatment modalities, have increased the clinical benefits

[12, 13]. In the current review, we discuss our novel vaccine strategy, the "personalized peptide vaccine (PPV)," in which vaccine antigens appropriate for individual patients are selected based on the preexisting immune responses to vaccine antigen candidates derived from TAAs [12–16].

21.3 Concept of the Personalized Peptide Vaccine (PPV)

In general, antitumor immunity is known to be dependent on both the antigen presentation of tumor cells and host immune cell repertoires. Considering that immune cell repertoires of the hosts are quite diverse and heterogeneous, antitumor immunity might be substantially different among individuals. Therefore, although single TAAs or combinations of TAAs that are highly and preferentially expressed on tumor cells are usually employed as common antigens for cancer vaccination, they might not always be appropriate for each patient. It is likely that vaccine antigens that are selected and administered without considering the host immune cell repertoires would not efficiently induce beneficial antitumor immune responses. To generate more clinical benefits from cancer vaccines, therefore, particular attention should be paid to the immunological status of each patient by characterizing the preexisting immune responses to vaccine antigens before vaccination.

Nevertheless, in most of the current clinical trials of cancer vaccines, common antigens have been employed for vaccination by disregarding preexisting individual immune responses. In contrast, we have developed a different approach, the PPV, for use in advanced cancer patients (Fig. 21.1) [12–16]. In this treatment, 2–4 suitable peptides are selected based on the patients' preexisting immune responses against vaccine antigen candidates. The PPV might thus be expected to efficiently amplify these preexisting immune responses, thereby contributing to the antitumor activity (Fig. 21.2).

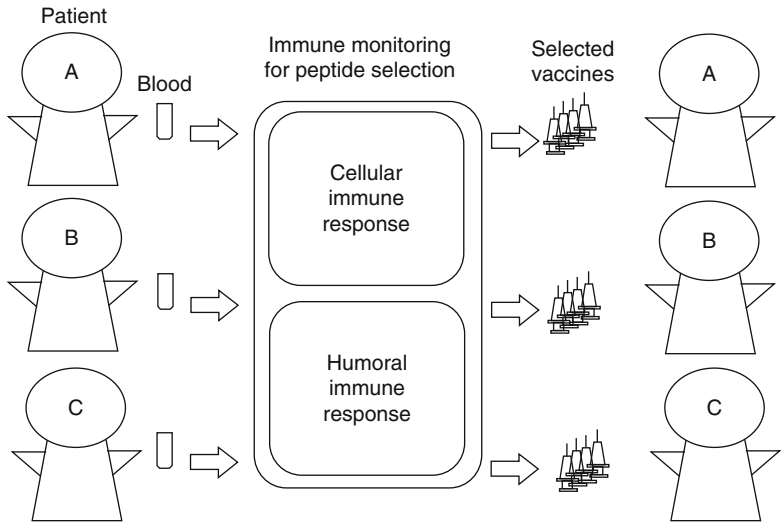

Fig. 21.1 Concept of PPV. Thirty-one antigens are employed as vaccine candidates for HLA-A2+, HLA-A24+, HLA-A3 supertype+, or HLA-A26+ cancer patients. Preexisting immune responses in the peripheral blood samples of patients are assessed by screening of antibody responses and/or cellular immune responses to vaccine candidates. A maximum of 4 peptides, which are selected based on the results of HLA typing and the preexisting immune responses specific to each of the 31 different vaccine candidates, are subcutaneously administered in complex with incomplete Freund's adjuvant weekly or biweekly

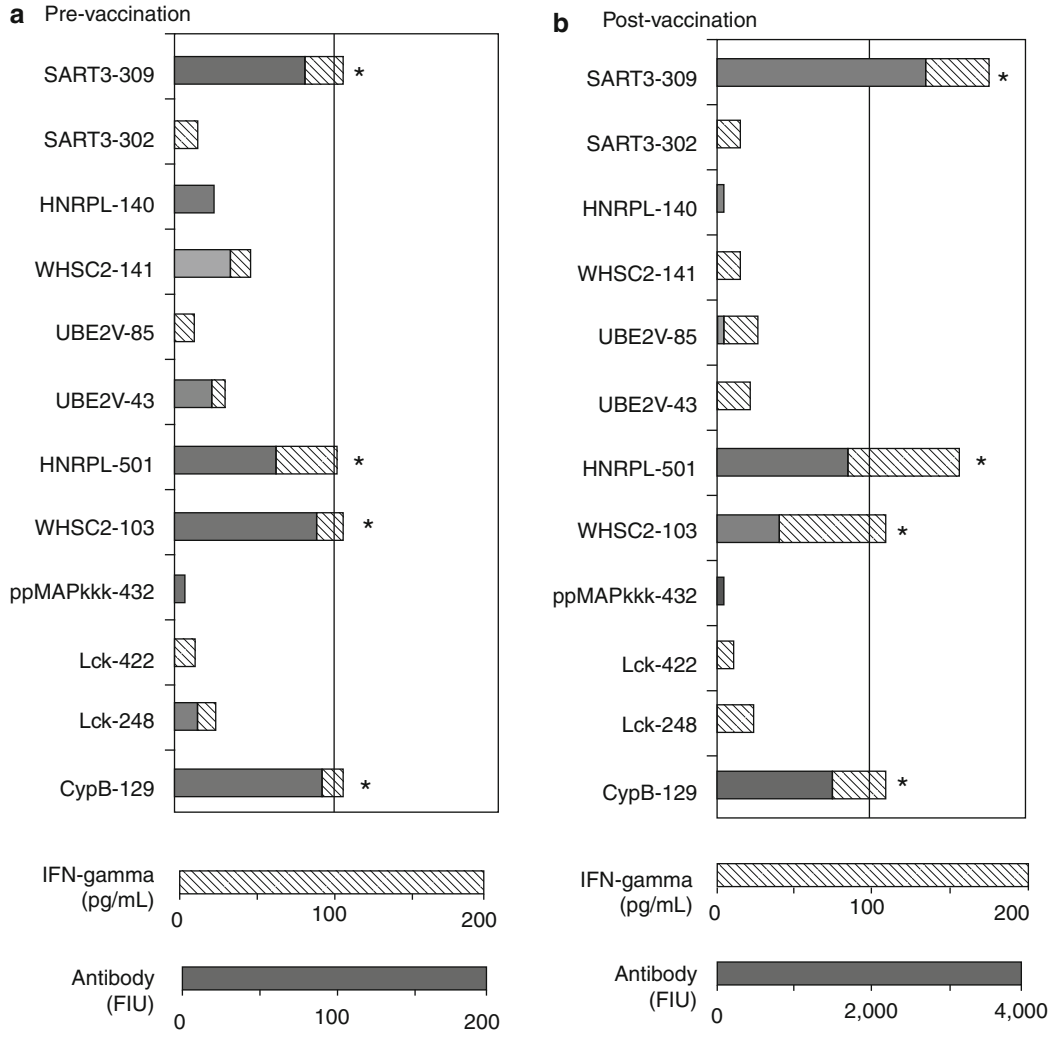

Fig. 21.2 Representative data of immune boosting by PPV. Representative results of the immune monitoring of blood samples collected before (**a**) and after (**b**) vaccination are shown. Antigen-specific antibody (IgG) responses were measured by the microsuspension array (Luminex® assay), and antigen-specific cellular immune responses were measured by the IFN-gamma secretion (ELISA). Four peptides for vaccination (SART3-309, HNRPL-501, WHSC2-103, and CypB-129, represented as "*") were selected by preexisting IgG titers specific to antigen candidates. IgG responses to SART3-309 peptide increased from 80 to 2,700 FIU in the post (6th)-vaccination sample. Similar boosting of humoral responses was observed in the other 3 peptides. Antigen-specific cellular immune responses were also increased in all 4 vaccinated peptides

21.4 Procedures of PPV

Currently, we employ 31 HLA class I-restricted peptide candidates, which were identified from a variety of TAAs by means of a cDNA expression cloning method with tumor-infiltrating lymphocyte clones/lines or a reverse immunology strategy; these include 12 peptides for HLA-A2+ patients, 14 for HLA-A24+ patients, 9 for HLA-A3 supertype+ (A3+, A11+, A31+, or A33+) patients, and 4 for HLA-A26+ patients (Table 21.1). The safety and potential immunological effects of these vaccine candidates have been reported in previously conducted clinical studies [12–16]. A maximum

Table 21.1 Information of peptide candidates used for PPV

Peptide name	Original protein	Position	Sequence	HLA-IA restriction	Reference
CypB-129	Cyclophilin B	129–138	KLKHYGPGWV	A2+/A3+ supertype	*Jpn. J. Cancer Res.* **92**, 762–767 (2001)
Lck-246	p56 lck	246–254	KLVERLGAA	A2+	*Int. J. Cancer* **94**, 237–242 (2001)
Lck-422	p56 lck	422–430	DVWSFGILL	A2+/A3+ supertype	*Int. J. Cancer* **94**, 237–242 (2001)
ppMAPkkk-432	ppMAPkkk	432–440	DLLSHAFFA	A2+/A26+	*Cancer Res.* **61**, 2038–2046 (2001)
WHSC2-103	WHSC2	103–111	ASLDSDPWV	A2+/A26+/ A3+ supertype	*Cancer Res.* **61**, 2038–2046 (2001)
HNRPL-501	HNRPL	501–510	NVLHFFNAPL	A2+/A26+	*Cancer Res.* **61**, 2038–2046 (2001)
UBE2V-43	UBE2V	43–51	RLQEWCSVI	A2+	*Cancer Res.* **61**, 2038–2046 (2001)
UBE2V-85	UBE2V	85–93	LIADFLSGL	A2+	*Cancer Res.* **61**, 2038–2046 (2001)
WHSC2-141	WHSC2	141–149	ILGELREKV	A2+	*Cancer Res.* **61**, 2038–2046 (2001)
HNRPL-140	HNRPL	140–148	ALVEFEDVL	A2+	*Cancer Res.* **61**, 2038–2046 (2001)
SART3-302	SART3	302–310	LLQAEAPRL	A2+	*Int. J. Cancer* **88**, 633–639 (2000)
SART3-309	SART3	309–317	RLAEYQAYI	A2+	*Int. J. Cancer* **88**, 633–639 (2000)
SART2-93	SART2	93–101	DYSARWNEI	A24+	*J. Immunol.* **164**, 2565–2574 (2000)
SART3-109	SART3	109–118	VYDYNCHVDL	A24+/ A24+/A3+ supertype	*Cancer Res.* **59**, 4056–4063 (1999)
Lck-208	p56 lck	208–216	HYTNASDGL	A24+	*Eur. J. Immunol.* **31**, 323–332 (2001)
PAP-213	PAP	213–221	LYCESVHNF	A24+	*J. Urol.* **166**, 1508–1513 (2001)
PSA-248	PSA	248–257	HYRKWIKDTI	A24+	*Prostate* **57**, 152–159 (2003)
EGF-R-800	EGF-R	800–809	DYVREHKDNI	A24+	*Eur. J. Cancer* **40**, 1776–1786 (2004)
MRP3-503	MRP3	503–511	LYAWEPSFL	A24+	*Cancer Res.* **61**, 6459–6466 (2001)
MRP3-1293	MRP3	1293–1302	NYSVRYRPGL	A24+	*Cancer Res.* **61**, 6459–6466 (2001)
SART2-161	SART2	161–169	AYDFLYNYL	A24+	*J. Immunol.* **164**, 2565–2574 (2000)
Lck-486	p56 lck	486–494	TFDYLRSVL	A24+	*Eur. J. Immunol.* **31**, 323–332 (2001)
Lck-488	p56 lck	488–497	DYLRSVLEDF	A24+	*Eur. J. Immunol.* **31**, 323–332 (2001)
PSMA-624	PSMA	624–632	TYSVSFDSL	A24+	*Cancer Sci.* **94**, 622–627 (2003)
EZH2-735	EZH2	735–743	KYVGIEREM	A24+	*Prostate* **60**, 273–281 (2004)
PTHrP-102	PTHrP	102–111	RYLTQETNKV	A24+	*Br. J. Cancer* **91**, 287–296 (2004)
SART3-511	SART3	511–519	WLEYYNLER	A3+ supertype	*Cancer Immunol. Immunother.* **56**, 689–698 (2007)
SART3-734	SART3	734–742	QIRPIFSNR	A3+ supertype	*Cancer Immunol. Immunother.* **56**, 689–698 (2007)
Lck-90	p56 lck	90–99	ILEQSGEWWK	A3+ supertype	*Br. J. Cancer* **97**, 1648–1654 (2007)
Lck-449	p56 lck	449–458	VIQNLERGYR	A3+ supertype	*Br. J. Cancer* **97**, 1648–1654 (2007)
PAP-248	PAP	248–257	GIHKQKEKSR	A3+ supertype	*Clin. Cancer Res.* **11**, 6933–6943 (2005)

All of the listed peptides, except for those derived from prostate-associated antigens (PSA, PAP, and PSMA) for prostatic cancer, are currently employed for vaccination in various types of cancers. A maximum of 4 peptides are selected and administered, based on the result of HLA typing and the pre-existing immune responses specific to each of the 31 different vaccine candidates

of 4 peptides considered appropriate for individual patients, which are selected based on the results of HLA typing and the preexisting immune responses specific to each of the 31 different vaccine candidates, are subcutaneously administered in emulsion with incomplete Freund's adjuvant weekly or biweekly.

For PPV, patients are treated by multiple peptides, since selection and vaccination of multiple CTL epitopes could substantially reduce the risk of antigen-negative clones escaping peptide-specific immune responses, compared to those of only a single epitope. Indeed, it would be relatively rare that tumor cells escape antigen-specific immune responses by simultaneously losing all of multiple antigens selected for vaccination. To prevent interaction/competition among multiple peptides at the vaccinated sites, each of multiple vaccine peptides is administered separately at different sites, but not in a mixture at a single site. Because previous feasibility studies showed that 4 peptides per vaccination seemed to be acceptable but more than 5 peptides per vaccination were intolerable due to adverse skin reactions, which sometimes caused unpleasant symptoms, such as itching and pain (unpublished data), patients are currently treated by up to 4 peptides per vaccination.

Although the clinical effects might be primarily attributed to the cellular immune responses to vaccine antigens, we currently evaluate humoral immune responses as a unique approach for selecting vaccine antigens before vaccination. Assessment of the cellular immune responses specific to antigen candidates is sometimes difficult when selecting the vaccine antigens for PPV, since antigen-specific effector T cells are generally present at very low concentrations in most cancer patients and the assay for determining cellular immune responses might be greatly influenced by the handling conditions of blood samples, such as the conditions for purification and storage of peripheral blood mononuclear cells (PBMC) [17–19]. This is why practical issues, such as the sensitivity, specificity, and reproducibility, must be taken into account when choosing an assay for assessing antigen-specific cellular immune responses.

On the other hand, the assays for assessing humoral immune responses, such as the measurement of antibodies specific to antigens, tend to be more stable than those for measuring cellular immune responses [19, 20]. In particular, the microsuspension array based on Luminex® technology that we have developed for monitoring humoral immune responses allows simple, quick, and highly reproducible high-throughput screening of IgG responses specific to large numbers of peptide antigens with only a tiny amount of plasma or serum [20]. Since the dynamic range of this assay is much wider than that of the traditional enzyme-linked immunosorbent assay (ELISA), it could be applicable for monitoring antigen-specific humoral immune responses in patient plasma or sera not only after vaccination but also before vaccination.

Although the biological functions of the antibodies specific to the TAA-derived peptides remain unclear, they may include functions for promoting antitumor immunity. For example, one of the functions of antigen-specific antibodies may be to promote tumor cell death. Indeed, Vaughan et al. reported that a monoclonal antibody mimicking T cell receptor (TCR) specifically targeted and killed tumor cells via recognition and binding to MHC-peptide epitopes [21]. Similarly, we also could detect antibodies specific to a peptide bound on MHC class I molecules in human sera (unpublished observation). Another possibility is that peptide-specific antibodies induce antibody-dependent cell-mediated cytotoxicity (ADCC), as reported by Frank et al. in breast cancer patients [22]. Further studies would be required to clarify which kinds of biological functions of antigen-specific antibodies are the most relevant to clinical responses to immunotherapy.

21.5 Clinical Outcome of PPV

The more than 500 patients with various types of cancers who were enrolled in the clinical trials for PPV during the past 10 years have shown promising results (Table 21.2) [12–15, 23]. The best clinical response observed in the 436 evaluable patients was a partial response (PR) in 43 patients (10 %), stable disease (SD) in 144 patients (33 %), and progressive disease (PD) in 249 patients (57 %), with a median overall survival of 9.9 months [14]. For example, a recent randomized phase II clinical trial showed a

Table 21.2 Clinical responses of advanced cancer patients treated with PPV

	Patient (*n*)	Best clinical response (*n*)[a]			Response rate (%)	Disease control rate (%)
		PR	SD	PD		
Total	**436**	**43**	**144**	**249**	**9.9**	**42.9**
Prostatic	155	29	36	90	18.7	41.9
Colorectal	68	1	23	44	1.5	35.3
Pancreatic	41	4	23	14	9.8	65.9
Gastric	35	0	8	27	0	22.9
Brain	30	5	11	14	16.7	53.3
Cervical	23	3	7	13	13.0	43.5
Non-small-cell lung	21	0	11	10	0	52.4
Renal cell	12	0	9	3	0	75.0
Melanoma	11	0	5	6	0	45.5
Breast	10	0	1	9	0	10.0
Uroepithelial	7	1	2	4	14.3	42.9
Others	23	0	8	15	0	34.8

PR partial response, *SD* stable disease, *PD* progressive disease
[a]Best clinical responses were evaluated by RECIST criteria (or PSA values in prostatic cancer)

potential clinical benefit of PPV in advanced castration-resistant prostate cancer (CRPC) patients [15]. In this study, 57 CRPC patients were enrolled and randomized into two groups: patients receiving PPV in combination with low-dose estramustine phosphate (EMP) or those receiving the standard dose of EMP alone. The patients receiving PPV in combination with low-dose EMP showed a significantly longer progression-free [median survival time (MST), 8.5 months vs. 2.8 months; hazard ratio (HR), 0.28 (95 % confidence interval (CI), 0.14–0.61); $P=0.0012$] and overall survival [MST, undefined vs. 16.1 months; HR, 0.30 (95 % CI, 0.1–0.91); $P=0.0328$] than those receiving the standard-dose EMP alone, suggesting the feasibility of this combination therapy.

21.6 Biomarkers for PPV

Only a subset of patients show clinical benefits from cancer immunotherapy, including PPV [14]. Therefore, for further development of PPV, it would be critical to identify biomarkers that accurately portray the antitumor immune responses and predict prognosis in vaccinated patients. With regard to postvaccination biomarkers, several factors, such as CTL responses, Th1 responses, delayed-type hypersensitivity (DTH), and autoimmunity, have been reported to be associated with clinical responses in some clinical trials of cancer vaccines [24–26]. In addition, we have reported that the overall survival of patients with increased levels of peptide-reactive IgG in plasma after vaccination was significantly more prolonged ($P=0.0003$) than that of patients without such an increase [14]. Assessment of the immune responses after vaccination might be thus useful as a biomarker for predicting the efficacy of cancer vaccines.

However, there has been little information available regarding biomarkers that are useful for predicting the clinical efficacy of peptide vaccinations before their use. Recently, to identify biomarkers useful for selecting appropriate patients before vaccination, we evaluated pre-vaccination prognostic markers in patients with several different types of advanced cancers who underwent PPV (Table 21.3). In CRPC patients who were treated with PPV ($n=40$), a comprehensive study of soluble factors and gene expression profiles by microarray analysis demonstrated that higher levels of IL-6 and higher frequencies of myeloid-derived suppressor cells (MDSC), which express inhibitory molecules such as ARG1 and inducible nitric oxide synthase [27, 28], and impair the immunological functions of T cells and other immune cells, in the peripheral blood before vaccination, were closely associated with poorer prognosis [29]. In patients with refractory non-small-cell lung cancer ($n=41$), Yoshiyama et al.

Table 21.3 Prognostic biomarkers for PPV

Type of cancer	Factor	Statistical analysis (HR, 95 % CI, *P* value)[a]	Reference
Miscellaneous ($n=500$)	Performance status	HR = 2.295; 95 % CI, 1.653–3.188; $P<0.0001$	*Cancer Biol. Ther.* **10**, 1266–1279 (2011)
	Lymphocyte counts	HR = 1.472; 95 % CI, 1.099–1.972, $P=0.0095$	
	IgG responses to antigens (after vaccination)	HR = 1.455; 95 % CI, 1.087–1.948, $P=0.0116$	
Non-small-cell lung ($n=41$)	C-reactive protein (CRP)	HR = 10.115; 95 % CI, 2.447–41.806; $P=0.001$	*Int. J. Oncol.* **40**, 1492–1500 (2012)
Biliary tract ($n=25$)	IL-6	HR = 1.123; 95 % CI, 1.008–1.252; $P=0.035$	*Exp. Ther. Med.* **3**, 463–469 (2012)
	Albumin	HR = 0.158; 95 % CI, 0.029–0.860; $P=0.033$	
	Numbers of vaccine peptides	HR = 0.258; 95 % CI, 0.098–0.682; $P=0.006$	
Pancreas ($n=36$)	Serum amyloid A (SAA)	HR = 1.21; 95 % CI, 1.05–1.40; $P=0.006$	*Oncol. Rep. in press* (2013)
Prostate ($n=40$)	IL-6	(Not determined)	*Cancer* **118**, 3208–3221 (2012)
	MDSC	(Not determined)	

HR hazard ratio, *CI* confidence interval, *MDSC* myeloid-derived suppressor cells
[a]Potential prognostic biomarkers for PPV were determined by multivariate Cox regression analyses

reported that the C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95 % CI = 2.447–41.806, $P=0.001$) [30]. In addition, in patients with refractory biliary tract cancer ($n=25$), multivariate Cox regression analyses showed that higher IL-6 and lower albumin levels in plasma before vaccination and less numbers of selected vaccine peptides were significantly unfavorable factors for overall survival [HR = 1.123, 95 % CI = 1.008–1.252, $P=0.035$; HR = 0.158, 95 % CI = 0.029–0.860, $P=0.033$; HR = 0.258, 95 % CI = 0.098–0.682, $P=0.006$; respectively] [31]. Furthermore, in PPV for chemotherapy-resistant advanced pancreatic cancer patients ($n=36$), the serum amyloid A (SAA) level was associated with overall survival [HR = 1.21, 95 % CI = 1.05–1.40, $P=0.006$] [32]. Collectively, these findings have demonstrated that less inflammation may contribute to better responses to the PPV, suggesting that evaluation of the inflammatory factors before vaccination could be useful for selecting appropriate cancer patients for PPV.

Based on these findings, gene expression profiling and/or measurement of soluble factors in peripheral blood might be used before vaccination for the monitoring of inflammation factors and immune suppression cells, which could help to predict the prognosis of patients undergoing cancer vaccines and lead to the development of better therapeutic strategies.

21.7 Summary

We have developed a novel approach for cancer vaccination, the PPV, which is designed to boost the preexisting immune responses to TAA-derived peptide epitopes in individual patients. Vaccine antigens considered appropriate for each patient are selected by measuring the humoral immune responses to each antigen before vaccination. Recent clinical trials of PPV have demonstrated the feasibility of this new therapeutic approach in various types of advanced cancers. To increase the clinical efficacy of PPV, we have identified predictive biomarkers in various cancer patients who enrolled in PPV trials. Inflammatory factors, such as IL-6, CRP, SAA, and immune suppressive cells, MDSC, are well correlated with clinical outcomes in the vaccinated patients.

In the near future, PPV could be recommended in combination with other therapeutic approaches to inhibit the inflammatory factors and MDSC frequently detected in advanced cancer patients.

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Molecular Immunotherapeutics and Vaccines for Renal Cell Carcinoma and Its Vasculature

22

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Contents

Abstract

Renal cell carcinoma (RCC) remains a significant health concern since many patients present with metastatic disease at initial diagnosis. RCC tumors are typically highly vascularized due to dysregulated angiogenesis, resulting in leaky, chaotic blood vessels that serve to limit immune effector cell access to the tumor, that may in part be responsible for the modest clinical success noted for cancer immunotherapeutic approaches. Current first-line therapeutics in the advanced-stage disease setting include anti-angiogenic small molecule drugs that have yielded high objective clinical response rates, although these tend to be transient in nature, with most patients ultimately becoming drug refractory.

The implementation of gene-based therapeutic vaccines targeting the tumor vasculature is a promising treatment option that may extend disease-free intervals and overall patient survival, either as a stand-alone therapy or in combination with existing treatment options. Such molecular therapies range from the delivery of ex vivo gene-modified antigen-presenting cells to the direct injection of recombinant viral vaccines. Here we discuss current and pending novel molecular therapeutic approaches germane to RCC patients.

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22.1 Renal Cell Carcinoma: A Need for Improved Molecular Therapeutics

Renal cell carcinoma (RCC) accounts for approximately 3 % of all cancers in adults, with metastases identified in 20–30 % of patients at the time of initial diagnosis. Metastatic RCC, if left untreated, has a 5-year disease-free survival rate of only 2–11 % [1]. Following nephrectomy, conventional treatments with standard chemotherapeutic agents, hormones, and radiotherapy have exhibited minimal success. This has prompted extensive evaluation of alternate treatment strategies, including immunotherapies, in the adjuvant and advanced-stage disease settings.

22.1.1 The Abnormal Tumor Vasculature Provides a Target for Therapeutic Intervention

Solid tumors of various tissue types, including renal, ovarian, and lung, are typically highly vascularized. Blood and lymphatic vessels in the tumor microenvironment (TME) support cancer progression by (1) delivering oxygen and nutrients, (2) providing a conduit by which primary tumors can metastasize, (3) recruiting/supporting cancer stem cells and vascular cell precursors that promote tumor neovascularization, and (4) recruiting immune regulatory cell populations (Treg, MDSC) that limit protective antitumor immune responses.

The vasculature in the TME is typically characterized as "abnormal," here defined as tortuous, irregular networks of blood vessels with highly unstable and permeable capillaries branching from the main vessel bed. Instability in the tumor vasculature increases interstitial pressure within the tumor, preventing the delivery of tumoricidal drugs and immune effector cells into the TME [2]. Tumor vascular endothelial cells (VEC) exhibit differential gene expression when compared to VEC isolated from healthy tissue, with such transcripts believed to underlie tumor blood vessel destabilization [3]. Silencing these genes using siRNA approaches may block VEC migration and vascular tube formation [4], thus supporting tumor VEC as a highly relevant therapeutic target. Another characteristic of the tumor vasculature is the aberrant mural cell coverage of the blood vessels. VEC recruit platelet-derived growth factor receptor (PDGFR)-β-expressing mural cells by secreting PDGF [5].

Mural cells support VEC survival and differentiation and can be subclassified into smooth muscle cell and pericyte populations. Normal mature pericytes have been found to play a major role in vessel stabilization, maturation, remodeling, and repair. Tumor pericytes are typically immature and loosely attached to the capillary and may even be absent in some areas, leading to vessel destabilization [6]. Such pericytes have been characterized phenotypically by overexpression of SMA, RGS5, and tumor endothelial marker-1 (TEM1) in a range of tumor models [7–9]. Interestingly, it has recently been shown that tumors in RGS5-deficient mice display a normal pattern vascular architecture, characterized by reduced mean vessel density (MVD) and mature pericyte coverage and reduced hypoxia and vessel leakiness when compared to tumors growing in wild-type hosts. These vascular changes are associated with an increased infiltration of adoptively transferred tumor-specific T cells into the TME and the dramatically prolonged survival of treated animals [10].

22.1.2 Immune Dysfunction in the TME

Solid tumors are frequently hypoxic resulting in increased expression of hypoxia-induced factors (HIFs) and impaired effector T cell and dendritic cell (DC) function [11]. Upregulated HIF expression also leads to increased VEGF expression, which can attenuate DC differentiation and increase STAT3 activation via VEGFR2-mediated signaling, thus promoting the intrinsic expression of immunosuppressive factors including IL-10 and TGF-β in these regulatory antigen-presenting cells [12]. In many cancer patients, Type 1 proinflammatory responses required for tumor destruction are inhibited or functionally dysregulated by the suppressive influence of Treg or myeloid-derived suppressor cells (MDSC) and/or by Type 2 inflammatory molecules [13–16].

Furthermore, Type 1 tumor-specific T cells may become functionally exhausted and undergo premature (apoptotic) death due to chronic stimulation by cognate tumor antigens in cancer-bearing patients [17, 18]. Such immune-evasion tactics assumed by the tumor serve to limit the protective host immunological responses and allow for tumor survival and progression. Additionally, due to the destabilized vasculature in progressively growing tumors, circulating Type 1 antitumor T effector cells may be unable to traffic efficiently to sites of tumor [10].

22.1.3 Immune Targeting of RCC

Optimism for the use of biologic response modifiers and vaccines has been fueled by past reports which indicate that, like melanoma, RCC progression and regression may be controlled immunologically [19]. Notably, a minor but significant frequency of RCC patients exhibit apparent spontaneous tumor regression [20], and patients on chronic immunosuppression regimens to retain kidney allografts exhibit increased risk of developing RCC [21]. Furthermore, the magnitude of tumor-infiltrating lymphocytes (TIL) has been suggested to represent a prognostic indicator for patient survival [22]. In this regard, Type 1 TIL (i.e., those producing IFN-γ and capable of mediating the cytolytic function) [23], appear to represent the primary immunological mediators of objective clinical responses.

Although administration of high-dose interleukin-2 (IL-2; aka T cell growth factor) has yielded durable complete responses in a minority of treated patients with metastatic RCC, severe toxicities have also been observed for this approach [24], suggesting the need to identify more specific/focused immunotherapy approaches.

22.1.4 Vascular Normalization in the TME Using Anti-angiogenic Small Molecule Drugs

Judah Folkman presented the idea of angiogenesis as a therapeutic target in cancer [25]. His theory spurred the development of several anti-angiogenic therapies including monoclonal antibodies reactive against angiogenic growth factors (and their cognate receptors) and small molecule inhibitors of pro-angiogenic receptor tyrosine kinases. Indeed in 2005, it was suggested that normalization of the tumor vasculature could be achieved via administration of anti-angiogenic agents as a novel cancer therapy [2, 10]. This approach coordinately allowed for the improved delivery of co-applied chemotherapeutic agents into the TME.

The humanized monoclonal antibody bevacizumab (Avastin) has been shown to induce a transient normalization of the tumor vasculature in treated patients, which when coupled with chemotherapy, promoted enhanced antitumor effects [26]. Targeting soluble VEGF increases the preponderance of PDGF in the TME, thus inducing pericyte recruitment and activation and capillary stabilization [27]. Huang et al. recently reported that at a given dose of anti-VEGFR2 antibody, the tumor vasculature became normalized in mice. Furthermore, this treatment reprogrammed the TME to be more pro-inflammatory and increasingly receptive to T effector cell infiltration [28]. Alternatively, pharmacological agents, such as sunitinib, targeting VEGF receptors (i.e., VEGFR1–3), as well as other pro-angiogenic tyrosine kinase receptors (i.e., PDGFR-β), can also inhibit dysregulated angiogenesis, leading to a temporary normalization of the vasculature.

Due to the highly vascularized nature of RCC, several clinical trials have utilized tyrosine kinase inhibitors (TKI) that antagonize angiogenesis as a means to treat this disease. One such inhibitor is sunitinib malate [29], which has exhibited pronounced (albeit transient) efficacy in phase I/II clinical trials and is approved as a first-line treatment for patients with RCC [30–32]. However, a phase III trial showed that while progression-free survival early on was higher in patients receiving sunitinib (11 months) compared to interferon treatment (5 months), the overall survival benefit associated with this approach was not dramatically different between the two groups (26.4 months vs. 21.8 months) [30].

Direct antitumor effects have not been determined for sunitinib, as no somatic mutations in RTK have been identified in human RCC [33].

Interestingly, in vitro studies have shown that sunitinib's mechanism of action involves the induction of an apoptotic death for tumor-associated endothelial cells rather than human RCC cells themselves. Like bevacizumab, in vivo studies have shown sunitinib to induce a temporary window of vascular normalization in the TME [34].

Although sunitinib was initially developed as an angiostatic agent, recent reports suggest that this TKI actually "normalizes" the tumor vasculature by selective pruning of immature and fragile vessels, leaving the more differentiated vessels intact with mature pericyte coverage. This normalization event leads to a decrease in interstitial pressure and improved delivery of chemotherapeutic drugs and effector T cells into the TME [35]. This corresponds with results from a study by Ganss et al. showing that inflammation of the TME induced by local radiotherapy, in conjunction with adoptive transfer of tumor-specific cells, can induce microvasculature remodeling towards a phenotype resembling normal tissue [36]. The normalized endothelium exhibited increased expression of Type 1 chemokines CXCL9 and CXCL10 as well as their cognate receptor CXCR3. CXCL10 is an endogenous angiogenic inhibitor that acts as a chemoattractant for Type 1 T cells and has been shown to induce apoptosis in human $CXCR3b^{+}$ endothelial cells but not tumor cells [37].

Additionally, patients treated with sunitinib exhibit reductions in MDSC and Treg populations and normalized Type 1 T cell responses in vitro [31, 32, 38, 39]. Murine tumor models suggest that sunitinib suppresses STAT3 activation [40] and boosts the efficacy of immunotherapy by promoting tumor-specific effector T cells while also suppressing MDSC and Treg in vivo [32, 38, 41]. Sunitinib treatment has been shown to skew expression of chemokines and their receptors towards a Type 1 profile [42] as well as increased expression of VCAM1 and CXCL9 (MIG) with increased IFN-γ-producing T cells within the TME and tumor-draining lymph nodes (TDLN), implying this TKI may represent a potent immune adjuvant. We anticipate that a similar and potentially more durable impact of vascular normalization might result from the specific immune targeting of tumor-associated blood vessels and their cell subpopulations.

22.1.5 Current Therapeutic Vaccines for RCC

Vaccines designed to promote specific adaptive immunity against RCC have traditionally involved the use of tumor cells themselves [43]. Autologous tumor cell vaccines have been modified by either coadministration with or genetically modified to express inflammatory cytokines including GM-CSF, IFN-γ, and IL-2 [44, 45]. Another tumor vaccine formulation is represented by RCC antigen-presenting cell (APC) fusion hybrids, which generate hybrid APC that are capable of expressing RCC gene products and presenting their derivative peptide epitopes to T cells [46].

An alternative approach to cellular-based vaccines (i.e., modified tumor cells, APC, T cells) is genetic vaccination using recombinant viral-based delivery systems. Various reports have shown virus-based vaccinations possess therapeutic advantages over protein antigen-/adjuvant-based approaches [47, 48], presumably due to the intrinsic pro-inflammatory properties of viruses (via activation of Toll-like receptors (TLR) expressed by APC) and their ability to infect professional APC, allowing for ectopic expression of the vaccine antigen within patient DC [49–51]. Additionally, high-titer recombinant viruses are easy to produce and when compared to cell-based therapies that require costly time-consuming methods due to their patient-specific nature, viral vectors can be administered to any given patient as an off-the-shelf treatment modality. Thus, despite minor concerns for replication-competent contaminant virus or for insertional mutagenesis in the case of retroviruses [52], genetic vaccines remain attractive treatment options in the cancer (RCC) setting.

The recombinant modified vaccinia virus Ankara (MVA) encoding RCC antigen 5T4, TroVax, has been tested in several clinical trials. Initial trials in RCC patients showed some objective clinical responses after administration of the TroVax vaccine [53, 54]; however, in phase III trials employing TroVax with or without cytokines

(IFN-α and IL-2) in combination with sunitinib, no significant difference in survival between the experimental and control groups could be demonstrated [55]. A second MVA vaccine was developed containing the recombinant MUC1 and IL-2 transgenes, administered with or without cytokines. Although some RCC patients exhibited anti-MUC1 T cell responses, treatment with this vaccine formulation did not result in objective clinical responses based on RECIST criteria [56].

It is important to note there are also nonviral molecular vaccines under development for RCC. Another approach involves DC pulsed or transfected with RCC-derived total mRNA (encoding the complete repertoire of RCC-associated antigens) [57–59]. More recently, a direct intradermal injection of mRNA-based vaccine encoding MUC1, CEA, Her-2/neu, telomerase, survivin, and MAGE-A1 has been evaluated in a phase I/II clinical trial involving 30 RCC patients. The reported results were promising, with some vaccinated patients exhibiting stable disease and coordinately increased tumor antigen-specific T cell responses [60].

A general summary of therapeutic vaccines targeting molecularly defined RCC-associated antigens is provided in Table 22.1. In general, these approaches have proven immunogenic, with postvaccine Type 1 T cell responses often being associated with improved clinical outcomes.

22.1.6 Genetic Vaccines Encoding Tumor Stromal Cell-Associated Antigens

Genetic aberrations can potentially develop within the evolving and heterogeneous RCC lesion over many months to years under immune selective pressure [72]. Vaccines based on whole tumor cells, tumor-APC hybrids, and/or tumor-derived mRNA or cDNA and tumor antigens derived from mutated or overexpressed proteins have thus far underperformed as therapeutic agents. These vaccine formulations may merely reinforce an existing, yet failing, immune repertoire given the immune dominance of certain tumor antigens over others and to immune-evasion paradigms assumed by the heterogeneous tumor mass. Competition by a myriad of peptide epitopes for loading into major histocompatibility complex (MHC) molecules expressed by (cross presenting) APCs in vivo could also limit effective immune activation against a broad range of otherwise therapeutic tumor antigen targets.

Recently, several groups [73, 74] have shown that while treatment with anti-angiogenic agents may lead to the transient normalization of the tumor vasculature and to at least transient tumor regression, ultimately, upon treatment cessation, the tumors often recur and may even exhibit more aggressive behavior with regard to their invasiveness and metastatic potential. Since these drugs only limit angiogenesis (i.e., via receptor tyrosine kinase (RTK)-signaling antagonism) rather than eradicating the vasculature outright, it is likely that tumor-associated vascular cells ultimately adapt to a state of drug resistance. A possible means to circumvent this problem is to specifically instigate immune targeting of tumor pericytes and/or vascular endothelial cells to provide a durable policing force capable of preferentially destroying/regulating tumor-associated stromal cells while sparing the vasculature in normal, tumor-uninvolved organs in the patient. By focusing Type 1 immune responses in the tumor vascular niche, one may also activate the TME (i.e., modulating its cytokine milieu and pattern/level/activation state of adhesion molecules expressed), leading to the enhanced recruitment and (poly) functionality of anti-RCC T effector cells.

We, and others, have recently advocated the implementation of vaccines promoting specific Type 1 T cell recognition of tumor vascular cell (i.e., pericytes, VEC) populations [75–77]; however, these vaccine formulations have been cellular based, utilizing adoptive transfer of DC presenting stromal antigen-derived peptides. Rosenberg's group has recently shown that T cells engineered to express a chimeric antigen receptor (CAR) specific for VEGFR2, which is overexpressed in the vasculature of many solid cancers, including RCC, were able to limit the growth of five different types of established, vascularized tumors in mice and to coordinately induce VEGFR2-specific host T cell responses [78]. This

Table 22.1 Clinical vaccines incorporating molecularly defined RCC-associated antigens

Vaccine format	Vaccine targeted antigen	Trial phase	Number of RCC patients treated	Vaccine immunogenicity	Clinical outcomes	References
Polypeptide vaccine +/− IL-2 or IFN-α	Selection of 4 defined TAA peptides from panel based on patient in vitro responses pretreatment	I	10	8/10 patients with positive DTH response to vaccine, 2/10 patients with increased Type 1 T cell response to peptides postvaccine	6 SD	[61]
AutoDC + MUC1 peptide + PADRE peptide + IL-2	MUC1	I	20	10/20 patients with Type 1 T cell response to peptides	1 CR, 2 PR, 5 SD, 2 MR; T cell responses correlated to OCR	[62]
MKC1106-PP gene prime/ peptide boost	PRAME, PSMA	I	2	2/2 patients with Type 1 T cell response to peptides	2/2 with SD > 11 months	[63]
CA-IX (G250) peptide + IFA	CA-IX	I	23	5/10 evaluable patients with Type 1 CD8+ T cell response to peptide	3 PR, 6 SD	[64]
Mature AutoDC + CA-IX (G250) peptides	CA-IX	I	6	No specific immunity to CA-IX	No OCR	[65]
Tumor antigen RNA + GM-CSF	MUC1, CEA, HER2/Neu, telomerase, survivin, MAGE-A1	I/II	30	8/10 evaluable patients with Type 1 T cell response to vaccine peptides	16 SD, 1 PR	[60]
Telomerase peptide + Montanide adjuvant	Telomerase	I/II	4	Not described, progressor tumor in 1 patient was a MHC-I loss variant	No OCR	[66]
AutoDC + telomerase peptide	Telomerase	I/II	10	2/8 evaluable patients with DTH response, CTL responses only in 3 patients with OCR	1 SD, 2 MR correlates with strongest T cell IFN-γ response to peptide	[67]
Mutant VHL peptide + Montanide adjuvant	Mutant VHL	I/II	6	4/5 evaluable patients with CD8+ T cell responses to antigen	No OCR, extended OS if patients responded to vaccination	[68]
IM901 + GM-CSF +/− CY	9 natural MHC-I presented TAA (PLIN2, APOL1 (2),CCND1, GUCY1A3, PRUNE2, MET, MUC1, RGS5) + 1 natural MHC-II presented TAA (MMP7) peptides	I + II	96	64–74 % of evaluable patients displayed CD8+ T cell responses to 1 or more vaccine peptides	Better OS observed if patients made immune response to multiple peptides	[69]

TG4010 (rVaccinia virus) +/− IFN-α/IL-2	MUC1	II	37	6/28 and 6/23 evaluable patients made CD4+ and CD8+ T cell responses to vaccine, respectively, immune responders with longer OS	6/20 patients with SD for more than 6 months	[56]
MVA-5T4 (TroVax; modified vaccinia Ankara) +/− sunitinib, IL-2, or IFN-α	5T4	III	288	56 % of evaluable patients developed anti-5T4 antibody responses postvaccine, T cell responses not evaluated	Magnitude of antibody response was associated with longer OS, greatest impact for smaller tumors	[53–55, 70, 71]
IMA901 +/− sunitinib	9 natural MHC-I presented TAA (PLIN2, APOL1 (2),CCND1, GUCY1A3, PRUNE2, MET, MUC1, RGS5) + 1 natural MHC-II presented TAA (MMP7) peptides	III	330	In progress, results expected in 2014	In progress, results expected in 2014	In progress, results expected in 2014

Abbreviations: *AlloDC* allogeneic dendritic cells, *APOL1* Apolipoprotein L1 antigen, *AutoDC* autologous dendritic cells, *CA-IX* carbonic anhydrase-IX antigen (also known as G250), *CCND1* cyclin D1 antigen, *CEA* carcinoembryonic antigen, *CR* complete response, *CTL* cytotoxic T lymphocyte, *CY* cyclophosphamide, *DTH* delayed-type hypersensitivity, *GUCY1A3* guanylate cyclase soluble subunit alpha-3 antigen, *HER2* human epidermal growth factor receptor 2 (also known as Neu), *IFA* incomplete Freund's adjuvant, *MAGE-A1* melanoma-associated antigen 1, *MET* c-MET proto-oncogene antigen (also known as hepatocyte growth factor receptor), *MHC* major histocompatibility complex, *MMP7* matrix metalloproteinase 7 antigen, *MR* mixed response, *MUC1* mucin-1 antigen, *OCR* objective clinical response, *OS* overall survival, *PADRE* Pan-DR presented epitope, *PR* partial response, *PRAME* preferentially expressed antigen in melanoma, *PRUNE2* protein prune homolog 2 antigen, *PSMA* prostate-specific membrane antigen, *RGS5* regulator of G-protein signaling 5 antigen, *SD* stabilization of disease, *TAA* tumor-associated antigen, *VHL* Von Hippel-Lindau protein, *5T4* trophoblast glycoprotein antigen (also known as TPBG)

treatment strategy is currently under investigation in phase I trials for RCC as well as melanoma patients. It has also been shown that treatment with fibroblasts genetically altered to express endostatin, another molecule critical for tumor angiogenesis, leads to reduced tumor burden with increased infiltration of $CD4^+$, $CD8^+$, and CD49b/VLA-4^+ lymphocytes in a metastatic RCC mouse model when combined with rIL-2 administration, indicating an additive immunomodulatory effect of the vaccine [79].

Current genetic immunization strategies for RCC have been limited to those using recombinant vaccinia viruses encoding tumor antigens, which have thus far met with moderate clinical success. Unfortunately, vaccinia vectors can only be applied once or twice due to the rapid host development of neutralizing antibodies that serve to limit the booster capacity of further administrations of this modality [80]. Given the ability of lentivirus to transduce endogenous DC in vivo in the absence of evoking neutralizing antiviral immunity [81], and therefore the potential of providing reiterated dosing to patients, we have recently developed a lentiviral-based vaccine designed to promote specific immune targeting of the tumor vasculature and, more specifically, vascular pericytes. Pericytes isolated from human and mouse RCC tumors differentially overexpress a cohort of antigens (compared to homologous cells in the normal kidney), including delta-like 1 homologue (DLK1). Therapeutic DLK1 peptide- and (lentiviral) gene-based vaccines were both observed to be immunogenic and effective in regulating the growth of RENCA (RCC) tumors in vivo. Effectively treated RCC tumors displayed vascular normalization based on reduced vascular leak and tissue hypoxia and were highly infiltrated by $CD8^+$ TIL in the perivascular space. Residual pericytes in the TME were tightly approximated to blood vessels and were deficient in expression of DLK1, supportive of vaccine-induced immunoselection of mature mural cell populations in vivo. We believe that vaccines targeting tumor-associated vascular antigens such as DLK1 may hold significant clinical promise in the setting of RCC and other vascularized solid cancers.

22.1.7 Existing Vaccine Platforms May Be Suboptimal, Mandating the Development of Combination Immunotherapy Approaches

Current single-modality treatments for RCC, either immune targeting of cancer cells or anti-angiogenic pharmacological agents, have thus far yielded limited clinical success. While functional tumor-specific T cells may be activated by vaccination, these cells typically do not effectively traffic into tumor lesions exhibiting an unstable vasculature, given the lack of recruitment signals and high interstitial pressures that serve as a physical barrier to lymphocytic extravasation [82]. The transient vascular normalization seen in tumors treated with anti-angiogenic treatments may circumvent the limitation of T cell trafficking if used in combination with vaccine-based therapies.

Vaccines promoting immune targeting of cancer cells can select for resistant (i.e., antigen or MHC loss) variants [83], leading to the maintenance of occult disease or the progression of tumors that are "invisible" to the adaptive immune system. The anti-angiogenic action mediated by a vascular antigen-specific $CD8^+$ T cell repertoire would be anticipated to delete specific vascular cell populations and therefore complement the specific inhibition of receptor signaling that is hallmark of alternative pharmacological anti-angiogenic treatment modalities such as anti-VEGF antibodies (i.e., bevacizumab) and small molecule tyrosine kinase inhibitors (i.e., sunitinib) [29, 84, 85]. Frequently, vascularized tumors treated with these agents rapidly become drug refractory due to their adoption of compensatory growth/progression pathways. Given such limitations, molecular vaccines targeting vascular stromal antigens could represent a logical second-line approach in the many cases of developed resistance to bevacizumab, sunitinib, or similar anti-angiogenic drugs. These vaccines may also represent effective co-first-line therapeutic agents (Fig. 22.1), since the specific activation, recruitment, and function of antitumor

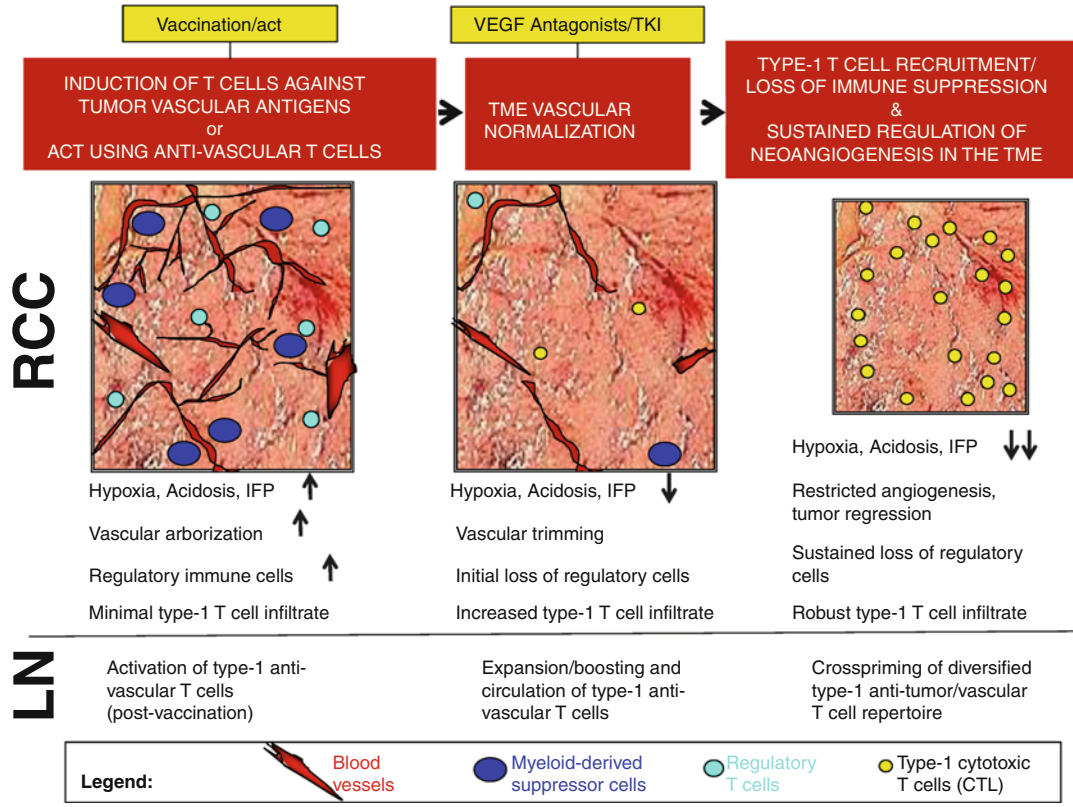

Fig. 22.1 Paradigm for the improved therapeutic benefit of combination vaccines targeting the RCC blood vasculature.
Untreated RCC tumor (*left panel*) is populated by regulatory immune cells (myeloid-derived suppressor cells [*MDSC*] and regulatory T cells [Treg]) with minimal Type 1 T effector cells present. An unstable, disorganized vasculature in the progressor lesion leads to hypoxia, acidosis and high interstitial fluid pressure (*IFP*). Active vaccination against tumor vascular antigens, and subsequent activation of specific T cells in the vaccine-draining lymph node (*LN*), or the adoptive transfer of vascular antigen-specific T cells (i.e., adoptive cell therapy, ACT) leads to circulating levels of Type 1 T effector cells that are inefficient in trafficking into RCC tumors. Staggered co-treatment with anti-angiogenic drugs such VEGF antagonists or TKI acts to at least transiently normalize the tumor vasculature, leading to reduced hypoxia/acidosis/IFP, as well as to remove regulatory cell populations (*middle panel*), allowing for the increased recruitment and effector function of Type 1 vaccine-induced or ACT T cells (*right panel*). Subsequent immune-mediated eradication of tumor cells and the presentation of tumor (and alternate stromal antigens) by locoregional DC populations may lead to the crosspriming of a diversified repertoire of therapeutic Type 1 T cells in tumor-draining lymph nodes, resulting in extended clinical benefit (*right panel*)

stroma-associated antigen T effector cells in the TME would be anticipated to be improved by the coadministration of anti-angiogenic TKI that reduce suppressor cell populations (most notably in RCC patients) and the activation of a pro-inflammatory TME in vivo [39–42].

22.2 Adoptive Cell Therapy (ACT)

Treatment with genetically modified recombinant T cell receptor (TCR) T cells has recently been shown to promote cancer regression in hematological malignancies [86]. Implementing

a similar strategy, tumor antigen-specific T cells can be isolated from RCC tumor-infiltrating lymphocytes (TIL) in vitro, with RCC antigen-specific TCRs subsequently isolated, cloned, and transduced into patient T cells to confer antitumor specificity [87]. Once expanded ex vivo, such antitumor effector cells may be adoptively transferred as a therapeutic agent.

Additionally, given the recent success in treatments using adoptive transfer of T cells genetically engineered to express CARs [88], there has been increasing interest in exploiting this strategy in the setting of solid tumors. For instance, clinical trials are underway to test the safety and efficacy of T cells engineered to express carcinoembryonic antigen (CEA)-reactive CARs in metastatic colon carcinoma [89] and folate receptor-alpha (FR-α)-reactive CARs in advanced-stage ovarian cancer [90]. In a phase 1 clinical trial for RCC patients, many individuals receiving T cells genetically engineered to express a CAR directed against carbonic anhydrase-IX (CA-IX, aka G250) RCC-associated protein developed corollary anti-G250 antibody responses and T cell-mediated immunity [91]. It will be of great interest to monitor the development and future clinical implementation of ACT using engineered T cells bearing CARs reactive against tumor-associated blood vessel antigens in patients with RCC. While such therapies are still in preliminary phases of evaluation in patients, we would predict that the treatment of RCC patients with anti-vascular CAR$^+$ T cells would greatly improve the magnitude and duration of preferred clinical outcomes, particularly when used in combination with other TME-normalizing drugs, such as bevacizumab and sunitinib.

22.3 Concluding Remarks

The heterogeneity of renal cell carcinomas, as well as most other solid forms of cancer, has made it difficult to develop uniformly successful therapies targeting tumor-associated antigens. Indeed, while several molecular strategies have been developed to induce immunity against tumor antigens, such approaches have met with minimal clinical success. As such, it has become necessary to identify common (immutable) features amongst tumors, such as the irregular blood vasculature that is a hallmark of macroscopic solid tumors. In this regard, subsets of immature vascular cell populations in the TME overexpress a range of antigens, such as receptor tyrosine kinases, that may be targeted by pharmacological drugs that lead to transient benefits or by immunotherapeutic approaches that offer the promise of sustained adaptive immune targeting of the tumor-associated vasculature.

Such strategies include vaccines incorporating tumor-associated vascular protein/peptide antigens, viral vaccines encoding full-length or subunit vascular antigens, and ex vivo expanded or molecularly engineered T cells reactive against tumor-associated vascular targets. We believe that while these molecular therapies targeting the tumor vasculature may provide therapeutic benefit in RCC patients, their effects may be further augmented if administered in combination with existing "vascular-normalizing" agents including monoclonal antibodies (i.e., bevacizumab) and receptor tyrosine kinase inhibitors (i.e., sunitinib).

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Lung Cancer Immunotherapy: Programmatic Development, Progress, and Perspectives

23

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Abstract

This chapter describes a decade of experience with lung cancer vaccine development at a single institution. Elements of review include rationale, developmental strategy, scientific approach relative to historical perspective, and clinical circumstance.

23.1 Lung Cancer

Lung cancer is the most common cancer worldwide and accounts for 1.3 million deaths annually, which is more than the next three most common

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cancers combined (colon, breast, and prostate). The insidious nature of lung cancer renders the vast majority of cases to advanced stage at the time of diagnosis. Since stage is most strongly associated with prognosis, late diagnosis is primarily responsible for the 15 % 5-year survival [1, 2].

Nearly half of the patients that can undergo potentially curative surgical resection ultimately succumb to their disease [1, 2]. The rate of second primary lung cancer following first cancer diagnosis is 1–2 % per year [1, 2]. Early detection and prevention initiatives may help reduce overall disease mortality, but the burden of improving outcomes following diagnosis falls more squarely on treatment.

23.2 Conventional Therapies

Lung cancer is divided into two main histologic subtypes, non-small cell (NSCLC) and small cell (SCLC), which describe distinct somatic origin, behavior, phenotype, and prognosis. Relative frequency of NSCLC: SCLC is 4:1 (80 % vs. 20 %). Conventional therapies are tailored to both stage and histologic cell type (NSCLC vs. SCLC); more recently, molecular phenotyping has been used to direct therapy towards the most effective regimen [1–4].

23.2.1 SCLC

For practical purposes, all cases of this aggressive tumor type are assumed metastatic at the time of diagnosis. Chemotherapy is the mainstay of therapy and surgery does not offer any therapeutic advantage. Radiation is added for local control in limited thoracic disease. SCLC mortality is roughly 95 % [2, 3].

23.2.2 NSCLC

Surgery is the primary treatment modality for early stage cancer and chemotherapy and radiation for more advanced disease. Combinations of surgical and nonsurgical therapies have incrementally increased survival rates over the past decade [4].

23.2.3 Early Stage Disease

Surgical resection continues to offer the best chance of cure for the minority of patients diagnosed with early stage NSCLC. Cure rates approach 80 % for small cancers and steadily decrease commensurate with tumor burden. Adjuvant chemotherapy is now conventionally offered to individuals with >35 % chance of recurrence following potentially curative resection [stage II and III disease], providing a 4–15 % survival advantage at 5 years. Other than pathological stage, there are no clinically validated predictive measures of recurrence to aid in the selection of appropriate candidates for adjuvant chemotherapy [4].

23.2.4 Advanced-Stage Disease

Eighty percent of patients with non-small-cell lung cancer (NSCLC) present with locally advanced or metastatic disease, and an additional 10 % develop mortal recurrence following potentially curative surgical resection. Median survival with treatment ranges from 7 to 12 months. Only 60 % of patients with advanced NSCLC will have clinical response to first-line chemotherapy, typically lasting 3–6 months; newer agents have reduced toxicities but have done little to produce durable responses. Maintenance chemotherapy is conceptually appealing, but only pemetrexed has yet proven effective in delaying time to progression in patients with adenocarcinoma. Upon progression second-line chemotherapies offer 8 months' increase in median survival over best supportive care [4].

23.3 Cancer Immunotherapy

This continually evolving field is based on knowledge that the immune system can recognize cancer cells as foreign [5]. The ability to selectively target disseminated disease with minimal impact on normal tissue is a fundamental precept. Incremental knowledge of immunobiology has given rise to myriad therapeutic strategies, each with the common objective of improving unacceptably poor treatment outcomes. Advanced

understanding of adaptive immunity, antigen discovery, and various effects of immune adjuvants from in vitro and animal studies spawned numerous early phase studies in lung and other cancers [5]. Despite decades of anticipatory promise, several phase II trials have given way to disappointing phase III results [5, 6].

A new era may have been ushered in by the 2010 Food and Drug Administration (FDA) approval of sipuleucel-T (Provenge), an autologous dendritic cell-based vaccine delivering a fusion protein comprised of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GMCSF), for metastatic hormone-refractory prostate cancer [6, 7]. The efficacy standard was 4-month increase in median survival in three randomized phase III trials [6]. Several other cancer vaccines and immune modulators are in the pipeline; a handful of ongoing randomized trials in lung cancer warrant cautious optimism. Most active immunotherapies for cancer have been, and continue to be directed, at generating antigen-specific T-cell responses. A more recent appreciation of functional immune regulation in cancer offers new avenues for modulating the immune system for clinical benefit [8, 9]. There has been much less evidence of therapeutic efficacy with tumor antigen-specific antibodies, although resurgence of this approach in recent years may change that historical precedent [10].

23.4 Lung Cancer Immunotherapy

Disease characteristics have certainly influenced developmental strategies for lung cancer. Antigenic heterogeneity, which is somewhat dependent on tumor histology, is a defining characteristic and significant challenge for antigen-specific therapies. Other considerations include the notable disparity in stage distribution, spectrums of conventional therapy, and poor prognosis. Targeting microscopic residual disease that leads to recurrence in nearly half the patients with surgically resected non-small-cell lung cancer (NSCLC) is a logical application. The risk-to-benefit ratio is especially relevant for these patients since recurrence cannot yet be predicted beyond statistical probability. Conversely, efficacy trials are encumbered by variable rates of recurrence and protracted clinical endpoints. Patients with advanced-stage disease have not been widely viewed as likely to benefit from immunotherapy, although slowing disease progression with minimal added risk is a meritorious objective. The terminal prognosis of advanced-stage NSCLC and the ability to accurately define tumor progression and survival make this population a logical choice for clinical trials. With the majority of those cases being diagnosed by scant cytological sampling, limited access to intrathoracic sites of disease presents secondary hurdles. Apart from being an obvious barrier to in situ approaches, availability of lung tumor tissue is an impediment to autologous vaccine production, antigen characterization, and immune monitoring; the risks of repeated sampling by invasive procedures can rarely be justified. Immunotherapy for small-cell lung cancer (SCLC) has not been as widely investigated. The disease tends to be very aggressive, and immunotherapy has not been regarded as having significant potential to affect outcomes. The lack of pathological specimens has also slowed preclinical investigation (in the same way readily available NSCLC specimens have facilitated study) because SCLC is not available for in vitro study or xenogeneic tumor modeling, and there is no appropriate SCLC animal model. Regardless, the poor prognosis associated with SCLC makes it a rational choice for further investigation.

23.5 Programmatic Development

Our university-based program is firmly rooted in the interdisciplinary collaboration between a clinician, an immunologist, and a dedicated nurse coordinator with common interest in novel therapeutics for NSCLC. From inception, objectives have been to develop universal vaccines for lung cancer, test central precepts in immunotherapy, and contribute information that leads to rational implementation of lung cancer vaccines. Although we benefited from ample preclinical and feasibility trials of a variety of agents in

different cancers, we were also confronted by several disappointingly negative efficacy studies and considerable speculation about basic precepts in immunotherapy [5]. Progress in NSCLC would require sequential confirmation of tangible endpoints and hinge on the immunological reliability of formulations. Options for vaccine preparation are multiple and varied. The most rational choices would offer potential for therapeutic activity while also functioning as a tool for exploring relevant questions in immunotherapy.

23.6 Choosing an Immune Adjuvant

A multitude of agents, ranging from nonspecific to highly selective, effectively promote antigen uptake, presentation, and/or recognition through a variety of intersecting mechanisms [11]. The innate capacity of dendritic cells (DCs) to fully orchestrate an adaptive response has led to their characterization as "nature's adjuvant" [12]. At the time, techniques for generating large numbers of dendritic cells (DCs) from peripheral blood had been established and the ability to generate potent vaccines by loading cultured DC ex vivo with antigen were just being translated from laboratory to the clinics. Promising preclinical data and apparent reliability of immune induction in a few early human studies suggested this approach was an emerging standard for cancer vaccines [12, 13]. With collective experience in NSCLC limited to a handful of patients bearing antigens common to other cancers, the open niche of DC vaccines for NSCLC seemed a logical entry to clinical investigations [12, 13]. The historical familiarity with cellular biologics also promised a more streamline regulatory process compared to other more complex, recombinant, or pharmacological alternatives.

23.7 Antigen

Beyond constraints of antigenic relevance, there is no ideal source of antigen. Distinctions between autologous vs. allogeneic, monovalent vs. multivalent, whole-cell vs. recombinant preparations are part theoretical and part pragmatic [11]. Autologous tumor antigen would offer the advantage of a patient-specific vaccine that circumvents the need to know the antigens patient's cancer expresses, but the volume of tumor tissue generally required to produce autologous tumor vaccines restricts that approach to individuals with a surgically resectable tumor. Monovalent approaches utilizing allogeneic recombinant protein (including gene therapy derived) or synthetic peptides would restrict usage to a subset of individuals bearing relevant antigens, and introduce a secondary hurdle for antigen characterization, with or without HLA restrictions. Allogeneic whole-cell preparations offer an array of well-described antigens, as well as a complement of minor determinants and mismatch antigens to enhance crosspriming [14]. Cell lines are a renewable source of tumor protein that provide a standardized, multivalent antigen preparation [14]. Recipient APCs process and present the most relevant epitopes via class I and class II pathways, thus generating the potential for both immune effector and immune memory responses.

23.8 Immune Monitoring

Immunological assays are measures of vaccine potency and immune competence. IFN-gamma ELISPOT is a mainstay of immune monitoring. This highly sensitive and reliable assay quantifies the number of CD4+ and CD8+ T cells, in mixed lymphocyte or fractionated populations, which recognize specific antigens. Cytotoxic T lymphocyte (CTL) assays by contrast are highly specific functional measures of adaptive immunity but have only moderate sensitivity and require cell numbers greater than that recovered from routine clinical blood draws. Tetramer assays that quantify HLA-matched T cells bearing selected antigen receptors are most widely used for monitoring HLA-restricted peptide vaccines. Delayed-type hypersensitivity (DTH) responses to intradermal antigen are commonly used to monitor allogeneic approaches, where antigens characteristically vary between individual vaccines [15].

It is generally accepted that a statistical increase in measureable reactivity following immunization

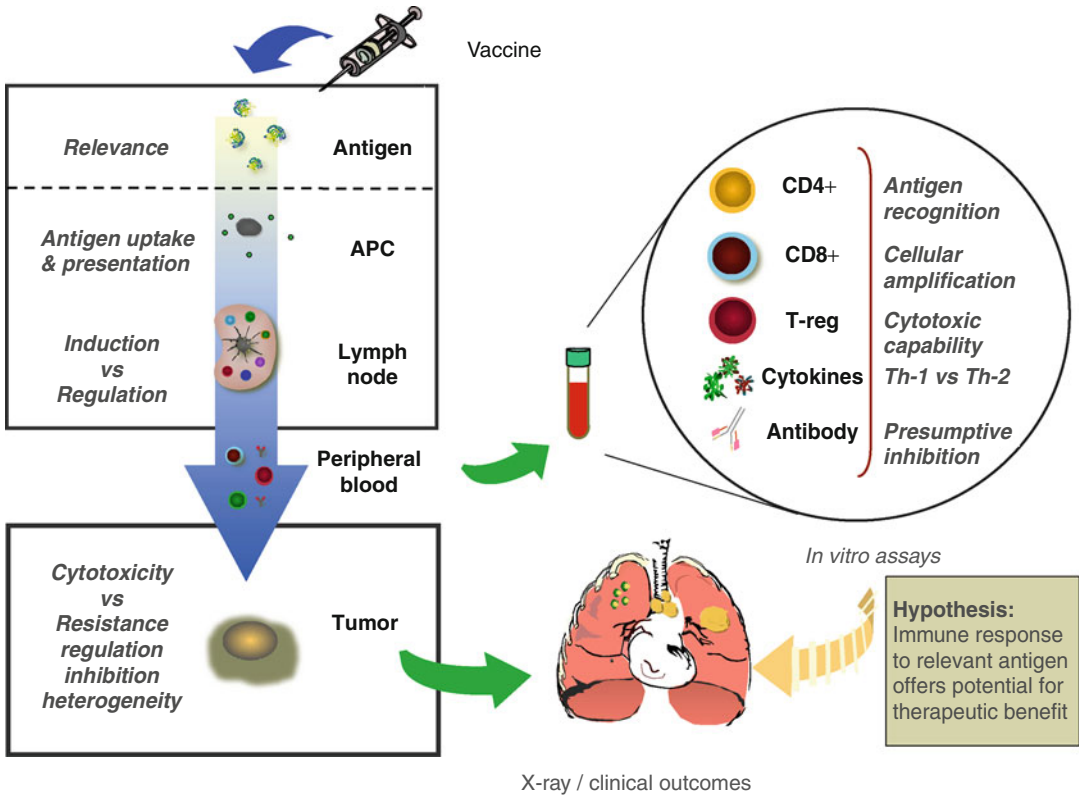

Fig. 23.1 Immune monitoring. The accompanying schematic characterizes the myriad known, measurable, and unknown elements of immune monitoring, including the somewhat speculative link between measurable immunological criteria and clinical outcomes. Putative mechanisms of adaptive immunity are depicted on the *left* of the diagram: Sequential sites of antitumor immune induction and actions of competing pathways are shown in *black boxes*. Vaccines, produced with either assumed or known antigenic relevance, are delivered into a biological crucible. Windows to the biological and therapeutic effects of vaccine delivery are shown on the *right*: Immunological and clinical endpoints offer quantifiable metrics. Immune status is extrapolated from batteries of in vitro assays that characterize the constituents of peripheral blood. Serial changes in measures are the fundamental basis of immune "response." Radiographic changes and clinical outcomes reflect the sum of cytotoxicity, inhibition, and resistance in the tumor microenvironment. The relationship of immune monitoring assays and therapeutic efficacy are recapitulated in the hypothesis that *immunological response to relevant antigens offers potential for therapeutic benefit*

reflects the mobilization of T cells to one or more antigens delivered by the vaccine [15]. The clinical relevance of "immunological response" continues to be speculative [15–17]. Problematically, assays are multiple, varied, and not standardized across laboratories [15, 18]. Further, in the notable absence of viable tumor from individual patients for assay production, most immune monitoring extrapolates antigenic relevance and specificity from allogeneic surrogates. Notwithstanding antigenic relevance to an individual's tumor, there are multiple downstream elements that can modulate effector function following induction. Nonetheless, at the current level of maturity in the field, immunological assays are the only practical measures of comparative potency and metrics of success during developmental phases of vaccines. Although it must be emphasized that immunological responses do not directly translate into therapeutic efficacy, multiple immunotherapy studies have observed an association of clinical response and induced immunity. The ability of most vaccines to induce measurable immunological responses suggests that many vaccines could have similar efficacy. Once efficacy is defined in NSCLC and the relevance of monitoring assays to outcomes better defined, standardized monitoring could facilitate development, provide comparative analysis, promote optimal

study design, and support strategies that enhance response rates [15–18] (Fig. 23.1).

23.9 Autologous DC Vaccines

23.9.1 Rationale and Trial Design

We initiated a clinical trial with DC vaccines to determine feasibility, to gain information with which to build future studies, and to work towards optimizing vaccines in NSCLC [19, 20]. Vaccine production and mechanisms of action are reasonably well understood (Fig. 23.2). Trial design focused on immunological responses to antigen-pulsed DC vaccines in a heterogeneous group NSCLC patients treated surgically and medically and with multimodality approaches. Any patient with histologically confirmed stage I–IIIB NSCLC was eligible, irrespective of prior therapy. Subjects would be immunized twice, 1 month apart. Blood was drawn prior to and serially following immunization out to 1 year. Safety parameters were continually monitored. The primary endpoint was the rate of immune induction by IFN-gamma ELISPOT in individual patients immunized with prime and boost vaccine 1 month apart. Comparative analysis between subjects would determine relative importance of tumor burden (surgically resected vs. measurable persistent disease) and effect of prior radiation or chemotherapy on immune induction. Data would be analyzed in context of host factors that may influence vaccine efficacy, including immune regulatory elements responsible for variable rates of induction. In context, immediate goals and longitudinal objectives were dependent on the reliability of immune induction, and momentum would be built on this definable metric. Anecdotal clinical outcomes would be noted.

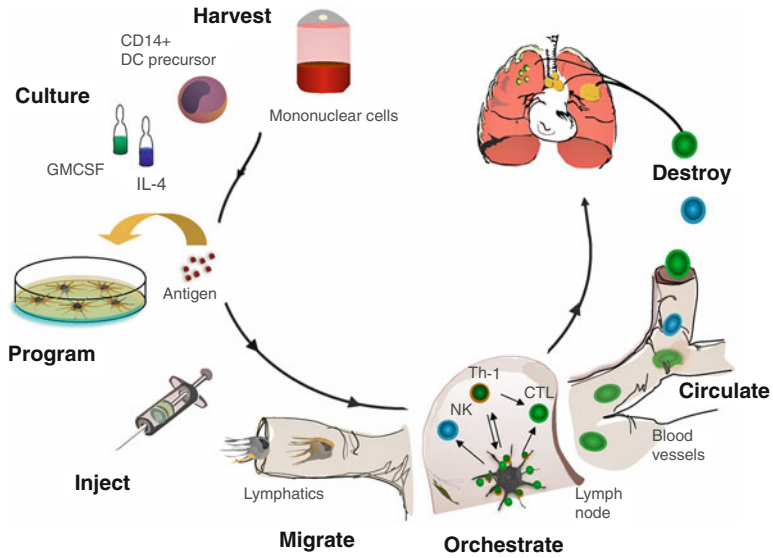

Fig. 23.2 Dendritic cell (*DC*) vaccines. Peripheral blood mononuclear cells from a patient are cultured in GMCSF and IL-4 to drive differentiation of CD14-DC precursors to immature myeloid DCs. Immature DCs are phagocytic and readily take up particulate antigen in culture. DCs process antigen into smaller peptides that are presented on the cell surface in context of MHC class I or class II molecules. Full antigen-presenting capacity is achieved when DCs are driven to maturity by addition of bioactive molecules in vitro or through cognate interaction with resident T cells in vivo. The DCs injected under the skin migrate to regional lymph nodes and interact with tumor-specific T-cell precursors. Effector cells are released into the peripheral circulation and migrate into tissues where they encounter and destroy antigen-expressing cells (e.g., tumor)

23.9.2 Formulation and Dosing

Vaccines were prepared according to FDA guidelines for cellular therapies. Patients underwent a 2-h leukapheresis procedure to harvest mononuclear cells in numbers adequate for vaccine production. Autologous dendritic cells were generated from peripheral blood monocyte culture in growth factor-enriched culture medium. Two different DC preparations were sequentially tested on a similar immunization schedule [19, 20]. In the first series of 18 patients, DC vaccines were matured in vitro after antigen pulsing using a cocktail of bioactive factors. The second series of 18 subjects received immature DC vaccines. All vaccines were prepared with antigen derived from the NSCLC tumor cell line TC1650. This line overexpresses CEA, Her2/neu, WT1, survivin, and NY-ESO-1, which promised at least one shared antigen with over 95 % of NSCLC. A master cell bank comprised of tumor cell line confirmed free of contaminants and adventitial virus was established as a renewable source of antigen.

Based on literature suggesting that apoptotic bodies offered a more highly immunogenic alternative to simple necrotic cell debris, cells were apoptosed with UV irradiation prior to lethal irradiation. Vaccines were produced over 8 days. The final vaccine product (autologous DCs pulsed ex vivo with processed TC1650) was aliquoted for two immunizations (prime and boost) plus several test vials for sterility testing and quality assurance. 24 h after the cryopreservation, a test vial was thawed, tested for endotoxin, and sent to the University's Clinical Microbiology Laboratory for bacterial and fungal testing with extended culture. After minimum 2-week sterility testing, the vaccines were released for injection. Each subject received two sequential intradermal injections of 10^8 pulsed DCs in the thigh 1 month apart. Subjects were monitored clinically for adverse events.

23.9.3 Results

Thirty-six individuals with definitively treated stage I–IIIB NSCLC were accrued and immunized with one of two DC vaccine preparations (matured and immature DCs). The dominant adverse event noted was local wheal and flare reaction at the site of injection 24–48 h following immunization. There were no serious adverse events related to the experimental therapy. Immunological response was measured by IFN-γ ELISPOT, comparing relative number of antigen-reactive T cells from pre-vaccine to serial timepoints post immunization. Measurable immunological responses by IFN-gamma ELISPOT assay were noted in 67 % of subjects and were irrespective of disease stage or prior therapy. Rates of induction were independent of phenotypic maturation state of DCs at the time of delivery, and both preparations showed rates comparable to other cancer vaccines in the literature. Immune induction inversely correlated with relative numbers of circulating T-regulatory cells at the time of immunization, and there was no apparent increase in T-regulatory cells in any subject following immunization. Numbers are too small to draw conclusions about therapeutic benefit, although anecdotal survival, especially in those with advanced-stage disease, is more favorable than would be predicted based on historical controls [19, 20].

23.10 Conclusions and Perspectives

Lung cancer patients could potentially benefit from immunotherapy. While DC vaccines clearly induce immune responses in a majority of patients, and might therefore be expected to show clinical efficacy in larger clinical trials, DC vaccine production is protracted, resource intensive, and costly; further, DC vaccines are individually produced, relatively non-transportable, and require each patient undergoes a leukapheresis procedure, restricting vaccine production for, and delivery to, selected patients at a major medical center. These factors make DC vaccines impractical for advanced-phase investigation and less than ideal for broad clinical use. Nonetheless, DC vaccines continue to be a valuable biological standard for solid tumor vaccines. This experience and these data would provide a valuable

prototype for further vaccine development. More specifically, we readily recognized that there is no standard vaccine approach and no comparison to suggest superiority of one cancer vaccine type, which offers the following working hypothesis: *any agent capable of inducing a measurable immunological response while availing the host of relevant tumor antigen would have similar potential for therapeutic benefit. Even so, it will undoubtedly be a long time before comparative therapeutic studies are done*.

23.11 Vaccine 1650-G

23.11.1 Rationale and Trial Design

Believing efficacy would depend more on the ability to induce an immunological response than on any specific vaccine formulation or adjuvant, we produced 1650-G, a simplified derivative of DC vaccines [21]. This vaccine combines the processed antigen component of our DC vaccine (tumor cell line 1650) with the recombinant GMCSF as an immune adjuvant. GMCSF, known to stimulate antigen uptake and presentation in vivo, would be used in lieu of antigen loading of autologous DCs ex vivo; the antigen profile of the allogeneic tumor cell line 1,650 would continue to confer multivalent coverage; the use of similar antigen, immunization schedule, and monitoring facilitated comparison to previously tested DC vaccines that would be used as a benchmark for immunological activity. In contrast to DC vaccines, 1650-G would be an economical and transportable alternative with which to pursue advanced-phase investigation.

The initial feasibility trial was an open-label nonrandomized multisite investigation in patients with stage I/II NSCLC and no evidence of disease following surgical resection. The study was conducted at four sites within the Commonwealth of Kentucky that represented a partnership between academic and regional community cancer centers. A prime vaccine and a single boost were given 1 month apart, similar to the prescribed schedule for autologous DC vaccines. Each immunization delivered 1.0×10^8 processed 1,650 tumor cells plus 100 μg GMCSF in 0.6 ml volumes; vaccine was injected intradermally at two locations in the thigh (0.3 ml/injection, 0.6 ml total); opposite thighs were used for prime and boost vaccine. Immunological response by IFN-γ ELISPOT was the primary endpoint. Consistent with literature and prior experience, immune responses were expected in only a percentage of study patients. Rate of immune induction relative to previously tested DC vaccines was the principal metric of vaccine potency; immunological comparability of 1650-G to DC vaccines would provide a foundation for further studies. Establishing infrastructure for vaccine collaborative group and testing the potential of the vaccine to be transported and delivered to cancer patients in community cancer clinics also fulfilled an important objective of research.

23.11.2 Formulation and Dosing

Vaccines are batch produced from a master cell bank. Each dose is comprised of 10^8 processed 1,650 cells (apoptosed and lethally irradiated) plus 100 μg of clinical-grade GMCSF in 0.6 ml sterile saline. Vials of prepared vaccine are stored in the gas phase of liquid nitrogen until delivery. Test vials were subject to sterility testing as previously described.

23.11.3 Results

Eleven individuals received two serial immunizations. The dominant adverse event was self-limited injection site reaction, and there were no serious unanticipated adverse events. Immune response measured by ELISPOT was seen in 6/11 of immunized patients, which is comparable to the more resource-intensive DC vaccine (Fig. 23.3). The observed biological activity coupled with ease of production, reduced cost, and portability make 1650-G an attractive candidate for randomized clinical investigation and subsequent clinical implementation [21].

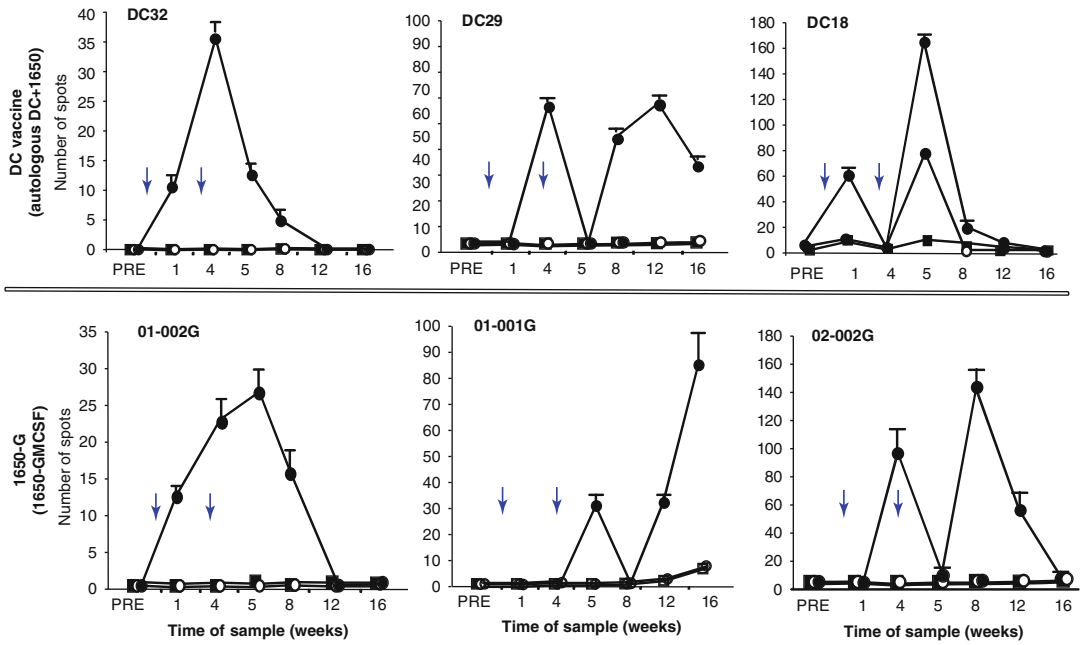

Fig. 23.3 IFN-γ ELISPOT: DC vaccine compared to 1650-G vaccine. Representative immunological responses are shown from individuals participating in a DC vaccine study (*top*) and patients receiving vaccine 1650-G (*bottom*). Both vaccines use the processed 1,650 cell line as a source of antigen. Immunization schedule was the same in both studies. *Graphs* show measurements of lymphocytes ("spots") from available timepoints (pre-vaccine to 16 weeks post) responding to (●) 1,650-pulsed autologous DCs, (■) DCs alone, (○) lymphocytes alone. *Arrows* indicate time of vaccine delivery. Comparable rates of immune induction and patterns of response across the study population indicate similarities that overshadow differences in immune adjuvant similarities in the kinetics indicate that either approach has potential for therapeutic benefit. These data are the basis for advanced-phase investigation using the simplified derivative of DC vaccines: 1650-G

23.11.4 Directions

Having been suitably encouraged by early phase results with vaccine 1650-G, ongoing interests are in defining parameters for implementation of immunotherapy in NSCLC. More specifically, two randomized clinical trials have been designed to evaluate the emerging concept of immunochemotherapy in advanced NSCLC; the primary endpoint in each is clinical response. The first trial, a randomized phase II/III study of maintenance 1650-G plus pemetrexed following first-line chemotherapy, was opened to accrual in 2010. That trial was placed on clinical hold because of lack of extramural funding. The second study, a randomized phase II/III study of second-line 1650-G plus docetaxel for progressive disease, has not yet opened to accrual, again for lack of funding. That trial is described below. A phase I trial examining the immune-potentiating effects of the dietary supplement β-glucan is ongoing.

23.12 Randomized Trial of Second-Line Immunochemotherapy (SLIC) for Advanced NSCLC

23.12.1 Rationale and Trial Design

Recognizing the challenges that vaccines would face as monotherapy in patients with large tumor burdens, we have been attracted by clinical anecdote and preclinical studies that indicate both immunological and therapeutic synergy between

chemotherapy and cancer vaccines [22, 23]. Compelling insights into the immune-potentiating effects of docetaxel are especially relevant to patients with advanced NSCLC [24, 25]. The primary objective is to show that the immune system can be mobilized for therapeutic benefit in a population with predictable outcomes from conventional chemotherapy.

23.12.2 Study Description

This study is an open-label 2:1 randomized trial to assess therapeutic efficacy of 1650-G and concurrent docetaxel (same treatment cycle) as second-line therapy for progressive stage IIIB or IV NSCLC not amenable to curative surgical or radiotherapy. A minimum study number of 120 patients and a maximum of 174 patients are based on an integrated phase II/III trial design. Patients are to be enrolled across eight sites in the Commonwealth of Kentucky from outpatient clinics, through self-referral from the community or referral from a primary treating physician. Chemotherapy (docetaxel) and procedures related to standard of care are the same in both study arms. All subjects would undergo four treatment cycles; each treatment cycle is 3 weeks. Vaccines (experimental arm) are administered day 1 of each cycle and docetaxel given day 5 of each cycle (Fig. 23.4).

23.12.3 Endpoints

The study specifically examines differences in outcomes between experimental and control arms. Primary endpoints include time to progression (TTP), survival, and immune-related response criteria (irRC). This latter measure includes allowance for moderate increases in tumor burden to accommodate protracted immune-mediated responses and inflammatory changes. Consistent with literature and prior experience with this vaccine, 50–70 % of individuals would be expected to show immune response by IFN-γ ELISPOT following immunization [11]. A secondary hypothesis is that clinical response will favor the group with immunological response. Correlative studies are designed to address the most understandable reasons for therapeutic success or treatment failure.

23.13 Strengths and Weakness

DC vaccines continue to have potential for therapeutic efficacy in NSCLC but are costly and resource intensive [12, 19, 20]. 1650-G is an immunologically comparable alternative. Beyond the obvious appeal of low cost, 1650-G is portable, uniform, and multivalent [21]. We continue to be attracted by theoretical, practical, and pragmatic advantages of this vaccine and are suitably encouraged by early phase trial results in NSCLC. In meeting the standard for more advanced investigation, vaccine 1650-G faces new challenges.

Without reservation, the availability of funding is the biggest impediment to further development. Simple nonproprietary strategies do not generally benefit from industry backing, and at the current level of extramural funding, the prospect of completing phase III studies with this agent seems remote. While the number of cancer vaccines described in the literature continues to grow and permutations continue to increase, enthusiasm for efficacy trials has appeared to

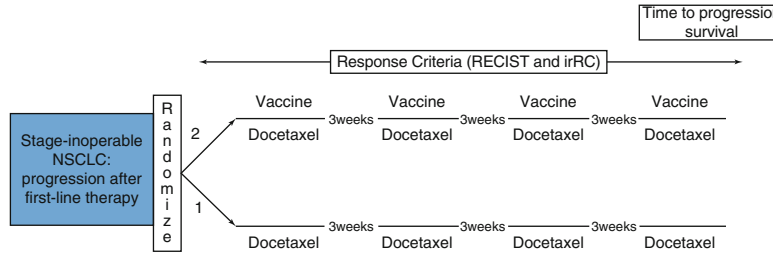

Fig. 23.4 Trial schema vaccine 1650-G: Randomized trial of second-line immunochemotherapy (SLIC) for advanced NSCLC

paradoxically decrease [26]. Understandably, efficacy trials, especially in advanced-stage patients, assume risk of negative results. Given the preparedness of the agent, severity of lung cancer, and notable therapeutic success of sipuleucel-T in prostate cancer, addressing questions of efficacy in NSCLC is far more compelling than exhaustive biological investigation. Neither can we justify the time and expense of developing alternate formulations without any demonstrable evidence that one vaccine would be more therapeutically active than another.

Although complexity is not a known requisite for efficacy, the simplicity of vaccine 1650-G has generated skepticism about its therapeutic potential. There are differing opinions on whole-cell vaccines, but no feature that precludes efficacy. GMCSF has not met with enduring success as an immune adjuvant, but differences in vaccine formulations, tumor types, schedules, monitoring assays, and doses and routes of GMCSF administration make comparison difficult and conclusion impossible [14, 27]. Despite these realities, historical bias is an appreciable hurdle, especially for funding. The therapeutic success of sipuleucel-T and renewed promise of GVAX vaccines, both formulated with GMCSF, may change opinions.

In pursuing efficacy this vaccine shares a common set of hurdles for cancer vaccines. The most well-recognized biological obstacles to optimal potential include a corrupted tumor microenvironment containing regulatory T cells and aberrantly matured myeloid cells, a tumor-specific T-cell repertoire that is prone to immunological exhaustion and senescence, and highly mutable tumor targets capable of antigen loss and immune evasion. Clinical factors that continue to impact development and implementation include non-standardized assays and uncertain relevance of immune monitoring, protracted time courses for therapeutic endpoints, and a lack of correlative biomarkers for assessment of clinical efficacy.

Perceptions about cancer vaccines are an implicit but very real challenge to the field. Opinions about therapeutic potential of immunotherapy run a full spectrum based on a handful of positive and negative statistically sized clinical studies [5, 6]. Although most investigators do not believe that immunotherapy will be a panacea for cancer, unrealistic expectations from the medical community continue to generate doubt and bias. It is however important to remember that over the past decades improved outcomes in advanced-stage lung cancer have been measured in weeks increase in survival, and the addition of adjuvant chemotherapy in surgically resected patients offers only minor reductions in relative risk of recurrence. Independent of reaching any global efficacy standard, there is tangible success in offering a percentage of patients with limited therapeutic options an opportunity to participate in, and contribute to, if not benefit from, lung cancer research.

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Melanoma: Perspectives of a Vaccine Based on Peptides

24

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Contents

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The standard treatments for patients with melanoma are surgery, chemotherapy, radiation therapy, and biologic and targeted therapy. The successful surgical removal of tumors depends on the early diagnosis of the disease. Metastatic melanoma has the poorest prognosis and a number of new agents, including cytokines, monoclonal antibodies, signal transduction inhibitors, oncolytic viruses, and angiogenesis inhibitors have been tried, generally with low percentages of positive response. Vaccines have not so far been successful in the treatment of metastatic melanoma but are a reasonable area of research.

Recently, ipilimumab, the humanized anti-CTLA-4 antibody, represents a promise in patients with metastatic melanoma. In all cases the toxicity of cancer therapeutics is high. New medications and protocols are needed and are actively being investigated in many centers. Presently, we show that peptides from readily available sources may provide a basis for the development of anti-melanoma therapeutics. Some are apoptotic or affect the migration of tumor cells in vitro, but their activation of dendritic cells and antimetastatic activity in vivo, in a melanoma experimental model, is encouraging and stimulates further investigation.

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24.1 Disease Description

Malignant melanoma is the most aggressive and treatment-resistant type of skin cancer, and its incidence has increased in fair-skinned populations worldwide for decades [1].

Despite the progress in the biology and molecular genetics knowledge of melanoma initiation and development, available treatment options have not been translated into significant improvement in the survival of patients with metastatic disease, with an estimated number in 2012, of 9,180 deaths in the United States [2].

The rapidly increasing incidence of cutaneous melanoma appears to result from changes in behavioral patterns related to sun exposure, but other factors may be involved in the pathogenesis of melanoma, such as heredity and chemical carcinogenesis [3].

The most important prognostic factor for patients with melanoma is the invasion depth of the primary lesion. A roughly inverse relationship between the diameter of the lesion and survival has also been established [4]. Treatment of early stage melanoma involves complete surgical excision of the primary tumor, which is usually sufficient to cure the disease. Lymph node mapping and sentinel lymph node biopsy indicate the extent of cancer cells spreading. Once melanoma spreads to distant and inner sites, no current treatment reliably limits the aggressive course of the disease [5]. Early detection, proper diagnosis, and new therapies are needed to restrain this potentially lethal tumor malignancy.

24.2 A Brief History

Melanoma is one of the oldest known forms of malignant tumors in humans, although historical evidence for its occurrence in antiquity is scarce. In 1960s, a reported examination of nine Peruvian Inca mummies, dated approximately 2,400 years old, showed apparent signs of melanoma, such as melanotic masses on the skin and diffuse metastases to the bones [6].

The first surgeon to reportedly operate on metastatic melanoma was John Hunter in 1787. He excised a recurrent mass behind the angle of the lower jaw of a 35-year-old male and described it as containing two distinct parts: one white firm section and the other spongy and dark in appearance [7, 8]. The French physician René Laennec described melanoma as a disease during a lecture at the Faculté de Médecine de Paris in 1804 [9]. In 1840, Samuel Cooper considered individuals with advanced stage melanoma untreatable and stated that the chance of survival depends on the early surgical removal. After about two decades, the general practitioner William Norris reported that there is a familial predisposition for melanoma development [10].

Since these initial observations, studies on the biology and treatment of melanoma have intensified, and efforts have been constantly encouraged to control the disease and prolong the survival of patients.

24.3 Origin and Etiology of Cutaneous Melanoma

Cutaneous melanoma is a tumor of neuroectodermal origin that results from the proliferation and malignant transformation of pigment-producing cells, the melanocytes, which originate from neural crest progenitors and migrate to the skin and hair follicles during embryogenesis [11].

Melanocytes are located to the basal layer of the epidermis, hair bulb, eyes, ears, and meninges, and among other functions, and contribute to photoprotection and thermoregulation by melanin production [12]. Homeostasis of melanocytes is tightly regulated by interaction with epidermal keratinocytes through a complex system of paracrine growth factors, adhesion molecules, and gap junctions [13]. Mutations in critical genes linked to cell proliferation control, production of autocrine growth factors, and loss of adhesion receptors cooperate to deregulation of intracellular signaling in melanocytes. Such mutations enable cells to escape from the control exerted by keratinocytes, creating a conducive microenvironment for the uncontrolled and independent proliferation of melanocytes [14, 15].

Although the precise etiology of malignant melanoma remains obscure, evidence strongly suggests that genetic and environmental interactions play a role in melanoma development. The list of host factors important in the onset of melanoma is long and includes family history of melanoma, melanoma susceptibility genes, number and type of nevi, skin type, and pigmentation [16].

Sunlight and ultraviolet radiation exposure have been attributed the most important risk factors for melanoma. In fact, epidemiological data indicate that the growing incidence of melanoma is closely related to changes in the pattern of sun exposure. The highly genotoxic UV radiation may cause DNA damage that, if not repaired, plays a central role in the pathogenesis and progression of melanoma [17].

Epidemiological studies have established that individuals with blond or red hair and pale skin, who are subjected more easily to sunburn and freckles, more likely develop melanoma than darkly pigmented individuals. Furthermore, studies have shown that exposure to UV radiation during childhood particularly increases the risk of developing melanoma [18, 19].

Multiple melanocytic nevi, dysplastic nevi, and atypical mole syndrome (AMS) are the main clinical phenotypes indicating melanoma susceptibility [20]. Accordingly, the presence of multiple common (banal, acquired) nevi, more than five dysplastic (atypical) nevi, large congenital nevi, and lesions larger than 6 mm in diameter have been shown to precede melanoma development [21, 22].

Familial melanoma refers to a frequency of two or more first-degree relatives diagnosed with this neoplastic disease. In general, patients have the first melanoma diagnosis at early age, with thinner lesions, different distribution patterns, and a high frequency of multiple primary melanomas. The lesions in family members are histologically indistinguishable from sporadic melanomas and have similar prognosis.

To date, two melanoma susceptibility genes, *CDKN2A* and *CDK4*, have been documented. *CDKN2A* encodes the cyclin-dependent kinase inhibitor 2A (CDKN2A, $p16^{Ink4A}$) also known as multiple tumor suppressor 1 (MTS-1). P16 has an important role in regulating the cell cycle. Mutations in this gene increase the risk of a variety of cancers, chiefly melanoma. The *CDK4* gene encodes the cyclin-dependent kinase 4, a component of the protein kinase complex that is important for cell cycle G1 phase progression. Three alternatively spliced variants encoding different proteins are known, as generated by *CDKN2A* transcription. Two of them encode isoforms that function as inhibitors of CDK4. The third includes another exon 1 that contains an alternate open reading frame (ARF), which encodes a protein that stabilizes p53 by interacting with MDM2. Therefore, mutations in these genes may affect relevant signaling pathways that control cell division and may confer high risk for melanoma [23, 24].

Other genes frequently mutated in melanoma include *BRAF* on the mitogen-activated protein kinase pathway and *PTEN* on the protein kinase B/Akt pathway. Nevertheless, these mutations have been reported as results of genetic disruption and not germline predisposing alterations that may contribute to melanoma-initiating events [25, 26].

24.4 Epidemiology

The incidence of cutaneous melanoma and the number of melanoma-related deaths have increased dramatically over the past few decades, and therefore, melanoma has become in many regions a public health issue. Although malignant melanoma accounts for less than 5 % of skin cancer, it causes 80 % of deaths, mainly due to its high metastatic potential [20].

Cutaneous melanoma occurs mostly in white populations and substantially less in populations of African or Asian origin with darker pigmentation. The cancer statistics in the United States revealed that 6 in 100,000 inhabitants were diagnosed with melanoma at the beginning of the 1970s as compared to 21 cases in 100,000 inhabitants reported in the late 2000s [27], thus being the fastest growing incidence of any form of cancer. Australia and New Zealand have the world's

highest incidence and mortality rates of melanoma [18]. Within Europe, a considerable variation exists in melanoma incidence and mortality among populations, but in general, the disease has also increased in this region [28].

Although male/female melanoma incidence varies in the databases in different countries, women generally have significantly longer survival than male patients [29]. The median age for melanoma diagnosis has been 55 years [18].

24.5 Signs and Symptoms

Major signs and symptoms of melanoma are skin lesions that increase in diameter or thickness, change in color, bleeding, itching, ulcer, and tenderness [30].

The ABCD criterion for melanoma recognition was proposed in 1985 and has been adopted for appraisal of potential melanoma lesions [31]. More recently, clinical data supported the ABCDE expansion to emphasize the significance of evolving pigmented lesions in melanoma progression [32]. Clinical findings of the ABCDE acronym are *A*, for asymmetry, when one half of the lesion differs from the other half; *B*, for border irregularity, which refers to notched, uneven, or blurred edges; *C*, for color variegation, regarding the presence of shades of brown, tan, and black lesions; *D*, for diameter greater than 6 mm (although some melanomas may be smaller in size); and *E*, time evolving progression of the lesion as a cardinal feature that characterizes malignant melanoma. Survey and monitoring of lesions exhibiting these features have improved and enhanced the ability of physicians to recognize melanomas at early stages during routine skin examination, thereby substantially contributing to definitive surgical treatment.

24.6 Staging and Classification

Based on changes during melanoma progression, a microstaging system was proposed by Clark et al. [33], reflecting melanocyte proliferation during the formation of nevi, nevi dysplasia, hyperplasia, invasion, and metastasis [34, 35]. Thus, according to the model, beginning and progression of melanoma involve a series of histologic alterations that are classified into five different stages: (1) development of benign nevus, a lesion characterized by limited growth of melanocytes; (2) dysplastic nevus, characterized by discontinuous and random arrangement of melanocytes; (3) radial growth phase (RGP), characterized by intraepidermal cell proliferation; (4) vertical growth phase (VGP), characterized by melanocytes penetration through the basement membrane towards the dermis and subcutaneous tissues; and (5) metastasis of melanoma, characterized by dispersion into other areas of skin and/or organs, more commonly liver, lung, bone, and brain [36]. Breslow's depth criterion is another method to assess melanoma microstaging [37]. By use of an ocular micrometer to measure the vertical thickness of the primary tumor, it evaluates the extent of melanoma invasion with inference on disease prognosis.

Currently, both Breslow's and Clark's staging parameters have been incorporated in the American Joint Committee on Cancer (AJCC) melanoma staging system [38]. It uses the widely used TNM (*T*umor, *N*ode, *M*etastasis) parameters: *T*, for features of the primary tumor, according to Breslow's depth, cell mitoses, and ulceration; *N*, for the involvement of regional lymph nodes; and *M*, the presence of metastasis at distant sites. Additionally, serum lactate dehydrogenase (LDH) has been chosen as an important marker to evaluate the metastatic potential of the disease [39]. Cutaneous melanoma is thus classified into the following stages according to the revised AJCC guidelines [40]:

- Stage 0, characterized by the presence of abnormal melanocytes restricted to the dermis, also referred to as melanoma in situ. Defined as Tis, N0, M0.
- Stage I, characterized by tumor thickness (<1 mm), mitotic index, and ulceration status. Defined as T1a, N0, M0; T1b, N0, M0; or T2a, N0, M0.
- Stage II, also characterized by tumor thickness (>1 mm) and ulceration status. There is no evidence of lymph node involvement or

distant metastasis. Defined as T2b, N0, M0; T3a, N0, M0; T3b, N0, M0; T4a, N0, M0; or T4b, N0, M0.
- Stage III, characterized by regional lymph node involvement and micro- or macrometastasis. Defined as any T, N1, M0; any T, N2, M0; or any T, N3, M0.
- Stage IV, characterized by the presence of distant metastases and the level of serum lactate dehydrogenase (LDH). Defined as any T, any N, M1.

The melanoma staging system accurately reflects the biology of melanoma on the basis of clinical outcome, predicts behavior of this challenging neoplasm, and provides a valuable tool for clinical decision making.

Analyses of the degree of sun exposure and the associated molecular alterations in combination with histomorphological features have defined distinct patterns of genomic mutations among different groups of primary melanomas [41]. Comparative studies showed that acral (palms, soles, and subungual sites) or mucosal melanomas exhibit higher frequency of chromosomal aberrations (e.g., focal amplifications and losses) than other groups of melanoma with or without chronic sun-induced lesions [42, 43]. Furthermore, BRAF mutations have been commonly found in melanomas arising in areas intermittently exposed to the sun and rarely in melanomas on skin with chronic exposure [44]. In melanomas with V^{600} -mutated BRAF, inactivation or loss of PTEN has been detected and associated with increased Breslow thickness of lesions [45]. Classification based on molecular biomarkers may redefine diagnostic and prognostic categories, thus providing additional information on malignant melanoma treatment and management.

24.7 Diagnosis and Classical Therapy

During routine skin or physical examination, the practitioner should be alert to signs of a potential melanoma lesion according to the ABCDE criteria. Because melanoma is potentially curable when detected at an early stage, any suspicious lesions should be surgically excised and submitted to histopathological evaluation [46]. Sentinel-node biopsy is recommended as a nodal staging procedure in patients with primary melanoma, 1–4 mm in thickness [47]. Melanoma diagnosis is based on general morphological, epidermal, dermal, and cytological features, which have been closely associated with prognostic information [48].

Most primary melanomas can be treated successfully by surgical excision when detected at early stages (0, I, II) [49]. Despite decades of clinical research, prognosis for patients with advanced melanoma remains extremely poor, and available treatment options are generally not effective. In patients with stage III melanoma with involvement of regional nodes, complete lymphadenectomy is typically recommended. Following surgical resection, adjuvant therapy with interferon-alpha (IFN-α) is an option [46]. More recently, treatment with anti-CTLA-4 monoclonal antibody (mAb) ipilimumab for patients with unresectable stage III melanoma has achieved statistically significant improvement in clinical trials [50] and thus has been indicated as first- or second-line treatment [51]. Also, the use of specific BRAF mutation inhibitor, vemurafenib, in patients with BRAF V^{600} mutation-positive and unresectable melanoma has shown partial responses and might be an alternative treatment for stage III disease [52].

Among patients with stage IV metastatic melanoma, the estimated median survival time is about 8 months, and the 5-year survival rate is less than 10 % [53]. There is no consensus on the standard management of metastatic melanoma [54], but systemic therapy is the only option [55]. Dacarbazine is the most widely used single chemotherapeutic agent and in combination with immunotherapy (e.g., ipilimumab, interleukin-2 (IL-2)) may slightly improve the response rate [48]. In addition, vemurafenib recently received approval for the monotherapy of patients with BRAF $V^{600}E$ mutation as detected by a US Food and Drug Administration (FDA)-approved test [52], but clinical studies have demonstrated only a discreet improvement

in progression-free survival and overall survival [56]. Other BRAF inhibitors, such as PLX4032 (Plexxikon/Roche), RAF265 (Novartis), XL281 (Exelixis), and GSK2118436 (GSK), are in clinical trials for metastatic melanoma. Particularly with PLX4032, encouraging effects have been obtained in stage IV melanoma carrying the V^{600} BRAF mutation, with complete or partial tumor regression in the majority of patients [57]. It has been reported, however, that tumors can acquire resistance to PLX4032 treatment [58]. Recent findings have suggested that chronic BRAF inhibition mediates melanoma survival through enhanced IGF-1R (insulin-like growth factor receptor 1) signaling, a pathway implicated in regulation of cell proliferation, prevention of apoptosis, and resistance to therapy in neoplasias [59]. Altogether, these observations may suggest that targeting a single pathway may not be sufficient to eradicate melanoma.

24.8 Emerging Antitumor Peptides

Natural peptides with antitumor activity have been described from a variety of sources. Some of them are free molecules and others are internal sequences from proteins that are liberated by proteolysis or are chemically synthesized. Their antimicrobial and antitumor activities resemble ancient molecules of innate immunity that have been effective in the protection against threatening conditions, infective or others, before the emergence of antibodies and adaptive T cell immunity.

Polonelli et al. [60] identified internal sequences in an anti-idiotypic antibody that exhibited anti-infective activities, and in a collaborative study we showed that CDRs from different monoclonal antibodies showed cytotoxic activities against *Candida* and HIV but also against the highly aggressive murine melanoma B16F10 [61]. Further work confirmed that, independently of antibody specificity, internal sequences of immunoglobulins, even from the constant (Fc) region, could display anti-infective and antitumor activities, therefore being a source of bioactive peptides [62, 63]. Specifically, V_H CDR 2 (H2) from mAb C7 (C7H2), directed to a mannoprotein from *C. albicans*, and V_L CDR 1 (L1) from mAb HuA (HuAL1), directed to human blood group A, tested as C-amidated synthetic peptides, led melanoma cells to apoptosis in vitro. Further, they were protective in a metastatic syngeneic model in vivo using intravenously challenged C57BL6 mice. C7H2 peptide (YISCYNGATSYNQKFK) exerted its apoptotic activity not only in B16F10 murine melanoma cells but also in several human tumor cell lines at similar EC50 concentration. Recently, Arruda et al. [64] showed that C7H2 bound to and caused polymerization of G-actin while acting on F-actin to stabilize it and, thus, altering actin dynamics which led to apoptosis following an intense production of superoxide anions. A typical caspase-dependent apoptosis ensued, documented by a number of cellular alterations and organelle disruptions. Similar effects were found with HuAL1 (RASQSVSSYLA), which apparently binds to a different receptor and causes tumor cell death with characteristics of necrosis (Arruda D.C., 2013 unpublished data).

Monoclonal antibodies to B16F10 murine melanoma (A4 and A4M) also provided CDR peptides with antitumor activity [63]. Interestingly, the V_H CDR 3 (H3)'s of both mAbs acted as microantibodies competing with the mAbs for binding to melanoma cells. A4H3 peptide in a linear or cyclic-extended form was as cytotoxic as mAb A4, targeting protocadherin β-13 (highly similar to human protocadherin β-6) on murine melanoma cells. The linear A4 H3 peptide (IRDGHYGSTSHWYF) was able to inhibit melanoma cells, at EC50 0.06 mM. Similarly with A4, it induced DNA degradation in B16F10-Nex2 cells and was also active in vivo against metastatic melanoma. CDRs L1 (RASGNIHNYLA) and L2 (NVKTLA) from mAb A4M inhibited growth of B16F10-Nex2 cells and induced DNA degradation in both melanoma and HL-60 cells, counteracted by Bcr-Abl, Bcl-2, and Bcl-X_L hyperexpression in the latter. The apoptotic effect of both L1 and L2 was accompanied by the anti-angiogenic activity of both peptides using HUVEC as target cells [63].

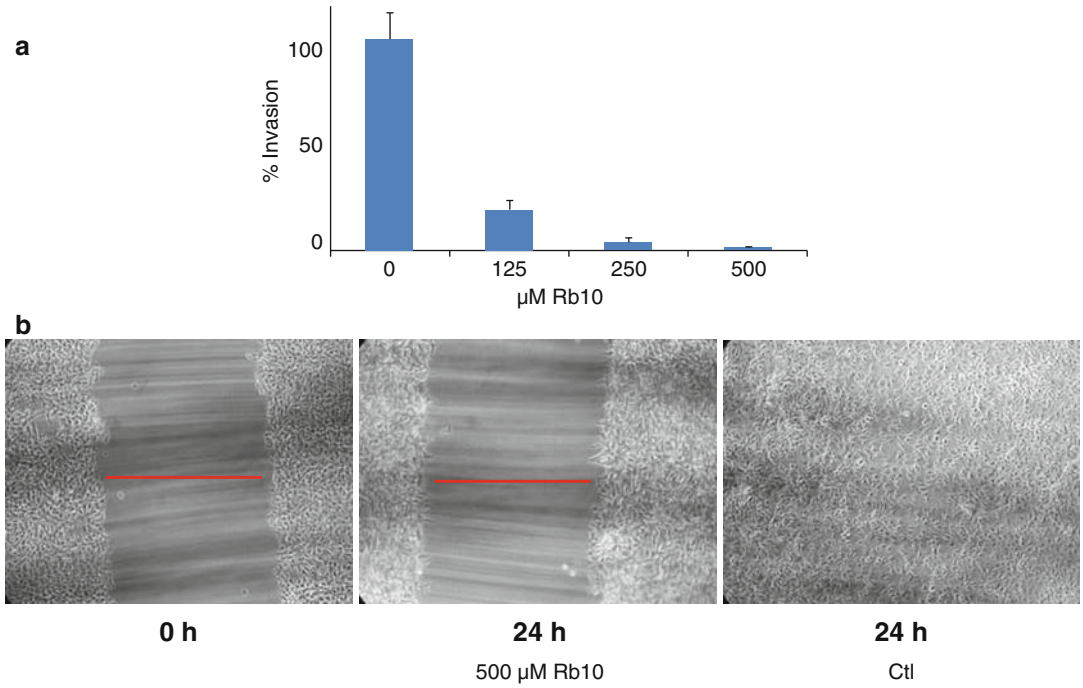

Fig. 24.1 Inhibition of melanoma cell invasion (Matrigel) and migration by Rb10 peptide (H3). B16F10-Nex2 melanoma cell invasion of Matrigel was measured in Transwell chambers, with 2×10^5 tumor cells suspended in serum-free medium added on the top chamber and 10 % FBS in the bottom chamber serving as cell attractant. Migrating cells were fixed, stained with Giemsa, and counted. (**a**) Inhibition of cell invasion by Rb10 at the indicated concentrations after 24 h. Values are from triplicate determinations. (**b**) Inhibition of B16F10-Nex2 melanoma cell migration (3×10^5 cells allowed to reach 70–80 % confluence in 12-well plates) by Rb10 using the scratch wound-healing assay. Complete inhibition was observed after 24-h incubation compared to the untreated control

The CDR H3 peptide from mAb REB 200 (humanized mAb MX35 directed to sodium-dependent phosphate transport protein 2b, NaPi2b), named Rb10, and its cyclic-extended derivative Rb9 are not apoptotic but induced a remarkable effect on tumor cells. They caused hyperadherence of cultured cells, thus inhibiting both migration and invasion through Matrigel (Fig. 24.1). Rb10 was antimetastatic in vivo using the murine melanoma model and apparently acted on tumor cells through HSP90 binding and inhibition of cell motility (PTC/1B2011/03053).

Another effect attributed to CDR (H3) of an anti-blood group A murine mAb (Ac1001) was the immunomodulation of macrophages [65]. The H3 peptide stimulated cytokine production, activated PI3K-Akt, and enhanced the expression of TLR-4. The different properties unraveled for the CDR peptides strengthened their potential use as medicinal drugs [66]. A list of apoptotic/necrotic peptides against several tumor cell lines is given on Table 24.1.

24.9 In Vivo Activity of Peptides and the Perspective of Antitumor Vaccines

The discovery of the apoptotic effect of immunoglobulin CDRs in vitro, targeting several tumor cell lines rather than nontumorigenic cells, led us to ask whether their protective antitumor activity in vivo could involve the same mechanism. Free peptides, even with the carboxy-terminal protected by amidation, are generally not very stable in the circulation being substrates of plasma peptidases and, depending on the size, cleared by renal filtration. Nevertheless, peptides of 16 (C7H2) and of 11

Table 24.1 CDR peptides from mAbs of various specificities which display apoptotic/necrotic antitumor activities

mAb	Immunogen	Ig	CDR	References
A4	Melanoma B16F10-Nex4	IgG	A4 CDR H3 (mic.mAb)	[64]
A4M	Melanoma B16F10-Nex4	IgM	A4M CDR L1 and L2	[64]
C7	Mannoprotein of *Candida*	IgM	C7 CDR H2	[62, 65]
Pc42	Hepatitis B/*P. falciparum* hybrid	IgM	pc42 CDR H2	[62]
HuA	Human blood group A (Fuc_2)	IgM	HuA CDR L1	[62]
AC1001	Mouse anti-blood group A (Fuc_2)	IgM	Ac1001 CDR H3	[66]
C36	Vaccinia	IgG	C36 CDR L1	–

The H3 may act as a micro (mic) antibody

(HuAL1) amino acids administered by intraperitoneal injections and in alternate days conferred significant protection against B16F10 tumor cells in a metastatic model [61].

An immune response to both peptides was suggested, and in the case of C7H2, the lack of activity in vivo of peptides with alanine substitutions at the N-terminal (Y1A and C4A) was compatible with specific amino acid recognition [64]. The apoptotic effect of C7H2 was associated to β-actin binding, but the C-terminal sequence rather than the N-terminal of C7H2 was shown to be involved in this reactivity.

The importance of the immune system was shown by the ineffectiveness of C7H2 to protect against melanoma in a metastatic model in NOD/Scid/IL-2rγ^{null} mice, implying that cells of the immune system were involved.

To explore the participation of the immune response, we examined the protective activity of dendritic cells stimulated or not by the in vitro-apoptotic CDRs. The same metastatic model of B16F10 melanoma cells was used. Syngeneic dendritic cells from C57Bl6 mice when primed with melanoma antigens were partially protective, but these cells pre-stimulated with the CDRs displayed a very significant antitumor effect (Fig. 24.2). It seems then that the antitumor effect in vivo does not depend on the direct targeting of tumor cells by the peptides but that dendritic cells may amplify the antitumor effect before the natural clearance of the CDRs. We hypothesize that CDR peptides, before degradation, are efficiently taken up by dendritic cells and that activated DCs most efficiently present tumor antigens for a competent T lymphocyte CTL response. A more extensive investigation has to follow these observations including other apoptotic/necrotic peptides to determine the effector cells and other characteristics of the protective immune response.

It is doubtful whether the protective in vivo effects of A4H3, a microantibody sequence from apoptotic mAb A4 and of Rb10 (and Rb9), CDRs that inhibit tumor metastasis (Fig. 24.3), are also dependent on DCs, but this has not yet been determined.

24.10 Antitumor Peptides Derived from Signaling Proteins

Apart from CDRs, which may concentrate their bioactivity owing to the high diversity and variability of their amino acid sequences, other peptides derived from signaling proteins have emerged as potential anticancer agents. Some of them are active in vivo and not in vitro like those of SOCS1 (Scutti J., 2013 unpublished results), and some others have several targets and complex mechanism of action still under investigation. Presently, we mention peptide pTj derived from the C-terminal zinc finger domain of WT1 [67]. WT1 is a transcription factor associated with melanoma oncogenesis and proliferation. The pTj peptide showed antiproliferative activity linked to irreversible G2/M cell cycle arrest and induction of senescence. In vivo, pTj

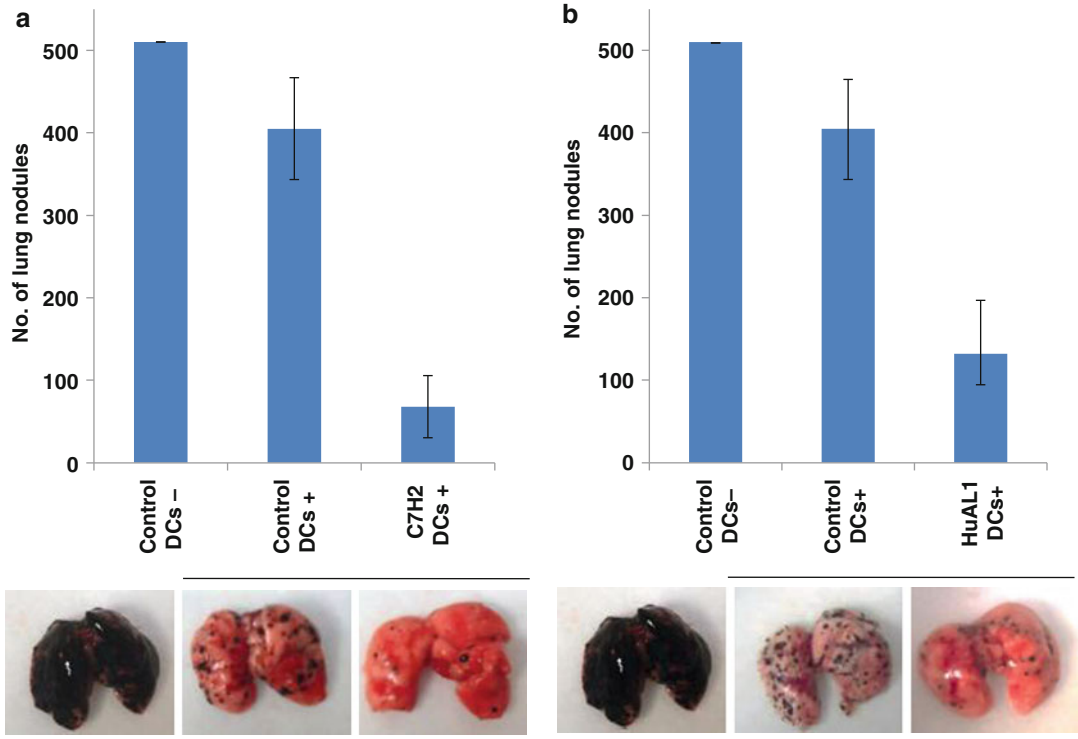

Fig. 24.2 Protective peptide-activated dendritic-cell vaccine against metastatic melanoma. Bone marrow dendritic cells (*DCs*) from syngeneic C57Bl/6 mice were primed with melanoma antigens (lysate from 5×10^5 B16F10-Nex2 cells incubated with 3×10^5 DCs for 1 h). Primed DCs were incubated with 20 μg/ml of either C7H2 or HuAL1 for 3 h. Peptide-stimulated DCs were injected subcutaneously in mice on days 1 and 4. Animals were challenged on day 11 with 5×10^5 melanoma cells injected endovenously. (**a**) protective effect of the C7H2-DCs primed (+) with melanoma antigens. Control primed (+) DCs but nonactivated with the peptide were poorly effective compared to control unprimed, nonactivated DCs. (**b**) the same as in (a), but with HuAL1 peptide-activated DCs. Images of the lungs with melanoma nodules as shown

showed a significant antitumor effect reducing the number of lung nodules in the B16F10 syngeneic model of metastatic melanoma and prolonging survival of nude mice challenged subcutaneously with human melanoma A2058 (Fig. 24.4).

Natural peptides derived from immunoglobulins and from domains of signaling proteins may represent a phylogenetic counterpart to peptide sequences from early innate immunity molecules that played an important role in the defense and regulatory mechanisms, with several reminiscent molecules still exerting important functions in our days. When tested as isolated peptides, they can act by themselves or through the activation of effector cells such as those of the immune system.

24.11 Antigen Characterization

Traditionally immune peptides are readily characterized after being mapped in antigenic proteins. In the case of human melanoma, MHC-restricted peptides from TRP2, NY-ESO-1, Melan A/MART-I, tyrosinase, and gp100/Pmel17 have been used as immunogens. Due to the heterogeneity of antigen expression, several groups tried whole melanoma cells expressing cytokines by gene transduction. Most promising is the use of genetically engineered lymphocytes reactive with NY-ESO-1 [68]. The NY-ESO-1 cancer/testis antigen is expressed in 80 % of patients with synovial cell sarcoma and approximately 25 % of patients with melanoma. Blockade of CTLA-4 increases CD8 (+) T cells

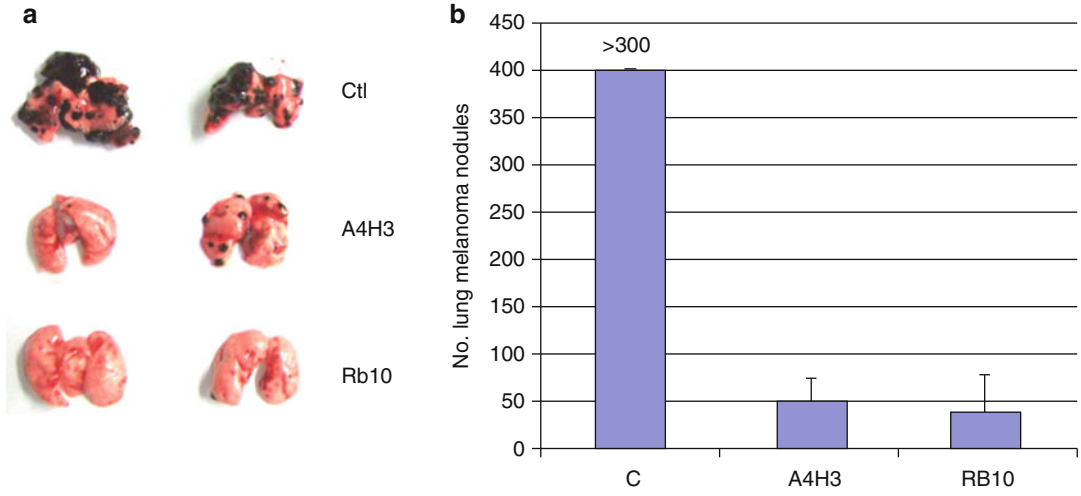

Fig. 24.3 Antimetastatic effect of peptides A4H3 and Rb10. Syngeneic mice (C57Bl/6) were challenged with 2×10^5 B16F10-Nex2 melanoma cells in 100 **µ**l, endovenously. Peptides were administered in six doses of 250 **µ**g/mouse/day intraperitoneally, starting on day 1 after challenge. Mice (5 per group) were sacrificed 22 days post-challenge and 10 days without treatment with the peptide. (**a**) lungs from untreated animals and from peptide-treated mice; (**b**) melanoma nodules in the lungs of control (untreated) and peptide-treated animals

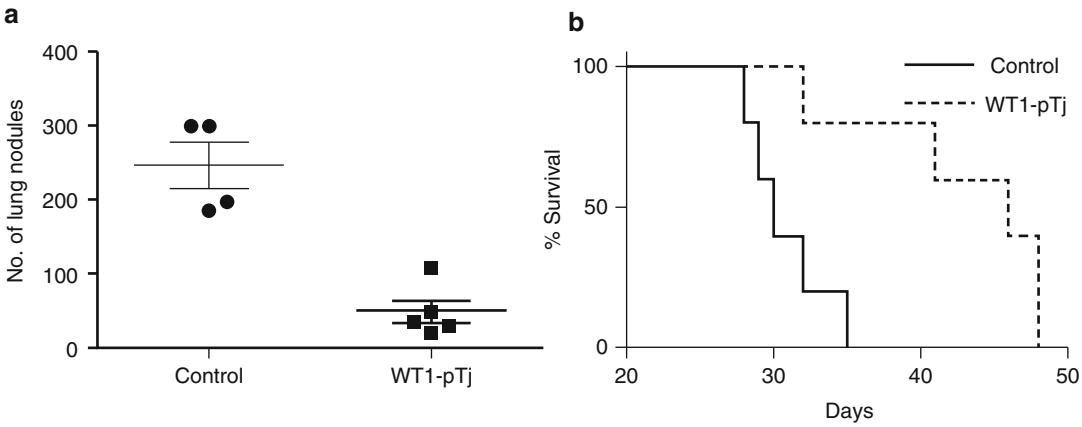

Fig. 24.4 Protective effect of WT1 pTj in vivo. (**a**) Lung colonization of B16F10-Nex2 cells in a syngeneic system was used to test the protective activity of WT1-derived peptide. Animals received 5 intraperitoneal doses of 300 **µ**g of WT1 pTj or vehicle (PBS) on consecutive days. The number of lung metastatic nodules was significantly reduced by 81 % after WT1 pTj treatment. (**b**) The survival curve of nude mice bearing human melanoma and treated with the WT1-derived peptide. Animals received 300 **µ**g of WT1 pTj or vehicle (PBS) on consecutive days. Peritumor therapy started the day after inoculation of melanoma cells and was extended for 5 days. Mean survival times were 31 ± 2.7 days for untreated tumor control group and 43 ± 6.7 for WT1 pTj-treated group

to a broader array of preimmunized melanoma antigens [69].

In the case of the peptides of the present review, they do not cross-react with tumor antigens to elicit a specific antitumor response. They act directly on cancer cells binding to different receptors or indirectly, activating cells of the immune system.

As said above, CDRs C7H2 (pc42H2) and A4H3 recognize β-actin and protocadherin β-13 (human β-16), respectively. Rb10M3, a derivative of Rb10 with dA at the second position,

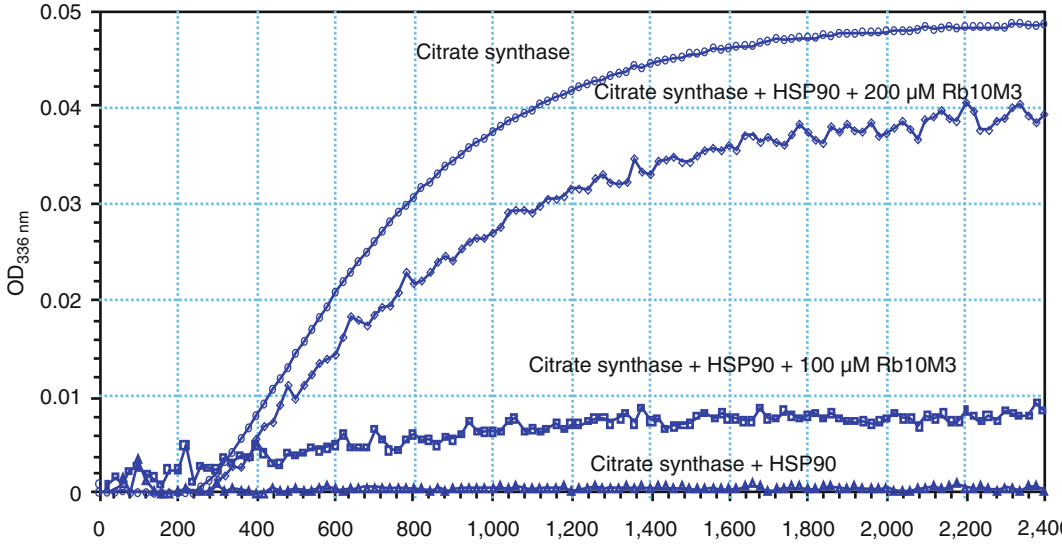

Fig. 24.5 Dose-dependent inhibition by Rb10M3 of HSP90 chaperone function using a citrate synthase aggregation assay. Rb10M3 is a derivative of Rb10 with dA (A2dA) to increase stability. Citrate synthase (0.115 **μ**M), in 40 mM HEPES, pH 7.5, was incubated at 43 °C for denaturation and aggregation. Aggregation was measured at OD 336 nm

reacts with HSP90, inhibiting its protein anti-aggregating activity (Fig. 24.5). Interference in the chaperone property of HSP90, which is highly expressed in tumor cells, affects a number of signaling pathways in the cell. In line with Rb10 inhibition of tumor cell metastasis, it was shown that actin dynamics-mediated cellular protrusion responsible for cell migration and invasion of matrix was associated with HSP90 inhibition [70]. HuAL1 binds to histone-3. WT1 pTj enhanced p53 activity and competed with WT1 protein for binding to p53 [67]. WT1 pTj may prevent important interactions between WT1 protein and its partners via a zinc finger motif. The peptide is, therefore, a promising agent against WT1-expressing malignancies (e.g., MCF-7, but not SK-BR-3 breast cancer or normal human fibroblasts, and also HL-60 acute myeloid leukemia).

24.12 Animal Model

C57Bl/6 mice and syngeneic murine melanoma B16F10 (subline Nex 2, developed at the Experimental Oncology Unit of Federal University of São Paulo, UNONEX-UNIFESP) are generally used for in vivo experiments. Melanoma cells are injected subcutaneously (4×10^4 to 10^5 cells) and tumor growth in mm^3 is measured applying the formula: $V=(d)^2\times D\times 0.52$ (wherein V is the tumor volume, D is the longest diameter, and d is the shortest diameter). For the metastatic model (lung colonization), 10^5 to 5×10^5 cells of B16F10-Nex2 melanoma are injected endovenously, and after 15–20 days the animals are sacrificed and the black nodules counted in the lungs. Peptides used in the protection experiments are injected intraperitoneally at 150–350 μg/mouse/day. Depending on the protocol, peptides are administered on alternate days, and treatment may be interrupted after 11–15 days for plotting the survival curve. For certain experiments with B16F10 and with human tumor cells, immunological-deficient animals are used. Nude$^{-/-}$, RAG 1/2$^{-/-}$, and NOD/Scid/IL-2rγ^{null} mice have been used with the tumor cells injected subcutaneously (A2058, SKMel28 human melanoma cells) or endovenously (B16F10-Nex2 murine melanoma cells). Peritumor injections were used for the protection experiments with WT1 pTj peptide.

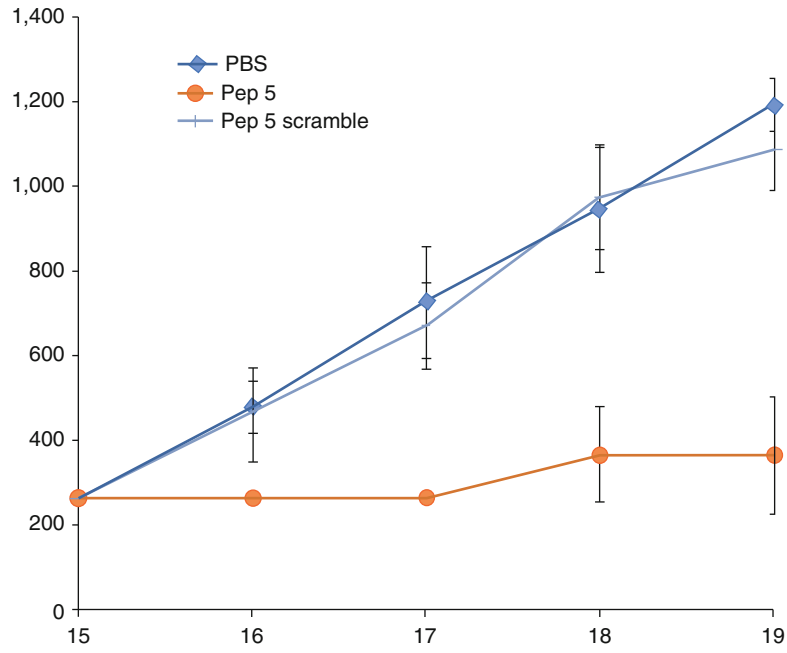

Fig. 24.6 SOCS-1-derived peptide (P5) arrests subcutaneous tumor development. Tumors were produced by injecting 5×10^4 B16F10-Nex2 cells into the right flanks of C57BL/6 mice ($n = 5$ per group). After 15 days (tumor size of 200 mm^3), the animals were treated i.p. for five consecutive days with 300 **µ**g peptide/mouse (*$p < 0.01$)

Prophylactic and therapeutic protocols were used in these studies. With SOCS-1 peptides, a therapeutic protocol started after the subcutaneously grafted tumor had reached 200 mm^3 (Fig. 24.6). In this model, 5×10^4 B16F10-Nex2 cells were injected into the right flanks of C57BL/6 mice ($n = 5$), and after 15 days the animals were treated intraperitoneally for 5 days with 300 µg peptide/mouse. As shown, tumor growth was arrested with SOCS-1 P5 treatment. The scramble peptide was used as a negative control.

24.13 Feasibility and Preclinical Development

Peptides listed on Table 24.1 and those derived from SOCS-1 and WT1 are readily obtainable and can be prepared in high degree of purity and reproducibility. Derivatives of these peptides will depend on the complexity of chemical synthesis. Conjugates with nanoparticles or macromolecules are more easily obtainable if validated in terms of antitumor activity.

Several peptides herein described depend on immune cells for their in vivo protective effects, and presently, we show two of them that acted via activation of dendritic cells (DCs). Activation in vivo of DCs by peptides and the activity of these cells as therapeutic agents is a possibility that has many precedents in DC-based vaccine trials using different propagation methodologies, antigenic load, and cell administration [71]. In the 1990s tumor peptides or whole-cell antigens have been used for ex vivo pulsing of DCs [72, 73]. In contrast, the peptides reviewed here are unrelated with the tumor cells and apparently act directly on DCs, rendering increased effectiveness to these APCs. They seem to activate DCs in vivo, which is an essential condition for triggering a protective immune response. In metastatic melanoma, vaccination with DCs loaded with melanoma antigen resulted in 9.5 % tumor regression compared to a maximum of 4.6 % using tumor cells, 2.7 % with immune peptide vaccines, and 1.9 % with viral vectors [74]. In our experiments in vivo simple injections of the peptides were sufficient to protect mice from metastatic melanoma, implying that tumor antigen presentation by activated DCs had been effective. As shown, protection was reproduced by peptide-treated tumor-antigen primed DCs ex vivo (Fig. 24.2). Further, it has been shown that intravenous or subcutaneous immunization with tumor

peptide-pulsed DCs in a mouse model of melanoma induced memory T cells that enabled control of metastasis in the lungs [75].

Comparison of in vivo DC-targeting and ex vivo antigen-loaded DCs has been made by Bonifaz et al. (2004) using B16 melanoma. Mice were challenged with B16 expressing OVA and then vaccinated with anti-DEC-205-OVA in the presence of anti-CD40. This was compared with ex vivo OVA-loaded mature DCs. The latter immunization did not inhibit tumor growth. Vaccination with anti-DEC-205-OVA was effective in controlling tumor growth. In the case of the antitumor peptides described in the present review, no targeting receptor has been identified so far, but there is direct evidence that DCs are activated by them. On the other hand, except for peptides derived from SOCS-1, the CDR-derived peptides and that derived from WT1 seem to recognize different ligands although, apparently, exerting similar stimulating effects.

If DCs are indeed those cells primarily targeted by the antitumor peptides, preclinical data in the mouse have to be translated into successful outcomes for patients. Identification of conserved targets in murine and human cells is an important step in these studies to understand the relation between immune modulation and vaccination.

24.14 Strengths and Weakness

Short-size peptides are usually poorly stable in vivo owing to proteolytic degradation and renal filtration. Yet, the antitumor peptides reviewed here, inoculated intraperitoneally in alternate days, are protective against metastatic melanoma. Such protection is not observed in immune-deficient, mice implying that cells of the immune system are necessary. Direct evidence was obtained for the activation of bone marrow dendritic cells which when primed with melanoma antigens ex vivo effectively protected against metastatic B16F10 melanoma. We hypothesize that injected peptides are rapidly taken up by dendritic cells, before being cleared from the blood circulation. Peptide-activated DCs would be quite efficient in the task of presenting tumor antigens even without targeting these cells in vivo with antibodies as discussed by Caminschi et al. [76]. Further, the types of DCs activated by these peptides are competent to induce T cells able to control tumor growth. Recently, Scutti in our laboratory (Scutti, JAB, 2013 unpublished results) showed that injection of a SOCS-1 peptide in mice challenged with B16F10 melanoma produced spleen T-CD8+ cells that were protective against metastatic melanoma growth in $RAG^{-/-}$ mice.

The main weakness of the present study is its early stage of development. Numerous questions have still to be answered mainly related to the mechanism of action of the peptides, their biodistribution, and the types of immune cells activated for antitumor effects. The peptide receptors and how the experiments in the mouse melanoma model can be translated into clinically effective procedures are other important concerns that will demand intense investigation. Nevertheless, the simple existence of these peptides, their easy production in purified state, and their anticancer effects warrant the present investigation.

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Parvoviruses: The Friendly Anticancer Immunomodulator

25

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Contents

Abstract

Oncolytic viruses represent versatile tools that through natural mechanisms or upon genetic manipulation can specifically target and kill tumor cells. In the last ten years it became clear that one of the major modes of action of these agents is their effect as an in situ (intratumoral) anticancer vaccine.

Parvoviruses (PVs) were recently approved for clinical use as an oncolytic drug to treat glioma. The chapter addresses several points of the immumomodulating mechanism of oncolytic PVs, such as the indirect (through immunogenic killing of tumor cells) or direct (abortive infection) activation of human immune cells. In addition, therapeutic strategies such as the use of cytokine modified or CpG DNA-enriched parvoviruses and immunomodulating combinations are also discussed.

The most recent research on that topic characterizes PVs as a silent anticancer immunomodulator.

25.1 Cancer as an Immunotherapeutic Challenge

In 1909, Paul Ehrlich has been the first to propose the concept that the immune system plays a critical role in protecting the host from cancer. Since then, the contribution of the immune system to effectively control cancer growth has

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been a controversial topic for many years. A major supporting evidence was provided in 2006 by Galon et al. who showed that the extent of the immune response to a tumor is positively associated with improved survival of patients with colorectal cancer [1]. However, the immune system and the progressing tumor mutually shape each other through the processes of tolerization and immunoediting. Exploiting stromal cells as their accomplices, cancer cells modify their immune cell environment to make it more "permissive" by either specifically eliminating tumor-reactive effectors or bringing them to a state of anergy. On the other hand, both the innate and adaptive immune responses edit the tumor, selecting for the least immunogenic and most resistant clones. Furthermore, each individual immune system and tumor has a unique set of genomic and epigenomic features that can influence the control of the former to the latter. This accounts for the difficulty in developing universal anticancer vaccines, except for a few tumor entities (e.g., cervical carcinoma) that express common epitopes derived from transforming viral proteins, to be recognized by the immune system [2].

25.2 Oncolytic Viruses (OVs) as Cancer Vaccines

Virotherapy recently emerged as an alternative to conventional treatments of cancer. Stemming from a few historical observations of tumor regression associated with virus infections, this new sphere of research is exploding due to the better understanding of virus and cancer biology and the mastering of DNA/RNA manipulation technologies. Over the last 20 years, the oncolytic activity of a dozen viruses has been characterized, and several have entered clinical trials. Most of them derive from modified human pathogenic viruses such as herpes simplex, adeno-, and poxviruses that possess large cloning capacities. This opens up the possibility of designing multifunctional virus-based products (therapeutic and imaging molecules) to be selectively expressed at high concentrations within human tumors. The initial idea of an oncolytic virus infecting the tumor and causing its eradication through successive rounds of infection-replication-oncolysis and reinfection has turned to be rather idealistic due to limitations on the efficiency of intratumoral virus replication and spreading in humans and the presence of a large percentage of normal stromal cells within most tumors. These limitations are however not necessarily reflected in a poor oncosuppressive effect of oncolytic viruses. Indeed, apart from their direct lytic activity, these viruses have the capacity to trigger both the cellular and humoral arm of the immune system within the tumor, representing in this way the perfect vaccine adjuvant.

The immune system plays a dual role in relation to cancer virotherapy.

On one hand, it contributes to defend the organism against infection, thereby antagonizing OV activity. Most OVs trigger a cellular defense mechanism that involves the induction of type I interferons and limits virus replication and propagation. This may not necessarily handicap OVs since IFN signaling is often impaired in tumor cells. The impairment is actually used to design OVs which are unable to block the interferon pathway and as a consequence replicate preferentially in tumors. Furthermore, the early innate immune responses mediated by NK cells, monocytes, macrophages, and neutrophils provide the initial line of defense of the host to limit OV infection, replication, and spread while facilitating the maturation of viral antigen-presenting cells and setting the stage for the subsequent adaptive immune response [3]. In particular, the recruitment of NK cells to infected tumors correlates with the production of IFN-γ which plays an essential role in the activation of macrophages that contribute to both OV clearance and later induction of T- and B-cell-mediated adaptive responses.

On the other hand, this pro-immune microenvironment arising from tumor infection with OVs is also favorable to the activation of anticancer innate response, cross-presentation of tumor-associated antigens, and induction of tumor-specific adaptive immunity [4]. In this regard, oncolytic viruses may act as in situ-personalized vaccine adjuvants by revealing the patient's own tumor antigens to

the immune system. This immune component of OV activity is especially important, since it should contribute to the destruction of noninfected tumor cells which may represent the majority of malignant cells in an OV-treated tumor. It should also be stated that the immunostimulating activity of OVs can be enhanced by arming them with appropriate regulating motifs or transgenes. This can be exemplified by the herpes- and vaccinia-based OncoVEX and JX594 vectors supplemented with a GM-CSF-encoding transgene, which can be assumed to play a role in the recently reported ability of these OVs to affect metastatic cancers after i.v. inoculation [5].

25.3 Parvoviruses (PVs) as Oncolytics

Parvoviridae are small (25 nm) icosahedral particles containing a single-stranded DNA genome of about 5 kb. Members of rodent origin belonging to the genus Parvovirus (PV) are of special interest due to their oncolytic properties. Among oncolytic viruses, rodent PVs fall in the category of the nonengineered (naturally oncolytic) animal viruses, together with Newcastle disease virus (NDV) (bird), myxoma (rabbit) Semliki Forest virus, and others [6–8]. These viruses can cause diseases in their natural hosts but are not pathogenic to humans. Furthermore, they show oncotropic and oncolytic features [9] in both animal and human cancer cells. The natural oncoselectivity of PVs appears to result, at least in part, from the dependence of viral DNA replication [10] gene expression [11] and nonstructural protein cytotoxic activity [12] on the proliferation and transformation of host cells. In particular, it has been shown in our laboratory that the rat parvovirus H-1PV selectively kills a panel of human tumor-derived cell lines [13–16]. Moreover, H-1PV systemic or intratumoral injection leads to the suppression of various human carcinomas, lymphomas, and nervous system tumors in animal models [17–21]. Importantly, H-1PV is infectious but devoid of significant pathogenicity for humans [22].

Studies of PV infection through different routes in animal models show that the viremia is transient and followed by a wave of virus-specific antibody production within 7–10 days p.i. A similar sequence of events was observed in humans experimentally infected with H-1PV [23]. Anti-PV antibodies are, at least in part, neutralizing and can thereby be expected to impede viral oncosuppression. Indeed, the ability of H-1PV to suppress hepatoma metastases in adult ACI rats upon virus administration shortly after tumor cell injection was impaired when animals were preinfected with H-1PV several weeks prior to treatment [24]. However, no or little preexisting natural immunity against PVs was found in the human population and even in laboratory personnel working routinely with these agents [22], unpublished observations. This data argues in favor of the low natural infectivity of PVs for humans and their suitability for clinical applications.

25.4 Activation of the Immune System by PVs

The possibility of PVs triggering anticancer vaccination was raised by the pioneering studies of McKisic et al. with the mouse parvovirus MPV-1. These authors showed that animals cured of a tumor upon virus infection are protected against subsequent challenges with the same tumor cells. This virus was shown to persistently infect mouse lymphoid tissues and to accelerate rejection of sarcoma allografts without directly infecting tumor cells [25].

Another hint as to the involvement of the immune system in PV oncosuppression was given by the comparison of mice differing in their immune status for their responsiveness to parvovirus MVMp-induced suppression of transplanted syngeneic gliomas. Immunocompetent mice were fully protected from tumoral outgrowth of GL261 glioma cells infected ex vivo with MVMp. In contrast, immunodeficient ($RAG2^{-/-}$) animals were less competent for MVMp-dependent tumor inhibition, with only 20 % of the recipients being protected, arguing for an additional adaptive immune component to allow full tumor suppression [26].

In a syngeneic immunocompetent rat model of pancreatic ductal adenocarcinomas (PDAC), parvovirus H-1PV could achieve striking tumor suppression under conditions where only a relatively minor fraction of the tumor cells underwent direct virus-induced lysis [19]. In this system, the virus appears to act mainly as trigger of an anticancer immune response. Indeed, adoptive transfer of splenocytes from H-1PV-treated tumor-bearing donors resulted in tumor regression in naïve recipients bearing preestablished orthotopic pancreatic cancers [27]. In another setup, H-1PV-induced oncolysis of an infected pancreatic tumor was found to lead to the regression of a noninfected distant tumor in the same animal. In both types of experiments, the regression of the recipient or distant tumor, respectively, occurred in the absence of virus transfer, presumably via a virus-triggered antitumor immune response. Another line of evidence for the role of the immune system in PV oncosuppression comes from immunodepletion experiments showing that the selective removal of CD8+ cells from immunocompetent rats correlates with reduced effectiveness of H-1PV to eradicate transplanted gliomas (unpublished observations) and hepatoma metastases [24].

25.4.1 Indirect Activation of the Immune System by PVs Through Killing of Tumor Cells

A number of experimental observations support the notion that PV-mediated oncosuppression is immunogenic (see Fig. 25.1, upper left panel). As mentioned above, the oncotropism of PVs is

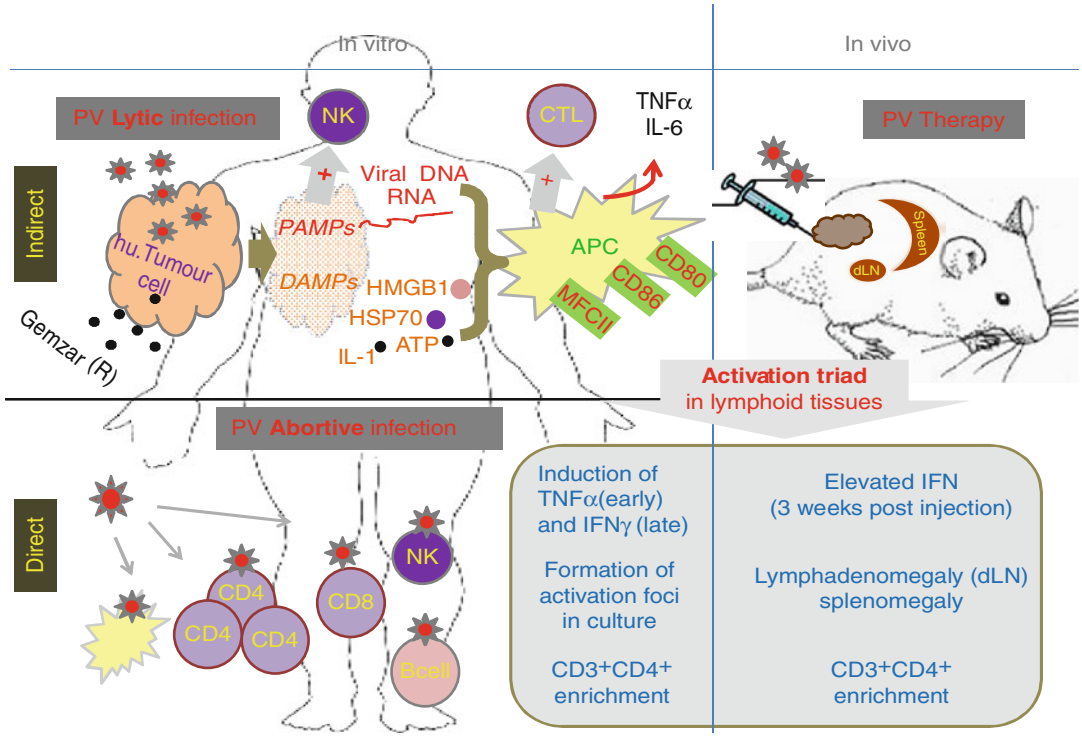

Fig. 25.1 Immunomodulation by PVs. *Upper panel* – indirect in vitro (*left*) immunomodulation by PVs in combination with standard pancreatic carcinoma chemotherapeutic. Tumor cells undergo immunogenic cell death (ICD), and the contribution of H-1PV-derived PAMPs (*DNA*, *RNA*) and H-1PV-Gemzar-induced DAMPs (*HMGB1*, *HSP70*, *ATP*, *IL-1*) to activate antigen-presenting cells (*APC*) is indicated. Immunomodulation in in vivo in PDAC model (*right*); tumor-draining lymph node (*dLN*). *Lower panel* – direct in vitro (human PBMCs) immunomodulation. The PV activation triad is observed in both systems; *CD4* Thelper, *CD8* cytotoxic T-cells, *NK* natural killers

not due to a better virus uptake by transformed cells but rather to the dependence of intracellular steps of the viral life cycle on the presence, absence, or activation of factors that are regulated as function of cell cycling and oncogenic transformation [28]. Due to small size and limited coding capacity of their genome, PVs exploit their nonstructural proteins to perform multiple functions. In particular, the activities of the major nonstructural protein NS1 are timely regulated through posttranslational modification and/or subcellular distribution. Phosphorylation of distinct NS1 domains controls both enzymatic (ATPase, helicase) and nonenzymatic (DNA and protein binding) functions of the viral product and varies during infection [29]. Cell death and eventual lysis are the final outcome of the infection of permissive malignant cells with PVs. Depending on the cell type and metabolic status, PV-infected cells can die through different mechanisms, namely, apoptosis, necrosis, or cathepsin spillage from lysosomes [13, 16, 19].

Several studies show evidence of an enhanced capacity of PV-infected versus noninfected tumor cells for activating APCs in human and rodent in vitro systems. This effect does not merely result from the PV-induced killing of tumor cells. Indeed, in vitro-matured dendritic cells (DCs) were found to be more efficiently activated by H-1PV-infected human melanoma cells than by melanoma cell lysates obtained by freezing/thawing. When DCs were co-cultured with PV-infected SK-29MEL cells, phagocytosis and cross-presentation of tumor-associated antigens by the DCs led to effective activation of melanoma-specific CTLs [30]. Mouse glioma cells (GL261) infected with the parvovirus MVMp (minute virus of mice) were also tested for their ability to activate dendritic cells (DCs) and microglia (MG), two distinct cell types that can act as APCs. Microglia and discrete DC subsets were activated after co-culture with MVMp-infected glioma GL261 cells, as evidenced by the upregulation of specific activation markers (CD80, CD86) and the release of proinflammatory cytokines (tumor necrosis factor-α and interleukin-6) [26].

In recent years, the dogma of apoptosis regarded as immunologically "silent" and necrosis as being the "alarming" way for cells to die has been revisited and reformulated in terms of immunogenic cell death (ICD). The emanation of danger-associated molecular patterns (DAMPs) such as calreticulin (CRT), high-mobility group box B1 (HMGB1), and ATP by dying cells was found to underlie ICD induced by some chemotherapeutics like anthracyclines [31, 32]. The mode of cell death does not seem to determine ICD profile, although CRT exposure has been linked more particularly to ER stress, release of HMGB1 to traumatic death, and the release of ATP to pyroptosis.

In this context, the view of apoptosis and necrosis as anti- and proinflammatory dying modalities, respectively, has been amended to relate immunogenicity of cell death to the induction of above three key ICD determinants [33]. Display of CRT, release of HMGB1, and ATP appears sufficient to enable paracrine maturation and activation of dendritic cells and consequent CTL priming – the cornerstone event of an adaptive immune response. Surface exposure of CRT mediates the uptake of dying tumor cells by dendritic cells, while the releases of HMGB1 and ATP are, respectively, involved in the activation of TLR2/TLR4/RAGE-mediated signaling and in the initiation of P2X7/inflammasome-mediated activation of caspase 1 (CASP1) leading to processing and secretion of matured interleukin-1 (IL1) [34].

Recent work from our lab points that H-1PV is a potent inducer of HMGB1 release from pancreatic carcinoma cells. In some of these cells, the combination of H-1PV with the standard PDAC chemotherapeutic gemcitabine can stimulate the full panel of ICD determinants including even IL-1 (ms in preparation). In an earlier study H1-induced apoptotic killing was reported to correlate with strong release of the immunogenic signal heat-shock protein (HSP72) of SK29-Mel-1 melanoma cells [35]. In this system also the combination of H-1PV infection and treatment with cytotoxic agents or sunitinib resulted in enhanced immunogenicity of melanoma cells, as revealed by their higher ability to induce the maturation of co-cultured DCs compared to singly treated tumor cells [36]. Altogether these studies highlight that the power of combined

chemovirotherapy can be used not only in its cytoreductive context but also as a local intratumoral adjuvant.

In addition to the cell-derived danger signals, viral elements such as double-stranded RNA or unmethylated CpG-containg DNA have been shown to serve as pathogen-associated molecular patterns (PAMPs) and trigger immune activation through pathogen recognition receptors (PRRs) (PKR, TLR 3, TLR 9). In the case of autonomous PVs, this issue is still under investigation, considering also the peculiar single-stranded nature of the viral genome, which offers unique opportunities to increase the genuine adjuvant effect of these viruses (see below).

Beside DCs, important effectors of innate immunity, namely, NK cells also appear to "sense" PV-mediated killing of PDAC cells, resulting in IFN-γ, TNF-α, and MIP-1α/β production. This activation was recently observed in cell cultures and may play an important role especially at the early stages of the immune response. NK cell-dependent killing of infected tumor cells in vitro could be traced back to the upregulation of the DNAM-1 ligand, CD155, and to the downregulation of MHC class I expression [37].

In summary, studies conducted with various human cancer models support the idea that PVs (in particular H-1PV and MVMp) can serve as indirect immunostimulators by killing human tumor cells through an immunogenic pathway leading to the activation of neighboring cells of the innate immune system. These approaches offer a tempting strategy for allogenic vaccination using PV-infected or chemovirotherapy-treated tumor cells.

25.4.2 Direct Effects of PVs on the IS

There is evidence that besides being able to indirectly activate immune cells, PVs may also have a direct impact on the immune system, as demonstrated in vitro in human PBMCs (see Fig. 25.1, lower left panel). Early work has shown that long-term cultured lymphocytes, monocytes, and immature and mature dendritic cells were not susceptible to the cytotoxic effect of H-1PV [35]. Furthermore, direct inoculation of H-1PV to human PBMC cultures caused only abortive infection [27, 38]. The virus could enter into the cells, achieve different levels of replication according to the nature and activation status of the cells, but no virus production could be detected. Upon mitogenic activation, the infection pattern (predilection of the virus to particular cell types) shifted from monocytes, B-, and NK cells towards T-cells and especially CD4 lymphocytes [27].

However, CD4 cells were quite stable, showing no impairment of proliferation and viability upon infection, in agreement with their low capacity for expressing the cytotoxic PV protein NS1. The fraction of CD4 cells was even increased in infected-nonstimulated PBMCs correlating with the formation of activation foci in the cultures. These changes were accompanied by early TNFα (24 h) and late IFNγ (72 h) production upon PBMC infection. Monocytes were identified as the source of TNFα, as established through isolation and separate infection of the adherent fraction of the cultures [27]. CD4+ T-cells also got activated in infected PBMC cultures, as revealed by activation marker expression and Th1 and Th2 cytokine secretion [27, 38]. In addition, the immunosuppressive properties of Tregs were reduced as a result of H-1PV infection, while the virus did not affect the viability of these cells [38].

Besides these in vitro data, some observations indicate that PVs can also interact in a direct way with immune cells under in vivo conditions. Significant MVMp gene expression was detected in lymphoid tissues from infected mice, whether or not with tumors (melanoma, lymphoma, hemangiosarcoma) [39]. This expression was especially obvious in lymph nodes draining virus-injected tumors (dLNs). In the hemangiosarcoma model, tumor cells could be easily identified using the transforming polyoma middle T oncogene (PymT) as specific tumor marker. The tumors of hemangiosarcoma-bearing mice showed strong accumulation of PymT, whereas

this marker was barely detectable in spleens and not detectable in dLN. Therefore, MVMp expression in lymph nodes is unlikely to be due to their invasion with infected metastasizing tumor cells and can be assigned to the infection of lymphoid cells instead. Indeed, myeloid DCs and B-1 lymphocytes were found to be the major source of viral mRNA expression in dLN, providing evidence for direct interaction of the parvovirus with immune cells.

Analysis of H-1PV-treated pancreatic carcinoma in a rat model (see above) confirmed that within the first days following intratumoral inoculation, PV expression is predominant in the tumor but can also be detected in lymphatic tissues and most abundantly in tumor-draining lymph nodes [19]. In both glioma and pancreas cancer models, immunocompetent animals treated with PVs showed spleno- and lymphadenomegaly, accompanied by an increase in the T-cell compartment with elevated CD4/CD8 ratios and induction of IFNγ in dLNs and splenocytes [27]. The fact that this same triad of changes was also observed upon direct infection of PBMC cultures (see above) suggests that in whole organisms, PV infection of immune cells takes place and results in a Th1 bias.

Altogether these observations lead us to propose that despite the abortive character PV infection of immune cells, the treatment of cancer-bearing organisms with PVs is not immunologically silent and results in both direct and tumor cell-mediated modulations that may confer adjuvant properties on these viruses. It is worth noting that induction of a vigorous local inflammatory reaction does not appear to be required for PV-induced priming of the immune system against tumors. No major burst of inflammatory cytokines was detected in either brain or pancreas of tumor-bearing animals undergoing PV-based oncosuppression. This low proinflammatory profile of PVs is consistent with their little-to-absent pathogenicity and indicates that PVs rather contribute to fine-tune the immune system [40]. The exact impact of this tuning on the abovementioned induction of splenocytes reactivity against an established tumor is currently investigated in our laboratory.

25.5 PVs as Part of Novel Immunotherapeutic Combinations

The fact, that intratumoral injection of an oncolytic virus generates an autologous in situ vaccine with the virus acting as an adjuvant, may provide a basis for designing a more effective virus. The silent immunomodulating features of H-1PV call for a certain increase in its immunogenicity, in order to create a more "dangerous" virus that can more potently revert the tolerogenic conditions prevailing in tumors.

25.5.1 Arming Propagation-Deficient Parvoviral Vectors with Immunostimulating Transgenes

Historically, the first attempts to increase the adjuvant properties of PVs were made by generating capsid replacement vectors from parvoviruses MVMp, H-1PV, and LuIII. These vectors were supplemented with transgenes encoding for toxic (Apoptin) or immunostimulatory (cytokines/chemokines) products [41–43]. Using an appropriate helper system, recombinant viruses transducing these heterologous genes were produced. Pilot animal experiments showed that recombinant PVs carrying the interleukin 2 (IL-2) cytokine gene have a reinforced anticancer capacity, in comparison with empty vectors [44]. Strong anticancer effects of recombinant parvoviruses expressing interferon gamma-inducible protein 10 (IP-10) and monocyte chemotactic protein 3 (MCP-3) were also observed against established hemangiosarcomas and melanomas in immunocompetent mice, respectively [42, 43]. Interestingly, MVMp vectors transducing TNFa and IP-10 were found to act synergistically in the suppression of mouse glioma [45]. It should be stated that due to DNA size restrictions for packaging, part of the capsid genes has to be removed from the vector for them to accommodate the above transgenes. In consequence, recombinant virions fail to generate progeny particles and to give rise to secondary rounds of infection.

25.5.2 Use of Replication-Competent Parvoviral Vectors as Vaccine Adjuvants

The abovementioned limitations of transgene-armed PV vectors prompted us to design another class of recombinants in which a short regulatory element is inserted in a noncoding region of the PV genome, in the absence of concomitant deletion of viral sequences. This novel group of replication competent PVs takes advantage of the minimal genome enlargement afforded for packaging by the viral capsid (aprx 150 bp) without affecting progeny production efficiency. The genomic region located between the stop codon and the polyA tail of the viral capsid genes proved to be most suitable for such insertions. Unmethylated CpG motifs in the DNA of bacterial and other microbial species have long been recognized as potent danger signal stimulators of antigen-presenting cells, through TLR-9 receptors [46].

Knowing that CpG motifs are underrepresented in the genomes of virtually all small eukaryotic viruses, including PVs, we assumed that enriching the genome of H-1PV with immunostimulatory CpG patterns would improve its immunomodulating properties [47]. The oncospecific replication of H-1PV should result in the selective amplification of these motifs within tumor cells, followed by their oncloytic release in the context of tumor antigens. This is expected to lead to a higher level of dendritic cell activation through the TLR-9 receptor, with cross-presentation of viral and tumor antigens to prime CTLs for killing both infected and noninfected tumor cells. Fully infectious CpG-armed H-1PV vectors were produced and tested for their adjuvant capacity in a typical therapeutic vaccination protocol (see Fig. 25.2, upper panel) [48]. Rat hepatoma cells with a known predisposition to metastatically settle in the lungs were intravenously injected in syngeneic immunocompetent ACI rats. The established lung metastases were treated by subcutaneous injection of irradiated autologous tumor cells infected with wild-type or modified H-1PVs. Under these conditions, the viruses did not reach the target metastases to exert direct oncolytic effects but replicated in and served as adjuvant to the autologous vaccine.

In keeping with the above evidence of parvoviral immunomodulation, infection with wild-type H-1PV (or GpC-supplemented control vector) improved the efficacy of the cell vaccine. This viral adjuvant effect was significantly enhanced by the H-1PV armed with CpG motifs, resulting in suppression of most metastases by the H-1PV-CG-infected autologous vaccine. This therapeutic vaccination effect was accompanied by strong induction of markers of cell-mediated immune response (IFNγ) and particularly of DC activation (CD80 and CD86) in the mediastinal lymph nodes draining metastases-harboring lungs. This is an exciting result, showing for the first time that non-defective parvoviral vectors can be engineered and used to generate oncolysates enriched in immunostimulatory CpG motifs and to boost vaccination against distant metastases even in the absence of virus spread. The same strategy (CpG genomic integration) was recently followed to modify oncolytic adenoviruses, improving significantly therapeutic effects [49].

25.5.3 Combining PVs with Interferon Gamma (Fig. 25.2, Lower Panel)

Our abovementioned studies of PDAC suppression by H-1PV strongly suggested that the immune system plays a major role in the therapeutic effect of the parvovirus in this system. Most PDAC patients have already distant (liver, peritoneum) metastasis at the time of diagnosis and are in need of aggressive chemotherapy at this stage. We assumed that a local application of PVs into the peritoneal cavity can be effective under these conditions [50]. The production of IFNγ in tumor-dLNs was found to systemically accompany the therapeutic effect of PVs, implying the importance of this potent cytokine in mediating PV immunomodulation. Intraperitoneal application of IFNy has been shown to achieve surgically documented responses as both first- and second-line therapy in randomized phase III clinical trials for ovarian cancer.

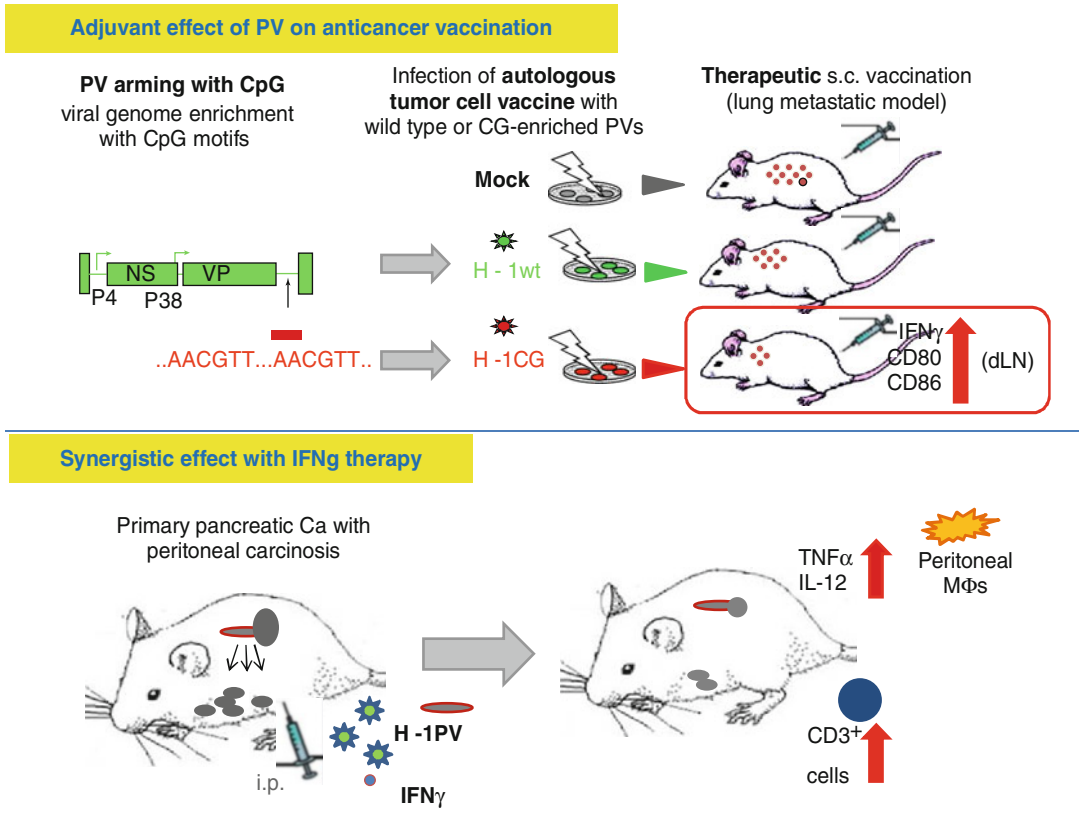

Fig. 25.2 Immunotherapy using PVs. *Upper panel* – improved vaccination approach with TLR-9 engagement. Treatment of established hepatoma lung metastasis using subcutaneous application of an autologous PV-infected vaccine. Infecting the vaccine with an engineered H-1PV virus genomically enriched with immunostimulatory CpG sequences improves the vaccination effect by reducing metastasis. IFN**γ**, CD80, and CD86 expression in mediastinal lymph nodes is boosted when the H-1PV-CpG-inoculated vaccine is applied. *Lower panel* – combined immunotherapy (*H-1PV* with *IFNγ*). Orthotopic pancreatic tumors complicated with peritoneal metastasis were treated by intraperitoneal injection of H-1PV + IFN**γ**. IFN**γ** enhanced peritoneal macrophage activation and CD3+ lymphocyte proliferation in spleens leading to retardation of primary tumor growth and carcinosis and prolonged survival

Altogether these data led us to reason that the therapeutic properties of H-1PV may be boosted with IFNγ for the treatment of late incurable stages of PDAC such as peritoneal carcinomatosis. Rats bearing established orthotopic pancreatic carcinomas with peritoneal metastases were treated through a single or intraperitoneal (i.p.) injection of H-1PV with or without concomitant IFNγ application (see Fig. 25.2, lower panel). This cotreatment with H-1PV and recIFNγ resulted in an improved therapeutic effect (prolonged animal survival), compared with i.p. injection of H-1PV alone. Interestingly, the cotreated group could also be distinguished by its immunological parameters, namely, (1) the proneness of isolated peritoneal macrophages to TNFα production, (2) the enhanced proliferation of spleen T-cells in presence of tumor cells under ex vivo conditions, and (3) a significant reduction in the titers of H-1PV-neutralizing antibodies in ascitic fluid. These results lend credit to our contention that PV oncosuppression induces immunological determinants which can be manipulated through appropriate combination treatments. To the best of our knowledge our study was the first to exploit the combination of IFNγ with an oncolytic virus.

Table 25.1 Preclinical data and ongoing clinical trial on parvovirotherapy

Model	Description and effects	
Glioma	Single intranasal instillation of H-1PV was sufficient to reach the brain and induce efficient regression of rat glioma. In rats with xenografts of human glioma, oncolytic activity of H-1PV was less pronounced, however, leading to significant prolongation of survival	[21]
Pancreas carcinoma (PDAC)	H-1PV monotherapy had strong anticancer effect both in vitro (human PDAC cells) and in vivo in rats and SCID mice (human xenotransplants) with reduction of tumor growth, prolonged survival of the animals, and absence of metastases on CT scans. H-1PV+ Gemzar combination had synergistic effect	[19]
Burkitt lymphoma	H-1PV productively infected and efficiently killed through necrosis, cell cultures originating from Burkitt lymphoma (BL) including resistant to apoptosis induction by rituximab cells. Parvovirus-based monotherapy efficiently suppressed established human BL at an advanced stage in a severe combined immunodeficient (SCID) mouse model of the disease	[20]
Cervical carcinoma	H-1PV caused a dose-dependent regression of subcutaneous tumors from human cervical carcinoma (HeLa) cells into immunodeficient rats and SCID mice	[17]
Breast carcinoma	Injection of parvovirus H-1PV at the site of human tumor cells' implantation in adult nude mice drastically inhibited tumor growth (greater than 80 %). When injected i.v. in animals bearing preformed tumors, H-1 virus was able to slow down and even in some cases to revert neoplastic growth	[18]
Metastatic cancer(lung, peritoneum)	Lung metastases from rat hepatoma were successfully treated with subcutaneous injection of a virus-infected (wild-type H-1PV or CpG enriched) autologous vaccine Concomitant i.p. treatment of H-1PV with recIFNγ immunotherapy resulted in improved therapeutic effect on peritoneal carcinosis from rat pancreatic cancer	[48, 50]
Phase I clinical trial glioma (GBM) (ongoing)	ParvOryx01 is an open, noncontrolled, two groups, intragroup dose escalation, single center, phase I/IIa trial. Eighteen patients with recurrent GBM will be treated in 2 groups of 9 patients each. Treatment group 1 will first receive H-1PV by intratumoral injection and second by administration into the walls of the tumor cavity during tumor resection. In group 2 the virus will initially be injected intravenously and afterwards, identical to group 1, into the surrounding brain tissue during tumor removal. The primary objective of the trial is local and systemic safety and tolerability and to determine the maximum-tolerated dose (MTD). Secondary objectives are proof of concept (PoC) and progression-free survival (PFS) up to 6 months	[51]

Conclusion

In summary, oncolytic viruses, and PVs in particular, represent versatile anticancer tools that can both specifically kill tumor cells and serve as adjuvants to hand the further tumor eradication over to the immune system. In comparison with human pathogenic viruses that have to be modified in order to become oncolytic (e.g., pox- and herpesviruses) and animal viruses that trigger overt immune reactions (e.g., NDV), PVs represent a safe and non-obtrusive invaders that rather achieve fine-tuning of the immune system to an anticancer state. However, this fact may allow the exposure and the rediscovery of cryptic tumor antigens in an immunogenic fashion, without strongly "attracting the attention" of the immune system towards inherent viral epitopes. Current and planned

parvovirotherapy clinical trials (Table 25.1) should give first indication, whether PV oncolysis can be translated into long-term protection by the immune system with improved tumor eradication and patient survival [51].

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Dendritic Cells Pulsed with Viral Oncolysate

26

Philippe Fournier and Volker Schirrmacher

Contents

Abstract

Glioblastoma multiforme (GBM) is an aggressive malignant tumour of the central neural system (CNS). Despite an improved surgical management and treatment by radiotherapy and adjuvant chemotherapy with temozolomide (TMZ), this form of cancer shows a poor prognosis with a median survival of less than 15 months. The use of dendritic cell (DC)-based active vaccination to bolster the otherwise impaired antitumour immune responses in glioma patients has received increasing attention. Early clinical trials demonstrated the safety and immunogenicity of autologous DCs loaded exogenously with various antigens. However, such DC vaccines have unfortunately rarely translated into strong clinical effects. The main reasons appear to be limitations in the induction of strong cellular antitumour immune responses able to counteract glioma-induced immunosuppression.

This may be due (1) to the immature/intermediate state of the used DCs that may induce tolerance and (2) to the lack of danger signals and type 1 polarisation signals, which create the proper context, in which tumour antigens should be presented by DCs to T cells. In this chapter, we introduce a new DC vaccine with reduced tolerogenic and enhanced immunogenic potential. The improved vaccine (NDV-DC) combines DCs with autologous tumour cells and danger

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signals provided by infection with an oncolytic strain of Newcastle Disease Virus (NDV). The uniqueness of this approach is associated with three important properties of NDV as bird paramyxovirus: tumour-selective replication, oncolytic potential and immune stimulatory capacity. Glioblastoma patients will be vaccinated postoperatively by intradermal applications of the NDV-DC vaccine, which is composed of ex vivo cultured patient-derived DCs loaded with viral oncolysate from NDV-infected autologous tumour cells.

When such tumour antigen-presenting DCs are applied to patients, their tumour antigen-specific T cells will receive several activation signals: (1) The tumour antigens will provide the T-cell activation signal 1, and (2) simultaneously, NDV-DC will provide so-called danger signals of importance for the induction of signal 2 (T-cell costimulation) and signal 3 (T-cell polarisation towards Th1). Compared to classical DC vaccines, NDV-DC presents improved T-cell activation signals required for induction of CD4+ and CD8+ T-cell-mediated immune responses resulting in the stimulation of strong and systemic tumoricidal T-cell immunity.

Through its capacity to induce interferon (IFN)-α and interferon (IFN)-ß, NDV also provides a link between the innate and the adaptive immunity system, thus further strengthening the anticancer immune response. NDV-induced molecular danger signals (viral RNA and HN protein) drive DCs to become Th1-directed immunogenic antigen-presenting cells able to overcome immunosuppression and tolerance mechanisms. Such a combination of oncolytic NDV with patient's tumour cells and autologous DCs will be evaluated initially against glioblastoma but it is applicable against virtually all types of cancer.

26.1 Introduction

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumour in adults and is a challenging disease to treat. The current standard of care includes maximally safe surgical resection, followed by a combination of radiation and chemotherapy with temozolomide (TMZ). Despite that, recurrence is quite common. That explains the search for more effective treatments for better prophylaxis against recurrence.

In contrast to the above standard treatments, which target tumour cells, immunology involves the host in the combat against the tumour. It aims at activating the patient's own immune system to help eliminate the cancer by attacking and rejecting the malignant tumour cells. T-cell-mediated antitumour immunity involves long-lasting memory responses and antitumour immunosurveillance mechanisms to prevent tumour recurrence. This area of research has made much progress in recent years. In 2010, the FDA approved a dendritic cell (DC)-based vaccine from Provenge (marketed by Denderon Corp) in the USA to treat refractory prostate cancer. This first vaccine against nonviral cancers [1–3] opens the door for T-cell-based immunotherapy to join the arsenal of standard cancer therapies.

While early phase immunotherapy trials with DCs against tumours of the central nervous system (CNS) showed encouraging outcomes, the DCs in use are believed to be only suboptimal for sufficient antitumour immune responses to mediate clinically meaningful changes in situ. This may be mainly due to the existence of numerous mechanisms, which are responsible for the highly immunosuppressive immunological microenvironment of the glioma tumour [4, 5].

We first present the characteristics of glioblastoma mutiforme as one of the most common and aggressive tumours of the gliomas. We also review advances in the diagnostics of such tumours as well as the classical treatment, which is used as the gold standard.

We will then relate to recent advances in the immunobiology of the CNS and its tumours. Such knowledge is essential to improve current treatment strategies harnessing the immune system to treat brain tumours.

We will then present a novel anticancer vaccine (NDV-DC), which is based on the combination of DCs, tumour cells and a virus and which aims to induce a glioma-specific Th1 response and cytotoxic T lymphocytes (CTLs). The chosen

virus is a bird paramyxovirus with particularly interesting properties: tumour-selective replication, oncolytic capacity and immunostimulatory properties.

We will finally discuss the pros and cons of such a novel immunotherapy strategy. This will be evaluated during a phase I/II clinical study in glioblastoma patients, which is in planning.

26.2 Glioblastoma Multiforme

Primary brain tumours, whose overall annual incidence is in the range of 5–6/100,000 [6], are defined as any intracranial tumour involving the brain parenchyma or surrounding structures such as the meninges, the pituitary or the pineal gland. Among those tumours, we can differentiate non-gliomas (meningiomas, pituitary tumours and medulloblastomas) from gliomas. These are tumours arising from non-neuronal glial cells, which are functioning to maintain homeostasis and to produce myelin, thereby providing both support and protection to neurons. Glial cells comprise astrocytes, oligodendrocytes and ependyma and can, upon cancerogenesis, form astrocytomas, oligodendrogliomas and ependymomas, respectively [7]. These represent approximately 36 % of all primary brain tumours and 80 % of malignant brain tumours [8].

Within the astrocytic tumours, GBM is the most frequent brain tumour and comprises the majority of all gliomas [9]. It accounts for 12–15 % of all intracranial neoplasms and for 60–75 % of astrocytic tumours [10]. A grading scheme proposed by the World Health Organization (WHO) distinguishes four different grades of gliomas, of which GBM has been categorised as WHO grade 4 and is the most malignant variant of all gliomas [11]. This grading is associated with cytologically malignant, mitotically active, necrosis-prone neoplasms with rapid disease evolution [12, 13]. GBM most typically affects adults and typically arises in the lobar white matter or in the deep grey matter of the brain. It is characterised as diffusely infiltrating growth [10, 14].

The development of this malignancy involves a multifaceted process that results in a loss of genetic or epigenetic gene control, in unregulated cell growth and in immune tolerance. It consists of highly proliferative and exceptionally migratory tumour cells: poorly differentiated round or pleomorphic cells, occasionally multinucleated, nuclei atypic, with a variety of acquired genetic alterations. The aetiology of primary tumours is still unknown and risk factors are poorly defined. Moreover, the cellular origins of GBM tumours remain enigmatic.

It may develop de novo from glial cells, typically has a clinical history of <6 months and is most common in older patients (primary GBM). It can also appear as the result of the malignant progression from a low-grade glioma (WHO grade II and III) from pre-existing low-grade astrocytomas and predominantly affects younger patients (secondary GBM).

The GBMs, much like other malignomas, are composed of a heterogeneous mix of neoplastic and non-neoplastic cells that include both native and recruited cells. The GBM stem cells (GBMSCs) can be defined as a slow-cycling, but highly tumorigenic, GBM cell subset that is selectively capable of indefinite self-renewal and differentiation to bulk populations of nontumorigenic cancer cells. These two basic properties make the GBMSC population the prime candidate for tumour maintenance and recurrence. They are also implicated in the development of chemo- and radioresistance and in tumour recurrence [15–20]. The disseminated nature of these neoplasms makes current therapeutic interventions highly ineffective at eradicating all residual intracranial tumour reservoirs. This leads to near universal tumour recurrence that, in turn, contributes to the lethality of this disease.

26.3 Diagnostics and Classical Therapy of Glioblastoma Multiforme

The symptoms exhibited by a GBM patient are dependent on the location of the tumour, its size and its growing rate. They can appear immediately, but in some cases, they only appear when the

tumour has reached a considerable size. They include headaches that differ in intensity; seizures, nausea and vomiting; hemiparesis, or paralysis on one side of the body; motor weakness and loss of sensation; a feeling of increased pressure within the cranium; cognitive impairment; changes in mood, personality, concentration and mental capacity; and loss of vision or aphasia. The presence of gliomas is primarily diagnosed with non-invasive methods such as magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) or modalities based on computed tomography (computed tomography (CT) scan, single photon emission computed tomography/X-ray computed tomography (SPECT/CT) and positron emission tomography (PET)). These allow imaging of anatomic changes within the patient and the detection of changes in molecular function. Because it can also be misdiagnosed as a benign brain lesion at this point, a histological analysis of the tumour biopsy sample is usually required to confirm the exact type and grade of a tumour. Primary GBM can be distinguished histopathologically from anaplastic astrocytoma by the presence of necrosis, microvascular hyperplasia and possibly thrombosis [21].

The addition of radiotherapy for the treatment of GBMs led to the first significant improvement in patient survival starting in the late 1970s. More recently, Stupp et al. [22] have shown that the addition of the chemotherapeutic agent TMZ can increase survival of GBM patients. Despite dramatic advances in surgical technique, imaging and adjuvant radiation therapy or chemotherapy, the prognosis for patients with malignant glial tumours remains poor. The current standard of care for high-grade glioma patients is maximum surgical resection combined with radiation and concomitant and adjuvant TMZ therapy [22]. This treatment results in a median survival of 14.6 months, a 2-year survival of 27.2 % and a 5-year survival of 9.8 % [22]. The main reason is the highly invasive nature of GBMs, which makes complete resection virtually impossible. This leads to a high rate of recurrences and low overall survival.

According to WHO, there are five main reasons accounting for the therapeutic resistance to aggressive treatments. One of the most important problems, when it comes to combating GBMs, is the vast genotypic and phenotypic heterogeneity. In clinical terms, this means that genetic heterogeneity is a major cause of acquired drug resistance and that the survival of only a very small subpopulation of cancer cells may still give rise to regrowth of the tumour or to metastases. Another very important factor that contributes to cellular heterogeneity is the presence of highly undifferentiated cells as well as cancer stem cells that most probably harbour resistance mechanisms. Moreover, the malignant cells have highly invasive properties that allow them to infiltrate into healthy tissue, as far as several centimetres away from the bulk tumour. Another obstacle that leads to poor response to different treatments is the blood–brain barrier (BBB) that is known to greatly reduce access of drugs to the brain. Last but not least, withdrawal of DNA repair systems abrogates the effectiveness of chemo- and radiotherapy [12].

The development of a successful treatment modality for malignant brain tumours will depend on devising a means of eliminating all intracranial neoplastic foci left behind after surgical resection of the primary tumour mass. This is a daunting task, given the highly disseminated nature of the disease process and our current inability to adequately visualise and therapeutically target every remaining tumour cell. However, treatment approaches aimed at using the patient's immune system to combat intracranial neoplasms hold promise for achieving this objective. Immunotherapeutic approaches aiming to bolster the arms of the immune system, which have been affected by tumour-induced suppressive factors, appear very promising. Conceivably, if effective, a therapeutically relevant immune-mediated treatment strategy for glioma should successfully overcome tumour-associated immunosuppression to the extent that the immune system can adequately recognise and clear all residual neoplastic foci within the brain following surgical resection of the primary tumour mass. The poor outcome of GBM has

spurred the search for novel experimental therapies that can address and overcome the basic biological phenomena associated with the lethality of this malignancy. The use of immunotherapy to boost the otherwise impaired antitumour immune responses in glioma patients has received increasing attention. One of the main advantages of generating an immune response towards cancer is that the immune effector cells can distinguish tumour from nontumour cells and destroy the tumour cells. These may be located in sites inaccessible for traditional surgery, radiation or chemotherapeutic drugs.

26.4 Immunological Aspects of the Brain and Implications for Cancer Immunotherapy

The brain is an organ composed of cells, which are extremely sensitive to toxic effects of exogenous substances. There are numerous factors limiting inflammation in the CNS. The blood–brain barrier (BBB) is a specialised structure of capillary endothelial cells, which protect the brain by using the selectivity of the tight junctions between them [23]. The CNS presents some unique characteristics and has been thought for many years to represent an immunologically privileged site [24] with no direct links to the immune system. This was supported by the absence of lymphatics [25], the presence of the BBB and a paucity of resident specialised antigen-presenting cells (APCs) within the CNS [26, 27]. Current data, however, demonstrate the fact that the brain is not an immunologically inactive organ and neuroinflammatory reactions can take place.

Cerebrospinal fluid (CSF) has been shown to drain via the Virchow–Robin spaces to the deep cervical lymphatics [28] via perivascular sheaths and through the nasal submucosa [29–31]. It is now recognised that, although naive T cells are not found within the CNS, under pathological conditions, such as inflammation or tumour growth, lymphocytes may infiltrate the brain and a systemic immune response may be elicited [32, 33]. In addition, microglia, macrophages but also vascular endothelial cells, smooth muscle cells, astrocytes, perivascular macrophages, choroid plexus epithelial cells, neurons and DCs have been shown to act as resident APCs within the CNS [34]. Microglia, as primary CNS antigen-presenting cells (APCs), with the phenotypic and functional characteristics of both macrophages and DCs [35, 36], express class II antigens and T-cell costimulatory molecules [36–38] and are capable of antigen (Ag) presentation [39]. DCs have been found to be present in the choroid plexus [40] and meninges [41] but not in parenchyma. All these observations explain that antigens within the CNS via entering the cervical lymph nodes (CLNs) by these routes [30] can induce an immune response. But this immune activation is characterised by a skewing towards a nontumour Th2 phenotype [30, 42, 43], leading to strong antibody responses and priming of cytotoxic T cells but an absence of delayed-type hypersensitivity (DTH) responses.

Within the glioma tumour tissue, the immune system appears as a driving force for cellular diversification. This leads to the successive accumulation of genetic and epigenetic changes. That means that glioma cells produce novel proteins and over-express molecules that are normally present only at low levels [44]. Increasing tumour heterogeneity also includes antigenic heterogeneity. While the immune system may exert pressure to eliminate certain glioma cells, other tumour cells may evade the immune response through modifications. This “immuno-editing” leads to tumours that develop various escape strategies for evading immune attack.

Malignant gliomas have been described to display local immune-suppressive characteristics (as shown in Fig. 26.1; [31, 45–48]).

It appears that certain types of immune cells from the tumour microenvironment actively participate in tumour development and progression.

In addition to the observation that the brain contains no or only a low number of natural killer (NK) cells, shows a very low amount of MHC-I molecules and lacks conventional lymphatic vessels [49, 50], tumour cells of the brain develop numerous passive and active mechanisms of immunoescape and immunosuppression

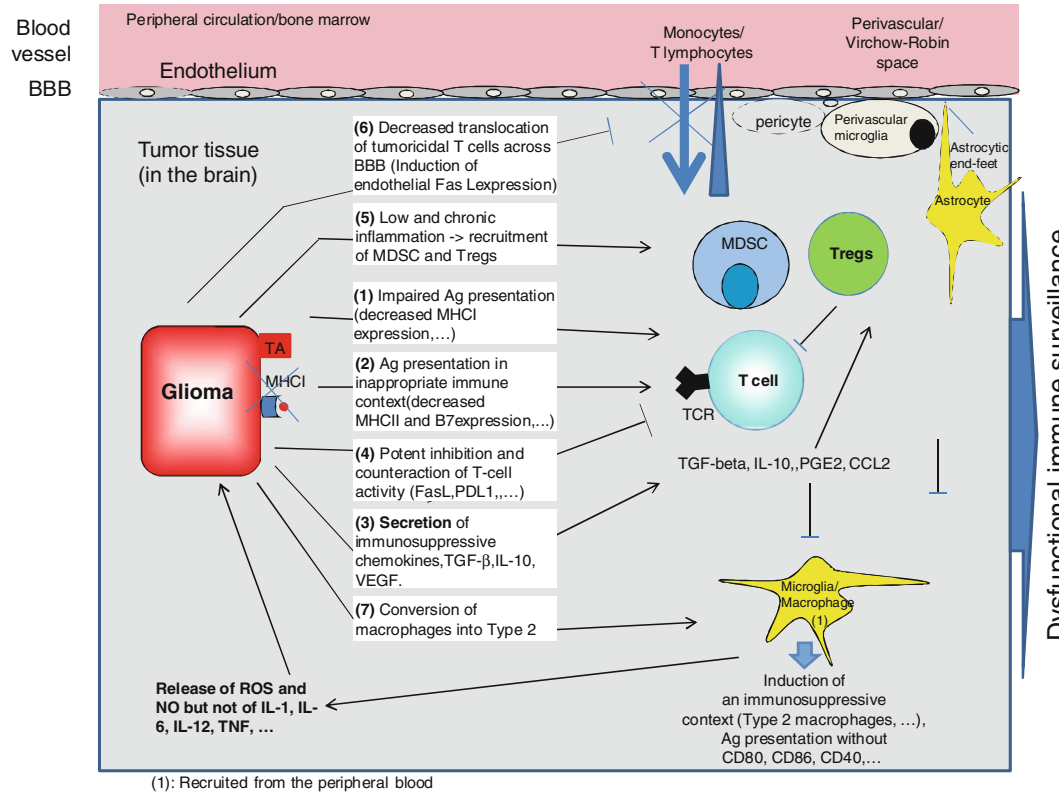

Fig. 26.1 Glioma and immunosuppression – complex immunosuppressive mechanisms employed by gliomas to blunt endogenous immune recognition and clearance Malignant gliomas avoid recognition and elimination by the immune system through numerous pathways. Some of them are shown on the figure. (*1*) Due to the lack of antigen presentation, via notably decreased MHC-I expression, a T-cell-mediated response is evaded. (*2*) Decreased MHCII and B7 expression (costimulation signals) impaired the induction of an immune response. (*3*) Tumours may inhibit T-cell activity by immunosuppressive cytokines and other factors (including TGF-**β**, IL-10, VEGF) and immunosuppressive cells (such as CD4+ CD25+ Tregs and MDSCs). (*4*) Tumours may also inhibit or counteract T-cell activity, for example, via upregulation of FasL and programmed cell death 1 ligand 1 (PDL-1) expression. (*5*) The presence of a low and chronic intratumoral inflammation leads to the recruitment of MDSCs and Tregs. (*6*) Glioma can decrease the translocation of monocytes and tumoricidal T cells across BBB at the Virchow–Robin spaces (implying pericytes, perivascular microglia and astrocytes). (*7*) The eventually recruited macrophages will be converted to type 2 macrophages, contributing to tumour progression by presenting tumour antigens to T cells without activating signal such as CD80, CD86 and CD40

(see Fig. 26.1). For example, they release/express several molecules with immunosuppressive properties, such as transforming growth factor beta (TGF-β), interleukin (IL)-10 and vascular endothelial growth factor (VEGF). TGF-β is known to suppress in T cells the production of interferon-γ and Fas ligand (FasL) production, which are necessary for cellular activation [51, 52]. In addition, TGF-β is known to downregulate the activating receptors on NKs and cytotoxic T cells. Another mechanism consists in the recruitment or conversion of several immunosuppressive cell types, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC). This leads to inhibition of T cells, DCs and NK cells [53].

This tumour microenvironment maintains the immunological intratumoral self-tolerance by actively preventing an immune response against the "foreign antigens" expressed by the tumour [54, 55]. This contributes to anergisation of the T cells, which are present at the tumour site, and to induction of immune tolerance towards the tumour cells.

Cancer immunotherapy, which aims at instructing the immune system to properly reject cancers, offers the premise to eliminate all

residual glioma cells after tumour resection. It has been shown that tumour-specific T cells can be primed in CLNs in murine glioma models [56–58]. Since the immunological microenvironment of the CNS and tumours arising in the CNS appear to be suboptimal for efficient antitumour immune responses to mediate clinically meaningful changes in situ [4, 5], the use of an immunotherapeutic strategy based on the generation of a strong cellular immune response against GBM outside of the brain, for instance, in the peripheral skin, appears as a good strategy. It has been shown that DCs present major dysfunctions in cancer patients. And, when isolated from cancer patients, DCs have a significantly lower effect in stimulating an immune response than do DCs isolated from healthy donors as it has been shown for breast cancer [59, 60].

To overcome the inefficiency of patient's DCs, the ex vivo preparation and activation of the DCs seem therefore appropriate. To this end, one of the most promising immunotherapeutic approaches consists in the peripheral vaccination of GBM patients with DCs pulsed ex vivo with tumour antigens in combination with microbial danger signals.

26.5 The NDV-DC Vaccine

The therapy with the NDV-DC vaccine consists in the intradermal application of DCs, which are generated ex vivo from blood cells of a cancer patient and which are activated via incubation with a viral oncolysate of the patient's tumour (Fig. 26.2).

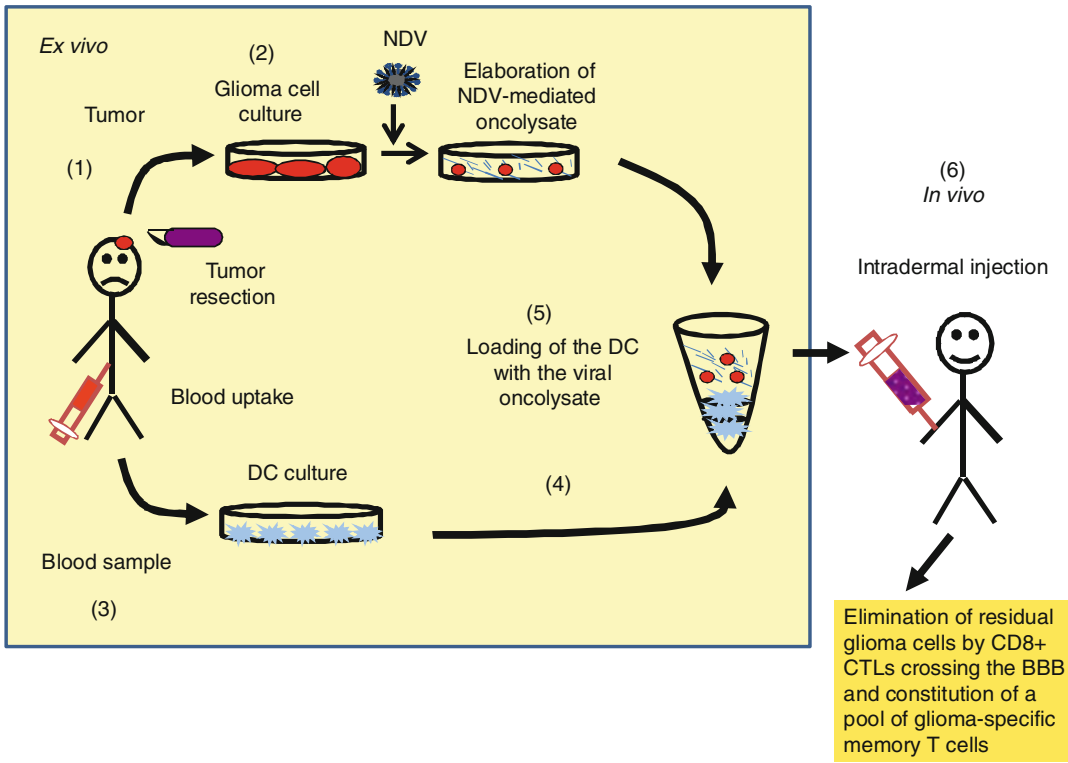

Fig. 26.2 Schematic representation of the NDV-DC therapy based on patient's derived DCs pulsed with lysate from NDV-infected autologous tumour cells (*1*) For preparation of the oncolysate, tumour cells are isolated from freshly operated tumour specimens. (*2*) Tumour cells are then grown in vitro. When the cells are growing exponentially, NDV infection is initiated by 1-h adsorption of the virus to the tumour cells. The cells are then left in culture until lysed. The oncolysate is then stored as frozen aliquots. (*3*) For each vaccination, monocytes are obtained from blood sample. (*4*) These cells are used to generate dendritic cells in the presence of IL-4 and GM-CSF. (*5*) DCs are then loaded with the viral oncolysate. (*6*) The activated DCs are then injected into the patient via intradermal injection

The objective is to induce at the periphery new antitumour immune responses by activating T cells from the T-cell repertoire against tumour cells and by restimulating potentially existing tumour-specific memory T cells, even if these may be partially anergised. Due to glioma-induced immunosuppression at the local site but also at the systemic level, the appropriate ex vivo preparation, information and activation of DCs are keys for treatment efficacy. To counteract glioma-induced immunomodulatory systemic effects, cancer patients may receive systemic NDV injection prior to NDV-DC immunotherapy. This can have a conditioning effect on the immune system due to the activation of a strong type I interferon response counteracting systemic tumour-induced Th2 polarisation effects.

The signals required for effective T-cell immunisation are, initially, antigen presentation and T-cell interaction ("signal 1"), which determines the specificity of the T-cell response. Further signals are required for costimulation and costimulatory molecule-mediated expansion ("signal 2"), which determines the magnitude of the response [61–63] of the selected tumour-specific T cells. Imprinting of additional features, which are critical for DCs to induce effective cancer immunity, is mandatory. This results in the polarising "signal 3" – which determines effector function (reactive or suppressive) and immunity type (Th1 or Th2)) in CD4+ and CD8+ T cells [64] – to selectively enhance Th1-, CTL- and NK cell-mediated type 1 immunity.

To this end, the rationale of the NDV-DC vaccine is based on:

1. The use of autologous DCs. These are the most potent APCs playing a central role in the initiation and regulation of T-cell-mediated immune responses. Such cells can be obtained ex vivo via differentiation of peripheral blood progenitors using recombinant cytokines [65].
2. The use of autologous tumour cells. This enables a close match between tumour antigens of the vaccine and those of the patient's tumour. Such antigens include common tumour antigens and also individually unique antigens, which are derived from somatic point mutations occurring in many different proteins expressed by tumour cells [66]. The latter represent the only true, tumour-specific antigens that are not expressed by any normal tissue. Unique (or mutated) antigens are postulated to present several potential advantages for immunotherapy:
   (a) Their T-cell repertoire should not be tolerised or deleted and they should be recognised as nonself by the immune system, as is the case with viral Ags [67].
   (b) Their potential resistance to negative selection in case the mutated protein is essential for cell survival [67].

   Recent gene profiling of breast tumours revealed their individual characteristics [68]. There is tumour heterogeneity also with regard to expression of tumour antigens. One million tumour cells, as are used for preparation of viral oncolysate for pulsing of DCs, may better represent such tumour antigen heterogeneity than well-defined common tumour antigens, as used in other vaccination trials [69, 70]. The logical extrapolation from these observations is an individualised approach for instruction ("priming") of DCs with patient-derived tumour material.
3. The use of Newcastle Disease Virus. This is a bird RNA paramyxovirus (see Fig. 26.3a).
   In humans, it induces a strong type I interferon response, which blocks any viral replication in normal cells. In tumour cells, NDV infection induces a weaker interferon response because such cells have various defects in their type I interferon response (see Fig. 26.3b; [72, 73]). Because of such defects, NDV replication in tumour cells is not inhibited [74]. This explains the three following properties, which could be assigned to this virus [75]:
   (a) Tumour-selective replication
   (b) Oncolytic properties for some strains
   (c) Immunostimulatory properties

In summary, the NDV-DC vaccine is composed of (1) *autologous DCs,* which are instructed with (2) *autologous tumour cells* expressing multiple TAs (as signal 1 or Ag signal) in association with (3) *multiple danger signals (DS)* (for the generation of the signals 2 and 3).

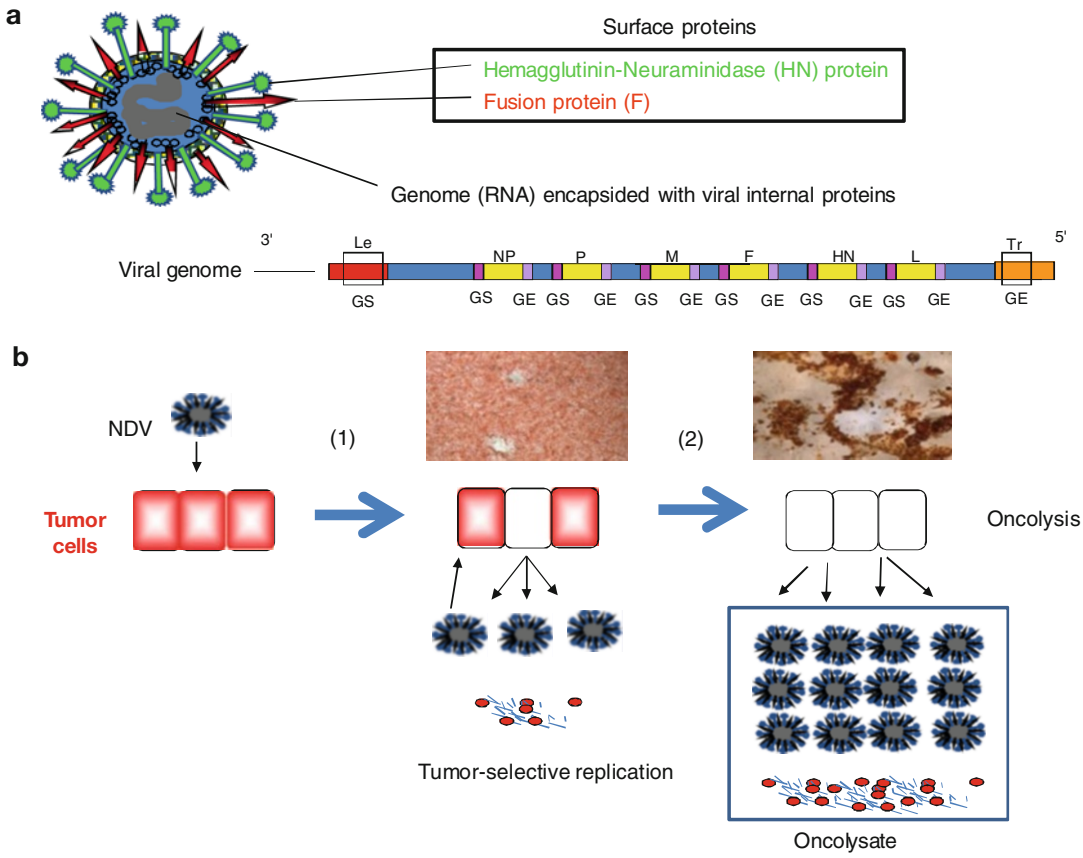

Fig. 26.3 NDV – a bird paramyxovirus inducing oncolysis via tumour-selective replication (**a**) Virus structure: NDV is an avian paramyxovirus of 150–300 nm diameter with an envelope containing viral proteins and a nucleocapsid with a negative strand RNA genome. The 15 Kb non-segmented genome has a leader (*Le*) and a trailer (*Tr*) sequence at each extremity allowing the binding and removal of the cellular ribosome. The transcription of the 6 viral genes (from the 3′ extremity to the 5′ end: nucleoprotein (*NP*), phosphoprotein (*P*), matrix protein (*M*), fusion protein (*F*), hemagglutinin-neuraminidase protein (*HN*) and large protein (*L*)) starts for each gene at a Gene Start (*GS*) sequence and terminates at a Gene End (*GE*) sequence. (**b**) Replication of NDV in tumour cells: (*1*) First round of binding/replication: The viral cycle is composed of two steps: binding and replication. (i) Binding: The first one involves the binding of the virus – via a lectin-like cell-binding domain of the HN molecule – to ubiquitously expressed host cell surface receptors expressing distinct carbohydrate side chains [71]. This is followed by the activation of the fusion protein F. The concerted action of HN and F leads to fusion of the viral and the host cell membrane. This membrane fusion event allows the viral genome to enter the cytoplasm of the host cell. There, the negative-stranded RNA genome is transcribed into messenger RNAs and translated into viral proteins. The proteins NP, P and L are required for nucleocapsid assembly. (ii) Replication (second step): The nucleocapsid as "anti-genome" is then used as a template for viral replication. The M protein and the envelope proteins HN and F, after post-translational modification, move to the membrane where virus assembly and budding occur. In this process, single copies of the NDV genome become wrapped into an outer coat envelope that is made from the host cells' plasma membrane. (*2*) Serial rounds of virus binding and replication lead to an oncolysate (containing viral particles and tumour cell fragments). The NDV strain used is virulent and has then a furin cleavage site in their F protein that allows its activation in a proteolytic medium. The cycle binding/replication can then be repeated, by infection of adjacent cells and their subsequent destruction by the same mechanism (multicyclic replication). This feature of viral replication provides virus production, which stops only when all the tumour cells are destroyed. The killing potential of lytic NDV strains is remarkable. Such strains have been shown to have a high capacity for killing tumour cells. One infectious particle leads in vitro to the death of approximately 10,000 cancer cells in 2–3 days

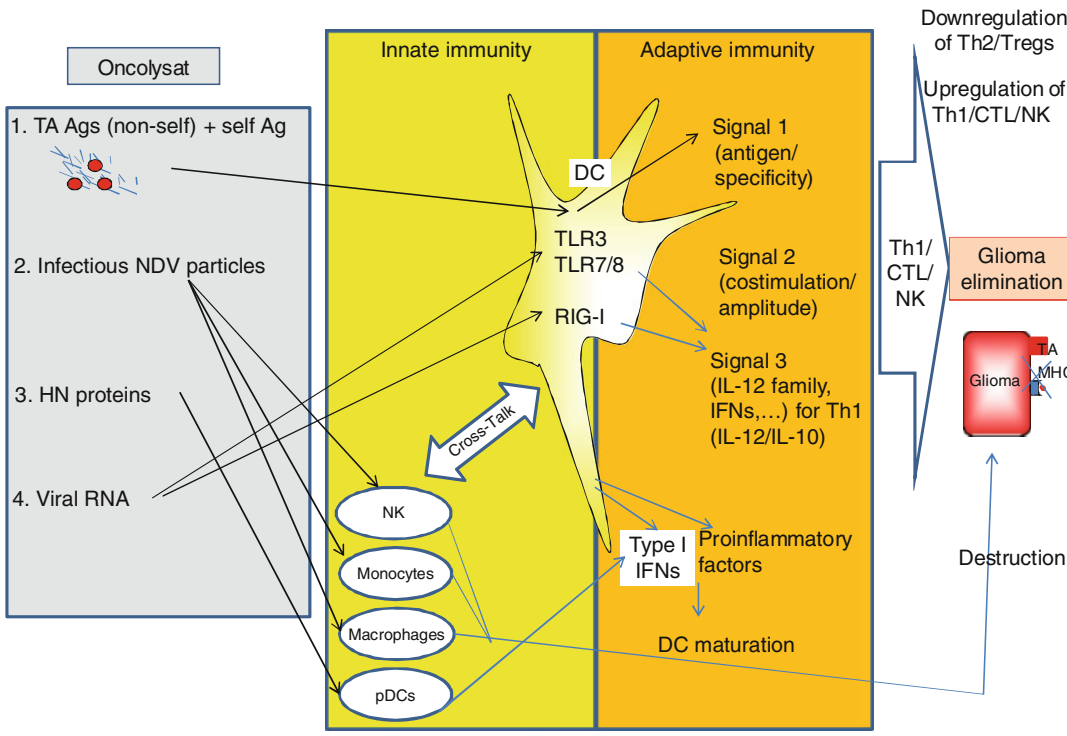

Fig. 26.4 Ex vivo imprinting of DCs by the oncolysate from NDV-infected autologous tumour cells for Th1-/CTL- and NK cell-mediated type 1 immunity To activate DCs, the lysate from NDV-infected autologous tumour cells was added exogenously to the patient's derived DCs. This oncolysate contains: (1) Tumour cell fragments (with tumour antigens (*TAs*) and nontumoural Ag (*self Ags*)) (2) Infectious NDV particles (3) HN proteins of NDV (4) Viral RNA. (1) TAs were processed into the MHC class I pathway and cross-presented as TA peptide-MHC (pMHC) complexes at the surface of the DCs. (2) Infectious NDV particles, which were present in the oncolysate, were shown to activate NK cells [76], monocytes [77, 78] and macrophages [79]. These activated cells contribute to tumour destruction. Crosstalks between NK cells and DCs [80] lead to type 1 DC activation. NDV stimulate also pDCs [81, 82] and monocytes [77] to release of type I interferon (IFN), which has a large effect on the immune system. Type 1 IFN plays an important role in the generation of CTL activity since such induction in mixed lymphocyte tumour cell culture (MLTC) assays could be blocked specifically by antisera to type 1 IFN [83]. Similar effects were observed in vivo during CTL priming suggesting that IFN-**α**/IFN-ß not only increases CTL activity but is also essential for the generation of CTL activity [83]. Type 1 IFN has been reported to induce the IL-12 receptor-**α** chain in T cells [84]. Together with IL-12, IFN-**α** polarises the T cell towards a cell-mediated Th1 response characterised by DTH and CTL activity. In addition, IFN-**α** induces the upregulation of molecules, which are important for antigen recognition (e.g. human leucocyte antigen (HLA)) and cell–cell interaction (e.g. cell adhesion molecules [85]). More recently, NDV was observed to polarise DCs to a DC1 phenotype [86]. (3) The HN protein induces the production of type 1 IFNs by pDCs [81, 82]. (4) Viral RNA activates DCs via TLR3 [87] and RIG-I [88] and contributes to the generation of strong danger signals in DCs. Whereas TAs activate DCs to induce signal 1 (antigen/specificity), the viral elements (*NDV*, *HN* and *RNA*) were shown to induce in DCs signal 2 (costimulation) as well as signal 3 (polarisation). This explains why addition of the oncolysates onto the DCs confers them with the three signals – *signal 1* (antigen), *signal 2* (costimulation) and *signal 3* (polarisation) – whose simultaneous expression is required for the generation of a strong tumour-specific Th1-/CTL- and NK cell-mediated-type immunity

The induction of danger signals in tumour cells upon NDV infection is key to the stimulation of DCs for the generation of strong antitumour effects (see Fig. 26.4).

NDV, through replication in tumour cells, leads to production of the viral hemagglutinin-neuraminidase (HN) proteins, a transmembrane protein, which is expressed at the surface of the

infected tumour cells [89]. This introduces to tumour cells new cell adhesion properties for lymphocyte interactions [90, 91] and for T-cell costimulation [92]. The HN protein has been shown to induce the production of IFN-α via paracrine interaction with pDCs [81, 82]. In addition, human tumour cell infection by NDV leads to upregulation of HLA and ICAM-1 molecules and to induction of IFNs, chemokines and finally apoptosis [85]. Human tumour cell infection by NDV leads also to by-products of viral replication such as dsRNA. These RNA derivatives can activate cytoplasmic PKR [72] as well as the cytoplasmic RNA-dependent helicase RIG-I [88] and also endosomal TLR3 [87].

All the danger signals, which derive from the infection by NDV (dsRNA and HN cell surface protein [93] but also IFN-α [81, 82]), allow activation of multiple innate immune responses (monocytes [77], DCs [94], macrophages [79] and NK cells [76]).

These activated innate cells play also an important role during interactions with cells of the adaptive immune system leading to effective adaptive antitumour immune responses including CD4+ and CD8+ T cells [83, 95] (summarised in [90, 96]; see Fig. 26.4).

As the normal brain parenchyma lacks DCs, it is virtually impossible to prime an adaptive immune response against antigens appearing exclusively in the brain. There are no professional APC cells available to transport tumour antigens from brain tumours to the CLNs with the microenvironment suited for optimal APC–T-cell interactions. The approach of generating patient-derived DCs ex vivo has circumvented this issue but also the one relating to the dysfunction of endogenous DCs in patients with cancer. It allows controlled "loading" of DCs with tumour antigens and the peripheral injection of such APCs at sites such as the dermis is superior to the brain parenchyma to allow for optimal APC–T-cell interactions.

Finally, the combination of autologous tumour cells with oncolytic NDV results in viral oncolysate, which, with the help of DCs, allows to deliver the key instructive signals (multiple and relevant TAs combined with multiple costimulatory and danger signals) needed for effective antitumour responses (see Fig. 26.5).

26.6 Strengths and Weakness

While this strategy holds much promise, various challenges remain at the scientific, technical or manufacturing level [100].

26.6.1 Scientific

Firstly, the use of corticosteroids, which almost always are part of the standard care for brain tumour patients, is known to suppress the immune response. Therefore, its use is contraindicated during active immunotherapy. Immunisation should be done as early as possible rather than late in a treatment protocol. In addition, it might be difficult to produce high-quality DCs from blood monocytes isolated at the time of corticosteroid treatment.

Secondly, the problem of induction of autoimmune responses by tumour lysate-pulsed DCs is not met. In general, the treatment was well tolerated. In none of the preclinical in vivo models nor in patients treated thus far with a previous generation of NDV vaccine based on autologous tumour cells and NDV [90, 96], which has been positively evaluated in glioblastoma patients [101], autoimmune reactions were observed. Reasons for this have been discussed elsewhere [102].

26.6.2 Technical

Challenges for clinical studies with GBM patients include relative difficulties in obtaining tumour tissues following immunotherapeutic treatments. Unlike other cancers, intracranial glioma tissue is readily accessible following vaccine treatment.

In addition, designing neo-adjuvant settings with vaccines is not always feasible because recurrent malignant gliomas, for which surgical resection is clinically indicated, typically do not allow to wait for weeks before surgery and often require treatment with high-dose corticosteroids.

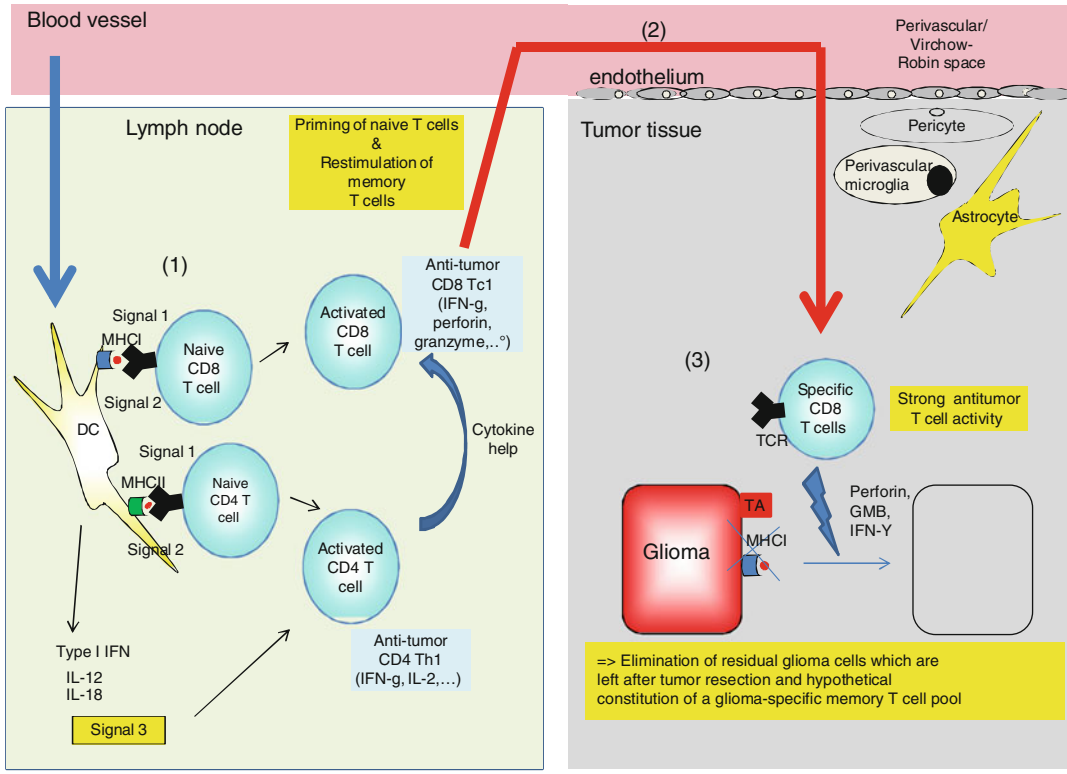

Fig. 26.5 Peripheral activation of T cells within peripheral lymph node and migration of tumour-specific CD8 T cells for the killing of residual tumour cells in the brain (*1*) In the lymph node, activated DCs deliver the three signals 1, 2 and 3 to CD4+ and CD8+ T cells. This leads to priming of naive T cells and to restimulation of tumour-specific memory T cells. CD4+ T cells help in the expansion of tumour Ag-specific CTLs [97] but also in activation of macrophages at tumour sites [98] and killing of tumour cells [99]. (*2*) The CD8+ T cells become tumour-specific CTLs and migrate to the tumour site via peripheral circulation and through the perivascular Virchow–Robin space. (*3*) In the brain, the intratumoral CD8+ T cells kill residual tumour cells via perforin and granzyme B (*GMB*) and contribute, via local IFN-**γ** production, to antitumour activity

Moreover, large randomised studies are often difficult to conduct since GBM is rare. Due to the need for individual highly patient-selective treatments, the patient accrual on clinical trials is likely slower than usual. The potential market for an approved product is smaller, and the development times for FDA or EMA approval are prolonged.

26.6.3 Manufacturing

There are significant manufacturing challenges facing the clinical development of the therapeutics presented here. Pulsing DCs with tumour lysate requires culture of autologous DCs and glioma cells and time-consuming procedures, for which Quality Control (QC)/Quality Assurance (QA) ratio is not always feasible. In addition, the current Good Manufacturing Practice (GMP) requirements for cells to be cultured for several weeks before being transferred to humans make the therapy extremely laborious and costly. To allow personalisation of the treatment, tasks of production for the vaccine need to be developed rigorously. All these requirements, in particular GMP, albeit necessary, require considerable investment. It necessitates also a centralised manufacturing facility. The vaccine as finished product of the culture and activation steps has to be stored in an appropriate fashion as shortly as possible. In addition, such therapies in autologous

necessitate an automate production. And there are also potential difficulties of standardisations. Overall, this slows down the pace of translation research.

As the obstacles to the efficiency of DC vaccines are removed and the standardisation of this vaccine moves forward, NDV-DC appears likely to become a viable option for GBM patients.

Conclusion

Glioblastoma multiforme is a cancer of the brain with very poor prognosis. The key obstacle for an adequate therapeutic antitumour immune response is the glioma-induced immunosuppression. Nevertheless, immunotherapy appears as a future option for new therapeutic intervention. With improved technology and novel, personalised, rational therapies, patient survival and quality of life can be expected to become improved.

The use of ex vivo-matured DCs, which acquire significant resistance to inhibitory factors, has become a therapeutic option. NDV-DC appears as a very good candidate to address this challenge. In this vaccine, DCs, by being combined with autologous tumour cells and NDV, are rationally instructed with key and potent instructions to simultaneously deliver the three signals (signal 1 (antigen), signal 2 (costimulation) and signal 3 (polarisation)), which are required for the activation and generation of strong antitumour activities in T cells.

Future DC-based immunotherapy approaches require a multipronged strategy to stimulate robust Ag-specific immune responses that are resistant to the inhibitory environment of tumours. NDV as a multivalent agent with tumour-selective, oncolytic and strong immunostimulatory properties appears as an outstanding agent to be combined with DC for such purposes. The scientific data presented in this review provide new insights into the rationale and the design of the NDV-DC tumour vaccine. This vaccine works as a potent Th1 response mediator, favouring the induction of DC maturation, the release of proinflammatory cytokines and the improvement of Ag cross-presentation, which are essential for the priming and activation of a CD8+ T-cell-mediated immune response resulting in antitumour clinical effectiveness. What is very important to note is the large diversity of mechanisms given by the use of NDV oncolysate for DC activation, which may ensure to overcome the broad resistance of GBM tumour cells against immune attack.

The previous generation of NDV tumour vaccine ATV-NDV, which was based on endogenous peripheral vaccination without ex vivo culture and activation of DCs, indicated in a prospective clinical study a clear benefit for glioblastoma patients in terms of survival [101]. Based on these data and the current knowledge in tumour immunology, combining DC vaccination with NDV oncolytic virotherapy paves the way for a new strategy of immunotherapy of tumours with poorly defined rejection antigens, for patients after primary or secondary tumour resection (with available tumour material).

The NDV-DC immunotherapeutic approach appears as a multipronged approach to stimulate robust Ag-specific immune responses that are resistant to the inhibitory environment of tumours. It offers a perspective to be combined with other treatments [103].

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Index

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