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Uday Kishore *Editor*

Microbial Pathogenesis

Infection and Immunity

Second Edition

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Prof. Robert Braidwood Sim (1951–2021)

Uday Kishore dedicates this book to Prof. Robert B. Sim for his unflinching and selfless mentorship. Bob sadly passed away on 6th February, 2021.

Foreword

Darwin was unfortunate when he wrote the *Origin of Species* not to be able to draw on a modern understanding of how the immune system recognizes and deals with pathogens. He never knew any of the fascinating stories of how organisms evolve both to defend themselves from infectious agents and to become more adept at bypassing their host's defences. It is possible to see written into immune system the result of millions of years of many Red Queen races, with host and pathogen continually evolving and adapting to ensure survival. Darwin would have found more to inspire him in the immune system than all the finches and animals that he studied in the Galapagos Islands.

In this book, Uday Kishore has pulled together contributions that show us some of the complexity and intricacy of the interactions between microbes and the immune system. This book covers a range of pathogens, which is important—the immune system must deal with viruses, bacteria, fungi, and parasites. This diversity of pathogens has shaped the immune system that we have.

At the time of writing this foreword, the world is battling with the SARS-CoV-2 pandemic. This is a very simple RNA virus with just four structural proteins and a genome of 30 kb. However, it has been able to wreak havoc across the world, locking down nations and disrupting economies. The worldwide death toll in August 2020 is around 750,000 and will rise to millions. This shows just how we need to increase our understanding of viruses to deal with newly emerging pathogens. There are contributions that cover SARS-CoV-2 and related viruses, which may be the first chapters that many readers turn to.

However, familiarity can breed contempt. There are many pathogens that humans have co-existed with for many millennia which continue to kill humans. Malaria has probably been an important human pathogen for around 10,000 years, once humans started to make settlements. It is a sobering statistic that probably around 400,000 people a year die from malaria (down from 1 million 20 years ago), about two thirds of whom are infants. This is happening year in, year out. The recent experience of developed countries with SARS-CoV-2 should make us more empathic to the impact of malaria on the health and economy of some of the poorest countries in the world. The chapter outlining our current understanding of malaria immunity and vaccine development describes a critical endeavour.

This book is going to be useful for all those who want to understand how microbes and the immune system interact, and how to use this knowledge to prevent and treat disease. I urge you to read chapters covering different pathogens, even those that you think you are not interested in. An understanding of immunity requires the breadth of scope contained in this book.

Twickenham, UK Andrew J. T. George

Preface

I am delighted to present to you the Second Edition of the book *Microbial Pathogenesis: Infection and Immunity*. The first edition was published nearly 7 years ago; the current edition is aimed at updating and complementing the first volume.

Understanding the pathogenic mechanisms used by a diverse range of infectious microorganisms, together with host's innate and adaptive immune responses, is so crucial for the development of effective chemotherapy as well as vaccination strategies and overcoming the concerning issue of drug resistance. Thus, it is important to investigate microbiological and clinical aspects of a pathogen with an eye on the host immune response against it and the escape mechanisms that can potentially be used to subvert anti-microbial immunity. Pathogens have co-evolved with us and, thus, they have been perfecting the art of avoiding, nullifying, and aggressively combating our immunity. This volume is going to the press at a time that is unprecedent in our recent memory due to Coronavirus Disease 2019 (COVID-19) pandemic. In less than one year, the scientific community has gone on from sequencing the viral genome, crystalizing the spike protein, discovering entry receptors/coreceptors, cracking pathogenic mechanisms, repurposing a range of drugs for treatment, developing several dozens' vaccine candidates, and finally, setting up promising clinical trials. Mass vaccination programme against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has gone ahead at full speed paving a way forward for life to attain some degree of normalcy. This whole exercise highlights why it is pertinent to appreciate a discipline that amalgamates microbiology and immunology.

This volume has a wide variety of chapters categorized, as expected, into the themes of viruses, bacteria, fungi, and parasites. At the outset, Alhamlan *et al.* describe various cancers caused by human papilloma virus, a great example of conquering the cancer by anti-viral vaccination. This is followed by Chapter 2 that addresses immune deviations orchestrated by Ebola virus. Chapter 3 examines the roles of innate immune cell surface and humoral pattern recognition receptors against human immunodeficiency virus-1 (HIV-1). This chapter highlights the importance of mucosal innate immune factors in the development of prophylaxis against HIV-1 infection. Chapter 4 assesses the diverse range of the innate and adaptive immune responses that shape infectivity as well as protection against respiratory viruses including respiratory syncytial virus and influenza viruses. Chapter 5 recaptures the nature of the host–pathogen interaction against the Middle-Eastern Respiratory

Syndrome (MERS) virus, a journey that offers a great insight and prelude to follow in terms of SARS-CoV-2. In Chapter 6, Yasmin *et al.* elaborate on almost all aspects of COVID-19, including receptor–ligand interaction, distribution of SARS-CoV-2 receptor/co-receptor in the host tissues, life cycle of the pathogen, innate and adaptive immune responses against the virus, various drugs repurposed for the treatment, and a range of vaccination strategies. It is largely expected that this chapter will act as a good primer for those readers who are keen on further understanding and researching about the SARS-CoV-2 and COVID-19.

McCarthy *et al.* in Chapter 7 allude to a major healthcare concern, i.e. antibiotic resistance in a range of bacterial pathogens. They take up the example of an opportunistic bacterial pathogen, *Acinetobacter baumannii*, which has a number of characteristics endowed within genomic resistance clusters, transposons, and integrons that facilitate acquisition of antibiotic resistance, in addition to modulation of enzymatic and transporter machineries. Chapter 8 dissects a classical, type IV hypersensitivity reaction-driven bacterial pathogenesis by *Mycobacterium leprae*, the causative agent of debilitating leprosy. This is also an excellent model to study granuloma formation, one of the finest examples of *in vivo* host–pathogen interaction, especially in lepromatous leprosy lesions. A largely ignored disease, leprosy, remains a major cause of morbidity in the developing countries. Chapter 9 evaluates the importance of pattern recognition receptors and their interaction with a range of ligands of *Mycobacterium tuberculosis*. Our longstanding efforts to enhance adaptive immunity, especially type 1 helper T cell response (interferon-gamma production), via various anti-tuberculosis vaccines, have fueled the growing realization that the innate immune components may have a bigger role to play in the anti-tuberculosis protective response. Given an intrinsic molecular dialogue between various innate and adaptive immune players, it is becoming evident that membrane, cytosolic and soluble pattern recognition receptors are likely to be important decision-makers in the latent tuberculosis. Ferluga *et al.* in Chapter 10 provide a detailed account of various vaccination strategies that have been trialed clinically against *Mycobacterium tuberculosis*, while expanding on larger-than-life virtues of the BCG vaccine.

Valand and Girija in Chapter 11 cover virulence factors of Candida, anti-Candida innate and adaptive immune responses, and most importantly, the immune escape strategies this fungal pathogen uses to thrive within the host. The last chapter in this volume (Chapter 12) examines immune response against the malaria parasite, in addition to critically evaluating various vaccination trials and outcomes.

We sincerely hope that this book provides an insight into the diversity and complexity of host–pathogen interactions. I am grateful to all the contributing authors who have taken time out of their busy schedule to write such well-crafted, well-illustrated, and stimulating chapters. I would also like to thank Alison Ball and Sofia Valsendur (Springer Nature) for their patience while I was editing this book during the COVID-19 lockdown period.

London, UK Uday Kishore

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About the Editor

Uday Kishore is a teacher and a scientist with special interest in innate immunity. He was the Founder Director of the Centre for Infection, Immunity and Disease Mechanisms, Brunel University London. He earned his BSc from S.P. Jain College, Sasaram, Bihar, India; MSc from Hindu College, Delhi; and PhD from the Department of Zoology, University of Delhi and CSIR Institute of Genomics and Integrative Biology, Delhi, India. After spending a year at the Salk Institute for Biological Studies, La Jolla, California, he moved to the University of Oxford for his post-doctoral training, first at the MRC Immunochemistry Unit, Department of Biochemistry, and then at the Weatherall Institute of Molecular Medicine, John Radcliffe Hospital. He is the recipient of fellowships of NASA (USA), Wellcome Trust (UK) and Alexander Humboldt Foundation (Germany), MRC Investigator Prize, European Commission Young Scientist Prize, and Mother Teresa Excellence Award. Uday Kishore holds several adjunct, visiting and honorary professorial positions nationally and internationally. He has altogether authored over 250 research papers, book chapters, patents, and books. His research focuses on how C-type lectins (especially collectins such as surfactant protein D) and individual complement proteins (C1q, factor H and properdin) resist a range of pathogens such as HIV-1, SARS-CoV-2, Influenza A Virus, *Mycobacterium tuberculosis, Aspergillus fumigatus and Plasmodium falciparum*.

Human Papillomavirus-Associated Cancers

1

Fatimah S. Alhamlan, Mohamed B. Alfageeh, Mona A. Al Mushait, Ismail A. Al-Badawi, and Mohammed N. Al-Ahdal

1 Introduction

Human papillomavirus (HPV) infection is now the most common sexually transmitted infection worldwide, including in the United States. An infected individual can directly spread these viruses to others through vaginal, anal, or oral sexual contact. The presence of HPV is associated with many types of subclinical and clinical infections, including anogenital, head and neck, and cutaneous infections. Of the more than 182 HPV genotypes that have been sequenced to date, 40 genotypes can infect the mucosa, including the mucosa of the anogenital tract. Although many of these are considered low-risk HPVs, that is they are associated with less severe disease, such as non-genital warts, there are currently 12 HPVs (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) that are considered by the World Health Organization (WHO) to be high-risk cancer-causing types, with several additional types including HPV-68 and HPV-73 identified as "possibly" causing cancer (Schiffman et al. 2009). More than 95% of all cervical cancer cases worldwide are caused by high-risk HPV types; thus, the epidemiology of anogenital HPV infection, especially cervical HPV infection, is well documented (Bosch et al. 2002). Fortunately, many, if not most, HPV infections are transient, and an individual's immune system may clear the infection before he or she becomes aware of its presence. Perhaps owing to this, HPV infection has received little attention from clinicians, the general public, or policy makers. However, this lack of attention may underpin a deadly and increasing problem because each newly acquired infection has the potential to persist. Persistent HPV infection may become an incurable, lifelong affliction that significantly increases the long-term risk of cancer not only for the afflicted individuals but also for their sexual partners (Shanmugasundaram and You 2017). Such HPV-related cancers may not manifest for decades, making successful treatment more difficult and costly, imposing a significant burden on the health care system (Workowski et al. 2002).

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Approximately, 4.5% of all cancers worldwide (630,000 new cancer cases per year) are attributable to HPV, with 8.6% of these occurring in women and 0.8% in men. One study by Martel et al. assessing the worldwide burden of cancer attributable to HPV by site, country, and HPV type showed that cervical cancer accounts for most of the HPV-attributable cancer, and two-thirds of those cervical cancer cases were found in less developed countries. The study also found numerous other HPV-attributable anogenital cancers, including 8500 cases of vulvar cancer, 12,000 cases of vaginal cancer, 35,000 cases of anal cancer (half occurring in men), and 13,000 cases of penile cancer. The HPV-attributable cancers of the head and neck included 38,000 cases, of which 21,000 cases were oropharyngeal cancers occurring in persons residing in more developed countries. The relative contribution of the high-risk types HPV-16 and HPV-18 to these cancers is 73% and that of other high-risk types (31, 33, 45, 52, and 58) is 90% (de Martel et al. 2017). Given these startling high percentages of likely preventable HPV-attributable cancers, a comprehensive strategy, based on HPV vaccination and screening, should be immediately implemented in both developed and developing countries.

2 HPV Genome

Human papillomaviruses are double-stranded circular DNA, composed of approximately 8000 base pairs. The HPV genome has a non-coding region, called a long control region, that contains the replication origin as well as post-transcriptional control sequences that contribute to viral gene expression. This non-coding region, which occupies approximately 10% of the HPV genome (about 850 base pairs), contains binding sites for cellular transcription factors and for the viral E1 and E2 proteins, which control viral replication and gene expression. Most HPV genomes also encode eight proteins. Six of these (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7*) are genes located in the early gene region, which occupies just over half of the 5′ end of the viral genome (Florin et al. 2002). The early proteins are regulatory rather than structural in function. Their roles primarily involve the control of HPV genome replication and transcription, cell cycle, cell signaling, and apoptosis as well as immune modulation and structural modification of the infected cell. The *E1* gene encodes an origin recognition and helicase protein, and the *E2* gene promotes the assembly of E1 complexes. In a productive infection, the expressed E4 protein associates with cytokeratin filament collapse. Expressed *E5*, *E6*, and *E7* are viral oncogenes, inducing cell immortalization and transformation. Expressed E6 and E7 proteins inactivate two cellular tumor suppressor genes, namely p53 and pRb, respectively (Zheng and Baker 2006). Most of these early proteins are expressed throughout the infectious cycle although their expression is decreased at later stages. The remaining two of the eight HPV genes (*L1* and *L2*) are located in the late gene region, which occupies almost 40% of the virus genome downstream to the early region. These *L1* and *L2* open reading frames encode two structural proteins, a major (*L1*) and a minor (*L2*) capsid protein; the capsid enables the transmission, spread, and survival of the virus in the environment (Zheng and Baker 2006; Florin et al. 2002). Except for the above-mentioned roles of the *E1* and *E2* genes, none of the remaining six genes encode for polymerases or other replication factors. Thus, viral replication relies primarily on host cell proteins to maintain viral genomes in undifferentiated cells and to enable replication or amplification in differentiated cells (Doorbar et al. 2012).

3 HPV Infection

HPVs can be grouped phylogenetically into five genera, α, β, γ, μ, υ with the α and β genera being the most studied, and can be further classified into species (Bernard et al. 2010). α HPV genus members infect genital epithelia and are the causative agents of many anogenital cancers. β HPV genus members infect cutaneous epithelia and are thought to be cofactors in the development of non-melanoma skin cancers (Galloway and

Laimins 2015). HPV infections are observed within specific regions of host transitional epithelial cells (e.g., the squamocolumnar junction). The up-regulation of these regions is complex and may leave host cells vulnerable to viral transformation (Herfs et al. 2012).

The mechanism of HPV infection is unique, occurring only in the basal cells of the stratified epithelium, which is the only tissue in which the virus replicates (Bernard et al. 2010). The life cycle of the virus is distinguished by the differentiation of these infected cells. The virus enters basal epithelial cells through epithelial microabrasions and attaches to these cells using common cell surface molecules, such as heparan sulfate proteoglycans. HPVs can also use α_6-integrin as a virus receptor, with virus entry through either caveolae- or clathrin-mediated endocytosis (Shafti-Keramat et al. 2003; Evander et al. 1997; McMillan et al. 1999; Smith et al. 2007a; Kines et al. 2009). When these infected cells divide, the daughter cells are pushed outward toward the epithelial surface. During this migration, various stages of the virus life cycle are triggered.

After a cell is infected, the virus is uncoated, and the circular viral genome is transported to the nucleus. When the infected cell divides, the nuclear episomal genome is replicated to 20–100 copies. These copies are maintained in the daughter cells during segregation by the attachment of the viral genome through the virus replication/transcription factor E2 to the host chromosome. During this first phase of basal layer cell infection, the expression of *E1* and *E2* genes maintain a low genome copy number (Ozbun 2002; Hamid et al. 2009).

Cells in the lower layers that are infected with HPV high-risk types express *E6* and *E7* genes. These cells are pushed through the cell cycle and are stimulated to divide. The *E6* and *E7* expression levels between high-risk and low-risk HPV types contribute to differences in carcinogenicity. In addition, differences in the regulation of host protein interactions have also been observed among high-risk types, and these differences may also contribute to carcinogenicity variability (Boon and Banks 2013; Egawa et al. 2015; Schiffman and Wentzensen 2013). In low-risk HPV type infections, basal cell proliferation is regulated primarily by the presence of growth factors, and the main role of the HPV E6 and E7 proteins in lesions of this type is to drive cell cycle entry above the basal layer, promoting HPV genome amplification (McLaughlin-Drubin et al. 2011). Proteins necessary for genome amplification increase in cells located in middle layers. These cells express viral E4 protein and are generally in the S or G2 phases of the cell cycle. However, the cells leave the cell cycle when they reach the upper epithelial layers. The E4-positive cells make viral L2 and L1 structural proteins, enabling packaging of the amplified viral genomes.

Persistent HPV infection is considered the major risk factor underlying cervical tumor progression. Infection may persist for up to several years in infected stem and daughter cells in the basal layer of the epithelium. While low-level viral genome replication occurs in the basal layer, the infected cells undergo terminal differentiation in the upper epithelial layers. The expression of E6 and E7 in those layers affects cell division, inhibits apoptosis, and abrogates epithelial differentiation. Thus, productive replication occurs during suprabasal layer differentiation. Once differentiation of suprabasal cells occurs, replication of the viral genome occurs at a high level. This amplification takes place in concert with the synthesis of the capsid proteins, followed by virion assembly and release (Doorbar et al. 2012; Galloway and Laimins 2015; Middleton et al. 2003; Klingelhutz and Roman 2012).

This ordered expression of viral gene products leading to virus particle production is disrupted in HPV-associated neoplasia. In cervical dysplasia, expression levels of *E6* and *E7*, genes which are generally considered viral oncogenes, increase from cervical intraepithelial neoplasia grade 1 (CIN1) to CIN3. These changes in gene expression are thought to underlie the neoplastic phenotype. CIN1 lesions, with relatively low levels of E6 and E7, appear to retain the ability to complete the HPV life cycle and produce virus particles. Such lesions resemble flat warts, which have a low level of cell proliferation in the basal and parabasal layers (Bodelon et al. 2016). The

low E6 and E7 levels do not appear to compromise their cellular targets sufficiently to facilitate cancer progression. However, the higher E6 and E7 expression levels observed in high-risk HPV infection are associated with CIN grade 2 or higher phenotype. These cells accumulate genetic changes that over time contribute to cancer progression. The viral dysregulation seen in CIN2, CIN3, or higher grade also appears to facilitate integration of the viral episome into the host cell chromosome, which can further deregulate expression of E6 and E7.

HPV replication is transient and depends on host cell differentiation. Once the virus enters basal epithelial cells, HPV genomes are established as autonomous replicating extrachromosomal elements. Thus, a low level of HPV expression is established. Once the infected cells differentiate, productive replication and expression of capsid genes is induced. This leads to the synthesis of progeny virions. Data obtained from immunosuppressed patients or patients with recurring laryngeal papillomatosis indicate that certain HPV types can exist in a latent state, wherein HPV DNA is present but no differentiation-dependent virion synthesis is observed. The presence of an HPV latent state may determine the effectiveness for treatment of infections (Stubenrauch and Laimins 1999). HPV DNA integrates into the host genome in most but not all cervical cancers (Vinokurova et al. 2008), and the mechanisms underlying this integration and promotion of carcinogenesis are not well understood. HPV DNA integration reportedly occurs in regions of genomic instability, or in short genome sequences, where the HPV and host are homologous. This latter observation suggests that DNA repair processes may contribute to HPV integrating into host cell genomes based on nucleotide sequence similarities (Akagi et al. 2014; Bodelon et al. 2016).

4 HPV Transmission

HPV is transmitted to an individual through direct contact with infected skin or mucosa. A strong and consistent association has been shown between mucosal HPV infection and sexual activity, with the number of infections increasing in proportion to the number of sexual partners (Winer et al. 2008; Alhamlan et al. 2016). HPV detection in the anal canal has been associated with anal intercourse for male having sex with male (i.e., MSM) and to a lesser degree for women (Dunne et al. 2006). However, HPV transmission is also associated with other sexual practices, including oral sex or the sharing of sex toys that penetrate the body despite standard cleaning of the toy (Gervaz et al. 2003; Anderson et al. 2014). Several HPVs genotypes can also infect the cutaneous epithelium. Transient skin lesions, such as common warts on the hands or verrucas on the feet, are generally benign. Such lesions may be embarrassing or unsightly to an individual, but if the lesion rapidly spreads, persists, or is in a site prone to injury, it may also become clinically relevant. Such lesions in patients who are immunosuppressed or who have received a diagnosis of epidermodysplasia verruciformis, an inherited recessive genetic disorder, may cause cancer (Howley and Pfister 2015; Patel et al. 2010).

HPV may also be vertically transmitted from mother to child, especially *in utero*, during delivery, or through contact after birth with the mother or relatives. Although the most likely route of transmission is from mother to child during a vaginal delivery when the child passes through the infected birth canal (Hahn et al. 2013; Freitas et al. 2013), HPV-induced lesions, such as laryngeal and anogenital lesions, have been observed at birth, suggesting intrauterine HPV transmission (Marcoux et al. 2006).

5 HPV Detection

Accurate molecular techniques for HPV detection and identification are of great importance for determining and diagnosing at-risk patients. There is currently no universal HPV detection assay. Instead, HPV types are detected based on pairwise nucleotide sequence identity within the highly conserved *L1* gene. Distinct HPV types are identified when nucleotides show a differ-

ence with other HPV types of at least 10% (Bernard et al. 2010; Schiller et al. 2010). Given the causal relationship between a persistent high-risk human papillomavirus (HR-HPV) infection and the possibility of developing cancer, HPV DNA-based testing has been advocated for the detection of clinically relevant cases especially lesions. Because of the confirmed the role of HR-HPV in over 70% of cervical cancer cases, HPV testing is necessary to determine whether a patient has an HPV infection. DNA testing has been used as a primary screening in conjunction with a Papanicolaou test, or has even been used alone because of its high sensitivity. Therefore, HPV testing is well-established in cervical-related research.

To date, the late gene (*L1*) region has been the most commonly used target for HPV detection assays. Indeed, the sequence of the L1 region from different HPV types is conserved enough to be utilized for primer design (Chan et al. 2012). However, HPV *E6* and *E7* genes are strong candidates for HPV detection and genotyping and have become the newly proposed targets for HPV detection in cancer (Fig. 1.1). *E6* and *E7* genes are oncoproteins in high-risk HPVs and have the ability to alter the function of cell cycle regulators. These two primary oncoproteins inactivate the two tumor suppressor proteins, p53 (inactivated by E6) and pRb (inactivated by E7) leading to cell cycle disruption (Narisawa-Saito and Kiyono 2007; Munger et al. 2004). They are proposed to be a candidate for detection and genotyping because they have the ability to be present in the episomal and in the integrated states of the virus, and are retained in the tumor. Therefore, these genes are strong candidates for HPV detection and viral load quantification. In fact, relying only on L1 primers could be unfortunately misleading because the L1 region is lost during HPV integration into human genome.

The best screening tests are practical, easy for the clinician to administer, safe for the patient and clinician, and accurate, which is generally measured by the sensitivity and specificity of the test. Culturing and *in vitro* propagation of HPV are impractical, and serological detection methods are insufficiently sensitive (Molijn et al. 2005). Thus, molecular biological methods, including DNA-based assays (e.g., polymerase

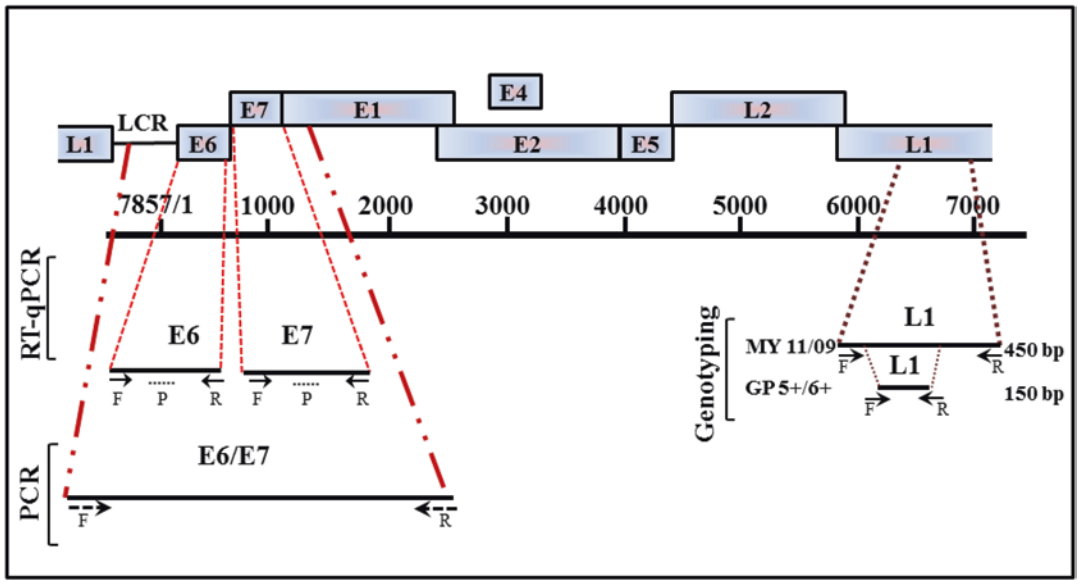

Fig. 1.1 Diagram of a linearized HPV 16 genome. Schematic diagram depicting the DNA and RNA target sequences for PCR, qPCR, qRT-PCR and genotyping. Arrows represent primers while the black dashed lines represent probes. *F* forward primer, *P* probe, *R* reverse primer, *LCR* long control region. (Figure is modified from Wang-Johanning et al. (2002))

chain reaction, in situ hybridization, reverse line plot, and Hybrid Capture 2) and RNA-based assays (e.g., mRNA gene or protein expression), have become the primary approaches for detection of HPV (Chan et al. 2012). At early stages, the best techniques for HPV detection and typing appear to be DNA based, whereas once disease progresses, greater accuracy is achievable via RNA based methods. Thus, for clinically relevant infections, both direct and indirect viral gene transcript detection methods are more specific approaches than DNA-based assays. In addition, HPV oncogene expression and deregulation evidence can be collected through direct detection of viral mRNA transcripts (Cuschieri and Wentzensen 2008). Figure 1.1 illustrates the assay (DNA vs. RNA) most accurate for detection of all HPV types at each stage of disease. Through the use of this suggested approach, HPV-16 and HPV-18 have been found to be responsible for over 70% of all invasive cervical cancers (Smith et al. 2007b). HPV-16 is the most carcinogenic of all the HPV types, causing about half of all cervical cancers, most of the other HPV-related anogenital cancers, and more than 80% of HPV-positive head and neck cancers (de Sanjose et al. 2010; Serrano et al. 2015; Mirabello et al. 2016). Advances in high-throughput next-generation sequencing, which have enabled large-scale studies examining HPV genome variability, have led to discoveries in HPV genomic research (Cullen et al. 2015; Cornet et al. 2013). Further empirical population-based studies will enable investigation at the intersection of molecular biology and epidemiology to markedly increase the knowledge and underlying mechanisms associated with HPV-related carcinogenesis (Jeantet et al. 2009).

6 HPV-Related Cancers

The National Program of Cancer Registries and the Surveillance, Epidemiology, and End Results program have reported an average of 33,369 HPV-associated cancers diagnosed annually in USA, with 21,290 of these detected in females (13.2 per 100,000) (Viens et al. 2016). Of all diagnosed human malignant neoplasms, approximately 4.5% are attributable to HPV, including 96% of cervical cancers, 93% of anal cancers, 64% of vaginal cancers, 51% of vulvar cancer, 36% of penile cancers, and 63% of oropharyngeal carcinomas. According to the Centers for Disease Control and Prevention, the annual incidence in the United States of new HPV-associated cancers is 26,800 (Brianti et al. 2017). The various HPV types infect specific tissue sites, that is, they display tissue tropism; thus, specific HPV types are associated with specific diseases. For example, HPV-16 is detected in squamous intraepithelial lesions, such as those of the cervix, vagina, vulva, anus, penis, and oropharynx; HPV-6 and -HPV-11 are detected in condylomata acuminate and respiratory papillomatosis; and HPV-1, 2, 4, 3, 10, and 7 are detected in cutaneous warts (Insinga et al. 2008). Mortality from HPV is caused by persistent infections with oncogenic HPV types that lead to dysplasia and cancer.

6.1 Cervical Cancer

Although cervical cancer is the fourth most frequent cancer among all women worldwide, it is the second most common cancer among those aged 15–44 (Serrano et al. 2018). Currently, 527,624 women worldwide receive a diagnosis of cervical cancer annually, and 265,672 die of this disease every year. A report assessing the incidence of cervical cancer by region found that of these 527,624 women, the vast majority, 444,546 or approximately 85%, reside in less developed countries, indicating that poorer countries bear most of this disease burden (Bruni et al. 2017). Cervical cancer in less developed regions accounts for nearly 12% of all female cancers. Globally, the annual number of newly detected cervical cancer cases continues to increase, but within developed countries, the number has fallen from its peak in 1975 of being the second most common cancer. This decrease has been attributed to early screening and vaccination programs (Parkin et al. 1984). Because cervical cancer is caused mainly by HPV, WHO issued a cervical cancer screening guideline in 2014,

recommending at least one screening for women who were between 39 and 49 years of age. The guideline further recommended extending screening to those women who were younger than 30 and at high risk of high-grade CIN (grade 2 or higher) (WHO 2014; Santesso et al. 2016). Resent research has shown that a negative HPV test result is more reassuring than a negative cytological test result because the latter has a greater chance of being falsely negative, leading to treatment delays (Koliopoulos et al. 2017). In addition, a recent randomized clinical study of 19,009 women, which investigated cervical cancer screening comparing primary cervical HPV testing with cytology testing, found that screening with primary HPV testing resulted in significantly lower likelihood of CIN grade 3 or higher (2.3 cases per 1000 women) than cytology testing (5.5 cases per 1000 women) at 48 months' follow-up (Ogilvie et al. 2018). Therefore, the majority of more developed countries use both HPV screening assays and cytology testing, a practice proven to be best for earlier diagnosis of high-grade CIN and more effective in prevention of invasive cervical cancer. However, cervical cancer screening or any clinical care found to be needed based on the screening is little used in low and middle income countries (LMICs) in which infrastructure and personnel requirements strain is existing health systems (Denny et al. 2006). Thus, LMICs have the highest burden of cervical cancer, about 85% according to a recent WHO report (Bray et al. 2015). Although cytology-based screening programs have improved cervical cancer control in developed countries, such programs in LMICs are constrained by cost, lack of infrastructure and trained staff, and the amount of time required between sample collection and test result, which is associated with treatment delays or management losses. Thus, trained midwives or nurses in low-resource settings frequently resort to conducting a visual inspection of the cervix using acetic acid or Lugol's iodine and simple tools (a speculum and a lamp). However, frequent training and supervision is required, and test interpretation is subjective, leading to variability in results accuracy. Still, one important advantage gained by using visual cervical inspection is that the results are quickly obtained; thus, any recommended treatment can be started immediately (Arbyn et al. 2008).

6.2 Anal Cancer

Anal cancer accounts for 27,000 new cancer cases worldwide where 90% have been estimated to be caused by HPV (de Martel et al. 2017; de Martel et al. 2012). Although there are many similarities, the natural history of HPV infection in the anus is less understood than the cervix. However, the recent studies found striking increase in the incidence of HPV-associated anal cancer especially among HIV-positive individuals. Indeed, the incidence of anal cancer has increased since the introduction of highly active antiretroviral therapy in HIV infected individuals (Palefsky 2009). Other studies have shown that five females are affected for every male, and the rates of anus, anal canal, and anorectal cancers among females of all races and ages has more than doubled in recent years (Galloway and Laimins 2015). Moreover, white woman show higher rates than black woman of anal squamous cell carcinomas, whereas black males have a significantly higher incident rate than white males (Joseph et al. 2008). Incidence of anal cancer is higher among men participating in male to male sexual (i.e., MSM) contact, women having a history of cervical or vulvar cancer, and patients with immunosuppression, which includes patients with HIV or a history of organ transplantation. These anal cancers are predominantly squamous cell carcinoma, adenocarcinomas, or basaloid (previously called cloacogenic) carcinomas (Crooms and Kovalcik 1985; Tougeron et al. 2009).

6.3 Vulvar and Vaginal Cancers

Despite the high numbers of all HPV-related cancers, vulvar and vaginal cancer are relatively uncommon globally, and HPV infection is not associated with all cancers of the external genitalia. It has been estimated that HPV-related

infections are responsible for 29–43% of vulvar cancer, 87% of vulvar intraepithelial neoplasia, 70% of vaginal cancer, and 69–100% of vaginal intraepithelial neoplasia (Forman et al. 2012; de Sanjose et al. 2013). Of the HPV-positive cancers, HPV-16 and HPV-18 are responsible for 35–77% of vulvar cancer, 75–80% of precancerous vulvar lesions, and 60% of vaginal cancer and precancerous vaginal lesions (De Vuyst et al. 2009). Compared with HPV-negative cancers of the external genitalia, HPV-associated vulvar cancers are detected in younger women. These cancers display basaloid, rather than the keratinizing pathology, which is observed in HPV-negative vulvar cancers. HPV-positive vulvar cancers are not associated with p53 mutations but are associated with sexual risk factors, unlike HPV-negative vulvar cancer (Hoevenaars et al. 2008). HPV-associated vaginal and vulvar cancers share many features although vaginal cancer is more likely than vulvar cancer to be associated with HPV infection (Smith et al. 2009).

6.4 Head and Neck Cancers

Squamous cell carcinoma of the head and neck is also HPV-associated (Mork et al. 2001; Villa and Hanna 2018; Li et al. 2013). The worldwide incidence of HPV-positive oropharyngeal cancer, which is found in the oropharynx and base of the tongue, tonsil and larynx, was 29,000 cases in 2012 and is on the rise, especially among younger men in Western countries, including the United States (Chaturvedi et al. 2013). Similar to HPV-positive vulvar cancer, HPV-positive oropharyngeal cancer is detected in a younger population than non-HPV-associated cancer. HPV-positive oropharyngeal cancer is also associated with sexual risk factors (Chaturvedi et al. 2011). According to 2011–2014 US National Health and Nutritional Examination Survey data, the prevalence in adults aged 18–69 years of any oral HPV infection is 7.3% and 4% for high-risk HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, or 68) (McQuillan et al. 2017). Persistent high-risk oral HPV infection, especially with HPV-16, is associated with progression to HPV-positive oropharyngeal cancers (Steinau et al. 2014).

6.5 Penile Cancer

Penile cancer is a globally rare malignant neoplasm although it comprises up to 10% of all male cancers in Africa, South America, and Asia (Mackenzie-Wood et al. 2001). The incidence of penile cancer has been associated with a number of risk factors, including history of phimosis, balanitis, chronic inflammation, penile trauma, lack of neonatal circumcision, tobacco use, lichen sclerosus, poor hygiene, and history of sexually transmitted diseases, especially HIV and HPV (Engelsgjerd and LaGrange 2018). Persistent HPV infection in men is associated with HIV infection, current and past sexual behavior, number of sex partners, absence of condom use, prior sexually transmitted infection, race, ethnicity, and circumcision status (Nyitray et al. 2011; Hernandez et al. 2010). Although HPV infection in not associated with all cancers of the external genitalia, HPV-16 and HPV-18 cause nearly 35–40% of penile cancers overall and cause 70–80% of HPV-positive penile cancers (Santesso et al. 2016). Compared with patients with HPV-negative cancers, HPV-positive penile cancers occur in younger patients and are associated with sexual risk factors.

6.6 Recurrent Respiratory Papillomatosis

Recurrent respiratory papillomatosis (RRP), a benign laryngeal tumor more common in children but also presents in young adults, is characterized by papillomatous lesions present along the aero-digestive tract. The incidence of RRP is 2 per 100,000 adults and 4 per 100,000 children (Carifi et al. 2015; Fusconi et al. 2014). RRP is thought to be caused by the transmission of, most commonly, HPV-6 or HPV-11 through sexual contact (oral sex) with infected external genitalia in adults or from a mother with genital warts to

her infant prior to birth through the placenta in about 12% of cases (Venkatesan et al. 2012) or during the infant's passage through the birth canal (Derkay and Wiatrak 2008; Fortes et al. 2017). A primary risk factor for RRP in adults is sexual activity with multiple partners (Fortes et al. 2017). The primary risk factor for juvenile-onset RRP is maternal anogenital warts, with the risk of RRP increasing approximately 230 times in the presence compared with the absence of maternal anogenital papillomatous lesions during pregnancy or birth. Approximately 0.7% of infants exposed to maternal anogenital warts develop the disease. Although RRP is benign, the warts can obstruct the larynx, requiring multiple ablative treatments, and is thus associated with considerable morbidity in children.

6.7 HPV-Induced Warts

Common, plantar, or flat warts are cutaneous HPV infections common throughout the general population, especially in children and adolescents; 10% of children have warts, with the incidence peaking between 12 and 16 years old (Beutner 2000). These nongenital HPV-associated warts spread through skin to skin contact. Not all nongenital warts are benign. For example, Bowen's disease, also called squamous cell carcinoma in situ, is a nonmelanocytic intraepidermal malignant neoplasia that transforms into invasive squamous cell cancer in approximately 3–5% of cases (Wozniak-Rito and Rudnicka 2018). Multiple HPV types, including HPV-16, 18, 31, 32, and 34, have been detected in finger, toe, palm, and foot skin lesions and from genital mucosa (Mackenzie-Wood et al. 2001).

6.8 Epidermodyplasia verruciformis

Epidermodyplasia verruciformis (EV) is a heritable defect in the cell-mediated immune response to HPV infection that leads to an increased susceptibility to infections of specific HPV types. EV is characterized by wart-like lesions and pityriasis versicolor-like spots covering parts of the body. Individuals with EV are at increased risk of developing cutaneous malignant neoplasms, Bowen's disease, or squamous cell carcinoma in particular (Shruti et al. 2017). Although several HPV types have been detected in these lesions, HPV-5 and HPV-8 are associated with the highest malignant potential (Karrer et al. 1999). Table 1.1 summarizes the most common HPV types in each cancer site.

Table 1.1 HPV-associated cancer with the most common HPV types

Cancer site	HPV-associated disease	HPV types
Cervix	Cervical cancer	HPV16, 18, 33, 35, 45, 52, 58, 59 and 73
Vaginal	Vaginal cancer	Mainly HPV 16
Vulva	Vulvar cancer	Mainly HPV 16
Penis	Penile cancer	Mainly HPV 16
Anus	Anal cancer	Mainly HPV 16
Oropharynx	Oropharyngeal cancer	Mainly HPV 16

7 Conclusions and Perspectives

Considerable progress has been made toward reducing HPV-mediated cervical carcinoma. The use of routine Papanicolaou testing and the prophylactic vaccines, Gardasil and Cervarix, have led to documented progress in decreasing the burden of this disease. More widespread use of currently available HPV vaccines among adolescent girls would further reduce the incidence and mortality of cervical cancer by two-thirds while establishment of cost-effective screening programs among adult women would more rapidly reduce cervical cancer mortality rates. However, the incidence of some noncervical HPV-related cancers, especially oropharyngeal, is rapidly increasing in more developed countries toward that of cervical cancer. Given this accelerated increase and that no approved screening programs for HPV-associated noncervical cancers currently exist, implementation of HPV vaccination programs is crucial. Reducing or preventing

male HPV cancers would require either male vaccination against HPV types or the development of "herd" immunity through high rates of HPV vaccinations among females. Such HPV vaccination would initially complement cervical screening in the fight against cervical cancer. Improved vaccines as well as better methods for early detection are being developed. Second-generation HPV vaccines against cervical cancer–related HPV types would prevent an even higher proportion of cervical precancer and may permit a safe reduction of screening intensity in more developed countries, reducing the HPV disease burden for future generations.

Information about the HPV life cycle, viral infection, and immune clearance have provided valuable insight emphasizing the biological and behavioral risk factors linked to cancer. However, there remains a distinct lack of awareness among those populations at greatest risk in both more and less developed countries. Because high-risk individuals belong to diverse ethnic groups and socioeconomic statuses, no single educational or interventional approach would benefit all of them. Thus, future initiatives for the prevention of cancer must aim to decrease existing inequalities, with a strong emphasis on providing targeted education about HPV transmission and screening for all ages of women, including adolescent girls, particularly in those groups in less developed countries where incidence and death rates are disproportionately high. Innovations in prevention must continue alongside development of better treatment options for populations that will not directly benefit from vaccine-associated therapies, including women who are currently infected with HPV, individuals who are immunocompromised, such as those co-infected with HPV and HIV, or organ transplant recipients. The treatment of cancer and patient prognosis depends on the ability to accurately diagnose and assign a clinical stage. Diagnostic imaging, surgery, radiation therapy, and chemotherapy are improving, allowing women to have more options and to make better-informed decisions. Molecular therapies are anticipated to reduce cancer cases as research on HPV early proteins reveal the viral mechanisms used to take control over cellular processes. Of these viral components, the E6 and E7 oncoproteins have long been recognized as the main mediators of the HPV-associated transformation to malignant neoplasms. Therefore, approaches targeting these two oncoproteins are likely to be anti-oncogenic.

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Mechanisms of Immune Evasion by Ebola Virus

2

Suchita Bhattacharyya

1 Introduction

Ebola virus, Marburg virus and Cueva virus constitute the Filoviridae family (Kuhn et al. 2013). Ebola virus causes hemorrhagic fever, with very high mortality rates (Feldmann et al. 2003). While Marburg and Cueva viruses have no known subtypes, Ebola virus has six species; Zaire, Sudan, Reston, Bundibugyo, Tai Forrest and Bombali. Out of the six species of Ebola virus, Zaire is the most virulent (Feldmann et al. 1994), while Reston is non-pathogenic in humans (Miranda and Miranda 2011).

Ebola is a zoonotic pathogen and its natural reservoir(s) remains a research hotspot. Fruit bats were earlier thought to act as a reservoir for Ebola virus (Leroy et al. 2005) and recent evidence has confirmed that bats are a natural reservoir for this virus (Goldstein et al. 2018). Devising strategies to eliminate these reservoirs would help to prevent viral transmission to humans.

Filoviruses are transmitted through contact with the blood or body fluids of infected patients or animals (Dowell et al. 1999). The first filovirus emerged in 1967 in Europe (Kissling et al. 1968) and the virus was designated Marburg after the city in Germany where it was first characterized. The first Ebola virus outbreak occurred in Africa in 1976, first in Sudan with a fatality rate of 53% and then in Zaire with a fatality rate of 88%. Subsequent outbreaks of Ebola virus occurred in Cynomolgus monkeys in 1989 in Reston where no human infection was reported and in 1994 in Ivory Coast where one patient was infected who subsequently recovered from the infection (Feldmann et al. 2003). Re-emergence of Ebola virus Zaire subtype in 1995 in Kitwit, the Democratic Republic of the Congo with a fatality rate of 92% drew the world's attention to this deadly pathogen. The 2013–2016 outbreak of Ebola virus in West Africa was the largest reported till date (World Health Organization. 2016). Meanwhile, the World Health Organization (WHO) had declared the recent Ebola outbreak in the Democratic Republic of the Congo as a "public health emergency of international concern" in order to draw worldwide attention to the disease and improve the health systems (World Health Organization. 2019. https://www.who.int/news/item/17-07-2019-ebola-outbreak-in-the-democratic-epublic-of-the-congo-declared-a-public-health-emergency-of-international-concern).

The lethal nature of Ebola virus infection makes it necessary to study the virus in a Biosafety Level 4 (BSL4) containment facility. Moreover, filoviruses have been classified as 'List A agents' and are considered as potential

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weapons for bioterrorism (Borio et al. 2002). Given the severity of the recent outbreaks, several researchers worldwide are actively investigating potential vaccine and therapeutic candidates against Ebola virus.

This chapter discusses the morphology, entry, replication, assembly, budding and pathogenesis of Ebola virus. The various mechanisms by which Ebola virus evades the host immune system and the specific viral proteins involved in these processes are also discussed. Further research focussing on targeting these mechanisms is needed to develop additional interventions against this deadly virus.

2 Morphology of Ebola Virus

Ebola virus is enveloped, non-segmented and filamentous in appearance. It is pleomorphic and can be either six-shaped, U-shaped or circular. Its length is highly variable and can extend up to 14 μm. The typical length of Ebola virus associated with maximum infectivity is 970 nm (Regnery et al. 1980). Ebola virus has a uniform diameter of 80 nm and density of 1.14 g/mL. Virions are composed of a helical nucleocapsid 50 nm in diameter, a closely apposed envelope derived from host cell plasma membrane and 5–10 nm long surface projection spikes composed of homotrimers of viral glycoprotein (GP) (Geisbert and Jahrling 1995). Virions contain a single molecule of RNA, which constitutes 1.1% of the virion mass (Regnery et al. 1980).

Virus infectivity is stable at room temperature (20 °C), but is completely lost within 1 h at 60 °C (Mitchell and McCormick 1984). Infectivity is also destroyed by ultraviolet and γ irradiation (Elliott et al. 1982), β-propiolactone, lipid solvents, commercial hypochlorite and phenolic disinfectants.

3 Endocytic Pathways Involved in Ebola Virus Entry

Ebola virus is known to enter host cells via pH-dependent endocytosis (Chan et al. 2000; Chazal et al. 2001). Treatment with microtubule disrupting agents impaired Ebola virus entry, while microtubule stabilizing agents enhanced entry. Also, disrupting the integrity of the actin cytoskeleton inhibited Ebola virus entry (Yonezawa et al. 2005). The endocytic pathways involved in Ebola virus entry have been extensively studied. Both clathrin-mediated endocytosis and macropinocytosis are known to be involved in Ebola virus entry (Bhattacharyya 2007; Bhattacharyya et al. 2010, 2011, 2012; Bhattacharyya and Hope 2013). The role of caveolae in Ebola virus entry has been supported (Empig and Goldsmith 2002) and refuted (Simmons et al. 2003) in different studies. Ebola virus was also suggested to simultaneously use multiple endocytic pathways for entry (Aleksandrowicz et al. 2011).

4 Replication, Assembly and Budding of Ebola Virus

Viral entry is followed by release of the genome and associated proteins into the cytoplasm. Transcription and genome replication takes place in the cytoplasm of host cells. Viral replication leads to the synthesis of seven viral proteins. Additionally, a non-structural, soluble glycoprotein (sGP) is expressed as a primary product of the glycoprotein (GP) gene in Ebola virus and RNA editing leads to the expression of membrane bound GP (Sanchez et al. 1996). Four proteins constitute the ribonucleoprotein complex: NP, VP35, VP30 and L, which are necessary for transcription and replication of the virus. The ribonucleoprotein complex generates positive-sense anti-genome, which serves as a template for transcription of messenger RNA (mRNA) encoding the viral proteins.

Electron microscopy studies have demonstrated that early filoviral infection is marked by the presence of viral precursor material in the host cell cytoplasm. The infection progresses with the formation of inclusion bodies, which increase in size and eventually occupy the entire host cell cytoplasm. The Ebola virus inclusion bodies contain preformed nucleocapsids that

mature and migrate to the plasma membrane and then exit the inclusion bodies. New virions are formed when the nucleocapsids associate with viral matrix proteins and GP molecules implanted in the cell membrane (Geisbert and Jahrling 1995).

Filoviruses are released by tearing away from the plasma membrane as opposed to gradual expulsion. Protracted formation of inclusion bodies leads to disruption of cellular organelles. The infected cells clear the degenerated cellular components by autophagosomal activity. The budding of nascent virions gradually declines and finally both the plasma and nuclear membranes rupture causing host cell death by necrosis (Geisbert and Jahrling 1995).

5 Pathogenesis of Ebola Virus

Ebola virus can infect several cell types across different species, with the exception of T and B lymphocytes (Wool-Lewis and Bates 1998). Numerous studies have examined the pathogenesis of Ebola virus infection using non-human primates as animal models because the symptoms of disease progression are similar to humans (Ryabchikova et al. 1999). Guinea pigs were not considered to be a suitable model for studying viral pathogenesis since they do not develop hemorrhagic syndrome (Ryabchikova et al. 1996).

Ebola virus causes an acute and often fatal infection, which usually lasts for 1–2 weeks after the onset of initial symptoms (Sanchez et al. 2004). Early infection is characterized by nonspecific flu-like symptoms such as fever, myalgia, diarrhea and fatigue. Often, patients develop maculopapular rash around day 5, which is an important diagnostic feature of the disease (Gear 1989). As the infection progresses, patients develop abnormalities in fluid distribution, hypotension and massive viremia that leads to disseminated intravascular coagulation (DIC) and hemorrhage. Serum enzyme levels, especially liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elevated, indicative of liver damage. The liver damage, hemorrhage and vascular permeability are caused by the release of tissue factor, mediators and overt cytokine production (Feldmann and Geisbert 2010). Fall in blood pressure ultimately leads to onset of severe systemic shock (Colebunders and Borchert 2000).

Monocytes and macrophages are the first cells to be infected by Ebola virus. Secondary target cells include hepatocytes and endothelial cells (Ryabchikova et al. 1999; Zaki and Goldsmith 1999). Infected monocytes and macrophages release cytokines and chemokines such as tumor-necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). TNF-α increases endothelial cell permeability (Feldmann et al. 1996), and decreases anticoagulant activity (Schleef et al. 1988), which could lead to DIC and systemic shock. TNF-α, Fas, Fas ligand, TNF-α related apoptosis inducing ligand (TRAIL) and nitric oxide released by infected macrophages are suggested to cause lymphocyte apoptosis (Hensley et al. 2002; Baize et al. 1999). Macrophages synthesize cell-surface tissue factor, which interacts with circulating factors VIIa and X, causing deposition of fibrin on the surface of infected cells, which also initiates DIC. Infected macrophages and dendritic cells (DCs) help the virus to spread throughout the body resulting in multifocal tissue necrosis. Additionally, infected DCs lack the ability to express co-stimulatory molecules, upregulate major histocompatibility complex (MHC) molecules or induce lymphocyte differentiation, thereby preventing an early immune response to the virus (Bosio et al. 2003).

The differential expression of cytokines has been linked to the viral disease outcome. The presence of interleukin-1β (IL-1β) and elevated levels of IL-6 following infection are associated with survival, while release of IL-10 and high levels of neopterin and IL-1 receptor A in the symptomatic phase are thought to be markers of fatal outcome. Survival has also been correlated with the development of antigen-specific immune response and appearance of specific antibodies against viral antigens (Hensley et al. 2002).

6 Genome Organization of Ebola Virus

Ebola virus contains a single molecule of non-infectious, linear, negative-sense, single-stranded RNA (Regnery et al. 1980; Kiley et al. 1982). The 19 kb Ebola genome is the largest known genome among all the negative-sense RNA viruses (Feldmann et al. 2003). Ebola virus genes have long non-coding regions at their 3′ and 5′ ends, which contribute to the increased length of their genome. The 3′ and 5′ ends of the transcripts are suggested to form stable hairpin structures that could enhance stability of the transcripts (Sanchez et al. 1993; Kiley et al. 1986).

7 Ebola Virus Proteins

Ebola virus is composed of six structural and one non-structural protein. The VP40, VP24, VP30 and VP35 proteins are named according to their molecular weights.

The envelope glycoprotein (GP) forms spikes on the virion surface and mediates viral entry into target cells. Ebola virus GP gene encodes different proteins by transcriptional editing. The edited transcript encodes the membrane bound GP. Ebola virus GP is known to cause cell rounding and cytotoxicity (Yang et al. 2000; Simmons et al. 2002; Sullivan et al. 2005). Full-length Ebola GP was found to accumulate in the endoplasmic reticulum in very close proximity to the nuclear membrane, which may be responsible for its cytotoxic effect (Bhattacharyya and Hope 2011).

The most abundant protein VP40 is also the major matrix protein of Ebola virus. VP40 is known to possess several key functions in the viral life cycle. Expression of VP40 alone in mammalian cells leads to the production of virus-like particles (VLPs), which exhibit the typical filamentous morphology of wild-type filoviruses (Noda et al. 2002), suggesting that VP40 possesses all the necessary attributes for particle assembly and budding. However, VP40 containing VLPs show a variable diameter of 50–70 nm as compared to the diameter of 80 nm for Ebola virus, and this shorter diameter is suggested to be due to lack of ribonucleoprotein complex (Bavari et al. 2002). Ebola VP40 was shown to oligomerize and bind to RNA, thereby suggesting that VP40 was essential for viral replication (Hoenen et al. 2005). VP40 recruits some cellular proteins such as the mammalian tumor susceptibility gene 101 protein (Tsg101), which is required for endosomal protein sorting and transport (Babst et al. 2000) and the neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4), which is a E3 ubiquitin ligase, to sites of virion budding at the plasma membrane, and this process is believed to facilitate viral egress (Martin-Serrano et al. 2001; Yasuda et al. 2003).

The membrane associated protein VP24 is suggested to be a minor matrix protein (Elliott et al. 1985). VP24 was detected in VLPs indicating that it may be involved in virus budding. VP24 associates with lipid bilayers and can oligomerize under physiological conditions, thereby suggesting a role in virus assembly (Han et al. 2003). Ebola VP24 was found to be essential for the assembly of functional nucleocapsids (Hoenen et al. 2006).

Ebola VP30 protein is phosphorylated and is closely associated with the virion RNA. It is suggested to play a role in transcription initiation of Ebola virus (Elliott et al. 1985). VP30 is also involved in virus assembly.

VP35 is a cofactor for polymerase L (Muhlberger et al. 1999). VP35, NP, VP30 and L constitute the active RNA-dependent RNA polymerase replication complex. VP35 is synthesized prior to VP40 (Elliott et al. 1985), and is thus believed to play a role in early events of viral replication. Along with NP and VP24, VP35 is involved in nucleocapsid formation.

The nucleoprotein (NP) self-assembles into large intracytoplasmic aggregates that contain nucleocapsid-like structures and are closely associated with the rough endoplasmic reticulum. Hence, NP is the principal determinant of the nucleocapsid structure. NP forms complexes with VP30 and VP35, and recruits them to the inclusion bodies. Ebola NP is phosphorylated (Elliott et al. 1985), and its size ranges from 96 to 104 kD. Interaction between Ebola VP30 and NP

is suggested to regulate viral RNA synthesis (Kirchdoerfer et al. 2016).

The non-structural protein RNA polymerase L is the largest (267 kD) and least abundant viral protein in the virion as well as infected cells. L is involved in transcription and regulation of viral genome. It is also known to play a role in mRNA editing. VP35, VP30 and L are essential for viral RNA synthesis.

Ebola virus produces another non-structural protein, which is the soluble glycoprotein (sGP). The 50–70 kD sGP is abundantly produced during the early stage of infection and secreted into the extracellular space.

8 Host Immune Evasion by Ebola Virus

Several proteins of the Ebola virus are known to participate in host immune evasion strategies to facilitate viral replication and pathogenesis. Ebola virus interferes with the cellular antiviral response by targeting the interferon pathway, thereby causing a decrease in secretion of IFN-α (Harcourt et al. 1999). Enveloped viruses that display phosphatidylserine on their membranes can activate Tyro3-Axl-Mer (TAM) receptors on DCs to inhibit type I interferon signaling and thereby evade the host innate immune response (Bhattacharyya et al. 2013; Bhattacharyya 2013). Phosphatidylserine expressed on the envelope of Ebola virus was shown to be responsible for entry (Moller-Tank et al. 2013; Yuan et al. 2015). Also, Axl was shown to enhance macropinocytic uptake of Ebola virus (Hunt et al. 2011). Thus, Ebola virus may also activate the TAM receptors on host cells to inhibit the innate immune response.

Ebola VP35 is known to suppress type I interferon production. VP35 can bind to dsRNA (Kimberlin et al. 2010), which suppresses the host innate immune response and promotes virulence. Moreover, VP35 competitively blocks PACT interaction with retinoic acid-inducible gene I (RIG-I), which impairs RIG-I activation to suppress the type I interferon response (Luthra et al. 2013). Furthermore, VP35 can block the interactions of inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKe) and TANK-binding kinase 1 (TBK-1) with interferon regulatory transcription factor (IRF)-3 and IRF-7 (Prins et al. 2009), which inhibits their activation. VP35 can also block the antiviral activity of protein kinase R (PKR) (Schümann et al. 2009). VP35 impairs maturation of DCs, which hinders the production of type I interferons and pro-inflammatory cytokines, resulting in failure of activation of naive T cells. Additionally, VP35 also blocks melanoma differentiation-associated protein 5 (MDA5)-mediated induction of type I interferon responses (Yen et al. 2014). Animal model studies have shown that mutant VP35 viruses had lower replication rates and were non-lethal at tested doses suggesting that VP35 was involved in Ebola pathogenesis (Hartman et al. 2008).

Ebola VP24 can bind the phosphorylated signal transducer and activator of transcription 1 (STAT1) binding site on some karyopherin alpha (KPNA) proteins, thereby functioning as a competitive inhibitor of phosphorylated STAT1 (Mateo et al. 2010). Hence, VP24 prevents nuclear trafficking of STAT1 to block interferon signaling (Reid et al. 2006). VP24 can also bind STAT1 directly, which is another innate immune suppression mechanism of this viral protein (Zhang et al. 2012). Moreover, VP24 was found to inhibit type III interferon-λ1 gene expression, and thereby hamper activation of the RIG-I-mediated antiviral pathway (He et al. 2017).

The cellular protein tetherin (also known as BST-2 and CD317) can prevent budding and release of enveloped viruses by tethering them to the cell surface. Tetherin is also responsible for activating the NF-kB pathway. Ebola GP is suggested to block tetherin function by affecting its stability. Ebola GP can also block VP40 interaction with tetherin and thus promote viral egress (Gustin et al. 2015). Moreover, Ebola GP was found to alter antiviral response of NK cells (Edri et al. 2018). Additionally, Ebola GP caused cytotoxicity of human endothelial cells and increased vascular permeability (Yang et al. 2000).

Large amount of sGP is found in the blood of Ebola virus infected patients. sGP is suggested to interfere with the host immune response by inhibiting the production of pro-inflammatory cytokines such as TNF-α and IL-6 by macrophages, and also preventing macrophage migration (Bradley et al. 2018). sGP may also prevent effective neutralization of Ebola virus during infection by a process termed as 'antigenic subversion'; which has negative implications for vaccine design (Mohan et al. 2012).

Ebola VP40, GP and NP are packaged into host cell exosomes. These exosomes contain pro-inflammatory cytokines and were suspected to increase apoptosis of bystander T-cells, thereby hindering adaptive immune response (Pleet et al. 2016; Pleet et al. 2019). Additionally, VP35, VP30 and VP40 act as suppressors of cellular RNA silencing system against Ebola virus infection (Fabozzi et al. 2011).

9 Conclusions

Several mechanisms by which Ebola virus evades the host immune system have been described. These diverse mechanisms facilitate viral replication, pathogenesis and virulence. Hence, devising effective strategies to block these immune evasion mechanisms of Ebola virus will help to prevent the spread of infection. The global public health and economic burden of Ebola virus disease is enormous, and extensive improvements are urgently needed in the public health systems of the affected countries. Future studies should focus on the development of additional novel drug and vaccine candidates to prevent Ebola virus disease as well as eliminating reservoirs of this lethal virus.

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Innate Immune Response Against HIV-1

3

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1 Introduction

The human immunodeficiency virus-1 (HIV-1) is a lentivirus. The HIV-1 infection is characterised by the depletion of $CD4^+$ helper T lymphocytes, leading to loss of cell-mediated immunity (Okoye et al. 2013). Activated $CD4^+$ T lymphocytes are the principal target of HIV-1 infection via interactions with several cell surface receptors, including CD4 and chemokine co-receptors to trigger viral fusion and facilitate viral entry into host cells (Okoye et al. 2013). The entry of HIV-1 is mediated by HIV-1 envelope (env) protein, which is comprised of heterodimeric gp120 and gp41 glycoproteins, organised as trimeric spikes (Checkley et al. 2011). Binding of CD4 to viral gp120 causes repositioning of variable loops, including V1-V3 and, thereby, exposing co-receptor binding sites (Trkola et al. 1996; Wu et al. 1996). The α-chemokine receptor, CXCR4, and β-chemokine receptor CCR5, are the two key secondary cellular receptors identified for T cell line-tropic and macrophage-tropic HIV-1 isolates; they belong to the members of the G protein-coupled receptor superfamily (GPCRs) (Allen et al. 2007). Upon co-receptor binding, conformational changes within trimeric gp120/gp41 complex leads to an insertion of gp41-mediated fusion peptide into the cell membrane (Kwong et al. 2000). As a result, uncoating steps occur through disassembling the viral capsid core, leading to entry of viral RNA into the cytosol, and then synthesis of the complementary DNA (Fig. 3.1).

The innate immune sytem of the female reproductive tract (FRT) offers resistance to HIV-1 transmission via both innate immune cells and non-cellular immune mediators. The innate immune recognition and activation upon HIV-1 (Shen et al. 2014) infection plays a critical role in the control of viral infection. The entry of HIV-1 in the FRT occurs across gential mucosal epithelium (Shen et al. 2014), and thereby, HIV-1 may translocate in the FRT via vagina, ectocervix, endocervix, uterus, and area with distinctive genital epithelial architecture. The innate effector mechanisms that contribute to the control of

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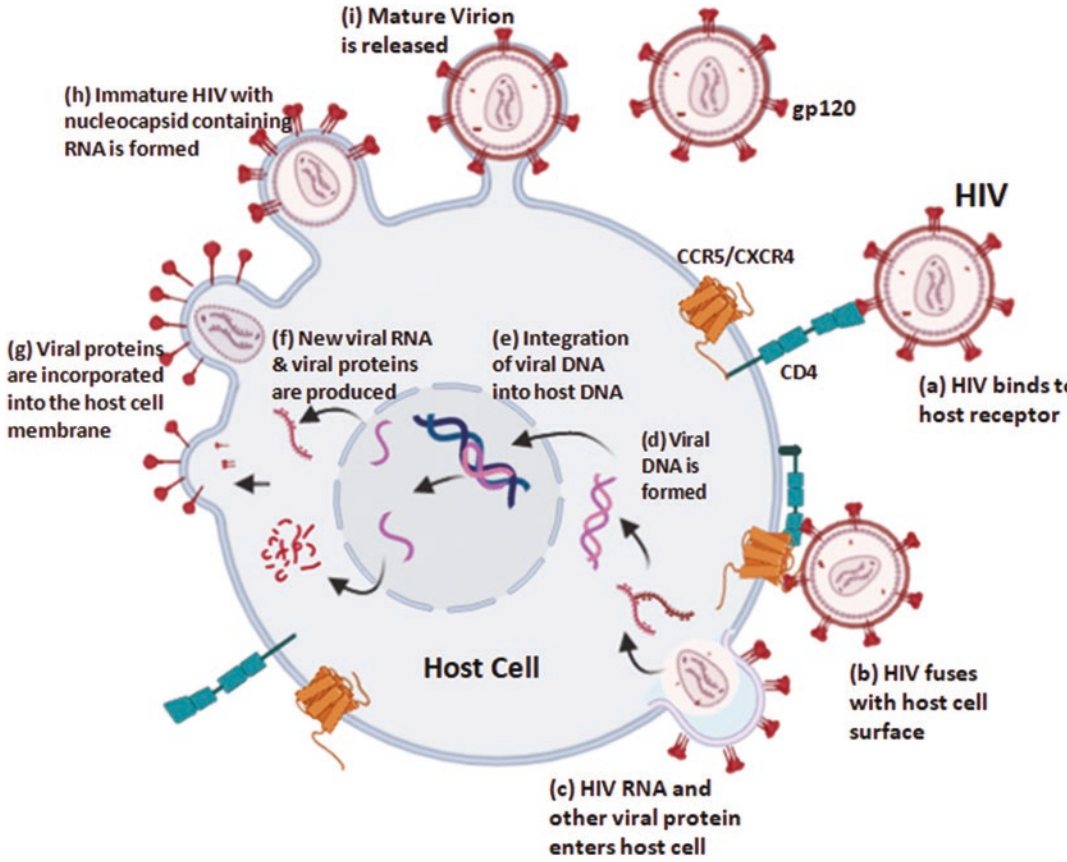

Fig. 3.1 A schematic representation of HIV-1 entry and capture. (**a**) HIV-1 entry is mediated by attachment of HIV-1 Env protein, composed of gp120 and gp41 structured as trimeric spikes on the viral surface. The viral glycoprotein gp120 binds to CD4 receptor, followed by repositioning of the variable loops V1, V2, and V3, which form a site for co-receptor binding, either with CCR5 or CXCR4. (**b**) This induces a subtle conformational changes in gp41 and leads to the insertion of gp41-mediated hydrophobic fusion peptide into the target cell membrane. HIV-1 virions and cell membrane become in close apposition to trigger viral fusion, (**c**) This facilitates the entry of viral nucleocapsid containing the RNA and other viral proteins into the target cell, (**d**) Nucleocapsid is degraded and the viral RNA is released into the cytosol, which further gets converted into double stranded DNA with the help of viral reverse transcriptase enzyme, (**e**) Viral DNA gets incorporated into the host DNA, (**f**) Upon incorporation it starts transcribing viral RNAs that is incorporated into the nucleocapsid for the formation of new virions and viral mRNAs get translated into necessary viral proteins, (**g**) Viral proteins are assembled to form capsid, spike and other necessary proteins required to produce a complete virion, (**h**) Immature HIV is formed with nucleocapsid containing viral RNA and outer capsid with envelope protein gp120 being incorporated into the host membrane, i) Complete mature virion buds off from the host membrane ready to infect other cells

HIV-1 infection include innate barriers such as epithium, mucus, pH, the complement system, and cells of the innate immune system. However, the virus can overcome these barriers to facilitate its entry and replication in target cells. The mucus in the endocervical epithelium is comprised of mucins that forms a physical barrier to pathogens and shields FRT tissues from ascending infections (Gipson et al. 1997; Vigil et al. 2009). The mucin is also produced by the stratified epithelium of the vagina and ectocervix, which may function as a barrier to provide protection for the upper tract against invading pathogens. The aqueous part of the mucin is rich in immunoglobulins as well as antimicrobial peptides (AMPs) (Ming et al. 2007). In addition, pH has also been established as a key innate component of the mucus that affects transmission of microorganisms. The

local presence of bacterium keeps the pH in the acidic form through lactic acid and hydrogen peroxide production, which provides anti-microbial environment (Martin et al. 2008; Ravel et al. 2011). Thus, an interplay of epithelial cells of FRT, mucus and lactic acid, together with complement proteins, forms a primary innate barrier that prevent progression of HIV-1 infection.

2 Mucosal Immunity and HIV-1

Mucosal immunity plays a central role in the pathophysiology of HIV-1 infection as well as sexual transmission. The most common route for HIV-1 infection is through sexual transmission across genital mucosa; over 90% of HIV-1 transmissions occur across the mucosal barrier. HIV-1 infection by a single virus variant/strain termed as transmitted/founder (T/F) virus have been found to be capable of establishing host infection upon mucosal exposure (Derdeyn et al. 2004; Keele et al. 2008; Pang et al. 1992; Wolinsky et al. 1992; Zhang et al. 1993; Zhu et al. 1993). By replicating within the exposed mucosal tissue for about a week or 10 days, they migrate to the draining lymph node and then into the blood stream. Once infection spreads and establishes at multiple sites, viral eradication becomes quite challenging. Thus, early events within the genital mucosa represent a vulnerable stage in the process of HIV transmission (Fig. 3.2).

2.1 Dual Roles of Dendritic Cells and Langerhans' Cells: DC-SIGN and Langerin

The HIV-1 transmission across mucosal barrier involves the interaction of the virus with dendritic cells (DCs) and/or $CD4^+$ $CCR5^+$ T cells. DCs have been established to be among the key cells that perform a pivotal role in encountering HIV-1 at the mucosa and regulating both innate and adaptive immune responses during HIV-1 infection. FRT mucosa consists of myeloid DCs, plasmacytoid DCs, and Langerhans' cells, which have distinct roles in encountering HIV-1 virions. In vitro studies have revealed the efficient capture and transfer of HIV-1 to T cells by DCs (Dopper et al. 2003; Yu et al. 2008). Thus, in vivo, HIV-1 utilizes mucosal DCs and causes HIV-1 transfer to T cells in lymph nodes. This early innate response favours DC maturation and its migration to secondary lymphoid tissues by creating an inflammatory microenvironment. During this process, DCs express co-stimulatory molecules on its surface required for efficient T cell priming.

HIV-1 is transmited to T cells by sub-epithelial DCs. In mucosal tissues, DC subsets can be distinguished by their expression of C-type lectins. DCs express dendritic cell-specific intercellular adhesion molecule-3-Grabbing non-integrin (DC-SIGN), also called CD209 (Geijtenbeek et al. 2000); Langerhans cells (LCs) specifically express Langerin (de Witte et al. 2007a, b). DC-SIGN is a type II C-type lectin receptor expressed on the surfaces of both DCs and macrophages; it binds gp120 (Curtis et al. 1992) and enhances HIV-1 infection of T cells (Geijtenbeek et al. 2000). The expression of DC-SIGN on DC cells highlights contrasting roles of DCs which includes immunostimulatory events that mediate the binding and transfer of HIV-1 to T cells. Several studies have revealed the role of DC-SIGN in the pathogenesis of HIV-1. DC-SIGN induction on activated B-lymphocytes can lead to an enhancement of HIV-1 transmission to $CD4^+$ T cells (Rappocciolo et al. 2006). The binding of gp120 to DC-SIGN also inhibits the anti-apoptotic activity of Nef and apoptosis induction in immature DCs (iDCs) (Sarkar et al. 2013).

HIV-1-DC-SIGN binding on DCs can activate and initiate signalling cascade that can induce HIV-1 replication and entry in DCs (Gringhuis et al. 2010). Blockade of DC-SIGN in iDCs (Wang et al. 2007) and in activated B lymphocytes (Rappocciolo et al. 2006) can lead to significant reduction of HIV-1 transfer to $CD4^+$ T cells. Over-expression of DC-SIGN correlates with an enhanced transmission of HIV-1 to $CD4^+$ T cells (Wu et al. 2004). However, low levels of DC-SIGN is expressed by LPS-matured DCs when compared to iDCs (Geijtenbeek et al. 2000a);

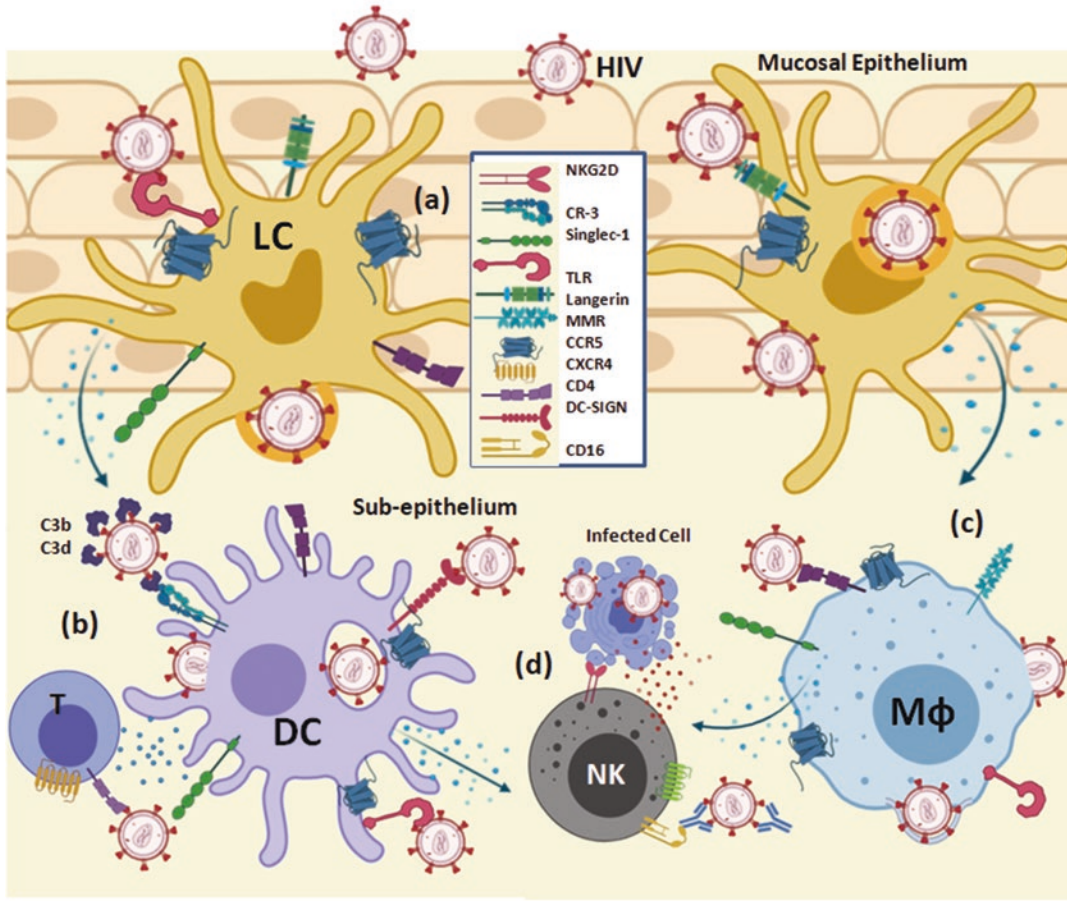

Fig. 3.2 Acquisition of HIV infection through vagina. (**a**) Cell free HIV-1 virions penetrate through the vaginal epithelium composed of multi-layered stratified squamous epithelial cells. There they get access to $CD4^+/CCR5^+$ Langerhans cells. TLR, Langerin, Singlec-1 cell surface receptors also facilitate HIV binding with Langerhan cells. Viruses get internalized by LCs, proliferate and then bud off as new virions. HIV through Langerhans cells then passes on to the mucosal subepithelium to infect other cells (dendritic cells, macrophages (Mϕs), $CD4^+$ T cells, NK cells). After exposure to HIV-1, cells start secreting several cytokines and chemokines that initiate an inflammatory response. (**b**) HIV-1-DC-SIGN binding on DCs activates and initiates signalling cascade that induces HIV-1 replication and entry in DCs. In mDCs, viral capture is enhanced and HIV-1 virions are stored in nonclassical endosomes enriched in tetraspanins or at invaginations of plasma membrane which protects them from endosomal or cytosolic degradation. C3 fragments, C3b/C3d attached to gp120, can bind to C3 receptor bearing cells such as DCs and Mϕs thus increasing the infectivity. TLRs and Singlec-1 present on DC membrane are also capable of binding to gp120 of HIV and can also present HIV to $CD4^+$ T cells. HIV can also directly infect $CD4^+$ T cells, (**c**) Macrophages expressing mannose-binding C-type lectins become infected with HIV. HIV infection also induces immunological dysfunction in mononuclear phagocytic cells, which also act as reservoirs of actively replicating virus, (**d**) Antibody recognizing HIV gp120 binds to NK cell via CD16 (FcγRIII), stimulating degranulation, mounting cytotoxic response towards infected cells. Stress related ligands such as ULBP-1, 2 and 3 are expressed on HIV-infected cells to which NKG2D receptor of NK cell binds for cell mediated killing. All the above mentioned events further cause the HIV-1 virions to penetrate into nearby lymphoid tissues for further infection

DC-SIGN blockade on LPS-matured DCs does not show any significant effect on HIV-1 transmission to $CD4^+$ T cells (Wang et al. 2007), since uptake of HIV-1 by mature DCs does not occur (Izquierdo-Useros et al. 2007). Complement-opsonised HIV-1 was shown to increase DC infection compared to non-opsonised virons, which further served as an endogenous adjuvant for DC-induced virus specific cytotoxic T lymphocytes (CTLs) (Dopper et al. 2003). Therefore,

DCs function in combination with complement-coated HIV-1 in mediating adaptive T cell responses. Yu et al. have proposed that the interaction between DCs and T cells promotes HIV-1 transmission by locally active receptors and chemokine-co-receptors during infectious synapse (Yu et al. 2008). Thus, dermal DCs do not only transfer HIV-1 from mucosal sites to the lymphatic tissue but also effectively shuttle HIV-1 to $CD4^+$ T cells.

Despite expressing HIV-1 receptor CD4 and coreceptors on their surface, the number of HIV-1 infected DCs are always low compared to activated $CD4^+$ T cells or macrophages (Granelli-Piperno et al. 1998; 1999; Cameron et al. 1992; Pope et al. 1995). The limited HIV-1 infection of DCs is possibly due to the host restriction factor SAMHD1 (sterile alpha motif domain–and HD domain–containing protein) which restricts infection by reducing the nucleotide pool available for reverse transcription (Lahouassa et al. 2012). In mature DCs (mDCs), viral capture is enhanced and HIV-1 virions are stored in non-classical endosomes enriched in tetraspanins (Garcia et al. 2005), or at invaginations of plasma membrane (Yu et al. 2008) which protects them from endosomal or cytosolic degradation (Kwon et al. 2002). HIV-1-infected macrophages also have budding compartments as invaginations of plasma membrane facilitating rapid transfer of HIV-1 through T cell contact zone (Welsch et al. 2007; Deneka et al. 2007).

In lymphoid tissues, compared to iDC, mDCs can effectively transfer HIV-1 to T cells through virological synapse which is also a major mode of transmission in the densely populated lymphoid tissue (Fig. 3.3). It has been found that sialyllactose-containing gangliosides in the viral membrane and the cellular lectin Siglec-1 are important for HIV-1 capture and storage by mDCs (Izquierdo-Useros et al. 2014). The HIV entry portals- CD4, CCR5, and CXCR4, on $CD4^+$ T cells are concentrated in the contact zone once interacting with mDCs, providing a suitable condition for viral entry and rapid infection (McDonald et al. 2003). The transmission of HIV-1 through synapse is potentially 10–1000 fold more efficient. Virological synapses contain interdigitated membrane surfaces, where the T cell extends its filopodia to make contact with the virions present inside the membrane invaginations on the DC membrane (Fig. 3.3) (Jolly et al. 2004; Martin et al. 2010; Sattentau 2008; Martin and Sattentau 2009; Sourisseau et al. 2007; Dimitrov et al. 1993; McDonald et al. 2003). These synapses are cytoskeleton-dependent and also act as a means to hide from immune response, thus, providing a potential mechanism to shield the virus, at least partially, from the immune system (Jolly and Sattentau 2004; Thao et al. 2014). Suppression of DC-SIGN can result in an irregular formation of the infectious synapse between DCs and T cells, leading to inhibiton of X4 HIV-1 trasmission to T cells (Arrighi et al. 2004a, b).

Binding of gp120 to DC-SIGN triggers Raf-1 via phosphorylation of p65 subunit of nuclear factor kappa B (NF-κB), causing elongated transcripts of HIV-1. This further results in HIV-1 transfer to $CD4^+$ T cells (Gringhuis and Geijtenbeek 2010). HIV-1 endocytosis by DCs and trafficking target HIV-1 for lysomal degradation via endosomal pathway (Yu et al. 2008). Despite the role of DCs during HIV-1 transfer, DCs are also shown to produce high levels of IL-12 other cytokines, aiding in the first line of host defense against invading pathogens. Thus, they activate natural killer (NK) cells through secretion of IL-12, IL-15, IL-18 and other factors (Fig. 3.2).

Mucosal substes of DCs have distinct roles during HIV-1 transmission. Epithelial Langerhans cells (LCs) and DC-$SIGN^+$-DCs are found in the genital tissues. LCs are the first type of DC subset, which are known to play a vital innate immune role in encounting HIV-1 (Cameron et al. 1996; Knight and Patterson 1997; Miller and Hu 1999). LCs reside in the epidermis of the skin and also in most of the mucosal epithelia, such as the ectocervix, vagina and foreskin; due to their location, they are among the first immune cells that encounter HIV-1 in genital tissue (Patterson et al. 2002; de Witte et al. 2007a, b). The maturation of LCs is represented by an enhancement of CCR7 and co-stimualtory molecules, such as CD80/CD86/CD40/CD83, and by down-regulation of

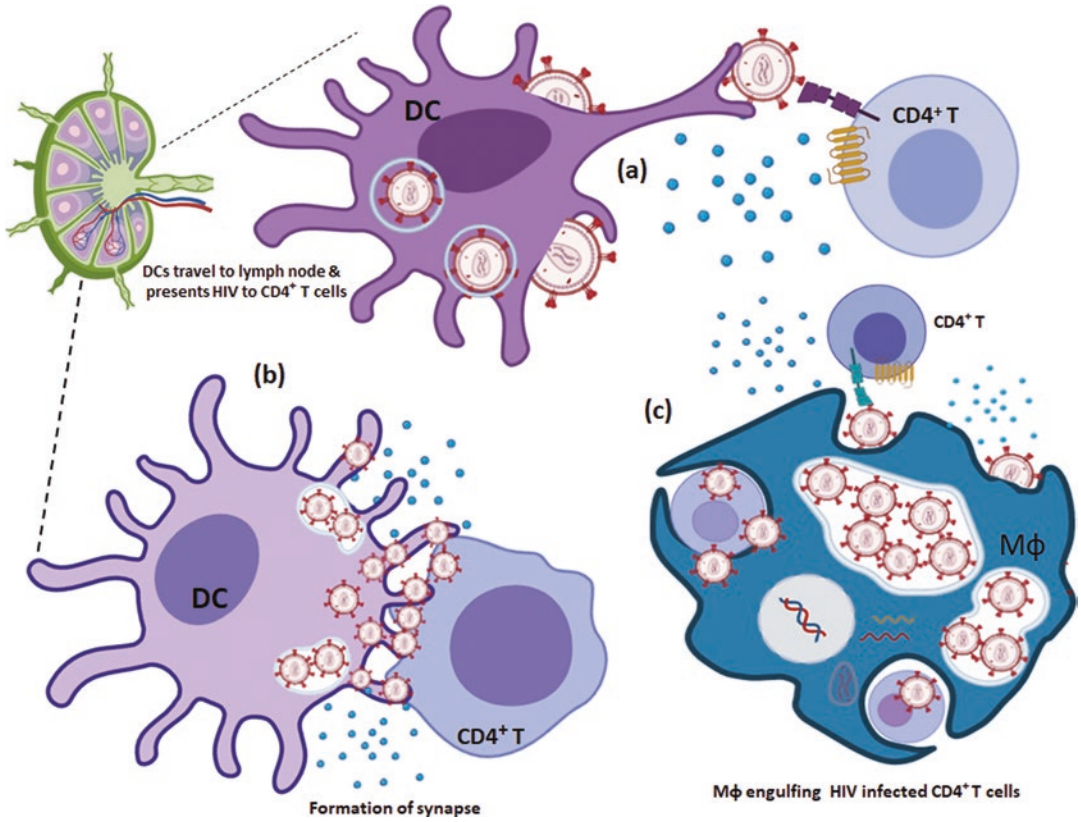

Fig. 3.3 T cell priming by DC and macrophages inside the lymph node. The early innate immune response inside the mucosa favours DC maturation and its migration to secondary lymphoid tissues by creating an inflammatory microenvironment; (**a**) During this process, DCs express co-stimulatory molecules on its surface required for efficient T cell priming. (**b**) In lymphoid tissues, mDCs can effectively transfer HIV-1 to T cells through virological synapse which is also a major mode of transmission. Cellular lectin Siglec-1 is important for HIV-1 capture and storage by mDCs. The HIV entry receptors CD4, CCR5, and CXCR4 on $CD4^+$ T cells get accumulated in the contact zone once in contact with mDCs. T cell extends its filopodia to make contact with the virions present inside the membrane invaginations on the DC membrane. Virological synapses contain interdigitated membrane surfaces facilitating HIV transmission; (**c**) HIV infection also induces immunological dysfunction in mononuclear phagocytic cells, which act as reservoirs of actively replicating viruses in its vesicular compartments. Macrophages engulf HIV-infected $CD4^+$T cells which further incorporate HIVs that proliferate and get released as new virions infecting other $CD4^+$ T cells

Langerin and E-cadherin (Merad et al. 2008). Thus, matured LCs (mLCs) induce an effective immune response by presenting the captured HIV-1 to T cells (Merad et al. 2008). LCs induce innate immune reponses (de Witte et al. 2007a, b) by expressing Toll Like receptors (TLRs), such as TLR-2,-3, and -5, and increased levels of CD1a, Langerin and intracellular Birbeck granules (BGs) (Valladeau et al. 2000; Flacher et al. 2006; Fahrbach et al. 2007; Romani et al. 2010). LCs bring about HIV-1 trasmission to T cells through Langerin at the cell surface, similar to transmission by DC-SIGN.

Cell lines expressing Langerin bind HIV-1 gp120 (Kedzierska et al. 2003). LCs expressing high levels of Langerin do not significantly transfer HIV-1, but LCs pre-incubated with 10E2 blocking antibody bring about increased HIV-1 transmission (de Witte et al. 2007a, b). However, internalization of HIV-1 through Langerin leads to inhibion of viral transmission and degradation of the virus (de Witte et al.

2007a, b). Thus, Langerin acts as a natural innate barrier against HIV-1 transmission by transporting endocytosed HIV-1 virons to Birbeck granules (BGs) for subsequent degradation (de Witte et al. 2007a, b). BGs are langerin-positive organelles exclusively present in LCs, where they originate at the cell membrane as langerin$^+$caveolin-1$^+$ caveolae, and subsequently develop into caveolin-1-positive BGs. BGs belong to caveolar endocytosis pathway and caveolin-1 mediated HIV-1 uptake is an intrinsic restriction mechanism limiting HIV-1 infection in LCs (van den Berg et al. 2014). Ribeiro et al. (2016) have shown that HIV-1 restriction in LCs is mediated through human E3-ubiquitin ligase tri-partite-containing motif 5α (TRIM5α). TRIM5α is capable of binding incoming retroviral capsid restricting retroviruses after fusion, and thus, interfers with the uncoating and reverse-transcription processes. TRIM5α promotes the assembly of an autophagy-activating scaffold to langerin, which further targets HIV-1 for autophagic degradation. Interestingly, HIV-1 binding to DC-SIGN$^+$ DCs abrogates TRIM5α restriction due to disassociation of TRIM5α from DC-SIGN (Ribeiro et al. 2016). Thus, binding of langerin with HIV-1 is possibly for the routing of HIV-1 into the human TRIM5α-mediated restriction pathway by LCs.

LCs have also been shown to inhibit T-cell infection via viral clearance by Langerin. However, anti-HIV-1 barrier of LCs are abrogated when sexually transmitted infections (STIs) are present, which promote HIV-1 transfer to CD4$^+$ T cells (Ogawa et al. 2009). In contrast, vaginal LCs do not seem to express langerin, suggesting that HIV-1 virions may bypass a langerin-mediated degradation pathway (Ballweber et al. 2011). Certain T/F viruses are also capable of infecting immature LCs, which are also found to retain these viruses. Not all T/F viruses are efficient in infecting LCs; this difference in infectivity possibly allows certain T/F viruses to escape LC restriction. Thus, HIV-1 potentially increases the range of available transmission routes (Hertoghs et al. 2019).

X4-tropic HIV-1 replicates vigorously in LCs and DCs ex vivo when compared to R5-tropic viruses (Cameron et al. 1996; Ganesh et al. 2004; Piguet and Steinman, 2007). HIV-1 virions are also known to interact with intercellular adhesion molecules (ICAMs) and their ligands which may promote DC-T cell intraction and HIV-1 transmission. Up-regulation of ICAM-1 by mDC is correlated with an enhanced mDC-mediated HIV-1 transmission (Sanders et al. 2002); inhibiting ICAM-1 on DCs causes impaired HIV-1 transmission (Sanders et al. 2002). HIV-1 incorporated ICAM-1 has been shown to enhance viral infection of cells expressing leukocyte function-associated molecule 1 (LFA-1) by promoting viral attachment and internalisation (Fortin et al. 1998; Tardif and Tremblay 2003). Therefore, ICAM-1:LFA-1 interaction is considered as a pivotal mechanism for HIV-1 infection (Sanders et al. 2002). In addition, interaction of cytoplasmic domain of ICAM-1 with immauture HIV-1 Gag (Beausejour and Tremblay 2004) results in ICAM-1 recruitment of HIV-1 particles.Virus incorporated ICAM-1 also results in low activity of neutralising antibodies against Env protein (Rizzuto and Sodroski 1997) and capability of ICAM-1 antibodies to block viral entry (Rizzuto and Sodroski 1997).

DC-SIGN interacts with ICAM-2 to mediate the transendothelial migration of DCs to the lymph nodes, or site of inflammation in peripheral tissue (Wang et al. 2003). Blocking ICAM-2 on DCs or CD4$^+$ T cells does not elicit any effect on DC-mediated HIV-1 transmission to CD4$^+$ T cells (Wang et al. 2009). In addition, DC-SIGN:ICAM-3 interaction has been shown to stabilise effective DC-T cell receptor engagement, further resulting in the internalization of soluble antigen ligands and antigentic peptide presentation to T cells (Halary et al. 2002; Yu Kimata et al. 2002). It is also known that the interaction between DC-SIGN and ICAM-2 regulates chemokine-induced DC transmigration across activated and resting endothelium. An increased replication of HIV-1 has been shown in ICAM-3 negative CD4$^+$ T cells, which may indicate that ICAM-3 plays an essential role in promoting replication of HIV-1 cis-infection (Biggins et al. 2007). Conversely, blocking ICAM-3 interaction with DC-SIGN does not

show any effect on HIV-1 cell to cell transmission (Wu et al. 2002; Wang et al. 2009).

DCs express TLRs that play a central role in innate immunity as they recognise and detect PAMPs on invading pathogens and trigger immune responses. Apart from TLRs, several other host proteins have also been identified as PRRs for HIV-1 PAMPs. Interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) are intracellular PRRs capable of recognizing viral reverse transcriptase products early in the viral replication cycle in HIV-1 infected cells (Altfeld and Gale Jr. 2015). HIV-1 infection of DCs leads to up-regulation of TLRs in DCs (Hernandez et al. 2011), thus promoting viral replication and cell-to-cell transmission (Gringhuis et al. 2010) via TLR signalling pathway. TLR2 and TLR4 are up-regulated during HIV-1 infection in DCs and monocytes from individuals co-infected with HIV-1 and *Mycobacterium tuberculosis* (Hernandez et al. 2011). The expression of TLR2/TLR4 has been positively correlated with an enhanced HIV-1 viral load in these individuals, which may indicate that increased levels of these TLRs may promote HIV-1 transmission to $CD4^+$ T cells (Hernandez et al. 2011). However, a direct correlation between HIV-1 and TLR2/TLR4 on replication of HIV-1 and DC-mediated cell to cell transmission is not been understood. Co-infection of HIV-1 with *M. tuberculosis* or *Candida albicans* stimulates levels of TLR2 homo- or heterodimers, resulting in an increased replication of HIV-1 involving kinase raf-1 through phosphorylation of the p65 subunit of NF-κB (Gringhuis and Geijtenbeek 2010). TLR2 signalling may thus endorse HIV-1 infection of DCs as well as DC-induced HIV-1 transmission. However, other studies have suggested that PAMPs recognition through TLR initiates anti-viral signalling cascades via NF-κB activation, further resulting in the downstream production of pro-inflammatory and anti-viral cytokines and chemokines (Kawai et al. 2007). Thus, activation of NF-κB serves as a vital innate factor during HIV-1 infection by facilitating replication of viruses via long terminal repeat (LTR) engagement (Barbeau et al. 1997). Soluble TLR2 (sTLR2) found in mucosal fluids has also been suggested to inhibit the production of pro-inflammatory cytokines (LeBouder et al. 2003; Kuroishi et al. 2007; Dulay et al. 2009) and to direct inhibition of cell-free HIV-1 infection in vitro (Henrick et al. 2014; Henrick et al. 2012). Furthermore, direct interaction of p17, gp41 and p24 of HIV-1 with sTLR2 leads to inhibition of viral protein induced activation of NF-κB and inflammation (Henrick et al. 2014). Therefore, HIV-1 structural proteins may act as PAMPs for cellular heterodimers of TLR2.

Treatment of DCs with fungus *Penicillium marneffei* has been shown to cause increased DC-mediated HIV-1 transmission to target cells (Qin et al. 2011). Stimulation of TLR2 dimers can activate DCs to induce DC-mediated HIV-1-transmission by recognising fungal PAMPs, either by direct signalling or up-regualtion of ICAM-1 (Qin et al. 2011). Carbohydrate-binding agents (CBAs) have been shown to prevent HIV-1 infection by targeting glycans of gp120 and inhibit DC-SIGN-mediated HIV-1 capture by DCs and transfer to $CD4^+$ T cells (Pollicita et al. 2008).

Plasmacytoid dentritic cells (pDCs) also have innate immune roles against invading pathogens. HIV-1 restricts pDCs-induced innate immune response by decreasing cell counts of pDCs in peripheral blood. Individuals infected with HIV-1 have lower pDC levels when compared to uninfected inviduals (Muller-Trutwin and Hosmalin 2005). Exposure of HIV-1 gp120 leads to suppression of pDC activation and TLR9-mediated inhibition of pro-inflammatory cytokines, thus, influencing expression levels of CD83 (a marker for DC-activation) (Muller-Trutwin and Hosmalin 2005). HIV-1 inhibits pDC function by TLR7/TLR8-mediated suppression (Martinson et al. 2007) and IFN-α restriction (Tilton et al. 2008). Prior to HIV-1 induced cell death, pDCs enhance the regulation of CCR7 production, leading to an increased production of IFN (Lehmann et al. 2010). HIV-1 virions induce pDCs into TRAIL-expressing killer pDCs and down-regulate HIV-1 mediated co-receptor binding by TLR7-induced IFN-α (Hardy et al. 2007).

2.2 Role of Monocytes and Macrophages

HIV-1 is capable of infecting a variety of myeloid cells. Monocytes are mononuclear phagocytic cell that are derived from $CD34^+$ myeloid progenitor cells within the bone marrow and act as common precursors of macrophages and myeloid DCs. HIV-1 infection also induces immunological dysfunction in mononuclear phagocytic cells, which also act as reservoirs of actively replicating virus. Phenotypic and functional changes have been found in monocyte population following HIV-1 infection, which includes expansion of $CD16^+$ monocytes (Trial et al. 1995; Nockher et al. 1994; Pulliam et al. 1997; Abel et al. 1992; van der Kuyl et al. 2007). Mococytes act as carriers of HIV-1 in semen, vaginal and cervical secretions (Mostad and Kreiss 1996) and DC-mediated transfer of virus to regional lymph nodes (Spira et al. 1996). Thus, these cells can accumulate large numbers of virions without cell death, and infected macrophage populations are preserved despite progressive T-cell depletion (Fig. 3.3) (Gendelman et al. 1989). Circulating monocytes in HIV-1 infection display marked alterations in surface markers and in their mode of functioning that is associated with progression to AIDS (Bender et al. 1988; Braun et al. 1988; Rich et al. 1988; Dudhane et al. 1996; Noursadeghi et al. 2006).

Macrophages are differentiated immune cells, which play an important role in the clearance of pathogens and cellular debris via phagocytosis; thus, macrophages are one of the important target cells for HIV-1 infection. Macrophages can also act as antigen presenting cells (APC) to $CD4^+$ T cells via major histocompatibility complex (MHC) class II pathway (Ackerman and Cresswell 2004; Koppensteiner et al. 2012), and trigger $CD8^+$ cytotoxic T cells (CTL) by cross-presentation of HIV-1 antigens (Ackerman and Cresswell 2004). Therefore, the interaction between macrophages and $CD4^+$ T cells is of paramount importance in HIV-1 transmission and immune response (Crowe et al. 1990; Groot et al. 2008). On the other hand, HIV-1 infected macrophages and monocytes exhibit a range of aberrant roles, inducing persistence of HIV-1 virions or delaying the adaptive immune responses, such as impaired T cell activation (Ennen et al. 1990; Twigg 3rd et al. 1991) and antigen presentation (Blauvelt et al. 1995), diminished expressions of Fc receptors (Dugast et al. 2011; Tyler et al. 1990) and surface molecules, e.g. CD36 (Olivetta et al. 2014), as well as impaired TLR response (Zhu et al. 2011).

In acute and chronic phases of HIV-1 infection, proliferation of $CD16^+$ monocytes were observed along with high plasma viral load (Kim et al. 2009). In the cases of terminal AIDS, depletion of $CD16^+$ monocytes were observed. $CD14^+$ monocytes, expressing CD16, expand during HIV-1 infection (Nockher et al. 1994; Thieblemont et al. 1995; Dunne et al. 1996). The expression of FcγR on the surface of monocytes (and other immune cells) may play a critical role in the immunopathogenesis of HIV-1 infection. A significant increase in the FcγRIII (CD16) expression whereas decrease in FcγRI (CD64) expression on blood monocytes were observed in HIV-1-infected patients (Allen et al. 1991; Miller et al. 2001) FcγRIII on monocytes may also mediate antibody-dependent enhancement of HIV-1 infectivity (Allen et al. 1991). Increased monocyte FcγRII (CD23) expression was also observed in AIDS patients (Miller et al. 2001).

Macrophages, infected with HIV-1 virus, transfer the virus to other target cells, for instance, $CD4^+$ lymphocytes, through T cell fusion (Crowe et al. 1990) and via the formation of viral synapse between HIV-1 Gag and Env on macrophages and CD4 on T cell (Groot, Welsch & Sattentau 2008; Gousset et al. 2008). Macrophage-mediated HIV-1 transfer to T cells may promote subsequent $CD4^+$ T-cell infection and their depletion (Garaci et al. 2003). Furthermore, mannose receptor (MR), expressed by monocyte-derived macrophage (MDM), was shown to capture both R5 and HIV-1 strains, which can be abrogated by manose-binding lectin (MBL) and MR antibody (Pollicita et al. 2008). Blood monocytes and lymph node macrophages have been implicated in the persistence and pathogenesis of HIV-1 infection (Kedzierska et al. 2003; Zhu et al. 2011; Crowe

et al. 2003; Sharova et al. 2005; Montaner et al. 2006). Both macrophages and monocytes cause HIV-1-mediated neuroinvasion and contribute to HIV-1 infection in the brain and neuronal injury (Soulas et al. 2011; Gras and Kaul 2010; Schnell et al. 2009). HIV-1 variants and macrophage-tropic HIV-1 env genes have been detected from brain tissues from individuals infected with HIV-1 (Gonzalez-Perez et al. 2012). HIV-1-mediated pro-inflammatory cytokine profiles of macrophages/monocytes increase viral replication and persistence in activated macrophages in vitro. Thus, up-regulation of IFN and NF-κB responses were found to be enhanced upon HIV-1 infection, which is possibly to promote viral spread by recruiting macropages and $CD4^+$ T cells to the site of infection (Woelk et al. 2004).

The roles of macrophages and monocytes have also been described in the neuropathogenesis due to HIV-1 infection. Thus, they are reported to contribute to HIV-1 mediated dementia as a result of pro-inflammtory cytokines and neurotoxin production (Kedzierska and Crowe 2002; Chakrabarti et al. 1991). Conversely, impaired effector functions of both monocytes and macrophages have been reported, including intracellular killing, phagocytosis, chemotaxis and production of cytokines and chemokines. In this regard, such malfunctions may contribute to the pathophysiology of AIDS through re-activation and expansion of other opportunistic infections (Kedzierska and Crowe 2002).

2.3 Role of Natural Killer Cells

Anti-viral effector functions of NK cells are critical in the innate immunity (Biron et al. 1989; Fleisher et al. 1982). NK cell-mediated anti-viral activity has been highlighted in HIV-1 infection (Orange 2006). Infection, activation and proliferation of NK cells result in both killing of infected cells as well as secretion of IFN-γ, TNF-α and chemokines, which drive Th1-antigen specific T and B cell mediated specific responses (Mocikat et al. 2003). IL-2, IL-12 and IL-15 produced during the initial phase of HIV-1 infection can also activate NK cells (Stacey et al. 2009a, b).

Killer immunoglobulin-like receptors (KIRs) play an important role in modulating the activity of NK cells against HIV-1-infected cells (Bashirova et al. 2001). Binding of inhibitory KIRs to their respective HLA class I ligands is necessary for NK cell licensing. Licensed NK cells are functionally active and capable of stronger anti-viral effector functions against HIV-1 through ADCC-mediated killing (and even direct killing of infected cells) (Kamya et al. 2011; Parsons et al. 2012, 2014; Song et al. 2014). Populations of licensed NK cells expressing KIR2DL1, KIR2DL2 or KIR2DL3 are preferentially expanded during primary HIV-1 infection as compared to unlicensed NK cells (Korner et al. 2014). Significant changes in NK cell subsets are associated with acute HIV-1 infection (Alter et al. 2005).

An impaired activity of $CD56^-$ NK cell subset in HIV-1-viremic individuals is associated with dysfunctional NK cell population (Mavilio et al. 2005). NK cells isolated from HIV-1-infected patients have been found to be dysfunctional in their ability to lyse HIV-1 infected cells (Ullum et al. 1995; Bonaparte and Barker 2003). Recently, the effects of HIV-1 viremia on NK cell phenotype and functions have been examined. The levels of both expression and acitivty of most inhibitory NK cell receptors (iNKRs) on the NK cell surface are significantly increased in viremic patients (Mavilio et al. 2003; Ahmad et al. 2001); a direct relationship with the levels of HIV-1 viremia has also been reported (Kottilil et al. 2004). Specific stress-related ligands such as ULBP-1, 2, and 3 by Vpr accessory protein, which are specific ligands for NKG2D receptor, are also upregulated due to HIV-1 infection that further aggrevates NK cell-mediated killing of infected cells (Richard et al. 2010) (Fig. 3.2).

Reduced surface expression of natural cytotoxicity receptors (NCRs), such as NKp44, NKp46 and NKp30, has also been seen in HIV-1 viremic patients, along with a reduction in NK-mediated cytolytic activity (Mavilio et al. 2003; De Maria et al. 2003). Direct correlation

between unusal expansion of NK cell subsets including $CD56^{-}/CD16^{+}$ ($CD56^{-}$) and enhanced HIV viral loads has also been reported (Mavilio et al. 2003; Scott-Algara et al. 2002). Furthermore, the ability of NK cells to secrete CC-chemokines is affected by HIV-1 viremia, these chemokines are well known supressors of HIV-1 replication in vitro (Kottilil et al. 2004; Oliva et al. 1998). Suppression of HIV-1 replication after treatment with anti-retroviral therapy (ART) results in a significant improvement in NK cell-mediated cytotoxicity, secretion of cytokines and NK cell receptor activity (Mavilio et al. 2003), as well as restoration of CD56 expression levels (Sondergaard et al. 1999). Activated NK cells secrete CCL3–5 chemokines, which are known ligands for CCR5 (HIV-1 co-receptor), and thus, block viral entry through binding to CCR5 (Kottilil et al. 2004).

3 Complement and HIV-1 Infection

Early defence against virus by the innate immune system is crucial in limiting viral invasion. The presence of complement in blood and other body fluids plays a significant role in virus infectivity and pathogenesis. Following the initial mucosal penetration, the complement system confers the foremost defence barrier to control HIV-1 propagation that relies on the recognition of patterns on the surface of invading pathogens and triggering complement activating effector functions via three pathways: classical, alternative and lectin. The complement system plays a central role in neutralising IgG and IgM-bound viruses and promoting phagoctytosis of HIV-1 virions.

The classical pathway is triggered by the binding of C1q to Fc regions of antigen bound IgG or IgM. The classical pathway can also be activated by the direct binding of C1q to the glycoproteins of certain viruses in the absence of specific antibodies; human cytomegalovirus (Spiller and Morgan 1998) and some retroviruses (Cooper et al. 1976; Solder et al. 1989) such as human T cell lymphotropic virus (Ikeda et al. 1998). However, HIV-1 defeats complement mediated inhibition via binding to C1q with HIV-gp41 envelope protein (Marschang et al. 1994). C1q and globular head receptor, gC1qR, are known to interact with gp41 of HIV-1 (Ebenbichler et al. 1991; Fausther-Bovendo et al. 2010). In addition, C1q has been shown to associate with ectodomain of gp41 via gC1q domain (Thielens et al. 1993) particularly via ghA chain (Kishore et al. 2003) (Thielens et al. 2002; Pinter et al. 1995). Recently, C1q was shown to suppress DC-SIGN-mediated HIV-1 transfer to activated pooled peripheral blood mononuclear cells (Pednekar et al. 2016). gC1qR on its own suppresses HIV-1 production in MT-4 and H9 human T cell lines, as well as $HIV\text{-}1_{IIIB}$ and $HIV\text{-}1_{Ba\text{-}L}$ infected macrophages (Szabó et al. 2001).

In vivo studies have demonstrated complement-dependent viral lysis (Sullivan et al. 1996; Sullivan et al. 1998). Complement can promote the effect of neutralising antibodies in vivo and in vitro (Posner et al. 1992; Gauduin et al. 1997). Binding of gp41 and/or gp120 to C1q (or MBL) leads to an enhancement of antibody-mediated complement activation (Ebenbichler et al. 1991; Thielens et al. 2002; Spear et al. 1991; Haurum et al. 1993; Thielens et al. 2002; Stoiber et al. 1994; Stoiber et al. 1995a, b; Susal et al. 1996) (Fig. 3.4). C1q- or C3-deficient serum from uninfected individuals as a source of complement show non-anti-HIV-1 activity. Thus, the classical pathways mainly contributes to anti-HIV-1 activity through anti-HIV-1 antibodies (Aasa-Chapman et al. 2005). HIV-1 infected patients demonstrate accumulation of C3 on the surface of the HIV-1 virions (Stoiber et al. 2001). The binding of gp120/gp41 of HIV-1 to MBL has also been shown to trigger complement activation via the lectin pathway (Haurum et al. 1993; Ezekowitz et al. 1989; Saifuddin et al. 2000). Purified MBL also inhbits HIV-1 infection of $CD4^{+}$ H9 lymphoblasts in vitro (Ezekowitz et al. 1989). Binding of MBL was seen in the case of U937 and H9 cells infected with HIV-1; MBL likely recognises high mannose glycans present on HIV-1 gp120, and thereby prevents viral entry to susceptible cells (Ezekowitz et al. 1989).

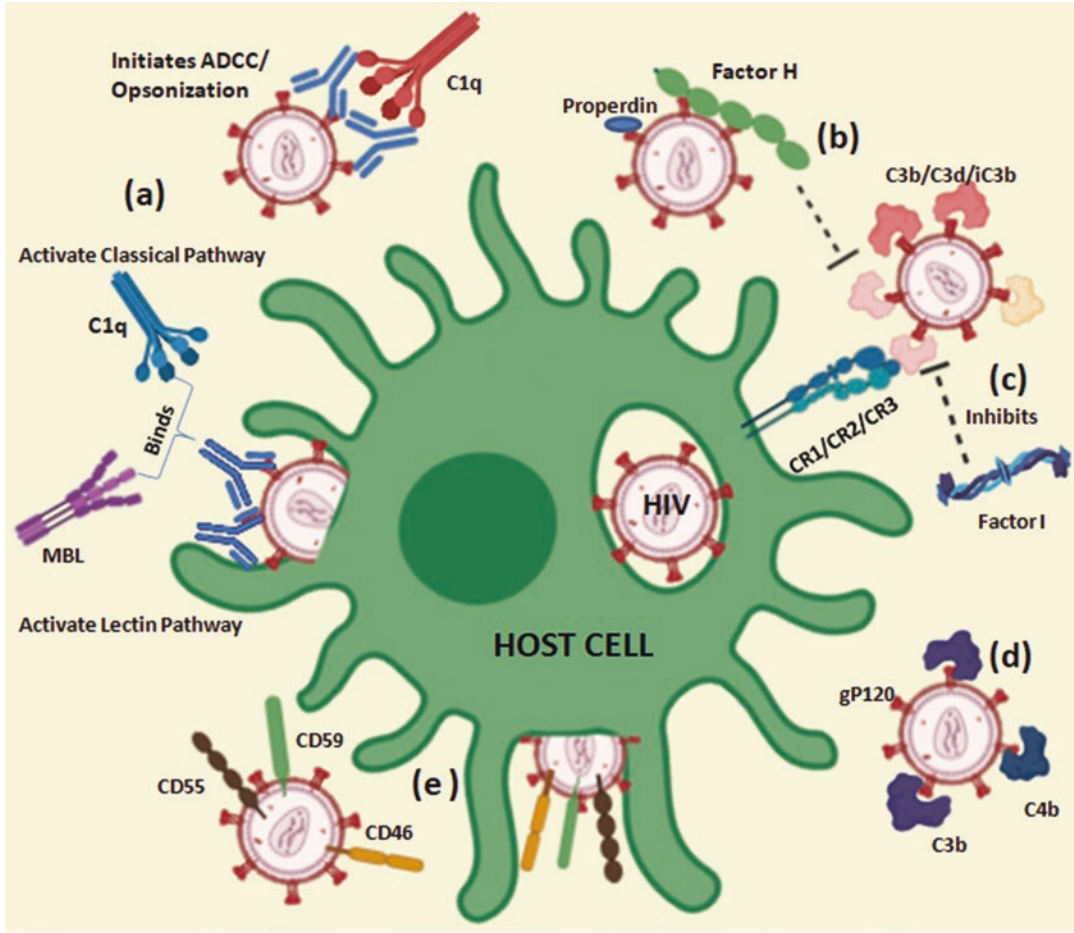

Fig. 3.4 Role of complement in HIV infection. (**a**) The binding of gp120/gp41 of HIV-1 to C1q or mannose-binding lectin (MBL) triggers complement activation, leading to an enhancement of Ab-mediated complement activation and opsonization. HIV-1 is also capable of defeating complement mediated inhibition via binding to C1q with HIV-gp41 envelope protein, (**b**) Binding of factor H to both gp41 and gp120 protects from complement mediated lysis, HIV-1 gp120 and gp41 can also bind to properdin; (**c**) HIV-1 mediated complement activation also leads to deposition of C3 fragments (C3b, C3d, iC3b) to gp120 and increases infectivity of C3-receptor (CR1, CR2, CR3) bearing cells. Factor I aids in the cleavage of C3b (iC3b or C3d) attached to HIV, thus can attenuate pathogenesis by inactivating HIV-conjugated C3b and C4b; (**d**) HIV gp120 and gp41 can also bind to C3b and C4b; (**e**) Complement regulator proteins such CD46, CD55 and CD59 are widely expressed on T lymphocytes, monocytes and macrophages that are incorporated into the budding HIV-1 enhancing resistance to complement-mediated lysis

Complement-mediated lysis of cells infected with HIV-1 is an important innate defence in clearing and neutralising HIV-1 virions. HIV-1 infected patients mount an effective anti-viral immunity, producing both neutralizing and non-neutralising antibodies. These antibodies contribute to anti-viral actitivity in four ways: direct neutralization of free residing virions, complement-mediated lysis through complement activation and antibody-antigen engagement, opsonisation and phagocytosis of virions by macrophages and other target cells, and viral destruction via antibody-dependent cellular cytotoxicity (ADCC) (Huber and Trkola 2007). Neutralising antibodies bind HIV-1 env sites, restricting binding of HIV-1 virions to target cells. However, non-neutralising antibody binding to either non-specific sites of HIV-1 proteins or env does

not have the abilitity to stop the virions from enaging with target receptor/co-receptor. However, non-neutralising antibodies can also block viral replication by restricting uncoating of virions or budding later in the HIV-1 life cycle (Huber and Trkola 2007). The antibodies specifically against gp120, gp41, core Gag and matrix p17 are detectable in the plasma from HIV-1 positive patients (Pincus et al. 1994; Belec et al. 1995; Binley et al. 1997; Pellegrin et al. 1996; Richman et al. 2003). However, HIV-1 virions can esape neutralizing antibodies by rapid mutations in viral env genes, mediating stumps of monomeric HIV-1 gp120 and gp41, and shedding neutralising epitopes and repositioning of the glycan-mediated shield (Wyatt and Sodroski 1998; Pantophlet and Burton 2006; Parren et al. 1999; Stoiber et al. 2008; Wei et al. 2003; Parren et al. 1997; Moore et al. 1990). Furthermore, the expression levels of host complement receptors are down-regulated by HIV-1, leading to an impaired monocyte-mediated chemotactic response to inflammatory stimuli; HIV-1 gp120 glycoportein exposure diminishes C5a (Speth and Dierich 1999). C3 and C5a deposition aids in HIV-1 interaction with cells containing complement receptors CR3 and CR4, including DCs and macrophages.

The binding of antibody and complement or complement alone to the viral surface leads to the loss of viral infectivity (Fig. 3.4). Complement receptors (CRs) present on macrophages or DCs can further opsonise viral particles with complement components present in blood or other body fluids. CR3 interaction with gp41 results in the enhancement of viral entry as well as spread in the cells (Stoiber et al. 1997). In addition, complement mediates the binding of HIV-1 to CR1 on erythrocytes and CR2 on B cells, exploiting these cells to produce C3d opsonised HIV-1 infectious reservoirs in order to infect other non-infected cells (Horakova et al. 2004). HIV-1-mediated complement activation leads to C3 fragment binding to gp120 complex and an increased infectivity of C3-receptor bearing cells. CR1 (CD35) and CR2 (CD21) contribute in an independent manner to facilitate infection of human T cells with complement-oponised HIV-1 virus (Delibrias et al. 1993). Furthermore, C3d-CR2 and CD4-gp120 interactions may enhance viral adhesion to target cells, an important step in viral entry (Lund et al. 1995).

Both HIV-1 envelope proteins, gp41 and gp120, recruit complement factor H, which has a role in protecting self-cells from complement mediated lysis (particularly alternative pathway) to various binding sites (Pinter et al. 1995; Stoiber et al. 1995a, b). This leads to reduction in complement-dependent lysis of virus and infected cells in vitro (Stoiber et al. 1996). Factor I aids in the cleavage of C3b (iC3b or C3d) attached to HIV-1, thus can attenuate HIV-1 pathogenesis by inactivating HIV-1-conjugated C3b and C4b (Banki et al. 2006). iC3b coated HIV-1 displays enhanced infection of macrophages and DCs expressing CR3 and CR4. Complement is capable of efficiently inducing CTL responses against different viral infections (Masaki et al. 1992; Banki et al. 2010) (Fig. 3.4). Complement opsonized retroviral particles can stimulate DCs to induce CTL responses both in vitro and in vivo (Bánki et al. 2010). DCs exposed to IgG-opsonized HIV-1 significantly decreases the HIV-1-specific $CD8^+$ T-cell response compared to DCs bound to complement-opsonized HIV-1 (Posch et al. 2012). Complement coating of HIV-1 can strongly influence intracellular transmission of the virus in DCs compared to non-opsonized HIV-1. At the same time, complement-opsonized HIV-1 can fruitfully bypass the SAMHD1 (Posch et al. 2015).

A number of host cell proteins are incorporated into virions of HIV-1 while budding out from the host cell. Complement regulatory proteins such as CD46, CD55 and CD59 are widely expressed on T lymphocytes, monocytes and macrophages (Terstappen et al. 1992) that are also incorporated into the budding HIV-1 enhancing their resistance to complement-mediated lysis (Saifuddin et al. 1997). Several pathogenic enveloped viruses, including HIV-1, influenza, herpes, Ebola and cytomegalovirus, are known to escape antibody-dependent complement mediated lysis by incorporating certain complement regulators such as CD59 and CD55 into their envelope, which may explain why some pathogenic human viruses are not neutralised by com-

plement in human fluids even when they induce a strong antibody response (Saifuddin et al. 1995). CD59, which can be found on the HIV-1 envelope, prevents complement mediated neutralisation of IgG bound viruses (Schmitz et al. 1995). HIV-1 infected patients have a low expression levels of both CD59 and CD55 on the membranes of lymphocytes (Lederman et al. 1989; Weiss et al. 1992). Thus, incorporation of these complement regulators in the HIV-1 envelope is to protect the virus against the complement attack.

4 Protective Role of Surfactant Protein D Against HIV-1

Surfactant Protein D (SP-D) is a pattern recognition innate immune molecule. SP-D is known to play a key role in the clearance of various pathogens via agglutination, enhanced phagocytosis and killing. SP-D structure mainly consists of N-terminal triple-helical collagen region, an α-helical coiled-coil neck region, and carbohydrate recognition domain (CRD). The trimeric CRD domain interacts with glycosylated moieties on the pathogen surface that brings about agglutination and inhibition of infection. The CRD and collagen domains in SP-D interacts with various receptors such as calreticulin/CD91, SIRPα, and CD14 on the cell surface of immune cells and regulate effector functions (Gardai et al. 2003). Human SP-D in its native form and also the recombinant fragment of human SP-D (rhSP-D containing neck and CRD) bind to gp120 of HIV-1 and inhibit viral replication in vitro in a calcium and dose-dependent manner (Pandit et al. 2014). SP-D also inhibits viral replication in U937 monocytes (Meschi et al. 2005) and PM1 T (Madsen et al. 2013) cells by binding to gp120. The increased serum SP-D levels has been reported in the AIDS patients, but not during early stages of HIV-1 infection (Jambo et al. 2007), which decreases following ART (Kunisaki et al. 2011). SP-D (and rfhSP-D) has been shown to reduce DC-SIGN-mediated transfer of HIV-1 to PBMCs (Dodagatta-Marri et al. 2017). Sensing of HIV-1 infection by PRRs such as SP-D results in the innate immune activation of both infected cells and bystander cells. This in turn causes production of proinflammatory cytokines and chemokines (Altfeld and Gale Jr. 2015), leading to activation of innate immune cells, such as macrophages, dendritic cells, and NK cells. Previous pre-clinical safety studies on SP-D coupled with its efficacy in restricting viral passage in the vaginal barrier through reversal of virus-induced gene expression also indicates possible application of SP-D as a topical anti-HIV microbicide (Pandit et al. 2019).

5 Innate Cytokines and Chemokines in HIV-1 Infection

Expression profiles of cytokines, chemokines and their respective receptors in HIV-1 infection are of great significance, which may influence HIV-1 pathogenesis and infection susceptibility. They contribute to the induction or inhibition of viral entry and replication. Altered activation of HIV-1-induced immune cells, such as macrophages, NK cells, DCs, and B cells leads to an increased production of both pro- and anti-inflammatory cytokines and chemokines, including interferons (IFNs), TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, MCP-1 and interferon gamma-induced protein (IP) -10 (Stacey et al. 2009a, b; Rychert et al. 2010).

HIV-1-gp120 attachment triggers production of CC chemokines, including CCL2, CCL3, CCL4 and CCL5, which serve as a chemoattractants for macrophages, DCs and lymphocytes. The expression levels of these CC chemokines are regulated by TNF-α, IL-6, IL-10 and IL-1β, which are also modulated by HIV-1 infection (Fantuzzi et al. 2003). Interaction between the negative regulatory factor (Nef) of HIV-1 and DCs results in an increased production of IL-12, IL-15, IL-1β, TNF-α and chemokines, including MIP-1α, MIP-1β and IL-8 (Quaranta et al. 2002; Guha et al. 2012). Human uterine epithelial cells up-regulate secretion of IL-6, IL-10, IL-1β and TNF-α in response to R5-induced HIV-1 infection (Nazli et al. 2010). The natural ligands for CCR5, including MIP-1α, MIP-1β, RANTES

(Combadiere et al. 1996) and monocyte chemotactic protein 2 (MCP-2) (Gong et al. 1998; Ruffing et al. 1998) suppress the entry of HIV-1 into target cells. In addition, CCR5 homozogous mutations in humans seem to confer resistance to HIV-1 infection (Deng et al. 1996). MIP-1α, MIP-1β and RANTES inhbit CD4/CRR5-HIV-1 mediated cell fusion, MCP-2 being a potent inhibitor of CD4/CRR5 induced HIV-1 entry and replication (Gong et al. 1998). Activated cervical $CD4^+$ T-cell subsets that express CCR5, IL-17A, IFN-γ and $\alpha_4\beta_7$ bind gp120 of HIV-1 in vitro (McKinnon et al. 2011); the levels of these cells are depleted during HIV-1 infection from the cervix, suggesting their role as HIV-1 targets during sexual transmission.

Innate type I IFNs, including IFN-*α*, IFN-*β*, IFN-*ε*, IFN-*κ*, and IFN-*ω*, are known to confer protection against virus infection via induction of immune activation, enhanced antigen presentation and anti-viral activity (Yamamoto et al. 1986). IFN-α-mediated inhibitory effects on HIV-1 replication in macrophages and monocyes are documented (Mace and Gazzolo 1991). During acute and late phases of HIV-1 infection, high level of plasma IFN has been observed; curiously, it correlates with an enhanced disease progression to AIDS (Herbeuval et al. 2007). Interferons stimulated genes (ISGs), which are triggered by type I IFNs, induce signalling events that restrict HIV-1 replication (Pitha 1994; Hou et al. 2009). Tripartite motif (TRIM) proteins also inhibit viral replication. For example, in Rhesus macaque, TRIM5α interferes with HIV-1 and other retroviral infections by restricting early stage of viral replication (Dutrieux et al. 2015).

In addition to pDCs-indced IFN activation, HIV-1 virions stimulate production of CXCL10, CCL4, CCL5, IL-6 and TNF-α (Megjugorac et al. 2004; Penna et al. 2002). Induction of TRIM22 by IFN-*β* blocks replication of HIV-1 (Barr et al. 2008). TRIM22 over-expression restricts HIV-1 replication in 293 T HEK cells and human macrophages (Bouazzaoui et al. 2006). Moreover, gp120 of HIV-1 can block IFN-α secretion and TLR9-mediated activation in pDCs (Martinelli et al. 2007). HIV-1 gp120 leads to suppression of pDC-induced cytolytic activity of NK cells. Thefore, the direct interaction between gp120 and pDCs may interfere with TLR9 activation, leading to reduced ability of pDCs to produce anti-viral inflammatory factors.

6 Anti-HIV-1 Innate Immunity by Peptide Antibiotics in Female Reproductive Tracts

The mucosal system of both male and female reproductive tracts utilizes a wide range of immune mechanisms to provide a protective environment against invading pathogens. The epithelial cells that line up FRT express innate immune sensors such as TLRs; endogenous secretions from these cells have anti-HIV-1 properties (Cole 2006; Keller et al. 2007; Fahey and Wira 2002; Schaefer et al. 2005; Ghosh et al. 2008). Human α- and β-defensins are the most well- characterised and abundunt anti-microbial peptides (AMPs), secreted by epithelial cells and neutrophils in the FRT (Wira et al. 2005; Klotman and Chang 2006).

Replication of HIV-1 was first shown to be inhibited by synthetic guinea pig, rat and rabbit α- defensins, which can block HIV-1 following viral load into transformed $CD4^+$ T cells (Nakashima et al. 1993). Similar anti-viral activities of human neutrophil peptide 1 (HNP1), HNP2 and HNP3, have been reported against HIV-1 primary isolates (Wu et al. 2005), HNP4 being more effective than HNP1–3 in preventing human PBMCs from X4 and R5 HIV-infection. HNP1–3 can restrict HIV-1 replication through direct interaction with the virus, or affect the target cells (Chang et al. 2005; Chang et al. 2003; Wang et al. 2004; Mackewicz et al. 2003). HNP1 can inactivate the virus directly before it infects target cells in the absence of serum at a low MOI (Chang et al. 2005). However, in the presence of serum, the direct effect of HNP1 was observed on infected cells which inhibited HIV-1 infection during nuclear import and transcription phase. HNP1 also interferes with protein kinase C (PKC) signalling in primary $CD4^+$ T cells. Furthermore, expression levels of CC-chemokines are up-regu-

lated by HNP1–2 in macrophages, which may contribute to HIV-1 blockade through receptor competition (Guo et al. 2004). CC-chemokines can also mediate HNP release from neutrophils via degranulation (Jan et al. 2006).

HNP1–3 can function as lectins; they bind to gp120 of HIV-1 and CD4 with greater affinity (Wang et al. 2004). HNP4 acts in a lectin independent manner and binding of HNP4 to CD4 or gp120 is not evident (Wu et al. 2005; Wang et al. 2004). The role of other α-defensins and their ability to inhbit HIV-1 capture and replication has been investigated. Rhesus macaque myeloid α-defensins-3 (RMAD3) can block HIV-1 infection at a high concentration associated with cytotoxicity. However, enhancement of viral replication is observed with mouse cryptdin-3 (Tanabe et al. 2004). Studies carried out using primary $CD4^+$ T cells and macrophages revealed that HNP1 caused a post-entry HIV infection, but not in some transformed T cell lines (Chang et al. 2005; Chang et al. 2003).

Anti-HIV-1 activities of human β-defensin 2 (HBD2) and HBD3 have been examined (Sun et al. 2005; Quinones-Mateu et al. 2003). HBD2 and HBD3 interact with HIV-1 virons (Quinones-Mateu et al. 2003). HBD2 blocks early stages of HIV-1 Bal and IIIB strains, but does not have any effect on cell-cell fusion and cellular proliferation (Sun et al. 2005). HBD1 and HBD2 do not modulate the expression of HIV-1 co-receptors in primary $CD4^+$ T cells. However, another study has indicated that both HBD1 and HBD2 mediate down-regulation of CXCR4 but not CCR5 in PBMCs and T lymphocytic cell line (Quinones-Mateu et al. 2003). HBD2 is expressed constitutively in healthy adult oral mucosa; HIV-1-infected individuals seem to show decreased expression levels of HBD2 (Sun et al. 2005).

Retrocyclins and θ-defensins from rhesus macaques including θ-defensin-1 (RTD1), RTD2 and RTD3 also act as lectins and induce anti-viral effects against primary isolates of HIV-1 (Wang et al. 2004; Munk et al. 2003; Wang et al. 2003; Cole et al. 2002). Retrocyclins seem to bind to CD4 and gp120 of HIV-1 with high affinity, instead of directly inactivating the virus (Munk et al. 2003). High affinity binding of Retrocyclins to gp120 (glycosylated) and CD4 is mediated through O- and N-linked sugar interaction (Wang et al. 1998). Thus, defensins can contribute to a novel anti-retroviral-mediated mechanism that can induce mucosal prevention of HIV-1 transmission.

Protease inhibitors are the second class of well-identified AMPs, including elafins, serine protease inhibitors (serpins), Secretory leukocyte protease inhibitors (SLPI) and cystatins (Wiesner and Vilcinskas 2010). Protease inhibitors exert anti-inflammatory properties by preventing proteases secreted by immune cells. Thus, they can activate the complement system and trigger production of other inflammatory mediators, leading to severe inflammation and inhibiton of proteolytic cleavage of protein precursors that are essential for production of virions. Epithelial cells of both upper and lower FRT produce Trappin-2/Elafin messenger RNA; recombinant form of Trappin-2/Elafin has been shown to inhibit both R5-M-tropic BaL and X4-T-tropic IIIB strain of HIV-1 in a dose-dependent manner (Ghosh et al. 2010). Vaginal fluid confers anti-HIV-1 properties against both R5 and X4 HIV-1 strains, thus, providing a protective barrier against HIV-1 infection as well as reducing integration of pro-viral genome in human cervicovaginal tissue derived organotypic cultures (Venkataraman et al. 2005).

Human apolipoprotein L1 (APOL1) as a key component of innate immunity has been reported to restrict HIV-1 transcription and cause degradation of HIV-1 Gag in the endolysomal compartment (Taylor et al. 2014), in addition to degradation of viral accessory proteins involved in targeting host restriction factors. For instance, APOL1-mediated viral degradation results in restoring levels of APOBEC3G (A3G) and reduced progeny virion infectivity. Endogenous APOL1 expression levels in differentiated U937 monocytic cells result in decreased production of HIV-1 virions. Other proteases such as cystatins and serpins exert anti-viral effects by blocking HIV-1 binding and viral replication (Aboud et al. 2014).

Lactoferrin, lysozyme and calthelicidin LL37 are the other anti-microbial products secreted by

neutrophils and lower FRT epithelium (Wira et al. 2005; Wiesner and Vilcinskas 2010). Lactoferrin, a well known conserved multifunctional protein of transferrin family, enhances the activity of NK cells (Damiens et al. 1998; Shau et al. 1992), further promoting neutrophil-mediated phagocytosis and production of reactive oxygen species (Kawai et al. 2007; Miyauchi et al. 1998; Ward et al. 2008). Lactoferrin also triggers macrophage activation (Wakabayashi et al. 2003) via enhanced production of cytokines and nitric oxide (NO) (Sorimachi et al. 1997) and regulation of the proliferation of intracellular pathogens (Actor et al. 2002).

Lactoferrin shows a potent anti-viral activity against HIV-1 replication (Harmsen et al. 1995); it inhbits HIV-1 infection of target cells through interference with viral fusion via V3 loop of gp120 (Harmsen et al. 1995; Cole and Cole 2008). Lactoferrin can restrict the replication of HIV-1 and formation of syncytium in a dose-dependent manner (Puddu et al. 1998). Glycolactin and angiogenin-1 proteins isolated from bovine milk suppress activity of HIV-1 reverse transcriptase, but weakly inhibit proteases and integrases of HIV-1 (Ng et al. 2001). Bovine lactoferrin prevents DC-SIGN-mediated HIV-1 capture and transmission through blockade of DC-SIGN-gp120 interaction (Groot et al. 2005). Decreased levels of lactoferrin correlate with an enhanced risk of HIV-1 transmission through breast milk from infected mother to her infant as they grow older (Ekpini et al. 1997). Therefore, supplement of lactoferrin may reduce the risk of HIV-1 transmission during this period. Moreover, both human and bovine lactoferrin have an effective anti-HIV-1 property when combined with zidovudine, suggesting synergism against syncytium-inducing and non-syncytium-inducing clinical isolates of HIV-1(Viani et al. 1999).

In addition, the binding ability of lactoferrin to larger quantities of iron has also been suggested to initiate protection against microorganisims and their metabolities by enhancing phagocytosis, cell adherence as well as regulating the release of pro-inflammatory cytokines and chemokines. In vitro studies have suggested the effective role of iron in HIV-1 replication. Thus, clinical studies also define an important correlation between the status of host iron and progression of HIV-1. For example, anemia has been reported as a consequence of HIV-1 that poorly impacts survival (Lundgren and Mocroft 2003; Moore 1999). Additionally, recombinant form of human erythropoietin (r-HuEPO) has been suggested to be an effective factor in enhancing levels of hematocrit and diminishing requirements of transfusion in HIV-1 infected individuals with < or = 500 IU/L levels of endogenous erythropoietin (Moore 1999). As a result, therapy in combination with r-HuEPO has been reported to be safe and well tolerated. Conversely, enhanced iron levels is positively correlated with viral load (Friis et al. 2003) and mortality (Gordeuk et al. 2006; McDermid et al. 2007). Furthermore, the role of iron metabolism in HIV-1 infection is supported by the gentic polymorphisms in iron regulatory genes, including Nramp1 and haptoglobin, thus these are effective predictors of mortality (McDermid et al. 2009). Anti-HIV activity of calthelicidin LL37, highly expressed by neutrophils and several mucosal epithelial cell types, has been explored. LL37 blocks the replication of HIV-1 in PBMC and CD^{+}T cells (Bergman et al. 2007).

7 Feto-Maternal Vertical Transmission of HIV-1

About 40% of HIV-1 infections in children (Hong et al. 2009; Luzuriaga 2007) occur through vertical transmission from mother-to-child either through transplacental or intra-uterine transmission, intrapartum transmission during delivery or during breastfeeding (Smith et al. 2003; Naarding et al. 2005; Boily-Larouche et al. 2009). DC-SIGN and DC-SIGN-related C-type lectin domain family 4, member M (L-SIGN) receptors are involved in HIV-1 transmission from mother to child (Baribaud et al. 2001).

Both DC-SIGN and L-SIGN are capable of binding to the gp120 of HIV-1 (Feinberg et al.

2001; Geijtenbeek et al. 2000a, b; Pöhlmann et al. 2001; Baribaud et al. 2001). Extracellular domain of DC-SIGN and L-SIGN receptors is divided into two structures: the neck repeat region and the CRD (Khoo et al. 2008). The neck repeats region plays a crucial role in tetramerization and supports carbohydrates' recognition, thus directly influencing the receptor's binding affinity to pathogens. The CRD region, both in DC-SIGN and L-SIGN, is flexibly connected to the neck repeat region (Wu and KewalRamani 2006). L-SIGN can bind to ICAM-3 in addition to other cell surface receptors, facilitating interactions between T cells and the endothelial cell surface. This helps in transmitting the virus to secondary lymphoid organs rich in T cells and increasing the infection of target $CD4^+$ cells (Geijtenbeek et al. 2000a, b). L-SIGN is capable of internalizing the virus and promoting virus degradation in a proteasome-dependent manner (Boily-Larouche et al. 2009).

Human placenta plays an important role in the transmission of HIV-1 infection exposing the virus to the fetus especially during the third trimester of pregnancy (Geijtenbeek et al. 2001). Hofbauer cells in the chorionic villi show high expression of DC-SIGN during pregnancy, thus enhancing the binding of HIV-1 on the surface (Soilleux et al. 2001). Hofbauer cells infected with HIV-1 may enter the fetus through the umbilical vein (Soilleux et al. 2001; Soilleux and Coleman 2003) or may release infectious viral particles, which further bind with the adjacent placental capillary endothelium through L-SIGN and to circulating lymphocytes travelling between the placenta and the fetus in umbilical cord blood (Soilleux et al. 2001; Soilleux and Coleman 2003).

Mammary epithelial cells and macrophages aid in transmission of HIV-1 to the newly born via breastfeeding. Breast milk contains huge number of macrophages (about 80% of total cells present in colostrum), which express CCR5 to which HIV-1 binds (Naarding et al. 2005; Ichikawa et al. 2003). Virus then reaches to the mucosal layer of upper intestine which contains large number of lymphocytes expressing CCR5 and CXCR4 that further helps in viral proliferation; this leads to depletion of $CD4^+$ T cells. The mucosal environment of gut also contains DCs expressing CD4/CCR5, DC-SIGN, and DC206 (Shen et al. 2010). HIV-1 inside the DCs are protected from gastrointestinal juice, they lose their infectivity once exposed to the acidic environment. HIV-1 can also attach to L-SIGN receptor to increase its infectivity (Naarding et al. 2005).

8 Mucosal Defence Factors in Clinical Trials

Several anti-HIV-1 agents have been identified and developed via clinical trials, specifically to target and block viral entry processes. ART is used to delay immune system-mediated destruction, lower severity and rates of opportunistic infections as well as progression of AIDS. Successful ART involves combination of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) (Gulick et al. 2003). These inhibitors reduce viral load below measurable levels in the plasma of HIV-1 infected patients, leading to reduced mortality and morbidity (Gulick et al. 2003; Barbaro et al. 2005; Hammer et al. 1996). However, the use of ART in patients only suppresses the virus, but do not eradicate it. In addition, multiple drug therapies using ART regimen lead to an adverse toxicities including drug-drug interaction (Piscitelli et al. 1996; Louie and Markowitz 2002).

Soluble polyanions, such as heparin, cyclodextrin sulphate and dextran sulphate, have been developed to block the non-specific adsorption and attachment between HIV-1 virions and cell membrane (Callahan et al. 1991). Cyanovirin-N, isolated from cyanobacterium *Nostocellipsosporum*, has been suggested to bind HIV-1-gp120 and potently inhibit HIV-1 attachment to target cells (Boyd et al. 1997; Botos et al. 2002). Additionally, PRO 2000, a naphthalene sulfonate polymer, binds non-specifically to CD4 receptor. Thus, vaginal PRO 2000 gel is well tolerated in phase I/II clinical trials (Van Damme et al. 2000; Smita et al. 2006). Furthermore, licorice root- isolated glycyrrhizin

has been shown to inhbit replication of HIV-1 through blocking viral absorption of $CD4^+$ cells (Huang and Chen 2002).

Besides ART, each step in the HIV-1 entry process provides several targets for chemotherapeutic attack, including pathways of viral entry, viral transcription, nuclear export and maturation (Pereira and Paridaen 2004; Yu et al. 2005). Among these potential targets, entry of the virus is one of the major effective target for the development of HIV inhibitors/drug. Enfuvirtide has been indentified as the first novel class of antiretroviral drugs to inhibit HIV fusion, which is approved by US FDA(Poveda et al. 2005). Recombinant forms of soluble CD4 (rsCD4) molecules were designed to target HIV-1 entry and rsCD4 has been reported to lack both transmembrane and cytoplasmic domain of CD4. However, these molecules were suggested to retain the ability of gp120 binding, thus, function as molecular decoys. In vitro studies have highlighted that these molecules have shown good anti-viral activity against tissue culture-adapted HIV-1 strains, but its activity in early phase of clinical trials was not effective (Schooley et al. 1990; Daar et al. 1990; Turner et al. 1992).

CD4-gp120 binding inhibitors have been developed. Expression and characterization of recombinant tetrameric CD4-IgG2, also known asPRO 542, was designed to replace both the heavy and light chain of Fv portions of human IgG2 by the human CD4 domains, including D1 and D2 (Allaway et al. 1995). It targets the binding site of CD4 on gp120 and mimicks CD4 receptor. Data from phase I-II clinical trials have demonstarted that PRO542 diminishes HIV-1 RNA levels after single intravenous dose in addition to being well tolerated and effective in HIV-1 infected individuals (Jacobson et al. 2004; Jacobson et al. 2000). Furthermore, human IgG4 monoclonal antibody, ibalizumab (TNX 355), was developed against CD4, which binds to D2 domain and blocks soluble post-binding of CD-4-induced conformational changes in the HIV-1 envelope glycoproteins (Moore et al. 1992). Ibalizumab has been effective in reducing up to a 1.5-log10HIV-1 RNA levels in plasma after a single dose for 14–21 days (Kuritzkes et al. 2004), but resistance was seen after 9 weeks administration (Jacobson et al. 2004). TNX 355 was effective in reducing plasma HIV-1 viral load and resulted in an increased levels of $CD4^+$ T cells (Kuritzkes et al. 2004). Combination of Ibalizumab and an optimised background regimen has been shown to result in decreased levels of plasma HIV-1 RNA than background regimen alone (Norris et al., 2006).

Small molecule inhibitors, including BMS-378806 and BMS-488043, have shown great promise in blocking gp120-CD4 interactions (Guo et al. 2003; Lin et al. 2003). BMS-378806 inhibitor is promising against HIV-1 subtype B and blocks CD4 receptor binding *in vitro* (Lin et al. 2003). However, BMS-378806 drug development was discontinued after phase I clinical trails due to poor target exposure was achieved. Development of BMS-488043 is being successfully studied in phase II clinical trials, which shows 1-log10 reductions of HIV-1 RNA levels in the plasma in treated individuals (Hanna et al. 2004.). NBD-556 (8) and NBD-557 (9) compounds have shown potent micromolar anti-HIV-1 activity (Zhao et al. 2005). In addition, Zintevir (AR177) was reported as an effective HIV integrase inhibitor but later its role was suggested to block the binding of CD4 to gp120 at a sub-micromolar concentration. However, studies of acute toxicity in mice demonstrated that AR177 to be toxic as evident from and histologic vacuolization in several organs (Wallace et al. 2000; Este et al. 1998). On the other hand, experiments using cynomolgus monkeys demonstrated that AR177 did not show any major hemodynamic toxicity (Wallace et al. 1996).

A range of approaches have been developed to block the interaction between HIV-1 and co-receptors, CCR5, CXCR4. The strategies to prevent CCR5 co-receptor binding includes CCR5 small molecule antagonists, mAbs and covently modified non-agonostic natural CCR5 ligands, such as AOP-RANTES. RANTES, MIP-1- α and MIP-1-β were identified as HIV-supressive factors to limit HIV infection. N-terminus modification of RANTES, AOP-RANTES and NNY-RANTES, are nanomolar inhibitors of HIV-R5 starins (Simmons et al. 1997). PRO 140 is another prom-

ising inhibitor of CCR5 that blocks gp120 interaction to CCR5, although it does not prevent CC-chemokine signalling. Anti-HIV activity of PRO 140 was shown to be potent and effective during phase II clinical trials (Trkola et al. 2001).

CCR5 small molecule antagonists, including maraviroc, aplaviroc, vicriviroc and INCB009471, exhibit significant inhibition of HIV-1 replication. Maraviroc as an antagonist of CCR5 has an anti-viral property against all CCR5-tropic HIV strains. Maraviroc also inhibits MIP-1- α as well as RANTES-induced singalling at lower nanomolar concentrations (Dorr et al. 2005). HIV-1 infected subjects achieve ≥1.6 $\log_{10}$ plasma HIV-1 reduction following 600 mg maraviroc daily doses (Fatkenheuer et al. 2005). Aplaviroc is another class of CCR5 antagonist that blocks MIP-1α (Watson et al. 2005), achieving 1.6-$\log_{10}$ reduction levels of plasma HIV- RNA during 10 day administration (Lalezari et al. 2005a, b). However, Aplaviroc has failed in phase II clical trials, because of drug-mediated hepatitis and severe hepatotoxicity (Nichols et al. 2008). Like maraviroc, vicriviroc exerts its anti-viral activity by blocking CC-chemokine signalling at nanomolar concentrations (Strizki et al. 2005). Fourteen-day administration of vicriviroc resulted in lower levels of HIV-1 RNA, down to 1.0–1.5 $\log_{10}$ copies/mL (Schurmann et al. 2007).

CXCR4 antagonists block entry of HIV-1 without affecting the downstream signalling pathway or causing internalization of CXCR4. Enfuvirtide and T-1249 are two well established fusion inhibitors designed to prevent gp41-mediated fusogenic conformation. Enfuvirtide (T-20) binds heptad repeat 1 (HR1) and mimics HR3 fragment of gp41, thereby, blocking viral fusion and entry (Wild et al. 1994). Phase III clinical trial studies have demonstrated the efficacy of enfuvirtide when combined with an optimized background regimen (Lalezari et al. 2003; Lazzarin et al. 2003). Additionally, co-administration of enfuvirtide with other agents such as darunavir, maraviroc, or tirapanavir significantly improved response rates in highly treatment-experienced HIV-1 patients in clinical trials (Hicks et al. 2006; Fatkenheuer et al. 2008; Clotet et al. 2007).

T-1249, is a second generation fusion inhibitor, which has ten-fold more anti-HIV activity (Lalezari et al. 2005a, b). T-1249 was also reported to be significantly active against HIV-1 enfuvirtide-resistant isolates as well as against HIV-2 and SIV (Briz et al. 2006; Melby et al. 2007). However, clinical development of T-1249 was discontinued due to formulation challenges (Martin-Carbonero 2004). Additional oligomeric HIV-1 fusion inhibitor peptides such as TRI-999 and TRI-1144, show better pharmacokinetic profiles. A series of oligomeric HR2 peptides, including T-2635, T-267221, T-267227, have been reported to be 3600 fold more active compared to compound 42 and T-1249, targeted against HR2-resistant viral isolates. Thus, studies using these peptides in cynomolgus monkeys have revealed 100-fold improved pharmacokinetics (Dwyer et al. 2007). The broadly neutralizing antibodies (bnAbs) are another new emerging class of therapeutics for HIV-1, which also participate in clearing viral reservoir cells through activation of host immune system (Grobben et al. 2019) Some of the promising candidates include 3BNC117, VRC01 that resulted in decrease of 0.8–2.5 Log_{10} copies/mL in responsive participants (Caskey et al. 2015; Lynch et al. 2015). The bnAbs have been shown to delay viral rebound in HIV-1 infected individuals post ART (Bar et al. 2016). However, de novo resistance to HIV-1 is one of the serious drawback for the bnAbs based therapy.

Recently, bnAbs 1-18, VH1-46-derived CD4 binders, have been explored (Schommers et al. 2020). These bnAbs have better potency than most of the classical VH1-46- and VH1-2-derived bnAbs. The bnAbs 1-18 effectively restrict viral escape and maintain both neutralizing activity against VRC01-class escape variants and full viral suppression in HIV-1YU2-infected humanized mice (Schommers et al. 2020).

9 Conclusions

The ability of innate immune system to recognise and respond to HIV-1 is a noteworthy approach to control HIV-1 transmission and disease progression. Complement system is recognised as a key mediator of innate immunity, which plays a number of roles in the pathogenesis of HIV-1, either contributing towards anti-HIV-1 activity or enhancement of infection. HIV-1 can directly activate the complement system; conversely, HIV-1 virions can also be resistant to complement-mediates lysis. After initial mucosal penetration, the direct binding of HIV-1-gp41 to C1q triggers activation of the classical pathway. Thus, HIV-1gp120 interaction also activates the classical pathway in an antibody dependent manner. However, HIV-1 utilises complement regulators such as CD59, CD55 as well as factor H, to overcome complement attack, leading to HIV-1 transmission and progression. The interaction between gp41 and CR3 enhances both viral entry as well as viral spread in the cells.

Innate immune response by mucosal epithelial cells lining up the FRT contributes to viral control, but the virus has multiple strategies to escape and overcome anti-viral resistance. Innate immune cells such as macropahges, DCs, NKs, and neutrophils play a significant role against invading HIV-1 virions. Langerhans cells and plasmacytoid dendritic cells have an important anti-viral role through virus degradation via Birbeck granules (BGs) or increased secretion of type I interferons. Type I and II-generated interferon stimulated genes (ISGs) induce signalling events that contribute to the inhibition of HIV-1 replication. TLR7/8 stimulation induces the production of multiple immunomodulatory and anti-viral cytokines. Inhibitory effects of IFN-α on replication of HIV-1 in macropahges and monocytes are well-documented.

ART has emerged as an effective anti-viral therapeutics with protective immune responses in HIV-1 individuals. ART, in combination with anti-microbial peptides (AMPs), including defensins and cathelicidins, suppress HIV-1 replication (Piscitelli et al. 1996; Louie and Markowitz 2002). Moreover, inhibitors that block the binding of HIV-1 gp120-CD4 and co-receptor related enagagment (including CXCR4 and CCR5 antagonists) have also been developed as an effective anti-HIV-1 measure, with improved the morbidity and mortality.

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4 Current Insights into the Host Immune Response to Respiratory Viral Infections

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1 Background

Respiratory viral infections are the leading cause of morbidity globally (Mizgerd 2006). In young children, they are responsible for 60% visits to the pediatricians and a leading cause of more than one quarter of hospitalizations (Tregoning and Schwarze 2010). For instance, respiratory viral infections lead to more than 400,000 hospitalizations per year in children less than 18 years of age in the United States (Miller et al. 2014). Common respiratory viral pathogens including the respiratory syncytial virus (RSV), rhinovirus (RV), influenza virus, parainfluenza virus (PIV), human coronavirus (hCoV), adenovirus and human metapneumovirus often cause acute infections localized to the upper respiratory tract. For most respiratory viral infections, the disease is characterized by usual annual winter or spring outbreaks except for PIV infection that can be prevalent throughout the year. Major factors that affect prevalence and severity of disease include age group and immune susceptibility of individual. From the epidemiological point of view, of all the respiratory viral infections, most studies have focused on RSV, RV and influenza as these are the major viral infections causing most of the hospitalization and illness worldwide. For example, RSV in newborns and infants can affect the lower airways along with the upper airways, resulting in wheeze, shortness of breath, bronchiolitis or pneumonia (Olenec et al. 2010). Rhinovirus is responsible for near about two thirds of asthma exacerbations besides causing the colds (Gern 2015). Influenza is the leading cause of respiratory illness and pneumonia related deaths in both developing as well as developed countries (Newton et al. 2016; Kumar et al. 2018; Khanna et al. 2008). It is ranked amongst the top ten causes of death in the USA (Newton et al. 2016). Influenza viruses, since time immemorial, have been in the spotlight due to genetic drift and genetic shift, that enables it escape the vaccine strategies, which works for most other viruses (Kumar et al. 2018) Similarly, the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), first reported in China in December 2019, spread across the globe with an unprecedented speed causing pandemic coronavirus disease 2019 (COVID-19) with more than 4 milion deaths reported till date (WHO, 2021).

In spite of considerable efforts towards vaccine research, we still do not have it for all respi-

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ratory viruses. Hence alternative approaches must be explored to be able to manage these respiratory infections, some of which often prove fatal to specific category and age group of patients. The human body is well equipped to face any viral infection generating a robust immune response as soon as the specific pattern associated with the pathogens is identified. The respiratory tract is the most important area of the pulmonary system that encounters and serves as the port of entry for a wide range of the environmental and pathogenic insults. It is very often invaded by some of the major respiratory viruses leading to a controlled and coordinated immune response. As soon as a virus infects, the human body activates the innate immune response which is the first line of defense, comprising of physical barriers (mucus and collectins), phagocytic cells, cytokines, interferons (IFNs), and IFN-stimulated genes (ISGs), that collectively defends against the invading virus and clear the infection. These rapid responses subsequently activate the adaptive wing of the host immune system that is mainly comprised of the B cells and T cells that helps in capturing and neutralizing the pathogen.

In summary, the host innate immune system provides the rapid, first line of defense and generates robust pro-inflammatory responses, while the adaptive immune system plays a critical role during the later stages of infection, by specifically clearing the viral pathogens. Both the wings of immunity work in coordination, in order to generate efficient antiviral responses, and a misbalance in any of the immune mechanisms may be detrimental for the host. In this chapter, we reviewed and highlighted the innate and adaptive immune response generated during major respiratory viral infections with an intention to provide more in-depth mechanistic details of host immune response to viral infections and opportunities of therapeutic interventions.

2 Innate Immunity Against Respiratory Viral Infection

The innate immune response, although nonspecific, is the rapid and first line of defense against invading viral pathogens which subsequently activates the adaptive arm of immunity. The host cells recognize the viral components through pattern-recognition receptors (PRRs) (Akira et al. 2006; Medzhitov 2007; Medzhitov and Janeway Jr. 2002). These PRRs then identify the pathogen-associated molecular patterns (PAMPs) (Janeway Jr. and Medzhitov 2002) and danger-associated molecular patterns (DAMPs) to activate the host immune system ultimately leading to secretion of cytokines and chemokines (Wilkins and Gale Jr 2010). Currently, four classes of PRRs, namely the Toll-like receptors (TLRs) (Kawai and Akira 2007), the retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) (Kato et al. 2005), NOD-like receptors (NLRs) (Ting et al. 2008) and AIM2-like receptors (ALRs) (Roberts et al. 2009), are known to be involved in detection of viral components such as the single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), genomic DNA, viral replicative intermediates and viral proteins (Pichlmair and Reis e Sousa 2007). While the TLRs are primarily expressed by the macrophages and dendritic cells (DCs) and sense the viral nucleic acid in the endosomes, the RLRs, NLRs and ALRs are ubiquitously expressed and sense PAMPS in the cytoplasm of infected cells (Kanneganti 2010). Several viruses induce the inflammatory response upon infection. The TLRs (TLR3/7, TLR8/9) and RLRs (RIG-I) senses the viral components and induces the production of inflammatory mediators and type I interferons (IFNs). In monocytes, macrophages and non-immune cells (endothelial cells and epithelial cells), RIG-I and/or TLR3 helps in the sensing of dsRNA; while in the plasmacytoid dendritic cells (pDCs), the TLR7 is highly expressed and acts as the major ssRNA sensor (Kato et al. 2005; Sun et al. 2009; Tsai et al. 2009; Diebold et al. 2004).

2.1 Toll-like Receptors (TLRs)

The TLRs are the key players of innate immunity to viruses by recognizing their PAMPs. The TLR2 and TLR4 senses the viral components such as the envelope glycoproteins /lipoproteins on the cell surface while the TLR3, TLR7, TLR8, and TLR9 are mainly endosomal and senses the viral nucleic acids (Fig. 4.1) (Finberg et al. 2007;

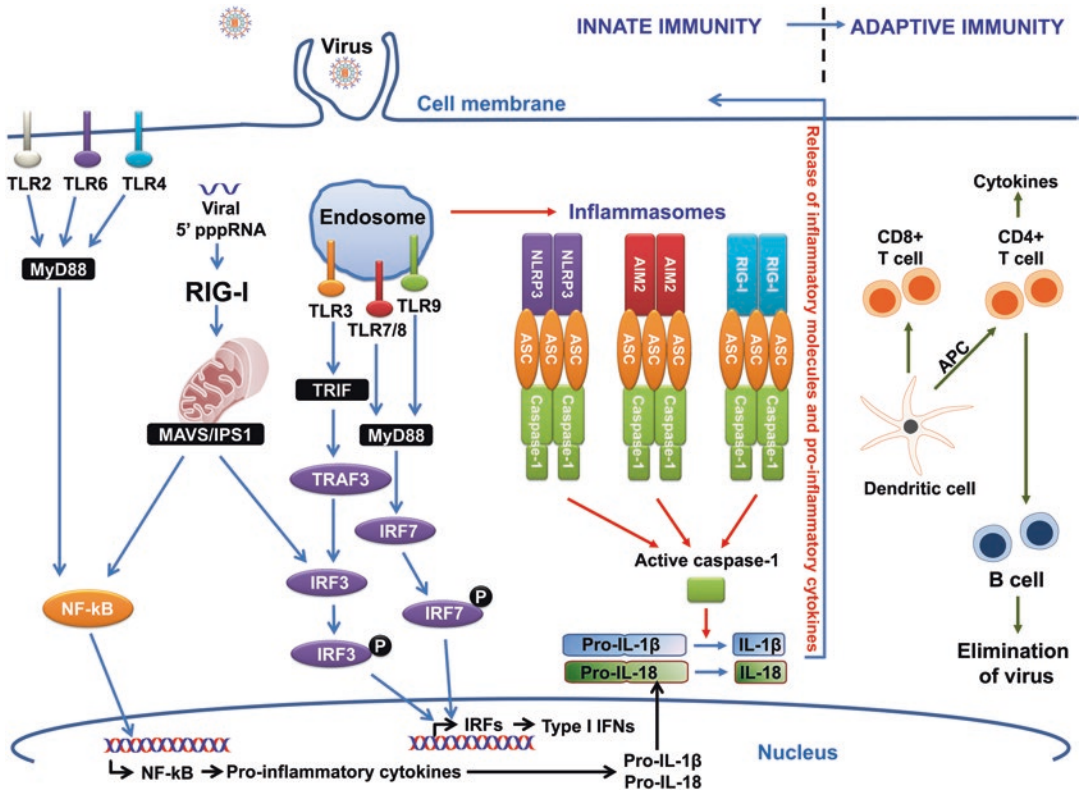

Fig. 4.1 Mechanism of immune response generation following viral infection. Infection with viruses leads to activation of several signaling cascades. TLRs located on either plasma membrane (TLR2, TLR6 and TLR4) or endosomes (TLR3, TLR7/8 and TLR9), sense the PAMPs/DAMPs and activate cellular pathways leading to the production of type I IFNs and proinflammatory cytokines. The viral nucleic acids are also sensed by the PRRs leading to the activation and assembly of different types of inflammasome. The NLRP3 and RIG-I senses the viral RNA while the AIM2 senses the viral DNA and form inflammasome complex with the adaptor ASC and effector caspase-1. An activated inflammasome leads to the production and secretion of proinflammatory cytokines that ultimately causes inflammation. The innate immune response subsequently activates the adaptive immune response where the T cells and B cells play vital roles in elimination of virus

Kawai and Akira 2011). These TLRs are known to recruit specific sets of adaptor proteins such as myeloid differentiation primary response protein 88 (MyD88), TIRAP, TIR-domain-containing adapter-inducing interferon-β (TRIF), or TRIF-related adaptor molecule (TRAM). These adaptor proteins further aid in the signaling pathways that activate NF-κB, interferon regulatory factor7 (IRF7) or IRF3, resulting in induction of antiviral response and secretion of cytokines. During IV infection, the TLRs often cooperate with the NLRs and RLRs to induce the innate immunity (Kawai and Akira 2011). The TLR7/8 binds to the ssRNA while TLR3 senses the dsRNA in the endosomes. A study showed that pretreatment of human monocyte-derived DCs with TLR3 ligand (poly I:C) conferred resistance to infection with highly pathogenic avian H5N1 influenza virus (Thitithanyanont et al. 2007). Another study demonstrated that a lethal dose of influenza virus in mice showed an enhancement of inflammatory reaction and CD8+ T cell response that was mediated by the TLR3. This event was associated with augmented viral clearance, in wild type mice compared to TLR3−/− mice (Le Goffic et al. 2006). The same study, however showed that TLR3−/− mice survived longer than wild-type mice thereby suggesting that the continued adaptive immunity is detrimental to the host (Le Goffic et al. 2006). There are other studies which show that TLR7-and MyD88 induce the high levels of IFN-α, and also inflammatory cytokines

in response to the live or inactivated influenza infection to pDC due to recognition of viral ssRNA (Diebold et al. 2004; Koyama et al. 2007). This TLR7 sensing in the pDC is required to confer protective primary adaptive immune response in mice (Koyama et al. 2007). However, another study by same group revealed that TLR7-induced type I IFN in pDCs was dispensable for induction of protective response to influenza virus in mice that were vaccinated with live-virus vaccine (Koyama et al. 2010). Also the IFN-α secretion in response to influenza virus infection in murine mDC/BMDCs was found to be dependent on live virus replication, but not on TLR7/MyD88- signaling (Koyama et al. 2007; Barchet et al. 2005). The murine BMDCs however, required the TLR7-signaling for the induction of pro-interleukin-1beta (IL-1β) and secretion of mature IL-1β after influenza virus infection (Ichinohe et al. 2010), thereby suggesting that there is a cell-specific role of TLR7 in the influenza virus infection as well as vaccination induced innate immune response. The ligand for TLR4 in influenza virus is not known, however, activation of TLR4-signaling has been shown to release a DAMP molecule (S100A9) in influenza virus-infected lungs that triggers TLR4-MyD88-signaling pathway in macrophages to induce exaggerated inflammatory response, cell-death, and virus pathogenesis following lethal infection (Tsai et al. 2014).

A number of TLRs such as the TLR2, TLR3, TLR4, and TLR7 have also been linked to the RSV infection. A study suggested that TLR2 is a functional receptor for RSV and further demonstrated that TLR2 and TLR6 signaling in leukocytes could activate innate immune response to RSV by promoting proinflammatory cytokines and chemokine production and DCs activation (Murawski et al. 2009). The TLR3 senses the dsRNA generated during the RSV replication cycle (Aeffner et al. 2011). Rudd et al. showed that TLR3 signaling pathways is activated upon RSV infection and regulate the expression of MyD88-independent chemokines, such as IP-10/CXCL10 and CCL5. The same group subsequently showed that upon RSV infection, the TLR3 activated a predominant Th1-type response, whereas the deletion of TLR3 led to increased pathogenic Th2-biased responses that resulted in the production of IL-13 and IL-5 along with accumulation of eosinophils in airways of TLR3−/− mice (Rudd et al. 2006). Another study in the same year showed that the TLR3 expression was enhanced upon RSV infection in the respiratory epithelial cells that further sensitized the cells to subsequent extracellular dsRNA exposure through NF-κB and IL-8 production (Groskreutz et al. 2006). The involvement of TLR4 has also been shown to have effect on RSV infection through its interaction with the RSV F protein using CD14 as a co-receptor (Kurt-Jones et al. 2000). The same study also showed that TLR4 null mice, compared to the TLR4-positive mice, demonstrated reduced pulmonary NK and CD14+ cell trafficking, deficient NK cell function, impaired IL-12 expression and delayed viral clearance in mice challenged with RSV (Kurt-Jones et al. 2000). Later studies demonstrated that the inhibition of the RSV-TLR4/CD14 interaction, suppressed the RSV-elicited production of proinflammatory cytokines IL-6 and IL-8 in epithelial cells (Numata et al. 2010). There are mixed reports for TLR4 expression and its effect on RSV disease severity. While one epidemiological study revealed that two single nucleotide polymorphisms (SNPs) encoding Asp299Gly and Thr399Ile substitutions in the TLR4 ectodomain lead to an increase in the severity of RSV bronchiolitis and subsequent risk for hospitalization (Mandelberg et al. 2006; Tal et al. 2004), another clinical study reported that upregulated TLR4 expression on blood monocytes in infants was found to be linked closely to the disease severity (Gagro et al. 2004). Similarly, the TLR7 can also recognize the RSV infection and regulate DC activation resulting in reduced IL-12 expression while promoting IL-23, that determine Th1 versus Th17 development, respectively (Lindell et al. 2011). The study also showed that TLR7 deficiency is associated with an alteration in T-cell responses.

Similarly, the adenovirus infection is also known to be associated with induction of inflammatory cytokines and the levels correlate with severity of the disease (Mistchenko et al. 1994).

The host inflammatory mediators are majorly enhanced by the adenovirus Early Region 1A (E1A) gene through NF-κB activation. Studies show that the immune response against adenovirus is generated post capsid detection by the PRRs followed by induction of chemokines (Liu and Muruve 2003). Another study conducted on the murine antigen presenting cells and lung fibroblasts demonstrated that adenovirus infection led to the production of type I IFN, IL-6 and TNF-α in an IRF3-dependent but MyD88-independent and TRIF independent manner (Nociari et al. 2007). Similarly the TLR9 also helps in adenovirus recognition. A study showed that the murine pDCs, produced high levels of type I IFN in response to adenoviral vectors in a MyD88 and TLR9-dependent manner (Zhu et al. 2007a). Another study further showed the role of TLR9 in IFN-α induction in the human pDCs (Iacobelli-Martinez and Nemerow 2007). Another supporting study showed the potential role for TLRs in inflammatory disease during adenovirus infection. The study revealed that the MyD88 knockout mice had lower amounts of inflammatory cytokines in their plasma during acute adenovirus infection compared to the wild-type mice (Hartman et al. 2007). Studies have showed that the adenovirus B is sensed by TLR9 in peripheral blood mononuclear cells and pDCs (Iacobelli-Martinez and Nemerow 2007; Sirena et al. 2004). In addition to TLR9, the TLR2 also contributes to the innate immune response against adenovirus as revealed by the reduced NF-κB activation and humoral responses to adenovirus vectors in TLR2 knockout mice (Appledorn et al. 2008). The adenovirus C, along with the coagulation factor X (FX), activates the TLR4 mediated innate immunity and initiates an IL1β inflammatory response (Doronin et al. 2012).

2.2 Retinoic Acid Inducible Gene-I (RIG-I)-like Receptors (RLRs)

The interferon (IFN) system comprises of a broad spectrum of sensors that recognize virus associated molecular patterns. The RLR family has 2 PRRs, MDA5 and RIG-I. The MDA5 recognizes dsRNA while the RIG-I recognize 5′-triphosphate ssRNA (Hornung et al. 2006). RIG-I functions as cytoplasmic sensors for viral RNA to initiate antiviral responses and is the most potent inducer of type I IFN. As soon as the RNA ligand is recognized, the RIG-I switches conformation and associates with the mitochondria based adaptor MAVS to initiate signaling events leading to activation of the transcription factors IRF3 and NF-κB, thereby stimulating the induction of IFNs (Yoneyama et al. 2015).

During influenza virus infection, the RIG-I senses the viral genome bearing 5′-triphosphate-RNA sequence that triggers the cellular innate immune responses during infection (Fig. 4.1) (Rehwinkel et al. 2010; Pichlmair et al. 2006). An interesting study revealed that during influenza virus infection the viral RNA, the antiviral proteins-protein kinase R (PKR) along with the RLRs are localized in stress granules (Onomoto et al. 2012). These stress granules serve as the site for 5′ ppp RNA-induced activation of RIG-I-signaling. The study further showed that influenza virus lacking non-structural protein 1 (NS1) efficiently generated much more of these antiviral stress granules and IFNs compared to the virus encoding NS1 (Onomoto et al. 2012). The authors also observed that the transfection of dsRNA resulted in IFN production in an antiviral stress granule-dependent manner (Onomoto et al. 2012). Similarly other studies also reported the influenza virus induced transient activation of RIG-I in bone marrow-derived DCs (BMDCs) (Koyama et al. 2007), respiratory epithelial cells (Le Goffic et al. 2007; Crotta et al. 2013), macrophages (Ohman et al. 2009; Wang et al. 2012) and mast cells (Graham et al. 2013). The RIG-I has also been shown to be activated by the avian-adapted strains of the PB2-627E genotypes. They show reduced PB2-NP affinity and thus better accessibility of the 5′ ppp-dsRNA panhandle. The study further documented that the avian adapted PB2-627E-type viruses exhibit an enhanced onset of infection in RIG-I knockout cells as well as in chicken cells (Lacks RIG-I) while the mammalian adapted PB2-627K-type viruses showed no difference in replication in

presence or absence of RIG-I (Weber et al. 2015). This could be possible because the PB2-627K-type viruses are less efficient activators of RIG-I due to their higher PB2-NP affinity (Weber et al. 2015).

Similarly the RSV infections have also been shown to upregulate both MDA5 and RIG-I and a strong correlation of RSV viral load and RIG-I mRNA levels have been observed (Scagnolari et al. 2009). The *in vitro* studies also showed that RIG-I mediated detection of the RSV infection leads to downstream NF-κB and IRF3 pathways by complexing with the adaptor MAVS, thereby generating the IFNβ, IP-10, and CCL5 expression in airway epithelial cells (Liu et al. 2007). The silencing of RIG-I also significantly inhibited the activation of NFkB, IRF3, and cytokine expression early during infection (Liu et al. 2007). Later studies also demonstrated that RSV proteins- NS1 and NS2 that directly decreases the RIG-I interaction with MAVS thereby poorly inducing the type I IFNs (Ling et al. 2009). A study further revealed the some strains of RSV are better inducers of IFN (Wright et al. 2006) and this variation in the RSV virulence could be attributed to the expression of the NS2 gene, a major inhibitor of IFN activity (Wright et al. 2006; Ramaswamy et al. 2006), however according to another study, the NS1 gene may also have a role in inhibiting IFN activity (Spann et al. 2004). Thus multiple parameters have been shown to be involved in shaping the innate immune response to RSV infections.

Adenoviruses encode two noncoding small RNAs, virus-associated (VA)-RNA I and VA-RNA II which are transcribed by RNA polymerase III at high levels during adenovirus replication (Mathews and Shenk 1991). In a study, these VA-RNAs have been reported to induce the production of type I IFN (IFN-α and IFN-β) but not the inflammatory cytokines (IL-6 and IL-12), in mouse embryonic fibroblasts (MEFs) and granulocyte-macrophage colony-stimulating factor–generated bone marrow-derived dendritic cells (GM-DCs). The study revealed that the IFN-β promoter stimulator-1 is involved in VA-RNA–dependent IFN-β production in the MEFs and is partially involved in the production of type I IFN in the GM-DCs (Yamaguchi et al. 2010).

2.3 Virus-Induced Inflammasomes

An inflammasome is a multi-protein complex comprised of a sensor, an adaptor and an effector molecule that have been shown to mature the proinflammatory cytokines, IL-1β and IL-18 via the caspase1 activity (Thomas et al. 2009). Among the various NLR family members, the nucleotide and oligomerization domain, leucine-rich repeat-containing protein family, pyrin domain containing 3 (NLRP3) is a key protein that is known to assemble a large protein complex comprised of ASC and caspase-1, called the inflammasome (Lupfer and Kanneganti 2013). Similarly the RIG-I and AIM2 can interact with ASC and caspase-1 to form the RIG-I and AIM2 inflammasomes respectively during viral infection. Several respiratory viruses such as the influenza virus, RSV, the adenovirus and the coronavirus have been known to induce inflammation and activate the inflammasomes (Table 4.1).

In addition, they are also reported to induce the gasdermin D-mediated pyroptotic cell death driven by the enzymatic activity of caspase-1. The detailed mechanism and signaling cascades required to activate an inflammasome leading to innate immune response has been shown in Fig. 4.1.

2.3.1 NLRP3 Inflammasome and Respiratory Viruses

NLRP3 inflammasome is one of the best characterized inflammasome and is comprised of a C-terminal leucine-rich repeats (LRRs), a central nucleotide-binding and oligomerization domain (NACHT) or the nucleotide binding oligomerization domain (NOD) with an ATPase activity, and a N-terminal pyrin domain (PYD) that recruits apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) (Kufer et al. 2005). The activation of a NLRP3 inflammasome is regulated at both transcriptional and post-translational levels. A priming signal induced by the TLR/ nuclear factor (NF)-κB pathway upregulates the pro-IL-1β and NLRP3 expression. A second signal from the PAMPs and DAMPs further initiates the assembly of the multiprotein

Table 4.1 Respiratory viruses that activate inflammasomes

Inflammasome	Respiratory virus	PAMPs recognized	Refs.
NLRP3	Influenza virus, RSV, Adenovirus, SARS-CoV2	RNA	Allen et al. (2009), Kuriakose et al. (2016), Kanneganti et al. (2006), Triantafilou et al. (2013), Segovia et al. (2012), Barlan et al. (2011a), Rodrigues et al. (2021)
RIG-I	Influenza virus	RNA	Graham et al. (2013), Pothlichet et al. (2013)
AIM2	Influenza virus, Adenovirus	Not determined, DNA	Zhang et al. (2017), Schulte et al. (2013), Eichholz et al. (2016)

complex containing the NLRP3, the adaptor protein ASC and the inactive zymogen form of cysteine protease casepase-1 that collectively activates the caspase-1 that executes the maturation and secretion of IL-1β and IL-18 cytokines (Fig. 4.1) (Martinon and Tschopp 2007). Several events, such as the ATP mediated efflux of PAMPs (He et al. 2016), lysosomal destabilization (Okada et al. 2014) and mitochondrial reactive oxygen species (ROS) generation (Sorbara and Girardin 2011), have been reported to activate the NLRP3 inflammasome. In addition to TLRs, the RIG-I-MAVS signaling has also shown to be the initiating step in the upregulating Nlrp3 expression subsequently leading to inflammatory condition (Pothlichet et al. 2013).

The NLRP3 inflammasome has also been shown to be activated and modulated by viruses belonging to different families. Influenza viruses are the most common activators of NLRP3 inflammasome. Soon after the identification of caspase-1 as the IL-1β processing enzyme, Pirhonen et al. showed that influenza virus infection induced the cleavage of caspase-1, caspase-3 and caspase-dependent secretion of IL-1 and IL-18 in human macrophages (Pirhonen et al. 1999; Pirhonen et al. 2001), however the mechanism of caspase-1 activation was identified only after the discovery of NLRP3 inflammasome (Martinon et al. 2002; Mariathasan et al. 2006). A study by Kanneganti et al. showed that influenza virus infection induced the NLRP3 mediated processing of caspase-1 and secretion of IL-1β and IL-18 in the bone marrow derived macrophages (BMDMs) (Kanneganti et al. 2006). They further showed that the synthetic RNA analogues also induced the NLRP3 activation in mouse and human cells (Kanneganti et al. 2006). Although several studies have shown the viral RNA as inducer of NLRP3 assembly, yet the molecular mechanism behind this event is still not clear. Further studies however, have shown a possibility of viral RNA interaction with RNA sensors such as the TLRs and RIG-I leading to the inflammasome assembly and release of inflammasome-dependent cytokines (Kuriakose et al. 2016; Pothlichet et al. 2013; Ichinohe et al. 2009). In addition, the influenza virus infection-induced activation of NLRP3 inflammasome has also been demonstrated in non-immune cells such as the primary bronchial epithelial cells, lung fibroblasts, and few other epithelial cells (Allen et al. 2009; Pothlichet et al. 2013; Ichinohe et al. 2009). Three crucial *in vivo* studies further strengthened the previous findings and identified the critical role of the NLRP3 inflammasome in the innate immune response to influenza virus infection (Thomas et al. 2009; Allen et al. 2009; Ichinohe et al. 2009). The studies collectively showed that influenza virus infection increased the expression of IL-1β in the bronchoalveolar lavage fluid (BALF) from wildtype mice but not in Nlrp3−/−, Casp1−/−, or Asc−/− mice thereby showing the requirement of inflammasome complex proteins in eliciting an antiviral response (Thomas et al. 2009; Allen et al. 2009). Further the mice deficient in inflammasome components also had reduced inflammatory cytokines (TNF, IL-6 and the chemokines KC and CXCL2) in their BALF 3 days post infection (Thomas et al. 2009; Allen et al. 2009). The study also showed that the Nlrp3−/−, Casp1−/−, or Asc−/− mice were more susceptible to infection with mouse-adapted pathogenic H1N1 A/PR/8/1934 strain of influenza virus and the viral sensing by the inflammasome mechanistically induced the inflammation (Thomas et al. 2009; Allen et al. 2009). Other related studies utilized a specific and potent

inhibitor of NLRP3, MCC950, and showed the importance of NLRP3 inflammasome in protection against influenza virus infection. The authors gave an intranasal treatment with MCC950 on day 1 post influenza virus infection and observed a significant decrease in the mice survival rate, however the same treatment on day 7 conferred protection against influenza virus and increased survival rate possibly due to decrease in the levels of proinflammatory cytokines including IL-1, IL18, IL-6, TNF and reduction in the recruitment of inflammatory cells into the airways, thereby demonstrating a detrimental role of NLRP3 inflammasome activation later during influenza virus infection (Tate et al. 2016). This is one of the major factors playing role in the influenza associated mortality. More recent studies also demonstrated the formation of ASC specks, representative of an inflammasome assembly, specifically in the influenza virus infected cells as observed by the co-staining of ASC with influenza virus nucleoprotein in the mice lung fibroblasts (Tzeng et al. 2016). Another interesting study performed on mice with old and young age groups infected with influenza virus, demonstrated that the DCs from elderly mice showed reduced expression of NLRP3 components (NLRP3, ACS, Caspase-1) and thus decreased IL-1β secretion, compared to young mice; which was rescued by the treatment with nigericin that augments inflammasome activation and thereby reduced the morbidity and mortality in elderly mice (Stout-Delgado et al. 2012). Similar studies have also been conducted on primate models to show that infection with the reconstructed 1918 pandemic influenza virus markedly enhanced the expression of both Nlrp3 and Il1b along with other genes associated with inflammation in the macaques (Cillóniz et al. 2009). This study also showed the detrimental effect of inflammasome activation leading the excessive recruitment of inflammatory cells into the lungs resulting in a cytokine storm (Cillóniz et al. 2009). A similar study conducted on macaques also reported enhanced levels of IL-1β upon infection with the highly pathogenic avian influenza virus (H5N1 strain) (Baskin et al. 2009). Recent studies have also shown that patients hospitalized with avian origin H7N9 influenza pneumonia demonstrated increased IL-18 mediated IFN-γ production in $CD161^{+}V\alpha7.2^{+}$ mucosal-associated invariant T cells (Loh et al. 2016). Another similar study reported a consistent finding that the BAL specimens obtained from patients infected with the H7N9 virus had 1000 fold increase in several proinflammatory cytokines including the IL-1β leading consequently leading to severe lung damage in those patients (Wang et al. 2014). A group of authors also showed that children infected with the H1N1 influenza virus had elevated levels of IL-1β (Chiaretti et al. 2013). Although the exact molecular mechanisms behind activation of NLRP3 inflammasome by the influenza virus need more detailed investigations, several studies do providing significant information related to this event. A study conducted on the primed macrophages and DCs showed that the strain specific M2 protein of influenza virus, that has channel activity, activates the NLRP3 inflammasome and its ion channel activity was required for the event (Ichinohe et al. 2010). Another study on DCs demonstrated that the activation of inflammasome could be blocked by inhibiting the activity of the M2 ion channel by amantadine, a known M2 ion channel blocker (Fernandez et al. 2016). Another potential pathogen-associated molecular pattern is the PB1-F2 protein found in several strains of influenza A virus, including the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemic strains, along with avian H5N1 and H7N9 subtypes. A study conducted on the human peripheral blood mononuclear cells (PBMCs) and mice BMDMs demonstrated an enhanced NLRP3 inflammasome activation *in vitro* as well as in mice challenged with the influenza A virus and recombinant PB1-F2 (McAuley et al. 2013; Pinar et al. 2017). More recent studies have shown that the RNA and the TRIM-25 domain of the NS1 protein of influenza virus can aid in binding with NLRP3 directly and inhibit the assembly of the NLRP3-ASC-caspase-1 complex and secretion of IL-1β fir its own survival and advantage (Moriyama et al. 2016). Influenza viruses have simultaneously evolved to utilize the inflammasome and they manipulate the host innate immune response for their own survival and advantage. Several studies have documented both protective as well as detrimental effect of the NLRP3 inflammasome activation upon influenza virus infection in both murine and primate models, including humans infected with influenza

virus. Thus, the therapeutic management of the inflammasome activation at different steps can provide protection against the excessive inflammatory response generated during infection. For example the therapeutically targeting TLR2 and TLR4 has been shown to provide protection against influenza A virus (IAV) infection. The studies showed that mice treated with TLR4 antagonist (Eritoran) or anti-TLR4 or anti-TLR2 IgG post influenza infection, reduced the pro-inflammatory cytokine production and overall mortality (Shirey et al. 2016; Shirey et al. 2013).

Similarly, the RSV, a leading cause of respiratory infections in infants and young children, has been shown to activate NLRP3 inflammasome (Triantafilou et al. 2013; Takeuchi et al. 1998). The study showed that RSV activated the IL-1β-converting enzyme (caspase-1) gene and its protein due to an increase in the IRF1 induction in the human alveolar epithelial cells (Takeuchi et al. 1998). Another study conducted on bone marrow derived macrophages revealed that reactive oxygen species (ROS)/potassium efflux (second signal) as well as the TLR2/MyD88/NF-κB pathway (first signal) was essential for the induction of NLRP3/ASC inflammasome assembly subsequently leading to the activation of caspase-1 and processing of IL-1β during the RSV infection (Segovia et al. 2012). Another study revealed the role of the RSV-Viroporin, SH, in triggering the signal for inflammasome activation (Triantafilou et al. 2013). Another interesting study evaluated the role of the TRIM33, a member of the tripartite motif (TRIM) family, in inflammasome activation during RSV generated dsRNA intermediates production (Weng et al. 2014). The study showed that upon RSV infection in THP-1–derived macrophages and human monocyte-derived macrophages, the TRIM33 binds to DHX33, which is a cytosolic dsRNA sensor for the NLRP3 inflammasome, and induces caspase-1–dependent production of IL-1b and IL-18 (Weng et al. 2014). A recent study also showed that inhibition of the inflammasome pathway shows better therapeutic effects on lung inflammation (Shen et al. 2018). The study showed the benefits of Jinxin oral liquid (JOL), derived from traditional Chinese medicine, to reduce the RSV induced excessive inflammation in BALB/c mice. The treatment with JOL also reduced the release of IL-1β, IL-18 and IL-33, in the serum and lung homogenate of the RSV-infected mice mainly through blocking the NLRP3/ASC/Caspase-1 signaling pathway (Shen et al. 2018). Thus the existing evidences show that the inflammasome plays an important role in viral recognition and the initiation of antiviral responses during RSV infection.

The human adenovirus also activates inflammasome upon infection. Studies have shown the sensing of viral DNA and subsequent assembly of the NALP3-ASC-caspase-1 complex upon infection of macrophages (Muruve et al. 2008). The study also demonstrated reduced innate inflammatory responses to adenovirus particles in NALP3- and ASC-deficient mice and that the sensing was due to ASC and not NLRP3 (Muruve et al. 2008). Barlan et al. showed that the adenovirus C5 activated NLRP3 inflammasome in THP1 cells conditioned with phorbol esters leading to production of IL1β and the release of lysosomal cathepsin B to the cytosol (Barlan et al. 2011a; Barlan et al. 2011b). A similar finding was revealed with adenovirus C5_dE1 vector or liposome-mediated transfection of purified adenovirus-C5_dE1 DNA to skin or HKT cells led to the expression of inflammatory cytokines and type 1 IFN-β (Schulte et al. 2013; Steinstraesser et al. 2011). SARS-CoV2 enters the target cells utilizing the hACE2 receptor (Kumar et al. 2021) and injuries caused to the alveolar epithelial cells have been reported to activate the NLRP3 inflammasome (Chen et al. 2019). Recent studies have also demonstrated the activation of NLRP3 inflammasomes in COVID-19 patients thereby suggesting its role in the pathophysiology of the disease (Rodrigues et al. 2021; Freeman and Swartz. 2020).

2.3.2 RIG-I Inflammasome

The RLR family member, RIG-I, is known to induce the type I IFN production collectively through its two N-terminal CARDs (recruits several adaptor proteins), a central RNA helicase domain (has ATPase activity) and C-terminal regulatory domain (CTD) that binds to the dsRNA (Kolakofsky et al. 2012). The dsRNA replication intermediates of several RNA viruses have been shown to be recognized by the RIG-I leading to inflammatory response (Yoneyama et al. 2004). Among the respiratory viruses, influenza virus is well known to activate the RIG-I. RIG-I, which is

a cytosolic sensor, detects influenza virus through recognition of 5′-triphosphates on genomic single stranded RNA (ssRNA), which is revealed after viral fusion and replication (Pichlmair et al. 2006). The RIG-I can either assemble into an active inflammasome or induce the type I IFNs to indirectly regulate the inflammasome assembly (Fig. 4.1) (Pothlichet et al. 2013; Poeck et al. 2009). The study for the first time demonstrated the relative roles of the RIG-I (DDX58), TLR3, and NLRP3 in the IL-1β response to influenza A virus infection in primary lung epithelial cells (Pothlichet et al. 2013). The influenza virus infection strongly demonstrated the RIG-I mediated type I IFN signaling and also activated the RIG-I inflammasome by interacting with ASC and caspase-1 (Pothlichet et al. 2013). The authors further demonstrated that influenza virus infection to normal human bronchial epithelial cells leads to formation of RIG-I/ASC and RIG-I/Caspase-1 complexes subsequently leading to IL-1 secretion, thereby also suggesting the assembly of inflammasome independent of NLRP3 (Pothlichet et al. 2013).

2.3.3 AIM2 Inflammasome

The AIM2-like receptors (ALR) senses the double-stranded DNA via the HIN200 domain of absent in melanoma 2 (AIM2) proteins, and interact with the caspase-1 protein via a PYD domain (Fig. 4.1) (Hornung et al. 2009). Influenza viruses have recently been shown to activate the AIM2 dependent inflammasome (Zhang et al. 2017). The study utilized the lethal dose of A/PR8/34 and A/California/07/09 strains of influenza A virus to infect the mice. The study revealed that influenza virus infection in wildtype mice significantly enhanced the AIM2 expression, induced the dsDNA release and further stimulated the caspase-1 activation and release of processed IL-1β in the lung, compared to the mice deficient in AIM2 (Zhang et al. 2017). The study also provides an interesting observation that AIM2 deficiency did not affect the transcription of caspase-1 and IL-1β, in fact the AIM2 deficient mice exhibited attenuated lung injury and significantly improved survival against influenza A virus challenges (Zhang et al. 2017). The AIM2 generates an efficient innate immune response, and it can be detrimental to influenza-induced lung injury and mortality.

The human adenovirus also activates the AIM2 inflammasome upon infection (Schulte et al. 2013). A recent study performed utilizing the immune-complexed human adenovirus revealed that protein VI-dependent endosomal escape is required for the adenovirus genome to be sensed by AIM2 in mDCs (Eichholz et al. 2016). The AIM2 then induces pyroptotic MoDC death via ASC aggregation, inflammasome assembly, caspase-1 activation and and IL-1β and gasdermin D cleavage (Eichholz et al. 2016).

2.4 Cyclic GMP-AMP Synthase (cGAS) Sensing of Virus

cGAS is a nucleotidyl-transferase that senses cytosolic DNA via the sugar backbone to produce a cyclic guanine-adenine dinucleotide (cGAMP) that binds to the adaptor protein STING (Sun et al. 2013) ultimately and leading to the activation of TBK1 and IRF3 and the production of IFN-β (Ablasser et al. 2013; Zhang et al. 2013). The human adenoviral DNA is also sensed by the cGAS triggering the IFN response in murine RAW 264.7 macrophage-like cells (Lam et al. 2014). In a more detailed study involving replication-defective adenovirus vectors and replication-competent wild-type adenovirus, a group of authors utilized permissive human cell lines (A549, HeLa, ARPE19, and THP1) to silence cGAS/STING and observed a loss of TBK1 and IRF3 activation, a lack of IFN-beta transcript induction, loss of IFN-dependent STAT1 activation, and reduced induction of interferon-stimulated genes (ISGs) (Lam and Falck-Pedersen 2014). A recent study utilized the murine model to understand how the cGAS/STING cascade influences the antiviral innate and adaptive immune responses (Anghelina et al. 2016). The authors observed that cGAS and STING were essential for the induction of the antiadenovirus response in the knockout BMDCs

and BMDMs (Anghelina et al. 2016). The study further demonstrated that the infection of cGAS$^{-/-}$ and STING$^{-/-}$ mice showed significantly lower levels IFN-β secretion and low levels of proinflammatory chemokine induction (Anghelina et al. 2016).

3 Adaptive Immunity Against Respiratory Viral Infection

The pathogenesis of respiratory viral infections is thought to be determined by cell tropism for human airway and alveolar epithelial cells, viral replication rate and the intensity and dynamics of the inflammatory response (Herold et al. 2015). Although a robust innate immune response becomes activated, it is not able to control the virus, often resulting in death due to direct viral damage to the airways (Korth et al. 2013). Thus a second wing of the immune system takes over the charge to manage the prevailing infection and deal with it more specifically and also make sure to have memory recalls for these viral infections. Lymphocytes are the key players during the induction and expression of adaptive immunity. They comprise of 2 classes of lymphocytes, the B cells and T cells, each bearing molecules on their surface that arm them to discharge their specialized role in the immune response. Host responses to viral infection in the lung are highly dynamic and comprise multiple sequential waves of gene expression (Pommerenke et al. 2012; Zhai et al. 2015). The first wave peaks around 2–5 days post infection, and is characterized by upregulation of innate immune networks, interferon and NK cell responses, and proinflammatory cytokines and chemokines. The next wave spikes on day 8 post infection and this is defined by upregulation of T cell activation and induction of apoptosis. This is followed by upregulation of B cell activation and proliferation, which is maximal around day 14. Around 30 days post infection, differentiation and tissue repair processes are upregulated, and these responses persist out to 60 days post infection, suggesting long term or permanent alterations to the lung in case of some viral infections (Pommerenke et al. 2012).

3.1 T Cell Responses

T cells are mainly of two types, CD4+ T and CD8+ T cells. Cytotoxic T lymphocytes (CTLs) differentiated from CD8+ T cells, produce cytokines and effector molecules to control viral replication and destroy virus-infected cells. Influenza viruses (IV) have also been shown to utilize the T cells and B cells as key players to activate adaptive immunity. Upon infection with IV, the infected respiratory tract becomes the primary site of initiation of adaptive immune T cell responses. The capture of viral antigen by resident respiratory DCs traveled from lungs to the T-cell region of the draining lymph nodes activates naïve CD8+ T cells leading to T-cell proliferation and differentiation into CTLs (Kreijtz et al. 2011; Ho et al. 2011). Type I IFNs, IFN-γ, IL-2, and IL-12 also assist CD8+ T cells to differentiate into CTLs (Pipkin et al. 2010; Whitmire et al. 2005). Not only this, but IFN-λs also enhances the T-cell proliferation during influenza virus vaccination (Egli et al. 2014). The migration of CTLs from lymph nodes to the lungs where they effectively kill influenza infected cells is aided by the decrease in expression of CCR7 and an upregulated expression of CXCR3 and CCR4. Once the CTLs reach the targeted infected cells they produce cytotoxic granules containing molecules like perforin and granzymes (e.g., GrA and GrB). Perforin forms pores on the cell membrane of infected cells and thereby facilitates passive diffusion of granzymes to induce successful apoptosis. Besides above stated mechanism, granzymes are also capable of restricting virus replication through cleavage of both, viral and host cell proteins involved in the process of protein synthesis (van Domselaar and Bovenschen 2011; Andrade 2010). Apoptosis can also be induced by recruitment of death receptors by cytokines (FASL, TNF, and TRAIL) produced by CTLs (Allie and Randall 2017).Once formed after the primary infection, these virus-specific CTLs and DCs circulate at the site of infection, in the blood and lymphoid organs (Rangel-Moreno et al. 2011). In case of secondary infection, the memory CTL cells are quick to response and the entire process of activation and differentiation completed during the first infection enhances the

overall proficiency and efficiency of CTLs altogether (van Gisbergen et al. 2011). The serotype cross-reactive cytotoxic T lymphocytes (CTL) recognizing the conserved epitopes of structural proteins, such as NP, M1, and PA contributes to heterosubtypic immunity to influenza A virus (Grant et al. 2016). Most recent studies suggest that antibodies (Abs) may make a significant contribution to these heterosubtypic immunity. Studies have suggested that the cytotoxicity of the memory CD8+ T cells decreases significantly due to decrease in cytolytic molecule expression leading to deteriorated target competence (Grant et al. 2016). This may be one of the reasons why influenza A virus -specific CD8+ T cells only lasts for 2 years in murine models (Valkenburg et al. 2012). Atg7-deficient mice are shown to be incapable of forming CD8+ T cell memory against influenza A virus infection making autophagy a critical factor for the establishment of memory CD8+ T cells (Puleston et al. 2014). Remarkably, influenza A virus—specific memory CD8+ T cells in the nasal epithelia stops the spread of the virus from the upper respiratory tract to the lung, consequently blocking the development of pulmonary disease (Pizzolla et al. 2017). Also, lung-resident memory CD8+ T cells restrain viral replication and facilitate viral elimination thereby defending against heterologous influenza A virus infection (Van Braeckel-Budimir and Harty 2017). During influenza A virus infection, lung-resident monocytes help upkeep lung-resident CD8+ T cell (Dunbar et al. 2016). CD4+ T cell is another component of adaptive immunity that targets influenza A virus-infected epithelial cells through MHC class II. Studies have shown expression of induced MHC class II in epithelial cells of infected cells in murine models (Brown et al. 2012; McKinstry et al. 2012). Numerous co-stimulatory ligands expressed by CD4+ T cells, including CD40 ligand (CD40L) activates B cell and stimulate antibody production (Swain et al. 2012). CD40L in particular has been shown to increase immune response against the highly mutated HA protein of influenza A virus (Yao et al. 2010). In influenza A virus infected cells, CD4+ T cells also get activated in a similar manner to CD8+ T cells. In short, the activation takes place by DCs migrating from the lung to the draining lymph nodes (Ingulli et al. 1997; Lukens et al. 2009). CD4+ T cells mature into Th1 cells in response to infection. This differentiation of CD4+ is decided by either stimulators, co-stimulatory molecules, or cytokines secreted by epithelial cells, DCs, and inflammatory cells (Magram et al. 1996; Pape et al. 1997). Th1 effector CD4+ T cells expressing antiviral cytokine, such as IFN-γ, TNF, and IL-2 (Szabo et al. 2000), activates alveolar macrophages (Liu et al. 2012). Differentiation of CD8+ is regulated by the IL-2 and IFN-γ produced by Th1 cells to clear the viral infection (Shu et al. 1995; Stuber et al. 1996). Recent findings have suggested that IL-2, produced by CD4 T along with IL-27 produced by innate cells in the lung, may induce IL-10 production by virus-specific CD4 and CD8 T cells, during acute influenza virus infection (Sun et al. 2011a). CD4+ T cells also differentiate into Th2, regulatory T cells (Treg cells), Th17, and sometimes as killer cells (Zhu et al. 2010; Lamb et al. 1982). Studies have shown that both Th17 and Treg cells are involved in regulating cellular immunity against influenza A virus infection (Mukherjee et al. 2011). Though studies have shown that CD4+ T cells can direct CD8+ T cell responses through secretion of various cytokines, studies in mice model has shown that primary CD8+ T cell response against influenza A virus infection could be initiated independently of CD4+ T cells (La Gruta and Turner 2014).

Similarly in case of a primary RSV infection, the host needs induction of an appropriate cellular immune response to clear the viral infection. The role of adaptive cellular immunity in reducing RSV viral titers is especially evident in children with defective T-cell responses that demonstrate prolonged virus shedding and experience increased disease severity (Fishaut et al. 1980; Hall et al. 1986). However, despite their critical role in viral clearance, CD8 T cells may also contribute to disease. In vivo depletion of CD8 T cells prior to acute RSV infection results in a significant reduction in weight loss in mice (Graham et al. 1991a). In contrast to the histological analysis of fatal RSV cases that indicated few CD4 and CD8 T cells could be observed in the lung as described above, virus-spe-

cific CD8 T cells can be readily detected in the bronchial alveolar lavage and peripheral blood of RSV-infected infants (Heidema et al. 2007). However, no comprehensive human studies to date have evaluated the relationship between the magnitude of the virus-specific CD8 T-cell response during a primary RSV infection and disease severity. Increased CD8 T cells in young children contribute to protective immunity against secondary infection with RSV (Mbawuike et al. 2001). Previous work in mice models have indicated that depletion of CD8 T cells delays viral clearance (Graham et al. 1991b). RSV M282–90-specific CD8 T cells have been known to mediate viral clearance, in BALB/c mice (Chang and Braciale 2002). IFN-c production by RSV-specific CD8 T cells is a critical determinant for their ability to eliminate virus. Data from recent studies suggested that during acute RSV infection FasL may also play a role in viral clearance. FasL-deficient mice exhibit delayed clearance of RSV as compared to wild-type controls (Rutigliano and Graham 2004).

Asthma yet another infection of airways shares some characteristics with RSV-induced pulmonary pathology such as the induction of airway hyper responsiveness and mucus production. Asthma and RSV, both diseases are characterized by the morphologic changes in the lung and production of cytokine such as IL-4, IL-5, and IL-13 along with development of pulmonary eosinophilia identifying Th2 responses in both diseases.

RSV peaks between 2 and 6 months of age in young children. At such young stage there are insufficient DCs signals to activate naïve T cells. There have been reports establishing a correlation between the levels of Th2 cytokines and RSV-induced disease severity in young children (Legg et al. 2003; Roman et al. 1997). However, other studies proved otherwise (Brandenburg et al. 2000; Garofalo et al. 2001). Many factors including the source/timing of the samples analyzed, age and time following initial infection, along with the role of Th1 and Th2 cells in RSV-induced disease may be reasoned for these observed differences. Recent data have indicated that Th17 cells may also affect RSV-induced disease severity (Mukherjee et al. 2011; Lukacs et al. 2010; Kallal et al. 2010). IL-17 and related family of cytokines have been shown to play pro-inflammatory role in various autoimmune diseases. IL-17A plays a crucial role in host defense against bacterial and fungal infections whereas IL-17F is involved in the development of asthma and airway inflammation. Tracheal aspirate samples obtained from RSV-infected infants have shown elevated levels of IL-17A protein (Mukherjee et al. 2011) in mice with acute RSV infection, neutralization of IL-17A results in a significant decrease in mucus production (Mukherjee et al. 2011). Thus, IL-17 may play an important role in RSV-induced disease. Acute RSV infection also results in an increase in the number of Tregs in the lung (Fulton et al. 2010; Ruckwardt et al. 2009). The kinetics of the Treg response in the lung seems to match with the kinetics of the RSV-specific CD4 and CD8 T-cell response (Fulton et al. 2010). Abolition of Tregs subsequent to acute RSV infection affects multiple aspects of the adaptive immune response by increasing secretion of the pro-inflammatory cytokine IL-6 and cellular infiltrate into the lungs (Fulton et al. 2010; Ruckwardt et al. 2009; Lee et al. 2010). Recent study has suggested that Tregs can prevent immunopathology via granzyme B without compromising host defense (Ruckwardt et al. 2009; Lee et al. 2010; Loebbermann et al. 2012a). Several works have established production of IL-10 by Tregs and RSV-specific CD4 and CD8 T cells during acute RSV infection (Loebbermann et al. 2012b; Sun et al. 2011b; Weiss et al. 2011). CD4 T cells make up the majority of the IL-10-producing cells in the lung following acute RSV infection (Loebbermann et al. 2012b; Sun et al. 2011b; Weiss et al. 2011). IL-10 produced by CD4 T cells, plays a significant role in limiting the development of pulmonary immunopathology in case of acute RSV infection. Recent findings, have suggested IL-2 produced by CD4 T along with IL-27 produced by innate cells in the lung may induce IL-10 production by virus-specific CD4 and CD8 T cells, during acute influenza virus infection (Sun et al. 2011a).

Similarly, the adenovirus is also reported to cause nonlethal disease in humans; however the infection may prove fatal in case of immunocompromised patients (Lynch 3rd and Kajon 2016). The infection by an adenovirus is sensed by the immune system as soon as it is in bloodstream. The viral infection triggers the proinflammatory signaling cascade followed by the attraction of cytotoxic immune cells to the site of infection (Atasheva and Shayakhmetov 2016). After few infections by adenoviruses, the immune system generates a long term humoral and T-cell responses reported to provide subsequent virus-induced morbidity (Flomenberg et al. 1995). Not many studies have focused on the adenovirus specific cellular immunity due to the limited permissiveness of infection in the murine models. The cellular immune responses, however, are consequently dominated by the CD8+ cytotoxic T cells (CTLs) specific for early proteins (Mullbacher et al. 1989; Rawle et al. 1991). A recent study conducted on primary human blood cells found that healthy adults harbor the adenovirus-specific Tregs (Tran et al. 2018). The study revealed that the adenovirus–antibody complexes are taken up by the DCs and favor the bystander DCs to become tolerogenic which subsequently helps in the formation of adenovirus-specific Tregs (Tran et al. 2018). The study also proposed that this mechanism likely favors the pathogen survival; however the same may be advantageous to the host too (Tran et al. 2018).

3.2 B Cell Responses

Antibodies play a vital role in preventing reinfection with viruses by either directly neutralizing or assisting in the opsonization of extracellular virus particles. Naïve B cells along with memory T cells are crucial for preparing the defense against heterosubtypic influenza virus strains (Rangel-Moreno et al. 2008). Non-neutralizing antibodies produced by B cells assist in viral elimination and fastens the expansion of memory CD8+ T cell after heterosubtypic infection (Rangel-Moreno et al. 2008). In addition, influenza specific antibody-dependent cell-mediated cytotoxicity (ADCC) also participates in the cross-reaction against different HA subtypes (Jegaskanda et al. 2014). IgG along with IgA plays important role in protecting against influenza A virus infection in the respiratory tract. IgG plays crucial role in inhibiting pathogenesis while IgA keeps a check on transmission of influenza A viruses (Seibert et al. 2013). Both, the lifespan and response time of memory B cells and plasma cells are crucial for the protective antibody response of influenza A virus vaccines. In elderly population, the memory B cells are maintained, but still multiple influenza A virus immunizations also fails to establish the antibody response. This may be caused due to a potential defect in the development of plasma cells, after certain age (Frasca et al. 2016).

The RSV infection also provokes antibody responses. The RSV attachment (G) and fusion (F) proteins are the major targets of RSV-specific neutralizing antibodies. Both IgA and IgG play important roles in protecting infection against upper respiratory tract infection. However, B cell depletion does not play significant role in viral clearance during primary RSV infection but significantly affects the rate of viral clearance after secondary infection (Graham et al. 1991a). Following acute RSV infection, the frequency of antibody-secreting plasma cells rapidly declines in the upper respiratory tract (Singleton et al. 2003). In case of acute RSV infection in young children, both the IgA and IgG titers decrease rapidly. In children, the immaturity of the immune system and the presence of maternal antibodies contributes to diminished antibody responses induced by acute RSV infection (Kasel et al. 1987). Since neutralizing RSV-specific antibody responses could only been detected in 50–75% of infants (Brandenburg et al. 1997; Murphy et al. 1986) population, thus antibody response generated after RSV infection fails to establish long-lasting immunity and prevent periodic reinfections throughout life. Several RSV-encoded proteins have been shown to modulate the innate and adaptive immune responses. For example, the two nonstructural proteins NS1 and NS2 have been

shown to act individually as well as simultaneously to inhibit type I IFN production as well as signaling (Ramaswamy et al. 2006; Spann et al. 2004). Inhibition of the type I IFN response by these structural proteins also negatively affects the CD8 T-cell response (Kotelkin et al. 2006). Naïve CD8 T cells require three signals: antigen, co-stimulation, and cytokines to become activated, proliferate and differentiate into effector cytotoxic lymphocytes. Thus, the inhibition of type I IFN by NS1 and NS2 may provide critical cell-survival signals to the CD8 T cell. The RSV G protein, available as both a membrane anchored as well as a soluble secreted protein has been shown to contain a number of structural features that serve to modulate the adaptive immune response. The soluble form of G protein acts as a decoy to bind neutralizing antibodies and prevent neutralization and/or opsonization of the viral particles. Purified RSV G protein has been shown to bind cells expressing the receptor and mediate their chemotaxis (Tripp et al. 2001) thereby modulating the local inflammatory environment in the lung by altering the infiltrating inflammatory cells. The presence of the G protein reduces expression of various chemokines thereby reducing Th1 and CD8 T-cell response and increasing Th2 responses (Mukherjee et al. 2011; Lukacs et al. 2010).

Recombinant adenoviruses have been known to be used as vehicles for gene therapy. A study showed that adenovirus infection induced the type I IFNs that was critically required for the multiple stages of adaptive B cell response including the B cell activation, germinal center formation, Ig isotype switching as well as plasma cell differentiation. The study also showed the importance of type I IFNs in the generation of protective neutralizing antibodies on both CD4 T and B cells (Zhu et al. 2007b).

4 Conclusion

Several past and recent studies have documented the activation of immune response to several viruses, including those that cause the major respiratory diseases. Influenza virus, RSV, adenovirus and few others have been a significant cause of public health concern and needs continuous worldwide surveillance. Over the past decade, considerable mechanistic observations have been made regarding the immune system's machinery to identify and eliminate acute respiratory viruses. These viruses are not only known to generate the innate immune response but also subsequently activate the adaptive wing of the host immune response during infections. Accumulating evidences have suggested the crucial role of inflammasome activation and generation of inflammatory response upon several respiratory viral infections. There is a balance between the beneficial and detrimental activation of this immune response that often becomes the deciding factor for a host survival during infection.

The discovery about the PRRs and how they distinguish between host and pathogen, show the complexity of the host immune system. The TLRs and IRFs play critical roles during the initiation of immune responses. Later the RNA helicases and DNA sensors were also shown to play crucial roles. The knockout mice models for MAVS, IRFs and MyD88-TRIF have provided potentials to elucidate these pathways in details and their contribution to both pathogenesis and immunity to these human respiratory pathogens. The advancements made in the area of T cell and B cell subsets have provided an in-depth knowledge about how a host is protected from a secondary challenge and memories are generated for a recall response during respiratory viral infections. Novel therapeutic antiviral agents as well as target host proteins need to be identified in order to efficiently manage these viral infections. The better understanding of the virus-host interactions, host immune response and the ability of the viruses to exploit host signaling mechanism will provide a comprehensive picture of antiviral immunity and further facilitate the development and implementation of new treatment strategies for pathogenic respiratory viruses.

Conflict of Interest The authors declare no conflict of interest.

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Immune Responses to MERS-CoV in Humans and Animals

5

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1 MERS-CoV

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is a group C betacoronavirus, with a positive stranded RNA genome of around 30 kb in length (van Boheemen et al. 2012). Its genome encodes structural proteins such as spike (S), envelope (E), membrane (M), and nucleocapsid (N) as well as non-structural proteins (van Boheemen et al. 2012). The virus was first identified in 2012 from a pneumonic patient in the Kingdom of Saudi Arabia (KSA) and subsequently has been introduced into 27 countries and caused numerous outbreaks, mainly in the Arabian Peninsula in large crowded hospitals, with one large outbreak in the Republic of Korea in 2015.

The origin of the virus is not known, but it may have come from bats because it shares genetic similarity with bat coronaviruses (van Boheemen et al. 2012). Dromedary camels (*Camelus dromedarius*) are the only confirmed animal intermediate host (Alagaili et al. 2014; Haagmans et al. 2014). More than 54% of primary human cases have reported contact with camels (Conzade et al. 2018); and the index patient in the Korean outbreak travelled back from the Arabian Gulf countries where MERS-CoV is endemic in dromedary camels (Korea Centers for Disease Control and Prevention 2015; Kasem et al. 2018a). Dromedaries have been infected with MERS-CoV by as early as 1983 according to serological testing of archived camel sera (Alagaili et al. 2014; Muller et al. 2014; Corman et al. 2014; Reusken et al. 2014; Hemida et al. 2014a; Kasem et al. 2018b; Meyer et al. 2014), indicating that MERS-CoV could have been circulating in camels for decades. In addition, several recent studies on camels confirmed that between 70 and 100% of dromedaries are seropositive for MERS-CoV in both Africa and the Arabian Peninsula; furthermore, the virus and/or its RNA have been isolated from dromedaries, confirming that these animals are a source for human infections (Kasem et al. 2018a; Reusken et al. 2013; Falzarano et al. 2017; Miguel et al. 2017; Deem et al. 2015; Perera et al. 2013).

In humans, MERS-CoV infects epithelial cells in the trachea, bronchi, and lungs and causes a respiratory illness with symptoms that may

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include fever, cough and shortness of breath. Pneumonia is common, but not always present. Gastrointestinal symptoms, including diarrhoea, have also been reported. At the cellular level, the virus enters the mammalian epithelial cells by binding to the receptor, dipeptidyl peptidase 4 (DPP4) (Raj et al. 2013a). In camels, the virus replicates and can be isolated from the upper respiratory track whereas in humans the virus is primarily found in the lower respiratory track. This is in part due to differential expression of DPP4 in different tissues in camels and humans (Widagdo et al. 2017; Eckerle et al. 2014).

Some laboratory-confirmed cases of MERS-CoV infection are reported as asymptomatic, meaning that they do not have any clinical symptoms. Most asymptomatic cases have been detected following aggressive contact tracing of a laboratory-confirmed case (WHO 2019). The infection has an unusually high case-fatality rate of around 35% but is not considered to pose an epidemic threat because the reproductive rate of the virus transmission (R_0 rate) is below 1, although super spreader events have occurred. There is currently no approved specific treatment, nor vaccines for MERS-CoV; and the cases are usually treated with supportive palliative care.

Both innate or adaptive responses against MERS-CoV occur following infection. While adaptive cell-mediated immunity can be initiated against any MHC-I or MHC-II presented epitopes, neutralising antibodies mainly target epitopes within the spike protein of MERS-CoV. The spike protein consists of 1353 amino acid (aa); with two subunits the S1 (aa 18–751), which contains the receptor-binding domain (RBD; aa 367–606); and S2 (aa 752–1353), which contains the fusion domain (Fig. 5.1). The RBD binds to DPP4 on epithelial cells in the respiratory tract of mammals such as humans and nonhuman primates, dromedaries and other camelids, rabbits and swine. Some mammals, such as the rodent family (mice and hamsters) have genetic sequence difference in their DPP4, that do not permit infection with MERS-CoV (Eckerle et al. 2014; Du et al. 2017; Wang et al. 2013; Raj et al. 2013b). Overall, the S protein is a main target for neutralising antibodies and several monoclonal antibodies against epitopes in the spike protein have been developed (Tang et al. 2014; Corti et al. 2015; Pascal et al. 2015; Chen et al. 2017; Jiang et al. 2014; Niu et al. 2018; Stalin Raj et al. 2018; Zhao et al. 2018). This chapter presents what is known, to date, on the immune responses to MERS-CoV infections in humans, camels, and experimental animal models.

2 Adaptive Immune Responses to MERS-CoV in Camels

2.1 Antibodies Against MERS-CoV in Camels

Camelid species produce three isotypes of immunoglobulin G antibodies: IgG1, IgG2, and IgG3. IgG2 and 3 are structurally unique, lacking light chains in the Fab region of the antibody. This results in the heavy chain domain of the Fab region being responsible for antigen specificity (Daley-Bauer et al. 2010). Most studies on camel immune responses to MERS-CoV have focused on evaluating the presence of MERS-CoV specific antibodies (seroprevalence studies); and the kinetics of antibody responses in camels have not been clearly defined. Antibodies against MERS-CoV can be elicited in dromedaries although the infection in these animals is usually asymptomatic. Anti-spike antibodies are usually detected from 1 month post-infection and continue to be detectable in the serum for more than a year (Meyer et al. 2016). Induction of antibodies following primary infection in young calves may require at least 1–2 months unlike older calves and adult dromedaries. A 1-year old calf mounted low neutralising antibody titre of 1/160 3 months after a MERS-CoV outbreak in a herd and this titre increased to 1/640 in the following (fourth) month post-outbreak (Hemida et al. 2017). When 2 year-old calves, were housed with many MERS-CoV shedding camels, the calves were infected within 4 days and mounted a detectable antibody response 1–2 months post-infection. However, in an experimental infection, naive camels challenged with a high dose of

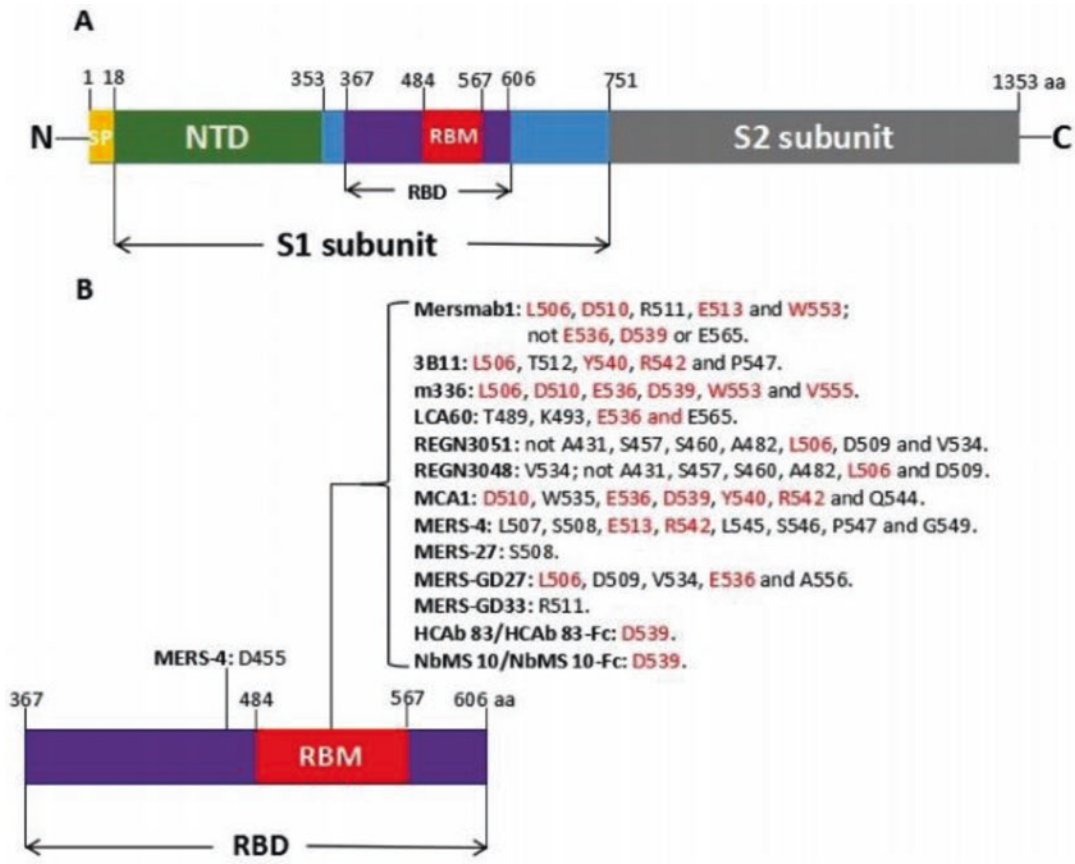

Fig. 5.1 A schematic representation of MERS-CoV spike protein, cited from Han et al. (2018) Viruses (Han et al. 2018)

MERS-CoV, mounted a detectable neutralising antibodies 1 week post-infection (Haagmans et al. 2016). Therefore, the induction of antibodies may take weeks to months, and would appear to depend on the infectious dose.

The antibodies decline rapidly 4–5 months following a single incident of infection (Hemida et al. 2017); therefore multiple re-infections enhance the antibody levels in camel sera that can be detected for longer time for more than a year (Ali et al. 2017).

2.2 Camelid Maternal Antibodies Against MERS-CoV

Newly born calves acquire maternal antibodies from their seropositive mothers and continue to possess these antibodies for 5 months (Meyer et al. 2016). Maternal antibody levels in calves are usually similar to their levels in the mothers, although this is not always the case, and these antibodies decline rapidly over the first 5 months post-parturition (Meyer et al. 2016; Ali et al. 2017). Infection of MERS-CoV is usually more predominant in immunologically naive calves than adult camels (that are usually seropositive). Infection in the calves generally results in higher virus replication than in seropositive adult camels. The antibodies in seropositive camels do not protect from the infection, but they may play a role in reducing virus replication and the magnitude of infectious viruses shed (Alagaili et al. 2014; Meyer et al. 2016; Hemida et al. 2014b). Therefore, re-infection of MERS-CoV occurs in seropositive camels as well as in naive calves or

calves with maternal antibodies (Meyer et al. 2016; Ali et al. 2017; Wernery et al. 2015).

2.3 Re-infection and Protection

Anti-S1 antibodies in serum that are induced following a natural infection do not completely protect camels from re-infection with MERS-CoV. It is suspected that either the level of these antibody is not sufficient for protection or that other immune responses, such as cell-mediated immunity, antibodies against other epitopes (such as the S2) and proteins (such as structural proteins), or mucosal antibody in the upper respiratory tract are required to achieve protection. One camel in a herd-based study was found to be re-infected despite having a high nAb titre of 1/40960 (Hemida et al. 2017). Although antibodies against MERS-CoV spike protein do not completely protect camels, they could significantly contribute to reducing virus presence in the camel respiratory tract. Seropositive camels that were co-housed with infected camels in one pen were infected, but the infection was transient and resolved significantly quicker than camels with no pre-existing anti-MERS-CoV antibodies (Alharbi et al., submitted manuscript). However, induction of protective immunity seemed possible when one seropositive camel with a defined anti-S Ab titre was vaccinated with a potent spike based vaccine and showed complete protection. Although this protection might be partially attributed to the pre-exisiting cellular immunity or antibodies against non-spike proteins, this finding could support establishing a protective titre based on anti-S1 antibodies level in camels (Alharbi et al., submitted manuscript).

2.4 Cellular Immune Responses

The lack of reagents specific to Old world camels has hampered the research in evaluating cellular immune responses in dromedaries. Currently, there is no data indicating what role cellular immunity might play in protection from infection. Although some reagents are available for New World camelids (e.g. Llama), these do not necessarily work in dromedaries.

3 Immunity of MERS-CoV in Other Animal Models

Several animal models for MERS-CoV have been developed including various mice models that express human DPP4, rabbits and non-human primates. Some of these animal models recapitulate the lower respiratory tract infection that is observed in humans.

3.1 Mouse Models

Several mouse models have been developed for MERS-CoV using a number of different strategies including transient expression of human DPP4, knock in, or substitution of mouse DDP4 with human or humanized versions of DPP4 (Agrawal et al. 2015; Zhao et al. 2014). The drawback of these models is they either do not results in consistent lethal disease (making pathogenesis and vaccine or treatment efficacy studies more difficult) or lethal disease is not consistent with the pathogenesis that is observed in humans.

3.2 Transduced Mice

Zhou et al. (2014) developed the first small animal model of mice for MERS-CoV by transient transduction of an adenoviral vector expressing human DPP4 and showed that the mice developed severe pneumonia with inflammation in the lower respiratory tract with extensive cell infiltration, comparable to that found in humans—but this model did not show any lethality. Virus clearance occurred 6–8 days post infection (Zhao et al. 2014). In transgenic mice with defective interferon signalling responses and infected with MERS-CoV, the disease was more severe. Absence of myD88 and MAVS pathways showed mild to severe outcomes, respectively following the infection.

In addition, in T cell and B cell deficient transgenic mice, infected with MERS-CoV, the virus was not cleared while passive transfer with immune sera raised against MERS-CoV showed that neutralizing antibodies were able to accelerate viral clearance. These findings indicate the importance of both nAb and T cells in viral clearance and potential protection. Several studies testing vaccine candidates were conducted in these models. For example, a study with vaccine using replication competent measles virus expressing spike in the transgenic mouse model showed induction of MERS-specific nAb and cytotoxic T cell responses (Malczyk et al. 2015). The Ad5-transduced rodent model was used to study MERS-CoV replication, vaccines and therapeutics as a small animal model (Zhao et al. 2014). However, uncontrolled expression levels of hDPP4 and tissue distribution limited their use in pathogenesis and immunology studies.

3.3 Transgenic Mice

Multiple transgenic (Tg+) mouse models have been developed. In one study a murine model by codon optimization with expression of hDPP4 (Zhao et al. 2015). Upon intranasal infection, virus replication was detected up to day 5 post infection with severe inflammation infiltration and lung damage. However, global distribution of human DPP4 receptor resulted in virus replication in the brain and kidney until day 9 after the initial replication in the lungs. While this model is very lethal at day 10 post challenge, it is not consistent with human disease, as virus dissemination to the brain occurs and is responsible for the lethal outcome. It has, however, proved suitable for vaccine and antiviral studies (Zhao et al. 2015).

Another hDPP4 Tg+ mouse model was generated by incorporating a CD26 cassette under the control of a CMV promoter. After infection with MERS-CoV, active replication and viral RNA was detected in lungs and brain at 2 and 4 days post infection, respectively (Agrawal et al. 2015). All mice in the study developed severe pneumonia and perivascular cuffing. Furthermore, this model showed activation of antivirals genes. Vaccine studies in these Tg+ mice with a single dose of recombinant adenovirus based S1 vaccine elicited specific IgG neutralizing antibodies and showed complete protection upon challenge infection of MERS-CoV and undetectable viral loads in lungs (Malczyk et al. 2015). Other transgenic mice, expressing hDPP4 under the control of cytokeratin 18, developed high virus titer in the brain and lungs at 2 and 6 days after infection. Vaccine studies with a Venezuelan equine encephalitis replicon particle expressing the S protein showed protection upon challenge with high antibody titers when compared to control mice (Li et al. 2016).

Additionally, knock-in (KI) mice with hDPP4 insertion by CRISPR-Cas9 in the Rosa26 locus of KI mice were generated (Fan et al. 2018). hDPP4 was expressed in lung tissues while expression was low in brain and other organs. Five days after infection with MERS-CoV, approximately 10^3 PFU virus was detected in the lungs of R26-hDPP4 mice. Serum neutralization antibodies were detected and correlated with protection in this model.

Li and colleagues developed several KI mouse models by replacing human exon 10–12 in the DPP4 locus of the mouse, which upon inoculation with MERS-CoV resulted in infection of lungs with absence of disease in mice (Li et al. 2017). After 30 passages in lungs of these KI mice, mouse–adapted MERS-CoV (MERS-MA) was developed which grew to high titres in the lungs of these mouse. Virus replication was evident in lung epithelia and macrophages. Histopathological studies showed alveolar damage with pulmonary edema with activated inflammatory monocyte derived macrophages and neutrophils in lungs. 13–22 mutations in spike protein conferred more rapid entry into host cells and were more virulent than the parental MERS-CoV. This mouse model showed severe signs of inflammation with activation of neutrophils. So far, this is the best mouse model for MERS-CoV in terms of pathogenesis (van Doremalen and Munster 2015).

3.4 Non-human Primates

Rhesus macaques and common marmosets have been developed as animal models for MERS-CoV. Viral replication occurs in the lungs of both models, but disease severity was different (Yu et al. 2017).

3.5 Rhesus Macaques

Rhesus macaques were described as the first animal model that supports MERS-CoV infection. Disease outcome was studied by inoculating MERS-CoV at mucosal surfaces including oral, tracheal, nasal and ocular routes, resulted in a lower respiratory tract infection (Yao et al. 2014). Elevation in body temperature was observed from 1 to 2 days post infection (dpi), but no significant change in body weight was recorded. All animals did not shed virus in upper respiratory tract. Imaging data in lungs showed immune cells filtration. All animals showed mild signs of disease within 24 h with resolution of signs between 3 and 6 dpi. Nasal and oropharyngeal swabs and bronchoalveolar lavage were positive for MERS-CoV RNA, in contrast to urogenital and fecal swabs (Yao et al. 2014).

A neutralizing antibody response was observed at 7 days post infection (dpi), but decreased eventually after 28 dpi. Transcriptome analysis showed that proinflammatory cytokines and inflammatory cells were upregulated in early infection and decreased in later stage of infection. Mild disease outcome in rhesus macaques recapitulates the disease observed in MERS patients with mild to moderate symptoms; and this model can be used as an infection model to study antivirals and vaccines.

A synthetic consensus anti-spike DNA vaccine induced protective innate and adaptive immunity in rhesus macaques when administrated intramuscularly (Muthumani et al. 2015). ELISPot analysis for IFN-γ showed robust T cell responses in vaccinated and protected (Muthumani et al. 2015). Dose dependent studies showed no significant difference in neutralizing antibody response between low and high dose. However, seroconversion appeared after first immunization in high dose group and protected animals from challenge whilst other group required multiple immunisations to seroconvert. High dose regimen of the vaccine was also successful to induce higher percentage of $CD8^+$ and $CD4^+$ response (Muthumani et al. 2015).

3.6 Common Marmosets

The common marmoset model was first developed with *in silico* analysis and confirmed that marmoset DPP4/S binding kinetics were predicted to be similar to human (Raj et al. 2013a; Lu et al. 2013). To establish marmosets as an animal model for MERS-CoV, 9 male marmosets were infected by intranasally, intraorally, intraoccularaly (Falzarano et al. 2014). The marmosets developed moderate to severe disease; and in contrast to rhesus macaques, a slower disease progression towards severity level was observed, with 2 animals being euthanized at 4 dpi due to clinical score. MERS-CoV RNA was found in nasal and oral swabs up to 13 dpi and was 3 logs higher than observed in rhesus macaques.

Viral RNA was also detected in blood samples as well as all investigated tissues (respiratory tract, conjunctiva, lymph nodes, gastrointestinal tract, kidney, heart, adrenal gland, liver, spleen and brain) in at least one animal. The highest viral load was found in the lungs, and did not significantly differ at 3, 4, and 6 dpi (Falzarano et al. 2014).

Although, the number of animals in this study was limited, the marmoset recapitulates the severe disease that can be observed in humans and therefore can be a better model to understand disease outcome and MERS-CoV pathogenesis. A marmoset treatment study with hyper-immune sera from seropositive marmosets showed decrease in viral load and reduced gross pathology. This suggests that antibodies play an important role in protection against MERS-CoV (van Doremalen et al. 2017) although no difference in lung pathology was observed.

3.7 Rabbits

Rabbit tissues can be infected in vitro with MERS-CoV indicating that rabbits can be animal models for MERS-CoV (Haagmans et al. 2015). Female rabbits inoculated via the intranasal and intratracheal route did not develop clinical disease (Haagmans et al. 2015). Further, New Zealand rabbits were described as a model of infection for MERS-CoV (Houser et al. 2017). They were inoculated via the same routes as previous models and were re-challenged on day 57. Virus replication was mild to moderate with RNA being detected in lungs but not in kidney. The serum IgG antibodies were detected but without neutralizing activity. These antibodies failed to protect upon re-infection (Houser et al. 2017). It seemed that the rabbit model is the only model to show exclusively non-neutralizing antibodies following the primary infection. In contrast to humans, viral antigens were predominant in the upper respiratory tract. Thus, rabbits may be considered as an asymptomatic animal model with upper respiratory infection of MERS-CoV.

3.8 Alpacas

MERS-CoV infects the upper respiratory tract in camels and other camelids including alpacas, in contrast to the infection in humans, which is primarily in the lower respiratory tract (Adney et al. 2016). Signs of disease in camels and alpacas range from asymptomatic to minor nasal discharge (Adney et al. 2016). Therefore, these animals cannot be used as animal models as they do not mimic the disease in humans. However, alpacas are useful for studying camelid immunology as a smaller model for camel infection. Infection challenge studies in alpacas showed that the infection occurs and both viral particles and viral RNA were detected through the first week post infection; virus nAb responses were also detected in alpacas (Adney et al. 2016; Adney et al. 2014).

A subunit vaccine with S1 showed reduced and delayed viral shedding in upper respiratory tract of alpacas and camels (Adney et al. 2019). High neutralizing antibodies induced by the vaccine were correlated with decreased viral shedding and protection, indicating the importance of nAb in protection from MERS-CoV in camelids. However, in this study, low nAb levels were detected in camels as compared to alpacas (Adney et al. 2019). This would suggest that although alpacas are potential animal models to study camel vaccines, vaccine efficacy has to be confirmed in camels.

4 Immune Response to MERS-CoV Infection in Humans

The spectrum of disease outcome and immune responses in humans is similar to other respiratory coronaviruses such as SARS-CoV. Current findings confirm that the neutralizing antibody response against spike protein along with cell-mediated immunity are required for virus clearance from host tissues.

4.1 Innate Immune Response

MERS-CoV has evolved a strategy to conquer innate arms of immune response by blocking IFN response and this may contribute to high fatality rate especially among immunocompromised individual (Balachandran et al. 2000). MERS-CoV downregulates downstream pathways of MDA-5 and RIG-1, important mediators of innate immune response (de Wit et al. 2016). Upon recognition of PRR (pattern recognition receptors) such as MDA-5 and RIG-1 by viral RNA, these receptors activate MyD88 following interferon response and NF-κB transcription factor (Siu et al. 2014). IFN-1 and MyD88 deficient mice showed severe outcome compared to MAVS (mitochondrial antiviral signaling protein) deficient mice (Zhao et al. 2014), indicating the critical role of these innate immune pathways in reducing the MERS-CoV infection. Accessory proteins like ORF4a, ORF4b, and ORF5 are responsible for downregulation of these pathways and therefore preventing synthesis of interferon beta, contributing to the viral pathogenesis

(Yang et al. 2013) as the interferon production leads to activation of JAK-STAT pathway, which leads to expression of IRF9 and further antiviral pathways. ORF4a and ORF4b also inhibit the downstream pathways of ISRE (interferon stimulated response element) by invading STAT complex and ISG (Interferon stimulated gene) (Yang et al. 2013). Therefore, among all accessory proteins, the ORF4a is considered a potent inhibitor of pathways including IRF3, ISRE and NF-κB (Yang et al. 2013). The MERS-CoV M protein is also known to block IFN promoter and IFN production, but the exact mechanism is not yet clear. In conclusion, structural proteins along with accessory proteins interfere with interferon signalling pathways and lead to severe inflammation responses which result into severe disease outcome.

4.2 Antibody Responses

Neutralizing antibodies are known to block infection of host cells by interfering with the interaction between antigens and receptors. Anti-MERS neutralizing antibody response occurs in sera at day 14 post-exposure to the virus and increase over time. Analysis using human immune sera confirmed that neutralizing antibodies binds to the receptor binding domain in the S protein and prevents binding to human DPP4. Serological studies in camel handlers indicated that more than 50% of camel workers had neutralizing antibodies against S1 protein. In a plaque reduction neutralization assay, antibody responses in those with mild disease was substantially lower than individuals with severe disease. In a study of 37 patients infected with MERS-CoV, seroconversion was observed after 2 weeks following onset of disease and was inversely proportional to viral RNA and had little role in virus clearance (Corman et al. 2015); and neutralizing antibodies did not correlate with the outcome of disease (Chafekar and Fielding 2018). Additionally, IgA levels were high in mucosal secretions with suggested late onset of IgA in serum. Another study on 9 healthcare workers showed that an antibody response was maintained 18 months following infection. However, patients with milder MERS-CoV disease showed variable antibody responses that either increased or decreased over time (Alshukairi et al. 2016). A study of antibody responses and neutralizing activity showed that anti-S antibodies were mounted 3 weeks post infection, and was sustained in survivors to a higher levels than patients with severe disease (Park et al. 2015). Delayed IgG neutralizing antibody response was correlated with severity of the disease; and viral shedding was detected with antibody response suggesting weak protection against MERS-CoV infection (Park et al. 2015).

4.3 Cell Mediated Immunity

Cell-mediated immunity (including $CD4^+$ and $CD8^+$ T cell responses) is a potent arm of adaptive immunity which is involved in antibody production and direct killing of infected cells. Current findings suggest that T cell responses along with antibody responses are important to clear viral infection from lungs. Apoptotic pathway in T cells can be induced upon MERS-CoV infection; however, this activation is stage-dependent and various at different stages of T cell development (Ying et al. 2016; Chu et al. 2016). By screening for MERS-CoV specific T cell responses in human sample, $CD8^+$ T cells were observed in many patients with mild infection prior to the detection of antibody responses in the acute phase of the infection. Convalescent samples showed moderate levels of $CD8^+$ T cells that did not change over time (Shin et al. 2019) while $CD4^+$ T cell responses were directed more towards E, M, and N proteins than S protein; and the same pattern was observed 1 year post-infection (Shin et al. 2019).

Patients with higher $CD4^+$ T cell response resulted in severe outcome than those with high $CD8^+$ T cell response (Zhao et al. 2017). Although antibody responses were absent in some patients, $CD8^+$ T cell response was detected and it may lead to early viral clearance from lungs. Overall, as $CD8^+$ T cell magnitudes are prominent during the acute phase, it might be important for disease prognosis (Fig. 5.2 and Table 5.1).

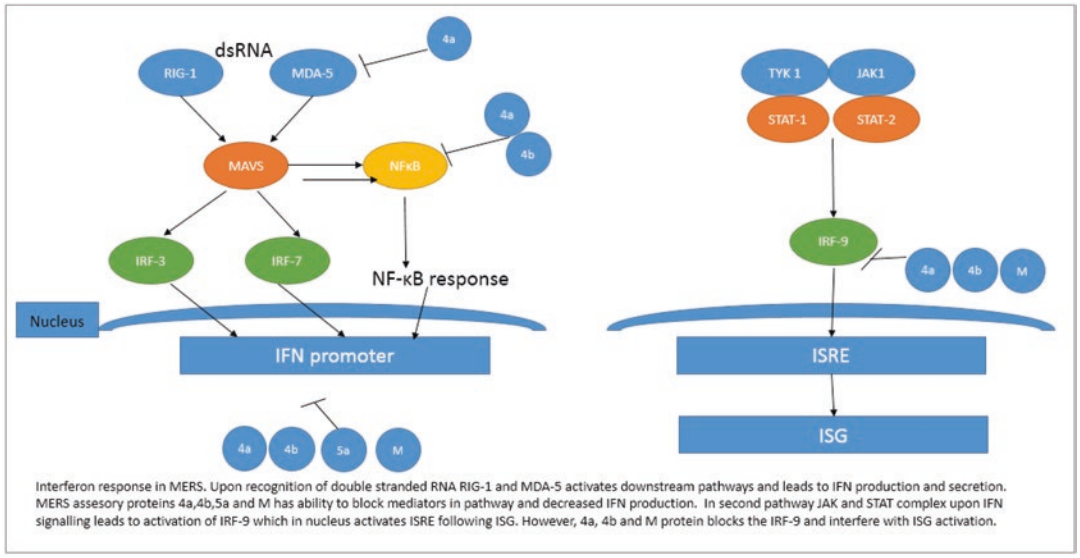

Fig. 5.2 Representative diagram on MERS-CoV proteins interaction with signalling pathways for innate immune responses

Table 5.1 Immune responses and vaccines evaluated in various animal models for MERS-CoV

Model	Disease outcome	Immune response to vaccination and infection	Vaccine tested	References
Ad5-hDPP4 transduced mice	Developed clinical disease and pneumonia, but no lethality	Induction of humoral and cell mediated responses	Replication competent measles expressing spike	Malczyk et al. (2015)
Transgenic mice (Tg+) model	hDPP4 expressed globally. Developed severe clinical disease with mortality	Induction of neutralizing antibody responses	Virus replicon expressing spike protein	Agrawal et al. (2015), Malczyk et al. (2015), Zhao et al. (2015)
Transgenic mice Rosa26-hDDP4 mice	Virus replicates in lungs	Induction of neutralizing antibody responses		Fan et al. (2018)
Transgenic mice hDPP4 under cytokeratin 18	High virus titer in brain and lungs	Induction of neutralizing antibody responses	Venezuela encephalitis replicon expressing spike protein	Li et al. (2016)
Rhesus macaques	Developed lower respiratory infection, mild to moderate clinical disease, virus replication in pneumocytes	Dose dependant in vaccine studies, induction of neutralizing antibody responses and CD4 and CD8 responses	Recombinant DNA and protein vaccines, expressing synthetic consensus spike protein	van Doremalen and Munster (2015), Yao et al. (2014), Muthumani et al. (2015)
Common marmosets	Developed severe pneumonia, showed partial lethality, viral RNA detected in lungs	Induction of neutralizing antibody responses to infection	Not studied for vaccines	van Doremalen and Munster (2015), Falzarano et al. (2014)
Rabbits	Developed asymptomatic disease, upper respiratory infection	Undetectable neutralizing antibodies	Not studied for vaccines	Haagmans et al. (2015), Houser et al. (2017)
Alpacas	Developed upper respiratory tract infection, viral shedding in nasal secretion, asymptomatic clinical disease	Induction of neutralizing IgG antibody responses and T cell based responses	Subunit vaccine	Adney et al. (2016, 2019), Crameri et al. (2016)

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6 SARS-CoV-2: Pathogenic Mechanisms and Host Immune Response

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1 Introduction

Coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 was first reported in Wuhan and related regions in Hubei province, People's Republic of China in December 2019 and subsequently spreading to most countries across the world. The infection clinically presents as atypical pneumonia which can progress to acute lung injury and acute respiratory distress syndrome (ARDS). SARS-CoV-2 was found highly homologous to the coronavirus (CoV) that caused the SARS (Severe acute respiratory syndrome) outbreak in 2003 in China (Zhu et al. 2020; National Health Commission of People's Republic of China, 2020). On 11 February 2020, The International Committee on Taxonomy of Viruses (ICTV) named it Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Simultaneously, the WHO named the disease caused by this virus COVID-19 (WHO report, 2020). This is the seventh coronavirus known to infect humans: SARS-CoV, Middle east respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 can cause severe disease. The other four, HKU1, NL63, OC43 and 229E, are endemic in the population accounting for up to 30% of annual respiratory infections and are associated with mild symptoms that are typically self-limiting. These cause seasonal infections in temperate climate during winter months (Charlton et al. 2018; Monto et al. 2020; Chan et al. 2020). CoV are associated with an increased risk of lower respiratory tract infections that are particularly debilitating in neonates, the elderly and in individuals with comorbidities (van der Hoek et al. 2005). Major symptoms of CoV infection include fever, sore throat and swollen adenoids (Liu et al. 2017) as well as viral or bacterial pneumonia or bronchitis (Forgie and Marrie 2009). NL-63 has been associated with onset of acute laryngotracheitis (van der Hoek et al. 2005). SARS-CoV-2 disseminates via asymptomatically infected individuals (Rothe et al. 2020; Ling et al. 2020; Pan et al. 2020b; Ghinai et al. 2020; Mazumder et al. 2020). The overall mortality rate is 0.5–3.5% (Guan et al. 2020; Wolfel et al. 2020; Wu and McGoogan 2020).

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2 Structural Organization of SARS-CoV-2

SARS-CoV-2, a β-coronavirus, is a non-segmented positive single-stranded RNA virus with a genome size of 29.9 kb (Wu et al. 2020a, b). The one-third of the viral genome encodes for structural proteins: Spike glycoprotein (S), Envelope protein (E), Membrane glycoprotein (M) and Nucleocapsid Protein (N). The remaining two-third of the genome is comprised of two open reading frames ORF1a and ORF1b that encode for non-structural proteins (nsps), which form the replication transcription complex (RTC); this controls the viral multiplication within host (Kim et al. 2020). The non-structural proteins that are translated include papain-like protease (PLpro), 3C-like protease (3CLpro), RNA dependent RNA polymerase (RdRp), helicase (Hel), and exonuclease (ExoN) (Tang et al. 2020a, b). The S genes of 2019-nCoV and RaTG13 (BatCoV) are longer than other SARS-CoV mainly found in bats (Zhou et al. 2020).

The most prominent viral envelope protein is the S-protein (Cavanagh 1995). It is heavily glycosylated to form large transmembrane homotrimeric spikes; this bulbous crown-like structure is what gives the name coronavirus. The S-protein is cleaved during viral internalization in endocytic vesicles to form two sub-units, S1 and S2, by host furin-like protease and assists viral integration into the host (Coutard et al. 2020; Walls et al. 2020a, b; Wrapp et al. 2020). The S1 sub-unit accommodating the receptor binding domain (RBD) determines the cellular tropism, while the S2 subunit containing the membrane binding domain (MBD) mediates fusion between cell and viral membranes for cell entry. The S1 subunit contains a signal peptide and two subdomains, the N-terminal domain (NTD) and the C-terminal domain (CTD), both domains can serve as the RBD (Tang et al. 2020a, b). RBD is a twisted five-stranded anti-parallel β-sheet (β1, β2, β3, β4 and β7) structure with an extended insertion region containing β5 and β6 strands, α4 and α5 helices and loops forming the receptor binding motif (RBM) (Lan et al. 2020). SARS-CoV-2 utilizes the CTD to bind angiotensin converting enzyme-2 (ACE2) for entry into the host cell (Zhou et al. 2020). The S2 subunit contains other regions; the fusion peptide (FP), HR1 (heptad repeat 1), HR2 (heptad repeat 2), transmembrane (TM) and cytoplasmic region (CP).

The E-protein and M-protein are conserved across the β-coronavirus (Bianchi et al. 2020). The E-protein is a small integral membrane polypeptide which can oligomerize and form ion channels-fundamental in the release of viral particles (Verdia-Baguena et al. 2012). The M-protein is prevalent within the viral membrane and maintains structural integrity of the virion envelope. It is important for budding process (Bianchi et al. 2020). It is a multi-spanning membrane protein with three trans-membrane segments with the major domain of the molecule being a large carboxy terminus situated in the interior of the virion (Rottier 1995). The M-protein is capable of interacting with other M, N, E and S proteins during the process of viral assembly (Alsaadi and Jones 2019; Neuman et al. 2011). The N-protein binds the RNA genome, continuously packaging it into the viral particle during assembly and also providing stability to the viral RNA. Moreover, it can antagonize antiviral RNAi and inhibit the activity of cyclin-dependent kinase (cyclin-CDK) complex, which results in the hypophosphorylation of retinoblastoma protein (pRB), inhibiting the genome replication (S-Phase) of the cell.

RNA dependent RNA polymerase (RdRp) is paramount in viral genome replication. It is a highly conserved protein between RNA viruses, hence a promising candidate for an antiviral drug development. Targeting the RdRp active site may inhibit viral replication (Aftab et al. 2020).

3 Transmission of SARS-CoV-2

Statistically, by the beginning of September 2020, there were an estimated 22,602,665 positive cases of COVID-19, with 852,758 confirmed deaths across 190 countries. This number increased to 127 million affected people with 2.7 million deaths globally by 31 March 2020.

SARS-CoV was the causative agent of the 2002–2003 SARS outbreak that originated in Guandong province, China and resulted in approximately 8098 cases and 774 deaths during a nine-month period, with an average mortality rate of 9%. In the elderly population, mortality peaked at almost 50% (Drosten et al. 2003). In 2012, a novel β-coronavirus emerged in Saudi Arabia, MERS-CoV, the causative agent in a number of highly virulent respiratory tract infections across the Middle East (Zaki et al. 2012). From 2012 to January 2020, there were 2506 cases with a 35% fatality rate, approximately four times higher than SARS-CoV (Killerby et al. 2020). The number of patients infected with SARS-CoV-2 is notably higher than SARS-CoV and MERS-CoV, suggesting a higher rate of infection per exposure for SARS CoV-2.

SARS CoV-2 is highly contagious and efficient at spreading. In nature, the lipid bilayer of the virion protects the virus from denaturation for a short time during which it can bind to a suitable target receptor. Transmission can occur from an infected individual through respiratory droplets in direct transmission, where they are expelled as aerosols while coughing, sneezing or talking in close contact, or saliva during intimate contact. Indirect transmission following deposition of the virus on fomites (surfaces) has also been observed (Chan et al. 2020; Li et al. 2020a, b, c; Ghinai et al. 2020). It has been suggested that airway secretions may protect the virus, enhancing its persistence and transmission via contaminated fomites (Pastorino et al. 2020). Aerosol suspension studies suggest that SARS-CoV-2 can persist for long periods in the aerosol form, with viral bioaerosols retaining infectivity and virion integrity for up to 16 h (Fears et al. 2020). Airborne transmission potentially occurs by inhaling aerosols containing a critical titre of the virus sufficient enough to cause infection, though the optimum and basal infectious doses of SARS-CoV-2 are yet to be ascertained. Droplets containing the coronavirus are heavy due to their large diameter, and therefore, are incapable of travelling long distances through air. Van Doremalen et al. (2020) have studied the stability of SARS CoV-2 in aerosols and various surfaces; it can remain viable in aerosols for 3 h, being more stable on plastic (for up to 72 h) and stainless steel (for up to 48 h) compared to copper (no viable virus after 4 h) and cardboard (no viable virus after 8 h). SARS CoV-2 RNA can also be detected in the urine and feces of some patients; however, due to low titres in plasma and serum, the potential of bloodborne transmission remains uncertain.

Hao et al. (2020) analyzed the transmission dynamics of the COVID-19 outbreak in Wuhan, and highlighted two key features: high covertness and high transmissibility. These features synergistically propelled the COVID-19 pandemic (Hao et al. 2020). In 40% of cases, the virus has been reported to spread via asymptomatically-infected individuals worldwide (Rothe et al. 2020; Ling et al. 2020; Pan et al. 2020b; Ghinai et al. 2020; Mazumder et al. 2020). Various statistical analyses were undertaken to ascertain the role of asymptomatic individuals in transmitting SARS-CoV-2. In a study involving cruise ship passengers off the coast of Japan carrying 3711 passengers and crew members, there were 634 confirmed infection cases: 306 symptomatic and 328 asymptomatic (Mizumoto et al. 2020). Similarly, a study on passengers flying from Wuhan to Japan up to the 6 February 2020, suggested half of the infected individuals were asymptomatic (Nishiura et al. 2020). Tong et al. (2020) identified two symptomatic COVID-19 cases after their exposure to a pre-symptomatic individual who was later diagnosed with laboratory-confirmed COVID-19. These two individuals later transmitted SARS-CoV-2 to three other family members, who also remained asymptomatic (Tong et al. 2020).

At the New York–Presbyterian Allen Hospital and Columbia University Irving Medical Center between March 22 and April 4, 2020, a total of 215 pregnant women who delivered infants were screened on admission for symptoms of COVID-19. Four women had SARS-CoV-2 related symptoms on admission while the remaining 211 women were asymptomatic and afebrile. Nasopharyngeal swabs indicated 33 patients were positive for SARS-CoV-2 at admission, 29 had no symptoms of COVID-19 (Sutton

et al. 2020). Similarly, in another study, 55 asymptomatic cases were identified with SARS-CoV-2 infection, their ages ranged from 30 to 49 years asymptomatic cases occurred more often in middle aged people in Shenzhen, China (Wang et al. 2020a, b, c, d, e, f, g). Although COVID-19 was found to have lower severity and mortality than SARS, it is highly contagious and affects comparatively more men than women (Jin et al. 2020; Huang et al. 2020; Mazumder et al. 2020).

A quantitative RT-PCR study showed that the viral load of SARS-CoV-2 in throat samples peaked around 5–6 days after the onset of symptoms (Pan et al. 2020a). SARS-CoV-2 can also be detected in deep throat saliva samples for 20 days or longer (To et al. 2020). In fecal samples, SARS-CoV-2 can be traced after 28 days from the first onset while the respiratory samples remained positive for around 17 days (Wu et al. 2020a, b). This possibly suggests that the virus may be actively replicating in the gastrointestinal tract, even when it is absent in the respiratory tract (Wu et al. 2020a, b). Viral RNA has also been detected in urine on 42 days post infection in very low quantities (Sun et al. 2020a, b). In a study involving 71 COVID-19 patients (68 cases were above 18 years) who were in the convalescence period, 32.5% patients were positive for viral RNA (results turned from negative to positive) and the longest RNA reversal phase time was 7 days (Liu et al. 2020a, b). In the same study, 52.9% of adults showed no obvious clinical symptoms, whereas the remainder exhibited mild and non-specific clinical symptoms (Liu et al. 2020a, b).

4 Epidemiology of SARS-CoV-2

Epidemiological studies have revealed that CoV are epizootic to bats; in particular, Chinese horseshoe bats harbor viral genomic sequences and serological evidence of prior infection with SARS-related CoV (Lau et al. 2005; Li et al. 2005). The coronavirus subfamily is genotypically and serologically divided into four genera; α, β, γ, and δ coronaviruses. The α- and β-coronaviruses both originate from bats and are mainly found in mammals such as bats, rodents, civets, and humans. Several exotic animals have tested positive for antibodies to SARS-related CoV, including hog badgers and raccoon dogs in Chinese wet wildlife markets. Moreover, masked palm civets inoculated with SARS-CoV develop lung pathology (Wu et al. 2005). Repetitive viral genome sequencing of SARS patients and suspected intermediary hosts produced a dendrogram suggesting that the first human SARS-CoV was related to a civet-derived virus; after several transmissions between human hosts, the virus had acquired point mutations augmenting its pathogenicity in humans (Song et al. 2005).

SARS-CoV-2 caused severe respiratory pathology in hosts, its symptomatology and incubation period resemble SARS-CoV and MERS-CoV. There are two notable features of the SARS CoV-2 genome: mutations in the contact residues of SARS-CoV-2 S-protein, and the inherent polybasic cleavage site at the two subunits of the S-protein. Genetic analyses of SARS-CoV-2 patient samples confirmed an 88% sequence similarity to bat SARS-related CoV, with a 79% similarity to SARS-CoV and 50% to MERS-CoV (Liu et al. 2020a, b). The similarity exhibited between SARS-CoV-2 and bat SARS-CoV suggested bats to be the possible reservoir. Zoonotic reservoirs are well maintained due to their population structure, migration patterns and life span (Calisher et al. 2006). They are capable of transmitting CoV, which has seen the re-emergence of this infectious disease globally. These findings corroborate that SARS-CoV-2 is a novel coronavirus with significant tolerance to genetic variability and is unlike previously known CoVs.

SARS-CoV-2 is a rapidly evolving RNA virus which is continually exhibiting genomic mutations as its transmits. Thus, the mutational landscape has been under constant global scrutiny to understand the infectivity and antigenicity of the new variants.

United Kingdom, on December 14, 2020, reported a SARS-CoV-2 variant of concern (VOC 202012/01), B.1.1.7 lineage. This B.1.1.7 variant became the dominant circulating SARS-CoV-2 variant in England since its emergence in September 2020. It has also been detected in other 30 countries including the United States.

Compared to ancestral viruses containing the D614G mutation, the B.1.1.7 variant has accumulated several other mutations where six nucleotide deletions in the S-gene resulted in the loss of two amino acids, H69 and V90 (Kemp et al. 2021; McCarthy et.al. 2020; Galloway et al. 2021). Several mathematical modelling and epidemiological studies predicted that variant can spread 56% faster than other lineages resulting in higher nasopharyngeal viral loads compared to the wild-type strain (Davies et al. 2020).

On 18 December 2020, another highly transmissible variant of SARS-CoV-2 named B.1.351 was reported by the authorities from Republic of South Africa. Compared to the Wuhan reference strain, the B.1.351 variant has 12 non-synonymous mutations and one deletion (Gómez et al. 2021). This variant has three mutations in the S-protein: K417N (a lysine to asparagine substitution at amino acid position 417), E484K (a Glutamic acid to lysine substitution at amino acid position 484) and N501Y (an asparagine to tyrosine substitution at amino acid position 501). The N501Y mutation is common in both B.1.1.7 and B.1.351 variant (Gómez et al. 2021).

P.1 (B.1.1.28.1) is the third variant of SARS-CoV-2 that was detected by Japan's National Institute of Infectious Diseases on 6 January 2021, which was isolated from the four travellers who arrived in Tokyo from Brazil. Later on, P.1 variant was identified in Brazil, as the widely transmitted variant (Candido et al. 2020; Gómez et al. 2021). The patient samples collected during October 2020 form the municipal region of Reo De Janeiro State identified the first variant individual with the S-Protein mutation E484K. This 484K.V2 variant has been transmitted to various other countries such as England, Norway, Singapore, Denmark, Ireland and Canada (Gómez et al. 2021; Resende et al. 2021; Vasques et al. 2021). The B.1.617.2 (Delta) variant of SARS-CoV-2 was identified in India in late 2020 and has subsequently been detected in around 60 countries (CDC. 2021). The B.1.617.2 variant has a potentially higher rate of transmission than other variants and currently account for approximately 95% of sequenced and 92% genotyped cases from 7 to 21 June 2021 in the UK (Public Health England, 2021) and became the dominant variant in the UK.

The rapid establishment of a national sequencing collaboration by the United Kingdom, the COVID-19 Genomics UK consortium (COG-UK, 2020) facilitated the robust systematic sampling of the viral genome. A considerable attention has been drawn on the D614G mutation, becoming the dominant form worldwide as the virus spreads from Asia into Europe and USA (Volz et al. 2021). D614G mutation in SARS-CoV-2 is a non-synonymous mutation resulting in a replacement of aspartic acid with glycine at position 614 of the virus spike protein. D614G has been found to be associated with higher viral load and with younger age of patient and not with higher mortality or clinical severity of the disease (Volz et al. 2021). Different demographic events such as population growth, random genetic drift, founder effects, positive selection and several other factors can be the reason for the spread of viral mutation that need to be monitored globally.

5 Clinical Aspects of COVID-19

COVID-19 typically begins with a mild, self-limiting respiratory tract illness, progressing to severe ARDS, and then leading to multiple organ failure in some cases. Within approximately 5.2 days of incubation, SARS-CoV-2 infection presents its first symptoms (Li et al. 2020a, b, c). The period from initial symptoms to potential fatality ranges from 6 to 41 days with a median of 14 days (Wang et al. 2020a, b, c, d, e, f, g). This variable time span is contingent on a number of co-factors, sex and immune status being the main issues. At the onset of the disease, most patients exhibit common symptoms such as headache, fever and dry cough. Other symptoms include muscle pain/fatigue, chest pain, diarrhoea, nausea, vomiting, and less often haemoptysis and anosmia (Huang et al. 2020; Chen et al. 2020a, b, c, d; Wang et al. 2020a, b, c, d, e, f, g; D'Amico et al. 2020; Kerslake et al. 2020). It was also observed that diabetes, hypertension, and cholesterol levels possess an apparent relation to COVID-19 severity (Wang et al. 2020a, b,

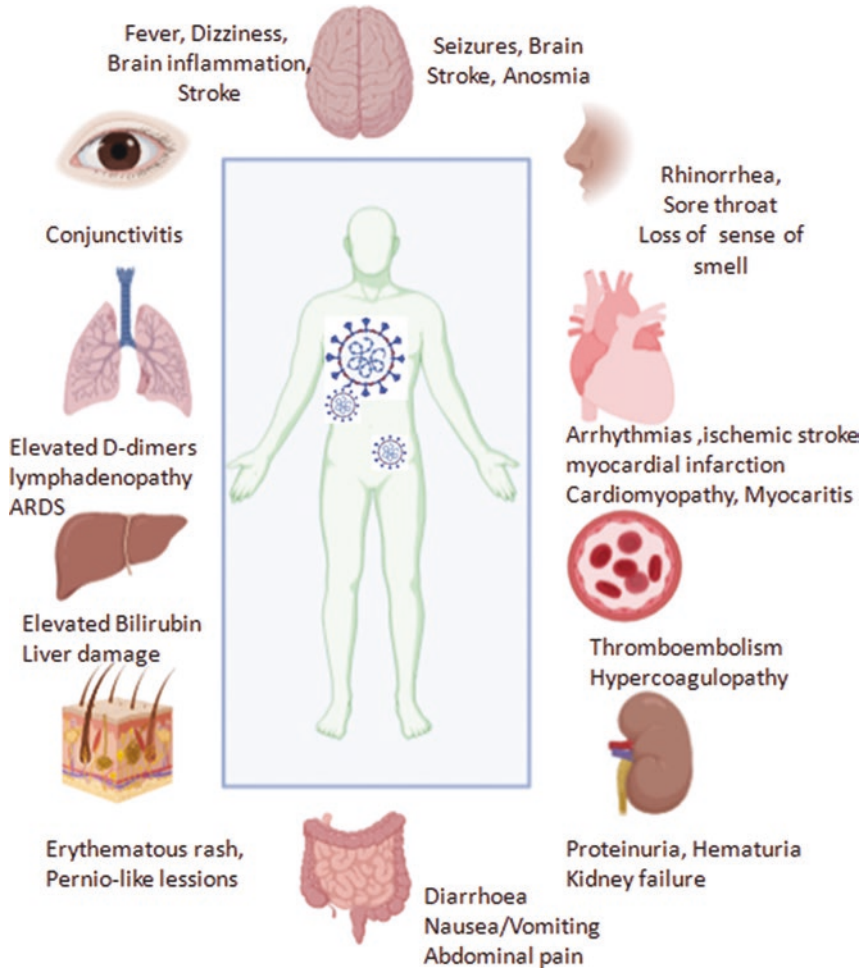

Fig. 6.1 Pulmonary and extrapulmonary manifestations of COVID-19

c, d, e, f, g) (Fig. 6.1); these patients also show high levels of IL-6, IL-10, TNF-α, and lactate dehydrogenase (LDH) in serum (Li et al. 2020a, b, c).

Mortality is higher in adults above the age of 65 years (approximately 6.4%) (WHO situation report, 127, 2020). Amongst the elderly population, the virus spreads rapidly into the gas exchange regions of lung possibly due to reduced muco-ciliary clearance (Ho et al. 2001). Pathological features of COVID-19 resemble those of SARS and MERS. While the virus is in the airway, it may also present symptoms such as hoarseness, ulceration and edema in the epiglottis and subglottis (Oliver et al. 2020). In the lungs, viral infection shows as multiple infrahilar airspace opacities on chest X rays (Lei et al. 2020); chest CT scans reveal ground-glass opacities, bilateral multifocal infiltrates, lymphadenopathy and invasive lung lesions with thoracic tissue injury (Ghinai et al. 2020; Ren et al. 2020) and may even lead to fibrosis (Mason 2020). Elevated D-dimers that are associated with inflammation suggest high risk of ARDS as observed in COVID-19 patients (Tang et al. 2020a, b). The risk of developing a lethal form of COVID-19 increases in the elderly, amongst adults with underlying health conditions and in individuals

with compromised immunity (Gralinski and Menachery 2020).

The neutrophil-to-lymphocyte ratio (NLR) can be a predictive factor for identifying those at risk of critical illness following COVID-19, patients aged ≥ 50 and with NLR ≥ 3.13 being at high risk (Liu et al. 2020a, b). Out of the first 41 patients diagnosed with COVID-19 in Wuhan, 5 had myocardial injury, which mainly manifested as an increase in high-sensitivity cardiac troponin I (Wang et al. 2020a, b, c, d, e, f, g). Laboratory tests showed elevated C-reactive protein (CRP), transaminases and LDH, and lymphopenia (Bonomi et al. 2020). The myocardial zymogram showed high levels of creatine kinase in several patients (Wang et al. 2020a, b, c, d, e, f, g). COVID-19 patients may predispose to thromboembolic disease due to excessive inflammation, hypoxia and diffuse intravascular coagulation (Wang et al. 2020a, b, c, d, e, f, g; Chen et al. 2020a, b, c, d; Guan et al. 2020). The majority of the ICU patients admitted with COVID-19 exhibited thrombotic complications, such as symptomatic acute pulmonary embolism, deep vein thrombosis, ischemic stroke and myocardial infarction (concomitant with high plasma levels of IL-2, IL-7, IL-10, GSCF, IP-10, MCP-1, MIP-1A, and TNF-α) (Klok et al. 2020). Kidney damage in COVID-19 patients was observed mainly due to sepsis, hypovolaemia, and nephrotoxins. Cardiorenal syndrome may also lead to acute kidney injury in COVID-19 patients (Wang et al. 2020a, b, c, d, e, f, g). Abnormal liver function was further documented in COVID-19 patients with alanine aminotransferase (ALT) or aspartate aminotransferase (AST) above the normal range (Wang et al. 2020a, b, c, d, e, f, g). Symptoms such as olfactory and gustatory dysfunctions were also found (Vaira et al. 2020). Moderate conjunctivitis could be the first sign of severe respiratory distress in COVID-19 patients (Daruich et al. 2020). A case of brain damage by SARS-CoV-2 in Beijing Ditan Hospital (Xiang et al. 2020) and a another case of SARS-CoV-2 infection-related encephalitis were also reported (Ye et al. 2020).

Cancer patients are particularly susceptible to severe form of the disease (Xia et al. 2020; Onder et al. 2020; Wang and Zhang 2020) and are significantly at higher risk of death from COVID-19 (Deng et al. 2020). An Italian population-wide study showed that out of 430 cancer patients, 118 had prostate cancer in a total of 4532 COVID-19 patients; the study also highlighted that male cancer patients were 79% more likely to test positive for SARS-CoV-2 (Montopoli et al. 2020). Studies on COVID-19 patients from a New York Health System revealed that the mortality rates were 55% for lung cancer, 14% for breast cancer, 20% for prostate cancer, and 38% for colorectal cancer (Mehta et al. 2020). Thus, cancer patients accompanying COVID-19 infection were recommended to avoid treatments causing immunosuppression (Zhang et al. 2020a, b). What is interesting is that the new data from UK and Italy seems to show that chemotherapy is not particularly a risk factor. It is suspected that there is a metabolic issue involved, consistent with susceptibility in cancer patients, elderly and male sex.

6 Pathogenesis of COVID-19

6.1 SARS-CoV-2 Attachment and Entry

SARS-CoV-2 infection ensues when the S-protein binds to ACE2 for cellular entry into the target host cell. The internalization of the virus is facilitated by TMPRSS2 protease activity and cathepsin B/L (cat B/L) activity which may substitute for TMPRSS2 (Hoffmann et al. 2020). ACE2 receptors contain two lobes at their N-terminal peptidase domain which is the peptide substrate binding site. The extended receptor binding motif (RBM) in the RBD of S1 attaches with the lower side on the small lobe of ACE2 accommodating its N-terminal helix. RBM contains most of the contact residues of SARS-CoV-2 that bind with ACE2, an estimated 17 residues of RBD interact with 20 residues of ACE2. The upper side of the RBM is capable of forming salt-bridge interactions with ACE2, which is unique to SARS-CoV-2 (Lan et al. 2020). S-protein of SARS- CoV-2 is capable of binding ACE2 with 10–20 times greater affinity than SARS-CoV

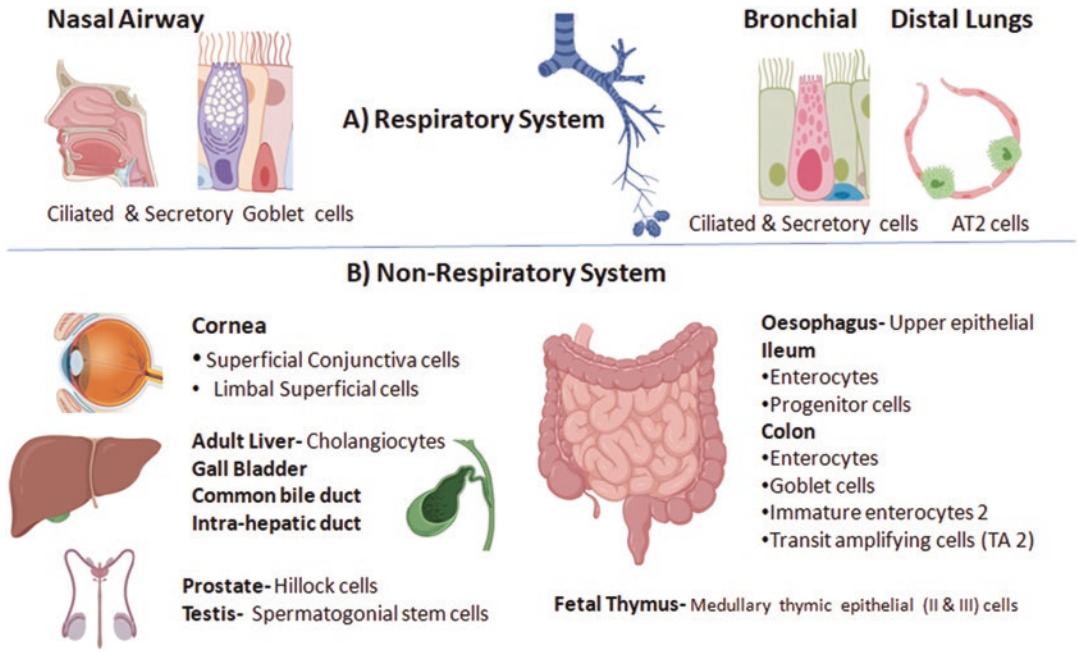

Fig. 6.2 Cells co-expressing ACE2 and TMPRSS2. Cells present in respiratory as well as non-respiratory systems can bind SARS-CoV-2 through its ACE2 and TMPRSS2. In the respiratory system, their co-expression is observed in ciliated and secretory cells of nasal and bronchial airways; in the distal lungs, they are co-expressed in alveolar type-2 cells (AT2). Different cell types present in the cornea, oesophagus, Ileum, colon, liver, gall bladder, prostate, testis and fetal thymus have been found to co-express ACE2 and TMPRSS2 necessary for SARS-CoV-2 infection

(Wrapp et al. 2020). The ACE2-binding ridge in SARS-CoV-2 RBD has a more compact conformation with two virus-binding hotspots at the RBD–ACE2 interface compared to SARS-CoV RBD (Shang et al. 2020a).

SARS-CoV-2 uses two different pathways for its entry, depending on the protease availability; it can either fuse with the plasma membrane (early pathway), or with the endosomal membrane (late pathway). Upon binding with the S-protein of SARS-CoV-2, the S2 subunit is primed by type 2 transmembrane protease TMPRSS2 that expedites coalescence enabling entry at the plasma membrane surface (Hoffmann et al. 2020; Matsuyama et al. 2010). This leads to cleavage of the ACE2 receptor, thereby facilitating viral entry into the target cell. A recent study on gene expression of ACE2 in multiple scRNA-seq datasets suggested that it is expressed in multiple tissues, such as the airways, oesophagus, ileum, colon, liver, cornea, heart, kidney and testis (Sungnak et al. 2020). A study of single cell gene expression matrices revealed that ACE2 is mainly expressed in alveolar lung type II cells (AT2), oesophagal keratinocytes, liver cholangiocytes, colon colonocytes, ileum endothelial cells (EC), rectum EC, stomach epithelial cells and renal proximal tubules (Qi et al. 2020). Across the airway, ACE2 was expressed in multiple epithelial cells, including alveolar epithelial type II cells in the parenchyma, where nasal epithelium clusters of goblet cells and ciliated cells indicated the highest expression. TMPRSS2 was also highly expressed in nasal goblet and ciliated cells which suggests that these cells may act as loci of original infection and possible reservoirs for dissemination within and between individuals (Sungnak et al. 2020) (Fig. 6.2). In single-cell RNA-sequence datasets of adult human testis, ACE2 was found to be expressed in both germ cells and somatic cells; Sertoli cells, spermatogenic stem cells, and Leydig cells showed ACE

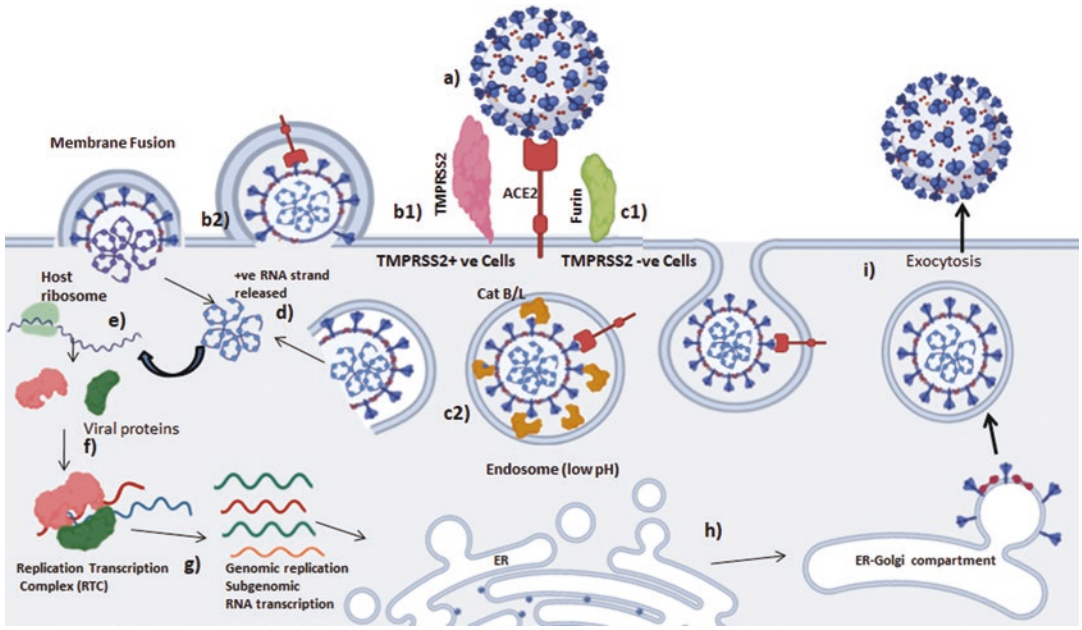

Fig. 6.3 Proposed model for SARS-CoV-2 entry and release from the host cell. (**a**) SARS-CoV-2 spike protein binds ACE2 by the amino terminal region (S1 portion) for cellular entry; (**b1**) Upon binding with the S-protein of SARS CoV-2, it is primed by type 2 transmembrane protease TMPRSS2 that enables entry at the plasma membrane surface, the S2-portion of the S-protein fuses with the TMPRSS2; (**b2**) The fusion peptide (FP) is inserted into the host cell membrane to trigger the fusion event with the host cell. The HR (HR1 and HR2) of the S2 unit adopts a hydrophobic interface to drive membrane fusion and the TM region next to HR2 anchors the S-protein in the viral membrane, (**c1**) For TMPRSS2^{-} cells, SARS-CoV-2 enters the host cell via CatB/L endosomal pathway. In case of cells with lower expression of TMPRSS2 and CatB/L proteases, furin pre-activation can facilitate SARS-CoV-2 entry. (**c2**) A low pH environment activates CatB/L cleaving S2′ site, thus triggering the fusion pathway, (**d**) SARS-CoV-2 genome is released inside the host cytoplasm, (**e**) Once the genomic RNA, which is a positive sense strand enters the cell, its two ORFs (ORF1a and ORF1b) translate into several nsps, (**f**) Coronavirus replication and transcription are mediated by a replication-transcription complex (RTC) which is virus-encoded, (**g**) RNA positive strand generates negative RNA intermediates that act as a template for the synthesis of a new positive sense RNA (gRNA) and sub-genomic RNAs (sgRNA). (**h**) The S glycoprotein oligomerizes in the endoplasmic reticulum and is incorporated into budding virions in a pre-Golgi compartment. The structural protein helps in packing the gRNA during virion assembly. (**i**) Eventually, the vesicles containing the virion fuse with the plasma membrane releasing them to infect other cells

abundance (Shen et al. 2020). Co-expression of ACE2 and TMPRSS2 in superficial conjunctival cells suggests the possibility of the spread of SARS-CoV-2 through the nasolacrimal duct (Sungnak et al. 2020) (Fig. 6.2). Other type II transmembrane serine proteases (TTSP) have also been found to play a role in CoV infection, such as TMPRSS11a that can cleave and activate SARS-CoV S-protein for fusion (Kam et al. 2009) and TMPRSS11d, also known as a human airway trypsin-like protease (HAT) that can activate MERS-CoV infection (Bertram et al. 2011; Zmora et al. 2018).

The S1/S2 cleavage site of SARS-CoV-2 S-protein possesses several arginine residues rendering it susceptible to cleavage (Hoffmann et al. 2020). The S-protein trimer is cleaved into S1, containing the RBD and S2 subunit, S2 is further cleaved into S2′ to form the viral membrane fusion peptide which is inserted into the host cell membrane (Walls et al. 2020a, b). Heptad repeat (HR1 and HR2) of the S2 unit adopts a hydrophobic interface to drive membrane fusion and the TM region located next to HR2 anchors the S-protein in the viral membrane (Tang et al. 2020a, b). In TMPRSS2^{-} cells, the low pH envi-

ronment activates cathepsin L cleavage of the S2′ site, thus triggering the fusion pathway and is responsible for viral egress from endosomes in SARS-CoV-2 (Tang et al. 2020a, b). Therefore, protease activity possibly encourages virus infiltration by one of the two pathways. The first mode is direct fusion of the S-protein through proteolytic cleavage by the host cell surface TMPRSS2 serine protease. The second route of entry is endocytosis; cleavage results in a conformational change and promotes fusion of the viral envelope with the endosome.

In cells with low expression of TMPRSS2 and pH-dependent CatB/L proteases, furin preactivation can facilitate SARS-CoV-2 entry by acting on furin-like cleavage sites at the S2 domain proximal to the fusion peptide site (Shang et al. 2020b; Coutard et al. 2020) (Fig. 6.3). The proprotein convertase (PPC) motif is also present at the S1/S2 boundary which is critical for SARS-CoV-2 entry into the host cell (as shown in Hela, Calu-3 and MRC-5 cells). Both TMPRSS2 and cathepsin have cumulative effects with furin favoring SARS-CoV-2 entry (Shang et al. 2020a, b) (Fig. 6.3).

6.2 SARS-CoV-2 Genome Translation, Replication, Assembly and Release

Following entry into the host, the SARS-CoV-2 genome is released into the cytoplasm of the host cell. The 5′ methylated cap and 3′ polyadenylated tail in the coronavirus RNA genome aid attachment of the viral replicase gene to host cell ribosomes where two-thirds are translated. ORF1a and ORF1b employ papain-like protease (PLpro) and 3C-like protease (3CLpro) to act on the polyprotein structures and cleave them at specific sites to produce several non-structural proteins (nsps). ORF1a translates into a 440–500 kDa polypeptide which gets cleaved into 11 nsps, whereas ORF1b translates into a large 740–810 kDa polypeptide which is cleaved into 15 nsps (Kim et al. 2020). The nsps assemble into the replication transcription complex (RTC) to upregulate RNA synthesis (Fig. 6.3). The viral RTC stimulates RNA synthesis of genomic and sub-genomic RNAs, which are required for accessory genes of the replicase polypeptides. RNA-dependent RNA polymerase (RdRp), also known as nsp12, is the main protein facilitating replication and transcription of viral RNA (Gao et al. 2020a, b, c, d). Papain-like protease (PLpro) and 3C-like protease (3CLpro) perform the proteolytic cleavage. RNA positive strand generates negative RNA intermediates that act as a template for synthesis of new positive sense RNA (gRNA) and subgenomic RNAs (sgRNA). RdRp, also known as nsp12, catalyzes the synthesis of viral RNA, possibly with the involvement of nsp7 and nsp8 as cofactors (Gao et al. 2020a, b, c, d). SARS-CoV-2 expresses nine canonical sgRNA (S, 3a, E, M, 6, 7a, 7b, 8 and N) along with gRNA. The structural protein helps in packing the gRNA during virion assembly. Like other coronavirus, SARS-CoV-2 RNAs also carry poly (A) tails (Kim et al. 2020). The S glycoprotein oligomerizes in the endoplasmic reticulum and is incorporated into budding virions in a pre-golgi compartment (Tooze et al. 1984). Eventually, the vesicles containing the virions fuse with the plasma membrane releasing them to infect other cells (Fig. 6.3).

7 Host Immune Response Against SARS-CoV-2

The time between exposure to SARS-CoV-2 and appearance of noticeable symptoms is the incubation period, which ranges between 2 and 14 days (median 4–5 days) (Guan et al. 2020; Li et al. 2020a, b, c; Backer et al. 2020; Wang et al. 2020a, b, c, d, e, f, g). The inhaled SARS-CoV-2 virus most likely first binds to the epithelial cells (ciliated and goblet cells) of the nasal airway through ACE2 receptors that are primed by TMPRSS2 protease. At this time, the virus can be found in nasal samples. Once inside the cell, the

virus starts replicating and activates the innate immune arm of the host.

7.1 Innate Immune Response

The innate immune response is most likely initiated when the virus reaches the airways where it is detected by toll-like receptors (TLRs), this induces expression of type I interferon (IFN). ACE2 is known to regulate the Renin-Angiotensin System (RAS), thus, a reduction in ACE2 expression due to viral infection results in RAS dysfunction. This potentially modulates blood pressure and induces inflammation and vascular permeability in respiratory airways. In approximately 80% of infected patients, the virus remains restricted to the upper and conducting airways exhibiting mild symptoms (Wu and McGoogan 2020).

The virus goes on to reach the alveoli, infecting alveolar type II cells (AT2) in the lungs. The viral infected AT2 cells undergo apoptosis and/or pyroptosis leading to vascular leakage and alveolar damage releasing the virus (Huang et al. 2020; Yang 2020). IL-1β is elevated during SARS-CoV-2 which is a pivotal cytokine released during pyroptosis (Huang et al. 2020). Pyroptosis leads to the release of damage associated molecular patterns (DAMPs), which are recognized by nearby epithelial cells, endothelial cells and alveolar macrophages, triggering production of pro-inflammatory cytokines and chemokines. This sudden increase in the local and circulating levels of pro-inflammatory cytokines leads to a cytokine storm. A severe inflammatory response can result in mass macrophage death within the lungs (accounting for more than 95% of the leukocytes) due to pyroptosis, necroptosis and necrosis leading to advanced lung damage (Vincent et al. 2005; Huang et al. 2020) (Fig. 6.4).

7.2 Cytokine Storm

Cytokine storm is an aggravated inflammatory response, which causes significant immunopathology involving widespread tissue damage. Cytokine storm has been reported in several viral infections including influenza (Kalaiyarasu et al. 2016), SARS-CoV and MERS-CoV (Channappanavar and Perlman 2017). In alveoli, the cytokine storm leads to acute lung injury and may set up ARDS, a major cause of morbidity in SARS-CoV-2 infection. Along with IL-1β, several other cytokines and chemokines such as IL-7, IL-8, IL-9, IL-10, basic fibroblast growth factor (FGF), granulocyte colony-stimulating factor (GCSF), GM-CSF, IFN-γ, interferon-γ-inducible protein-10 (IP-10) (also known as CXCL10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1A and 1B (MIP-1A, MIP-1B), platelet derived growth factor (PDGF), TNF-α, and VEGF, have been identified as constituents of this rogue response. IL-6 and IFN-γ levels are significantly higher in both ICU and non-ICU cases of COVID-19 patients compared to healthy adults (Huang et al. 2020). Plasma concentrations of IL-2, IL-7, IL-10, G-CSF, IP-10, MCP-1, MIP-1A, and TNF-α are higher in ICU patients than non-ICU patients, suggesting the severity of COVID-19 and its associated morbidity is possibly due to virally driven hyperinflammation. IL-6 is one of the frequently reported cytokines elevated in COVID-19 patients whose level is significantly higher in severe cases than in mild cases (Ruan et al. 2020; Gao et al. 2020a, b, c, d; Chen et al. 2020a, b, c, d). In addition to IL-6, IL-10 and TNF-α are also linked with severe COVID-19 cases (Chen et al. 2020a, b, c, d). This uncontrolled and overwhelming systemic inflammatory response leads to vascular permeability, pneumocyte desquamation, plasma leakage into interstitial and alveolar spaces, and pulmonary infiltration of leukocytes such as macrophages and neutrophils (Martines et al. 2020). At this stage, chest CT scans exhibit bilateral glass opacities with multifocal infiltrates due to alveolar collapse and edema (Ghinai et al. 2020). Alveolar collapse causing hypoxemia and dyspnea is initiated potentially due to increase in alveoli surface tension as the level of surfactant protein in lungs drops. Lung autopsies in severe cases of COVID-19 show bilateral alveolar damage with cellular

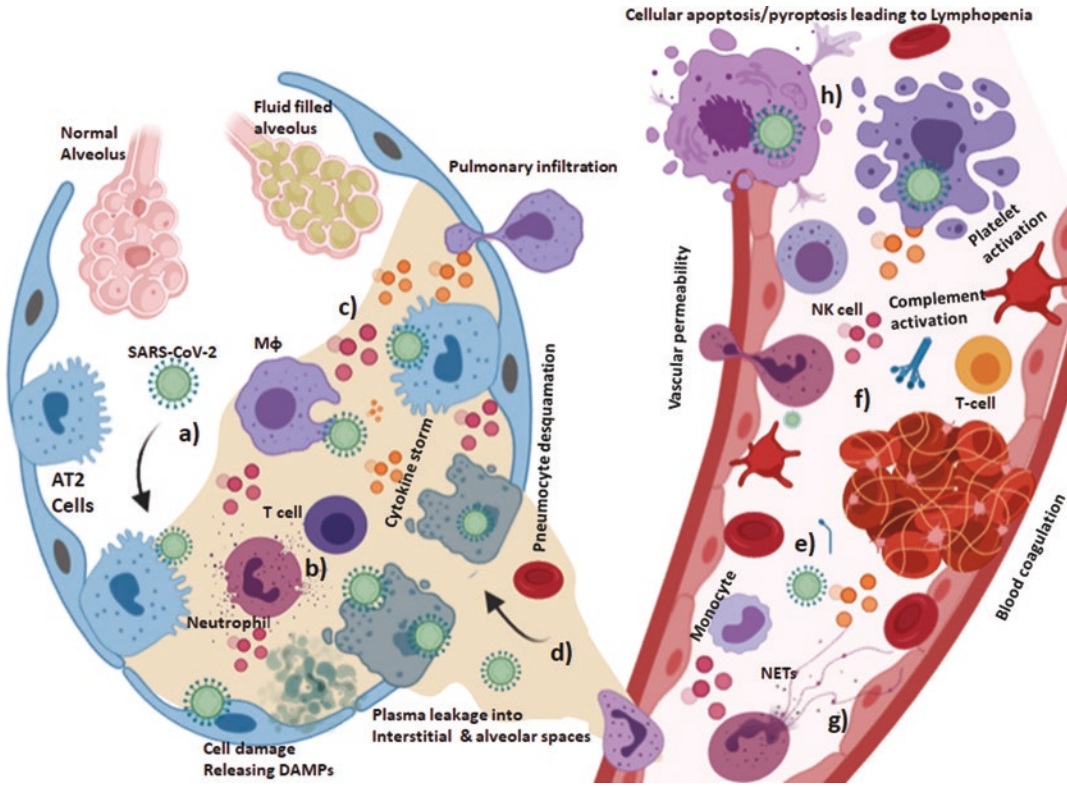

Fig. 6.4 Innate Immune response (proposed). (**a**) SARS-CoV-2 reaches the alveolar airways and infects the alveolar type II cells (AT2). (**b**) The infected AT2 cells undergo apoptosis and/or pyroptosis leading to alveolar damage releasing the virus. Damage Associated Molecular Patterns (DAMPs) released from the damaged cells are recognized by nearby epithelial cells, endothelial cells and alveolar macrophages triggering production of pro-inflammatory cytokines and chemokines, (**c**) This sudden acute increase in the levels of pro-inflammatory cytokines and chemokines leads to a cytokine storm and is the main cause of ARDS. Several cytokines and chemokines such as IL-1β, IL-7, IL-8, IL-9, IL-10, FGF, GCSF, GMCSF, IFN-γ, IP-10, MCP-1, MIP1A, MIP1B, PDGF, TNFα, VEGF, IL-6 and IFN-γ contribute to the cytokine storm, (**d**) This uncontrolled systemic inflammatory response leads to vascular permeability, pneumocyte desquamation, plasma leakage into interstitial and alveolar spaces, and pulmonary infiltration of leukocytes such as macrophages, neutrophils and lymphocytes. Neutrophils and macrophages release enormous amounts of reactive oxygen species (ROS), (**e**) Excessive inflammatory reactions lead to several pathological changes, such as coagulation pathway activation as well as disseminated intravascular coagulation (DIC), (**f**) Activation of platelets is often linked with elevation of complement activation products, leading to systemic inflammatory response syndrome (SIRS), (**g**) Cellular apoptosis/pyroptosis of virus infected cells leads to endothelial destruction and enables plasma flooding into alveoli; (**h**) Neutrophil extracellular traps (NETs) are produced in response to infection where extracellular DNA fibers are extruded by neutrophils allowing them to trap and kill extracellular microorganisms

fibromyxoid exudates and mononuclear inflammatory lymphocytes (Xu et al. 2020a, b, c; Bonomi et al. 2020) (Fig. 6.4).

Surfactant proteins, SP-A and SP-D, are involved in innate immune responses at the mucosal surfaces, especially in the lungs, against various pathogens including viruses (Yasmin and Kishore 2021). In case of SARS-type pneumonia, SP-D levels were significantly elevated (Leth-Larsen et al. 2007; Wu et al. 2009). HCoV-229E, a common non-SARS human CoV binds with SP-A and SP-D; pre-treatment of HCoV-229E with SP-A or SP-D inhibits viral infection. SP-D is more effective in inhibiting 16HBE cells infection whereas SP-A is more in inhibiting infection of alveolar macrophages (Funk et al.

2012). A recent work showed recombinant SP-D (rfhSP-D) was capable of competing with ACE-2 for binding to the S1 spike protein of SARS-CoV-2. rfhSP-D treatment inhibited viral replication by ~5.5 fold and a 2-fold reduction in viral infectivity was also observed in SARS-CoV-2 positive clinical samples (Madan et al. 2021). In an another study, rfhSP-D showed a dose-responsive binding to S1 spike protein and its receptor binding domain of SARS-CoV-2. rfhSP-D was capable in inhibiting interaction of S1 protein with the HEK293T cells overexpressing ACE-2 (Hsieh et al. 2021). These results highlight the possible therapeutic potential of rfhSP-D in SARS-CoV-2 infection.

Severe COVID-19 patients show symptoms related to secondary haemophagocytic lymphohistiocytosis (SHLH), which is typically characterized by sudden fatal hypercytokinaemia with multiorgan failure (Ramos-Casals et al. 2014). Approximately 50% of SHLH patients show clinical features similar to ARDS (Seguin et al. 2016). The cytokine profile (IL-2, IL-7, MIP-1A, G-CSF, TNF-α) elevated in severe COVID-19 also draws parallels with SHLH (Huang et al. 2020). The host repair system in many cases restores normal function but excessive tissue damage can often trigger wound healing through fibrosis that can eventually result in persistent organ dysfunction. The pulmonary cytokine storm circulates to other organs (systemic inflammatory response syndrome) causing increased capillary permeability in systemic circulation leading to decreased blood pressure (hypotension). This hypotension reduces the organ perfusion pressure causing multi-organ failure. SARS-CoV-2 infection often becomes life-threatening by inducing multi-organ injury involving the heart, liver, kidney, brain, intestine, and eyes (Li et al. 2020a, b, c; Klok et al. 2020).

7.3 Complement Associated Pathogenesis

Excessive inflammation precipitates several pathological changes, such as coagulation pathway activation, disseminated intravascular coagulation (DIC), cellular apoptosis/pyroptosis, increased vasopermeability and hypermetabolism, which finally proceeds into a septic pro-inflammatory microenvironment. Cardiovascular complications in COVID-19 patients such as acute thrombosis of the abdominal aorta and pulmonary embolism have been observed (Le Berre et al. 2020). Coagulation and complement, though being two distinct systems, are similar in how they are controlled and interact with each other. These controls occur at two basic levels, either by inhibiting the enzyme activity or by blocking the binding of a cascade component (Oikonomopoulou 2012). In critical COVID-19 cases, there is an increasing recognition of a hypercoagulable condition with possible complement activation noted in some patients (Magro et al. 2020).

Activation of the complement cascade is correlated with thrombosis and the development of multiple organ failure. Both C3 and C5 can be proteolytically activated by several components of the coagulation cascade in addition to thrombin. C5a, exhibiting chemotactic activity towards neutrophils, is produced by the enzymatic action of thrombin. Activation of complement components both upstream and downstream of C3 and C5 convertases can also be initiated by the coagulation cascade (Ghebrehiwet et al. 1981). For example, coagulation factor XIIa can activate C1 initiating the classical pathway; C1q as well as C1 inhibitors, C4b-binding protein and factor H can bind to platelet surfaces (Ghebrehiwet et al. 1983; Hamad et al. 2010). Further hyperactivation of complement can also be linked to excessive septic inflammation leading to systemic inflammatory response syndrome (SIRS). Activation of platelets is a common event during sepsis along with elevation of complement activation products, such as C3a, C4a, and C5a (Younger et al. 2010; Hack et al. 1989). C5a is a major player in the pathogenesis of several diseases and is capable of activating the coagulation and TLR pathways (Hajishengallis and Lambris 2010; Rittirsch et al. 2008; Hawlisch et al. 2005). Activated platelets can release a serine/ threonine protein kinase that is able to phosphorylate C3 (Ekdahl and Nilsson 1995; Gulla et al. 2010). This modification can result in the generation of

a phosphorylated C3b fragment that is resistant to further proteolytic processing into iC3b by factor I. Complement Factor H has an inhibitory effect on the Hageman FXII contact plasma activation by acidic phospholipids (Ferluga et al. 2014). Factor H can downregulate the complement classical pathway, by competing with C1q binding to anionic phospholipid surface (Tan et al. 2010; Kishore and Sim 2012). Endothelial cell injury in tissue factor-dependent thrombosis, where activated platelets were found to secrete granular polyphosphates can further activate FXII inducing occlusive thrombosis (Muller et al. 2009; Renne et al. 2012).

Severe COVID-19 patients show a procoagulant profile characterized by increased clot strength, elevated D-dimer levels, hyperfibrinogenemia, and increase in CRP, factor-VIII and von Willebrand factor (Panigada et al. 2020; Ranucci et al. 2020). AMY-101, a C3 inhibitor, has been evaluated for its anti-inflammatory response in severe cases of COVID-19 infection. Intravenous administration of AMY-101 showed a dramatic improvement with CRP and LDH getting normalized progressively, while leukocytosis and lymphocytopenia improved more gradually. A significant improvement in the respiratory performance was also observed. Treatment with AMY-101 was found to be safe with no side effects and with no further worsening of renal and hepatic function (Mastaglio et al. 2020). High fibrinogen levels are also associated with IL-6. With increased thromboprophylaxis, the pro-coagulant profile attained normalization and depreciated the D-dimer levels in COVID-19 patients (Ranucci et al. 2020).

A thin layer of endothelial-epithelial septum separates the alveolar cavity from blood. Endothelial destruction due to pyroptosis/apoptosis allows large amounts of plasma and cells to flood into alveoli causing ARDS (Fig. 6.4). Endothelial injury can also cause microvascular angiopathy and thrombosis, this damage can activate the complement lectin pathway. The lectin pathway effector enzyme, mannan-binding lectin-associated serine protease-2 (MASP-2), aids in the activation of thrombin (Krarup et al. 2007; Gulla et al. 2010). SARS-CoV-2 nucleocapsid protein can activate MASP-2; it has also been traced in the lung tissue of COVID-19 patients along with C4d and the membrane attack complex, C5b-9 (Gao et al. 2020a, b, c, d; Magro et al. 2020). Narsoplimab is a high-affinity humanised monoclonal antibody, which is capable of binding with MASP-2 and blocking the lectin pathway, was found to be effective in treating COVID-19 patients with no adverse drug reactions (Rambaldi et al. 2020).

Neutrophil infiltration in capillaries with fibrin deposition is observed in COVID-19 patients (Zuo et al. 2020). Neutrophil extracellular traps (NETs) are produced in response to infection where extracellular DNA fibers are extruded by neutrophils allowing them to trap and kill extracellular microorganisms. NETs cause platelet adhesion (often associated with deep vein thrombosis) (Costanzo et al. 2020). Sera of COVID-19 patients showed elevated levels of myeloperoxidase-DNA (MPO-DNA) and citrullinated histone H3 (Cit-H3), which are specific markers of NETs (Zuo et al. 2020). COVID-19 patient's plasma showed spontaneous formation of NETs expressing functional tissue factor (TF) and considerable increase in plasma level of sC5b-9 (terminal complement component). Thrombin or NETosis inhibition or C5aR1 blockade could attenuate platelet-mediated NET-driven thrombogenicity in COVID-19 patients. Cp40-mediated C3 inhibition was capable of disrupting TF expression in neutrophils, thus preventing complement activation and impairing thrombogenicity (Skendros et al. 2020). Thus, complement activation during SARS-CoV-2 infection can possibly influence the platelet-NETs-TF-thrombin axis.

7.4 Adaptive Immune Response

COVID-19 patients show high level of SARS-CoV-2 specific IgM at early time points which decline over time while the IgG antibodies remain relatively stable (Sun et al. 2020a, b). However, antibody responses is not detectable in all patients, especially those with less severe forms of COVID-19 (Long et al. 2020a, b; Mallapaty

2020; Woloshin et al. 2020). COVID-19 patients have anti-viral IgG within 19 days of symptom onset, however both IgM and IgG titres reach plateau within 6 days of seroconversion (Long et al. 2020a, b). Zhou et al. (2020) reported that COVID-19 patients exhibited nucleocapsid protein (NP)-specific antibody responses, with IgM peaking at the ninth day post disease onset and then switching to IgG by week 2. Sera from COVID-19 patients were capable of inhibiting SARS-CoV-2 entry in target cells (Zhou et al. 2020). In another study, the median seroconversion time for IgM and IgG were day 11, 12 and 14 post symptom onset. Within 1 week of onset, the presence of antibodies was low but increased considerably from 15 day onwards (more IgG than IgM) (Zhao et al. 2020). COVID-19 patients mounted IgG and IgM responses to N-protein and spike-RBD proteins, and infected patients could maintain IgG levels for at least 2 weeks (Ni et al. 2020). Most COVID-19 convalescent individuals have a detectable level of neutralizing antibodies, as judged using the pseudovirus particle-based neutralization assay. The anti-S-RBD IgG might be predictive of serum neutralization capabilities in COVID-19 patients (Ni et al. 2020). In a recent study, antibody responses to 15 different SARS-CoV-2 antigens in COVID-19 patients was assessed with a luciferase immunoprecipitation system (LIPS) (Hachim et al. 2020). Antibodies representing the structural and non-structural viral proteins [four structural proteins (S, N, M and E), three S subunits (S1, S2 and S2′), the seven available ORFs (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8 and ORF10) and one relevant NSP within ORF1ab (NSP1)] were considered for the LIPS assay. Elevated antibody responses were seen against 11 antigens (the structural proteins full S, S1, S2′, N and M and the ORFs: NSP1, ORF3a, ORF3b, ORF7a, ORF7b and ORF10), with nucleocapsid, open reading frame (ORF)-8 and ORF3b eliciting the strongest specific antibody responses. ORF8 and ORF3b antibodies are therefore potential serological markers for SARS-CoV-2 infection, identified in 96.5% of COVID-19 samples at early and late time points of disease with 99.5% specificity (Hachim et al. 2020). Anti-RBD IgM and IgA were also detected in the majority of recovered COVID-19 patients (Grifoni et al. 2020).

A recent detailed study has sought to characterize humoral and circulating follicular helper T cell (cTFH) immunity against the S-protein in COVID-19 recovered patients (Juno et al. 2020). Comparatively low frequencies of B cells and cTFH specific cells for the RBD of the S-protein were found. The frequency and specificity of class-switched ($CD19^+IgD^-$) B cells were examined using an S or RBD flow cytometric probe, where populations of B cells binding spike (S^+RBD^-), spike and RBD (S^+RBD^+) or RBD alone (S^-RBD^+) in convalescent COVID-19 patients were compared to a healthy control. The majority of S^+RBD^- B cells were IgG^+ with smaller proportions of IgM^+ and IgA^+. The activation phenotype of antigen-specific B cells was examined using CD21 and CD27. The S^+RBD^- or S^+RBD^+ B cells were found predominantly in the resting memory phenotype ($CD21^+CD27^+$), consistent with the median time since infection. A considerable population of activated memory B cells ($CD21^-CD27^+$) was observed for both S^+RBD^- and S^+RBD^+ populations. Thus, S-specific antibodies, memory B cells and cTFH are consistently elicited after SARS-CoV-2 infection, exhibiting robust humoral immunity that positively correlates with plasma neutralizing activity (Juno et al. 2020) (Fig. 6.5).

A flow cytometry study of peripheral blood monocytic cells (PBMCs) showed an increased frequency of NK cells in discharged patients while the percentage of T cells remained unchanged. An S-RBD induced T cell immune response was identified with a higher percentage of IFN-γ-secreting S-RBD specific T cells compared with healthy donors (Ni et al. 2020). Patients with acute, moderate or severe COVID-19 showed low frequencies of $CD4^+$ and $CD8^+$ T cells (Liu et al. 2020a, b). A study was carried out involving unexposed individuals, exposed family members, and individuals with acute or convalescent COVID-19 to understand the functional and phenotypic landscape of SARS-CoV-2-specific T cell responses. Memory $CD8^+$ T cells from patients with acute, moderate or severe COVID-19 were found to express CD38, HLA-DR, Ki-67,

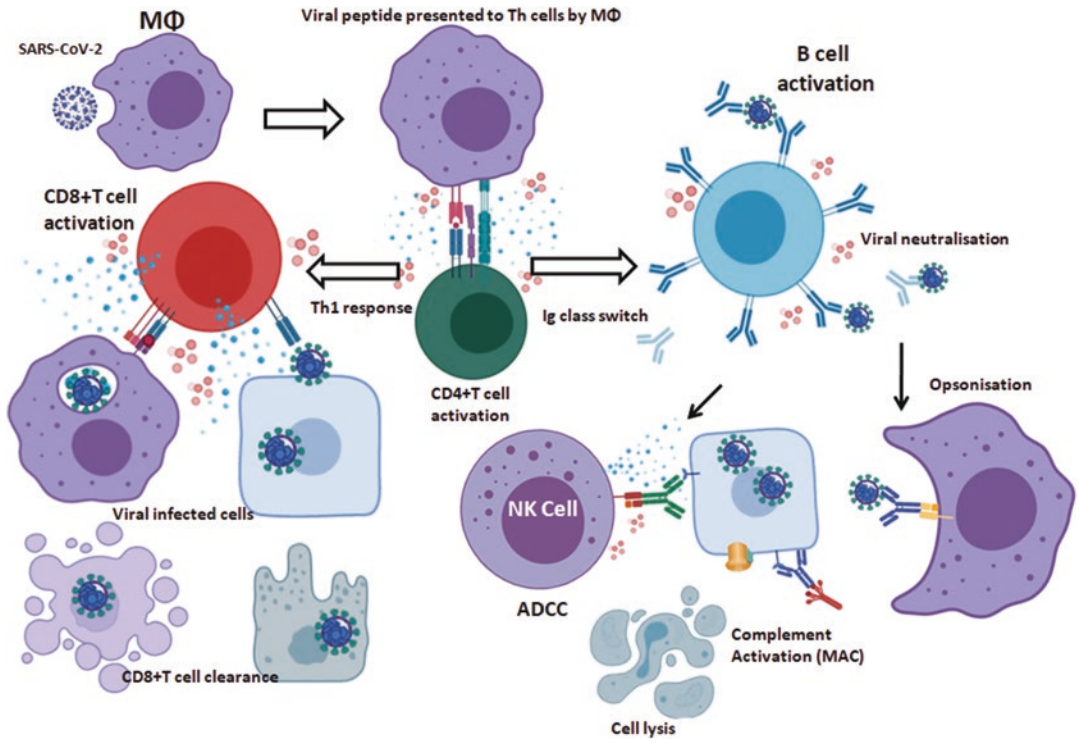

Fig. 6.5 Adaptive Immune response against SARS-CoV-2. SARS-CoV-2 viruses are engulfed by phagocytic cells such as macrophages (MΦ). These phagocytic cells further express the viral peptide on their surface to present $CD4^{+}$T cells. This binding activates $CD4^{+}$T cells, which secrete cytokines to further activate (a) $CD8^{+}$T cells which mount a cytotoxic response towards virally infected macrophages or other cells by secreting perforin and granzymes (b) B cells which undergo Ig class switching and secrete virus specific antibodies which are capable of neutralizing the virus. Antibodies secreted by these activated B cells can also mount a response through ADCC by activating NK cells and complement pathways leading to MAC formation that eventually destroys the virally infected cells. Antibodies can also enhance opsonization by binding with the Fc portion of macrophages, and/or clearance from circulation by Kupffer cells

and PD-1, markers that are associated with activation and cell division. This suggests that T cells may establish a more robust early SARS-COV-2-specific adaptive immune response in COVID-19 patients (Sekine et al. 2020). The results also revealed a clear segregation between memory T cells from patients with acute, moderate or severe COVID-19 and those from convalescent individuals and healthy blood donors and more importantly T cell activation, characterized by the expression of CD38. SARS-CoV-2-specific T cells displayed a highly activated cytotoxic phenotype in the acute phase of the disease that correlated well with various clinical markers of disease severity such as age, hemoglobin concentration, platelet count, and plasma levels of alanine aminotransferase, albumin, D-dimer, fibrinogen, and myoglobin (Sekine et al. 2020).

CD4 and CD8 T cell responses were recognized against multiple regions of the N proteins of SARS-CoV-2 in patients convalescing from COVID-19 (Le Bert et al. 2020) (Fig. 6.5). In a group of individuals who recovered from SARS (2003), the data illustrated long-lasting memory T cells and displayed robust cross-reactivity towards the N-protein of SARS-CoV-2. Notably, ORF1-specific T cells were traced in a few individuals who were not exposed to SARS-CoV-2, at the same time T cells from individuals who recovered from COVID-19 could preferentially recognize structural proteins. ORF1-encoded proteins are produced as soon as viral RNA enters

the host cells and are essential for formation of the RTC, thus, it can be assumed that ORF1-specific T cells can potentially mount a cytotoxic response towards SARS-CoV-2 infected cells prior to the formation of mature virions (Le Bert et al. 2020). The presence of SARS-CoV-2 cross reactive $CD4^+$ T cells specific to S-protein were also observed in unexposed healthy individuals, suggesting some degree of cross-reactive pre-existing immunity to SARS-CoV-2 in the human population (Grifoni et al. 2020).

8 Increased Susceptibility to SARS-COV-2 in Men

Recent studies with single-cell RNA-sequence datasets of adult human testes appear to suggest that SARS-CoV-2 may also infect the testis. ACE2 receptors are expressed in both germ cells and somatic cells, among which major clusters are found in Sertoli cells, spermatogenic stem cells, and Leydig cells. The difference in fatality rate between males and females is underscored by the fact that ACE2 is located on the X chromosome; oestrogen and testosterone sex hormones have different immunoregulatory functions that may contribute to protection or severity of the disease (Taneja 2018; Tay et al. 2020). In a study with 9280 SARS-CoV-2-positive patients, males developed more severe complications and had a worse clinical outcome than females (Montopoli et al. 2020). It is known that androgen receptor (AR) mediates the effects of male sex steroids and simultaneously AR regulates TMPRSS2 expression in non-prostatic tissues (Mikkonen et al. 2010), which is a vital component for SARS-CoV-2 entry in host cells and possibly explains the increased susceptibility of men to developing severe infections.

Innate immune recognition markers are encoded by genes belonging to a family of TLRs located on the X-chromosome. SARS-CoV-2 contains a host of proteins/peptides that can be recognized by TLR7/8 (Moreno-Eutimio et al. 2020). Several other immune regulatory genes located on the X chromosome include TLR8, FOXP3, CXCR3, and CD40L that usually contribute to a stronger immune response against viruses in women (Kritas et al. 2020; Flanagan et al. 2017; Klein 2012; Klein and Flanagan 2016). Detection by TLRs leads to the expression of Type I IFN (Heil et al. 2004) which are expressed in high levels by females (Klein 2012, Klein and Flanagan 2016). Female COVID-19 patients clear SARS-CoV-2 significantly earlier compared to infected male patients (Xu et al. 2020a, b, c). A meta-analysis of COVID-19 patients demonstrated a prevalence of immune mediators that are associated with adverse outcomes of SARS-CoV-2 in men, including TNFSF13B, CCL14, CCL23, IL-7, IL-16, and IL-18 (Wei et al. 2020). Males with moderate COVID-19 disease demonstrated higher level of IL-8 and IL-18 compared to female counterparts. At the same time, more robust activation of non-classical monocytes was observed in males whereas female patients mounted significantly more robust T cell activation during SARS-CoV-2 infection, suggesting the possible explanation of worse disease outcome in males. This sex bias in COVID-19 can possibly be considered as a vital factor for remedial approaches in future (Takahashi et al. 2020).

9 Repurposing Drugs in COVID-19

Developing a vaccine is time consuming and may take a substantial amount of time to become available globally. Thus, repurposing an existing drug is a more viable route, which can expedite COVID-19 treatment. Several pre-existing drugs have now been tested for COVID-19 (Table 6.1). Further details of drug repurposing have been recently reviewed (Varghese et al. 2020).

10 Neutralizing Antibodies and Passive Immunization (Convalescent Plasma Therapy)

In patients with SARS-CoV-2 infections, severe respiratory symptoms may develop after a week of symptom onset; this is associated with the release of several pro-inflammatory cytokines.

Table 6.1 Mode of action and the present use of existing drugs that have been repurposed for COVID-19 treatment

Drug	Mode of action and its use in COVID-19	Reference
Remdesivir	• An adenosine analogue prodrug designed specifically to improve cell permeability • It inhibits viral RNA polymerase • *In vitro* efficacy against SARS-CoV-2 infected Vero E6 cells have been studied • It is converted into nucleoside monophosphate by intracellular hydrolases leading to the formation of active negatively charged nucleoside triphosphate which is incorporated into nascent viral RNA chain, causing pre-mature termination • Administered intravenously (200 mg on day 1 followed by daily dose of 100 mg for 5–9 days)	Grein et al. (2020); Beigel et al. (2020); Wang et al. (a, b, c, d, e, f, g); Warren et al. (2016); Sun (2020); Gordon et al. (2020); https://clinicaltrials.gov/
Favipiravir	• A purine nucleoside analogue prodrug (pyrazinecarboxamide derivative) • Acts as a competitive inhibitor of RNA-dependent RNA polymerase (RdRp) causing lethal mutagenesis when incorporated into viral RNA • Effective in treating influenza, oseltamivir, zanamivir-resistant influenza and Ebola virus • Capable of inhibiting SARS-CoV-2 but duration is twice as long as that used for treating influenza (tablets of 200 mg available) • Exhibits better efficacy in anti-viral activity and with lower adverse reactions compared to lopinavir/ritonavir drug combination	Cai (2020); Furuta et al. (2013); Oestereich et al. (2014); Shiraki and Daikoku (2020); Dong et al. (2020)
Ribavirin	• A guanosine analogue • Converts intracellularly into triphosphorylated (RTP) forms by cellular kinases. RTP binds to the nucleotide binding site of viral RNA polymerase and DNA polymerase, it is incorporated into the viral genome, leading to a reduction in viral replication • Inhibits inosine monophosphate dehydrogenase by mimicking ribavirin monophosphate and acts as a competitive inhibitor and reduces de novo synthesis of guanine • It was approved only for the treatment of severe respiratory syncytial virus (RSV) infection in minors, it has also been used in the treatment of Lassa fever viral infection, influenza A and B and other viruses • Triple combination of ribavirin and lopinavir-ritonavir given orally and interferon beta-1b as an injection can effectively suppress the shedding of SARS-CoV-2 within 7 days	Graci and Cameron (2006); Kristina et al. (2020); Krilov (2001); Andrei and De Clercq (1993); Van Voris and Newell (1993); Hung et al. (2020); Miller et al. (1997); Eriksson et al. (1977)
Lopinavir and ritonavir (combination drug)	• A viral 3CL protease inhibitor used specifically in the treatment of HIV-I infection • Lopinavir is marketed in combination with ritonavir to increase the plasma half-life by inhibition of cytochrome P450 • Combination treatment including Lopinavir-ritonavir, ribavirin (an oral hepatitis C virus drug) and IFN-β1b showed effective response in reducing COVID-19 symptoms with faster viral shedding	Sheahan et al. (2020); Cao et al. (2020); Hung et al. (2020)

(continued)

Table 6.1 (continued)

Drug	Mode of action and its use in COVID-19	Reference
Dexamethasone	• An immunosuppressive drug (corticosteroid) • Possibly capable of modulating a dysregulated immune system • 6 mg of dexamethasone (orally or intravenously) for 10 days, reduces the progression to respiratory failure and death • As per the updates by WHO, 26 March, 2021, dexamethasone provides no improvement for patients with mild symptoms and is only recommended (dose 6mg) for COVID-19 patients on ventilators for their health improvement.	Horby et al. (2020); National Institute for Health Research (2020); WHO Report 2021a
Chloroquine and Hydroxychloroquine	• An aminoquinoline derived drug specifically used as an anti-malarial drug • *In vitro* studies have shown that chloroquine increases endosomal and lysosomal pH which blocks SARS-CoV-2 fusion • Its derivative, like Hydroxychloroquine is capable of decreasing IL-1, IL-2, IL-6, IL-17, IL-22, IFN-α and tumor necrosis factor which is aggressively upregulated by SARS-CoV-2 • Chloroquine can affect heart rhythms, like QT interval prolongation, ventricular tachycardia and has shown other side effects including headache, rashes, nausea and abdominal pain in COVID-19 patients • As per the updates by WHO, 17 March, 2021, clinical trials confirm that chloroquine or hydroxychloroquine does not prevent illness or death from COVID-19. It shows little to no impact on illness, hospitalization or death	Vincent et al. (2005); Silva et al. (2013); Wang et al. (a, b, c, d, e, f, g); WHO Report 2021b
Anakinra	• A recombinant IL-1 receptor antagonist that has shown promise in treating severe COVID-19 disease • Beneficial in COVID-19 patients with cytokine storm syndrome and acute hypoxic respiratory failure (AHRF) preventing them from mechanical ventilation	Navarro-Millan et al. (2020)
Ivermectin	• A broad-spectrum anti-parasitic and antiviral drug • In invertebrate parasites it can bind to specific neurotransmitter receptors blocking chemical transmission across the nerve synapses that use glutamate-gated anion channels or γ-aminobutyric acid-gated chloride channels • It can also inhibit the proliferation of cancer cells • It is likely to inhibit IMPα/β1- meditated nuclear import of viral proteins and is capable of controlling SARS-CoV-2 replication within 24–48 h	Caly et al. (2020); Chhaiya et al. (2012)

(continued)

Table 6.1 (continued)

Drug	Mode of action and its use in COVID-19	Reference
Pirfenidone	• It is a pyridone derived (5-methy-1-phenyl-2-[1H]-pyridone) anti-fibrotic drug available in oral form to treat idiopathic pulmonary fibrosis • Can also downregulate the effects of cytokines and chemokines such as TNF-α, TGF-β1, connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF) • Has been found to downregulate ACE-2 receptors. Possibly can reduce fibrotic lung lesions caused by SARS-CoV-2 • An inhaled formulation is under evaluation for patients of COVID-19 • This drug is restricted if the patient has an estimated glomerular filtration rate less than 30 mL/min per 1.73 m^3	Chung et al. (2020); Ferrara et al. (2020)
Nintedanib	• An anti-fibrotic drug available in oral form, capable of inhibiting tyrosine kinase • It reduces the time to first acute exacerbation in COVID-19 patients. Due to its oral formulation it is restricted for use in patients with COVID-19 at ICU	George et al. (2020)
Azithromycin	• Upregulates production of type I and type III interferons, specially interferon-β and interferon-λ and genes involved in virus recognition such as MDA5 and RIG-I • It regulates/decreases several cytokines involved in COVID-19 pathogenesis like IL-1β, IL-6, IL-8, IL-10, IL-12, and IFN-α	Bleyzac et al. (2020)
Heparin (low-molecular-weight)	• An anticoagulant, with anti-inflammatory properties, inhibition of neutrophil chemotaxis, and protection of endothelial cells, and a potential antiviral effect • Used as an adjunctive therapeutic drug for the treatment of COVID-19 pneumopathy	Costanzo et al. (2020)
Umifenovir	• Derivative of indole carboxylic acids • Incorporates into cell membrane, can block viral fusion with the host cell membrane and endosome • Used in the treatment of influenza A and B • Treatment has not shown improvement in clinical prognosis or in accelerating viral clearance as of yet	Boriskin et al. (2008); Villalaín (2010); Liang et al. (2020)
Tocilizumab	• A recombinant humanized IL-6 receptor antagonist • Used in the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, giant cell arteritis, effective in treating cytokine storm triggered by CAR-T cell therapy (hematological malignancies) • The US Food and Drug Administration (FDA) has approved Roche's phase III clinical trial of the use of tocilizumab for severe COVID-19 pneumonia • Intravenously or subcutaneously administered it can reduce the risk of mechanical ventilation and death in COVID-19 patients	Chen et al. (2019); Xu et al. (2020a, b, c); Barbulescu et al. (2020); Guaraldi et al. (2020)

This can often be related to low avidity and poor neutralizing antibodies and can be compensated by passive administration of antibodies to the patient. Antibodies show their anti-viral activity by inhibiting entry of infectious viral particles into host through neutralization. Antibodies function by triggering simultaneous binding of its Fab portion with the viral epitope or with the infected cells and Fc portion with immunocompetent cells such as macrophages and NK cells. The complement pathway is also activated by the Fc region binding to C1q, resulting in opsonization of viruses or infected cells.

The passive transfer of neutralizing antibody has been shown to confer protection in hamsters against a high-dose of SARS-CoV-2 (Rogers et al. 2020). Hamsters immunized with recombinant SARS-CoV S-protein trimer could also induce the development of neutralizing antibodies and were protected against a viral challenge (Kam et al. 2007). In an experiment with rhesus macaques, SARS-CoV-2 immunity associated with neutralizing antibodies and antibody-mediated effector functions and provided protection upon viral re-challenge at 35 days (Chandrashekar et al. 2020).

For several infectious diseases involving SARS, MERS and H1N1, convalescent plasma (CP) therapy, a classic passive immunotherapy, has been applied for its prevention and treatment. Patients recovered from COVID-19 with a high neutralizing antibody titer may become valuable donors for CP. In a study with ten severe SARS-CoV-2 patients, plasma (200 mL) at a median of 16.5 days after onset was administered, the presence of viruses in the blood was no longer detected and clinical parameters improved within 3 days (Duan et al. 2020). Several clinical trials are currently being undertaken to understand the potential clinical benefit and risk of convalescent blood products in COVID-19 (clinicaltrials.gov). In other studies, patients with severe or life-threatening COVID-19 who had undergone CP therapy did not result in a statistically significant improvement (Li et al. 2020a, b, c).

11 Type 1 Interferon Treatment Against COVID-19

Type I IFNs constitute a group of low-molecular glycoproteins and are among the first cytokines produced during a viral infection. This group of cytokines is recognized by the IFN-α receptor present at the plasma membrane in most cell types (Samuel 2001). Due to its immunomodulatory properties, it has been used for the treatment of numerous diseases including MERS-CoV and SARS-CoV, often in combination with lopinavir/ritonavir (Chan et al. 2015; Sheahan et al. 2020), ribavirin (Chen et al. 2004; Morgenstern et al. 2005; Omrani et al. 2014), and/or remdesivir, or corticosteroids (Loutfy et al. 2003).

Clinical studies in children from China revealed that IFN-α is capable of reducing viral load and shortening the disease duration for viral pneumonia, bronchiolitis and acute respiratory tract infections (Chen et al. 2005; Shang et al. 2014; Shen et al. 2018; 2020). Recombinant human IFN-α2b spray prevents SARS-CoV-2 infection by inhibiting viral replication in rhesus monkeys (Gao et al. 2005). A clinical study has suggested that IFN-α can be used as a prophylaxis against SARS-CoV-2 (Lokugamage et al. 2020). Clinical trials have been recently registered to evaluate a combination of lopinavir/ritonavir and IFN-α2b (ChiCTR2000029387) or a combination of lopinavir/ritonavir with ribavirin and IFN-β1b administered subcutaneously (NCT04276688) (Sallard et al. 2020). In an open clinical trial safety and efficacy trials of COVID-19 (NCT04315948), hospitalized adults were assessed in which subcutaneous IFN-β1a in combination with lopinavir/ritonavir is being compared to lopinavir/ritonavir alone, hydroxychloroquine, and remdesivir (Clinicaltrials.gov 2020).

12 Vaccination Strategies for COVID-19

Most of the vaccines under development against COVID-19 target the S-protein to elicit robust T and B cell responses, along with high viral neu-

tralizing antibody production. Researchers have been in a race to develop ways to selectively target the most potent neutralizing epitopes likely to be critical for effective vaccines against SARS-CoV-2.

12.1 Viral Vector Vaccines

Adenovirus is an attractive vector candidate for the transfer of foreign genes because it is well characterized and comparatively easier to manipulate. Most adenoviruses are well tolerated and cause mild effects in immunocompetent human adults. Deletion of some crucial regions results in a replication-defective vector, which increases efficiency and reduces side-effects. For clinical use, they can be applied systemically as well as through the mucosal surface (Tatsis and Ertl 2004). Recombinant viruses can be used as vehicles for delivery of vaccines as the viral protein can act as potent adjuvants and can directly infect antigen-presenting cells (Rocha et al. 2004). The first report of using a chimpanzee adenovirus as a viral vectored vaccine demonstrated that chimpanzee adenovirus serotype 68 can express rabies glycoprotein and induce an immune response (Xiang et al. 2002). Viral vector vaccines induce cellular immune responses better than subunit vaccines (Draper and Heeney 2010).

12.1.1 Ad5-nCoV Vaccine

Ad5-nCoV vaccine is a genetically engineered vaccine which is delivered by a type-5 replication-defective adenovirus expressing the spike glycoprotein of SARS-CoV-2 (Sha et al. 2016). It contains the full length spike glycoprotein gene based on SARS-CoV-2 isolate Wuhan-Hu-1 with tissue plasminogen activator signal peptide into an E1 and E3 deleted Ad5 Vector. For phase 1 clinical trials, the vaccine contained 5×10^{10} viral particles per 0.5 mL/vial as a liquid formulation, injected intramuscularly into the arms of participants in three different dose groups (low/moderate/high). Systematic adverse reactions like fever, fatigue, headache and muscle pain or joint pain were observed which may be associated with viremia caused by Ad5 vector infection. RBD antibodies were observed from day 14 with a single dose eliciting a four-fold increase and showed higher antibody geometric titre based on infection assay using 1×10^{11} viral particles. Neutralizing antibodies against spike protein were found to be moderately at day 14. TNF-α was significantly lower in the low dose group and was higher in the high dose group. The vaccine was able to induce humoral and cellular response rapidly in most candidates; T-cell response peaked at day 14 and antibodies at day 28 after the vaccination. For phase 2 of the clinical trial, an intermediate dose was chosen and the trial is expected to be completed by 31 January 2021 (Zhu et al. 2020a, b).

12.1.2 ChAdOx1 nCoV-19

ChAdOx1 is a viral vector engineered as a replication-incompetent virus by Oxford University, UK. Previously, ChAdOx1 Chik has been tested for Chikungunya virus (CHIKV) which causes Chikungunya fever (CHIKF), an acute febrile illness leading to long-term arthralgia, especially in distal joints of the extremities (Kroon Campos et al. 2019). ChAdOx viral vector has previously been assessed for its safety and immunogenicity against a wide range of diseases such as influenza virus, plague, zika virus, tuberculosis, malaria, meningococcal group B bacteria (MenB) and MERS-CoV.

ChAdOx1 nCoV-19 vaccine is a chimpanzee derived adenovirus-vectored novel COVID-19 vaccine with replication deficient simian adenovirus expressing the full-length spike gene with a tissue plasminogen activator leader sequence inserted in to its genome. ChAdOx1 nCoV-19 vaccine was found to be immunogenic that elicited a robust anti-viral response in a murine model (van Doremalen et al. 2020). The vaccine has already completed phase 1 and 2 in a single-blinded, randomized controlled trial at six sites in the UK. Healthy adult participants aged 18–55 years with no exposure history of COVID-19 infection were

chosen for the trial. No serious adverse effects related to ChAdOx1 nCoV-19 were observed and exhibited S-specific effector T-cell as early as day 7 which peaked on day 14. Anti-S IgG increased by day 28 capable of neutralizing the live SARS-CoV-2 virus. The booster dose resulted in the induction of both humoral as well as cellular immune responses. In rhesus macaques, this vaccine was capable of protecting against lower respiratory tract infection in primates. Clinical trial results thus far suggest ChAdOx1 nCoV-19 vaccine to be safe, tolerant and immunogenic (Folegatti 2020).

During April 23 and Nov 4, 2020, 53 848 participants were enrolled for Phase 3 trial and 11636 participants (7548 in UK and 4088 in Brazil) were included in the interim primary efficacy study. The results show significant vaccine efficacy of 70.4% after two doses and protection of 64.1% after at least one dose against symptomatic disease. ChAdOx1 nCoV-19 showed acceptable safety profile and is efficacious against symptomatic COVID-19 patients. Vaccine is suitable for distribution as it can be stored and distributed in 2-8˚C (Voysey et al. 2021).

12.1.3 Sputnik V

Russia announced the launch of Sputnik V, heterologous COVID-19 vaccine consisting of two components, a recombinant adenovirus type 26 (rAd26) vector and a recombinant adenovirus type 5 (rAd5) vector, both carrying the gene for SARS-CoV-2 spike glycoprotein (rAd26-S and rAd5-S). The first interim analysis of Phase III trials of the Sputnik V vaccine revealed 92% efficacy in Covid-19 patients on 20 confirmed Covid-19 cases (Logunov et al. 2020). The interim data is based on the double-blind, randomised, placebo-controlled trials and is being conducted on 40,000 participants in Russia. Most adverse events were mild (pain at injection site, hyperthermia, headache, and muscle and joint pain) and no serious adverse events were detected. All participants produced antibodies to SARS-CoV-2 glycoprotein (Logunov et al. 2020). During September 2000, around 10,000 medics and other high-risk groups were administered Sputnik V under the civil use of the vaccine out of clinical trials. The III clinical trials of Sputnik V Phase are undergoing in Belarus, UAE, Venezuela and other countries, as well as Phase II-III in India (https://www.clinicaltrialsarena.com/news/russia-sputnik-v-efficacy). The preliminary results on the efficacy and safety of Gam-COVID-Vac (Sputnik V) of phase III trial shows that the vaccine is 91·6% (95% CI 85·6–95·2) efficacious against COVID-19 from the day of receiving second dose (day 21 after first dose). There were reports of serious adverse events in 45 (0·3%) of 16 427 participants, all of which were considered not due to the vaccine (Logunov et al. 2021).

12.2 Inactivated Vaccine Candidates

12.2.1 BBIBP-CorV

The strain 19nCoV-CDC-Tan-HB02 was considered for developing the inactivated SARS-CoV-2 vaccine, BBIBP-CorV. HB02 strain is homologous to other viral strains and the spike protein has 100% identity. BBIBP-CorV was capable of inducing high levels of neutralizing antibody in rats, mice, guinea pigs, rabbits, cynomolgus monkeys, and rhesus macaques, and found to be protective against SARS-CoV-2 infection. A lower two-dose immunization regime (2 mg/dose) provided highly efficient protection against SARS-CoV-2 in rhesus macaques with no immunopathological effects (Wang et al. 2020a, b, c, d, e, f, g).

12.2.2 PiCoVacc

PiCoVacc is a purified inactivated SARS-CoV-2 vaccine candidate developed from CN2 strains in conjunction with CN1, CN3-CN5, and OS1-OS6, which were used as pre-clinical challenge strains. PiCoVacc was able to induce SARS-CoV-2 specific neutralizing antibodies in mice, rats, and non-human primates. To assess the immunogenicity of PiCoVacc, BALB/c mice were injected

with various doses of PiCoVacc mixed with an alum adjuvant. No inflammation or other adverse effects were observed. SARS-CoV-2 S-specific and RBD-specific IgG antibodies were generated and the titer peaked at week 6 accounting for half of the S-induced antibody responses. Two immunization doses, 3 μg and 6 μg, provided partial or complete protection in macaques against a SARS-CoV-2 challenge without demonstrating any observable antibody dependent enhancement of the infection (Gao et al. 2020a, b, c, d).

12.3 Nucleic Acid Vaccine Candidates

12.3.1 mRNA 1273 (RNA Vaccine Candidate)

This is an mRNA vaccine that encodes for S-2P antigen which is a SARS-CoV-2 spike glycoprotein trimer with a transmembrane anchor and an intact S1-S2 cleavage site. S-2P is stabilized by two consecutive proline substitutions at amino acid positions 986 and 987, at the top of heptad repeat 1, which prevent structural rearrangements of the fusion S2 subunit and retain its prefusion conformation. The nucleoside-modified messenger RNA (mRNA) is encapsulated in a lipid nanoparticle capsule, formulated in a fixed ratio of mRNA and lipid. The mRNA is suspended in a sterile liquid for injection at a concentration of 0.5 mg per mL. This vaccine is developed by Moderna in collaboration with the National Institute of Allergy and Infectious Disease Vaccine Research Centre. The vaccine underwent an open-label phase 1 clinical trial which started for 6 weeks in three dose cohorts (25 μg, 100 μg and 250 μg) via intramuscular injection in the upper arm. A Phase II trial with 600 healthy participants in two cohorts treated with a placebo, a 50 μg or a 250 μg dose also started recently. The vaccine was found to be immunogenic in murine models, capable of inducing IgG2a and IgG1 antibodies. It could also stimulate higher secretions of IFN-γ than IL-4, IL-5 or IL-3 upon re-stimulation with peptide pools and induce robust $CD8^+$T cell response to the S1 peptide pool with balanced Th1/Th2 antibody isotype (Corbett et al. 2020).

On 26 January, 2021 WHO announced Moderna mRNA 1273 vaccine to have an efficacy of approximately 92 % in protecting against COVID-19, 14 days after the first dose. The vaccine has been found to be safe and effective in people with medical conditions associated with increased risk of hypertension, diabetes, asthma, pulmonary, liver or kidney disease, as well as chronic infections that are stable and controlled. The new variants of SARS-CoV-2, including the B.1.1.7 and the 501Y.V2, do not alter the effectiveness of the Moderna mRNA vaccine (WHO Report 2021c).

12.3.2 BNT162 (RNA Vaccine Candidate)

BNT162 is a Pfizer licensed BioN Tech mRNA vaccine candidate: there are four vaccine candidates under this program, two for coding the SARS-CoV-2 S-protein and two for the RBD of the S-protein made up of three different mRNA formats. During the preclinical studies among the four BNT162 mRNA vaccine candidates, BNT162b1 and BNT162b2 emerged as strong candidates on the basis of their immune response and safety. In clinical phase 1 and 2 trials, results conducted on up to 120 patients exhibited that BNT162b2 had a favorable tolerability profile over BNT162b1 and also showed high $CD4^+$ and $CD8^+$ T cell responses. BNT162b2 has been chosen for phase 2 and 3 trials where participants were chosen between the age of 18 to 85 years (BioNTech n.d.).

BNT162b2 is a lipid nanoparticle formulated nucleoside modified RNA vaccine. In phase 2/3, two-dose regimen of BNT162b2 given at an interval of 21 days to 43,548 participants, of whom 43,448 received injections (21,720 with BNT162b2 and 21,728 with placebo). A two-dose regimen of BNT162b2 conferred 95% protection against Covid-19 in persons 16 years of age or older. Systematic reactogenicity was more common and severe after the second dose. Severe fatigue was also observed in 4% of BNT162b2 recipient. These reactogenicity events (short-term, mild-to-moderate pain at the injection site, fatigue, and headache) were transient and resolved within a couple of days.

(Polack F. 2020). Children, pregnant women and immunocompromised persons were not included in this 2/3 phase trial.

12.3.3 DNA Vaccine

A prototype DNA vaccine expressing six variants of the SARS-CoV-2 spike protein: (a) full-length, (b) cytoplasmic tail deleted, (c) transmembrane domain deleted and cytoplasmic tail reflecting the soluble ectodomain, (d) S1 domain with a foldon trimerization tag, (e) RBD with a fold-on trimerization tag, and (f) a prefusion stabilized soluble ectodomain with furin cleavage site deleted, was constructed. The vaccine was tested on rhesus macaques which developed humoral and cellular immune responses, including neutralizing antibody titers that were comparable to macaques infected with SARS-CoV-2. The vaccine elicited neutralizing antibody inducing protection (Yu et al. 2020).

12.3.4 INO-4800

INO-4800, is an optimized S- protein of SARS-CoV-2 viral DNA plasmids developed by Inovio (Pharmaceuticals 2020). Phase I trial started on April 3, 2020 to evaluate the safety, tolerability and immunogenicity of the vaccine. INO-4800 with a regime of three different doses are being administered intradermally followed by electroporation in healthy volunteers (120 participants).

12.4 Protein-based vaccines

SCB-2019 is a protein subunit vaccine candidate containing a stabilised trimeric form of the spike (S)-protein (S-Trimer) combined with two different adjuvants. The difference of SCB-2019 with other vaccine is that it uses a stabilised protein trimer as the antigen. The Trimer-Tag is a protein, derived from the C-terminus of human type I procollagen which preserves the trimeric conformation of the SARS-CoV-2 spike protein (Blakney and McKay 2021). The efficacy and safety of SCB-2019 was assessed as the S-Trimer protein alone (non-adjuvanted), or as one of two adjuvanted formulations with either AS03 or CpG/Alum. The Phase I trial suggested non-adjuvanted SCB-2019 to be poorly immunogenic, but in combination with the adjuvant system (AS03 or CpG/Alum) robust increase in functional immune responses were observed with SARS-CoV-2 neutralising activity that correlated well with IgG antibodies against SCB-2019 or ACE2-competitive blocking antibodies (Richmond et al. 2021).

13 Perspectives

Last 18 months have seen an explosion of information about SARS-CoV-2 genome, virulence factors, mode of entry into the target cells, receptors and co-receptors/proteases, and host immune response. The fact that a large proportion of individuals can be infected and remain asymptomatic bodes well for the role of the innate immunity in engineering a host-pathogen stand-off. Whether this reflects on the threshold of virus latency remains to be understood. Whether the complement system has a protective role is yet to be established: there are viral proteins other that Spike protein that can activate complement, specifically the lectin pathway. What is remarkably clear is that the complement activation contributes considerably in microangiopathy and coagulopathy in severe COVID-19 patients. Complement activation also seems to contribute to coagulopathy seen in very few individuals who were administered AstraGeneca (Oxford) vaccines. The roles that T cells, NK cells and DCs play in mounting a protective response against SARS-CoV-2 are being recognized as well. The presence of specific antibodies, some of them being neutralizing, is also well-documented. Vaccine trials have relied mostly on the use of Spike protein: results have been promising and several countries have double vaccinated a vast majority of their adult population. However, levels of neutralizing antibodies and persistence of B cell response remain a concern. These limitations apply to infection as well as vaccination trials. To continue to assess likely alterations in the viral genome (with or without a fitness cost), uncertaintly of neutralizing antibody titres, and human-to-human transmission rates, are going to be major issues in short- and medium-terms However, like other vaccines, one would expect that SARS-CoV-2 vaccines will also work following the principle of

immunology, i.e. after two doses of vaccinations, subsequent virus exposure should recall memory cells and hence swift protective immune response. There are discussions about giving third dose of the vaccine in winter that may coincide with rise in flu.

There is a terrible beauty about how viruses such as SARS-CoV-2 have evolved to be so efficient at causing infection. The intricate molecular interactions between the viral and host cellular molecules and the tango between the immune response and the viral mechanisms that seek to subvert it are complex and fascinating. It is also truly amazing that in such a short period of time, we have learnt so much about the both SARS-CoV-2 and COVID-19. An understanding of COVID-19 requires us to look at the molecular level (for example during replication of the virus), the cellular (internalization of the virus), at the level of the tissue or organ (local inflammatory response) and the organism (adaptive immune responses). We also need to understand interactions between people, and between people and their environment, and social issues such as a flow of people across the world and the impact of socioeconomic factors on viral propagation. Interventions on all these levels are needed—there will not be a single 'magic bullet'.

Some will argue that this is pessimistic. Vaccination will allow us to deal with SARS-CoV-2. However, while development of an effective vaccination is an incredibly useful tool in helping us cope with the virus, we should not rely on it. In part, because it may be more difficult than we hope to produce an effective vaccine that results in long term immunity (the failure to produce effective vaccines to other forms of CoV should give us cause for concern). However, even with an effective vaccine, it took many years for us to eradicate smallpox. Salk developed the first effective polio vaccine in 1952, and despite a worldwide effort, there are still some pockets of polio in Asia. These are diseases with no animal reservoirs of infection.

This is not a message of gloom. We will be able to control the virus, but it will need a multi-pronged approach to do so. We also need to develop our science base and understanding so that when the next pandemic strikes (which it will do), we can rapidly mobilise to contain and control it. We need to develop public understanding of the science (for example, to counter the ant-vaccination movement) so we learn from SARS-CoV-2 to be more resilient to future threats.

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7 Antibiotic Resistance Mechanisms and Their Transmission in *Acinetobacter baumannii*

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1 Introduction

The discovery of penicillin over 90 years ago, and its subsequent uptake by healthcare systems around the world revolutionised the treatment of bacterial infections. It marked the beginning of a golden age in antibiotic discovery with new classes of antibiotics being routinely discovered and saving millions of lives globally. However, towards the end of the last century the rate of discovery slowed significantly. This decline in discovery coincided with the rapid emergence and spread of bacterial pathogens that exhibit resistance to multiple antibiotics. Research into antibiotic discovery is now a global priority in order to maintain sustainable access to effective treatments for bacterial infections. The rise of antibiotic resistance is closely linked to their indiscriminate use. Antibiotics can be acquired without the need for a prescription or clinical advice in many parts of the world. The Infectious Diseases Society of America (IDSA) have grouped the most problematic antibiotic-resistant pathogens and called them the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp)* (Pendleton et al. 2013; Boucher et al. 2009) So-called due to their abilities to 'escape' the biocidal actions of antimicrobial treatment (Pendleton et al. 2013). A 2018 report from the World Health Organisation (WHO) placed carbapenem resistant *A. baumannii* (CRAB) at the top of a global priority list of bacteria in urgent need of novel therapeutic intervention strategies whilst the CDC has categorized multidrug resistant *A. baumannii* as a serious threat (Tacconelli et al. 2018; Harding et al. 2018).

A. baumannii is a Gram-negative, aerobic coccobacillus that is ubiquitous in nature. It is an opportunistic pathogen that can colonise a range of anatomical sites in usually immunocompromised individuals leading to a variety of life-threatening clinical complications (Harding et al. 2018). Prior to the 2000s, *A. baumannii* infections were relatively infrequent and typically susceptible to front line antibiotics. However, there has been a rapid increase in the number of these infections, initially due to the high rates of MDR strains recovered from veterans or soldiers from the Iraq and Afghanistan conflicts in the 2000s (Weintrob

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et al. 2018; Camp and Tatum 2010). The clinical impact of *A. baumannii* has continued to grow, with studies suggesting that up to 10% of all nosocomial infections in the United States, and 2% in Europe, are due to this organism. The rates of hospital associated infections due to this pathogen in Asia and the Middle East are reported as almost twice as high (Harding et al. 2018; Kröger et al. 2016; Karlowsky et al. 2017; Badave and Kulkarni 2015). The associated clinical manifestations include ventilator-associated pneumonia (VAP), bacteraemia, meningitis, urinary tract infections, septic shock and surgical wound infections (Kröger et al. 2016; Karlowsky et al. 2017; Peleg et al. 2008; García-Quintanilla et al. 2013). The greatest concern associated with this pathogen however is that between 44 and 70% of isolates exhibit multidrug resistance (MDR) (resistant to at least 3 classes of antibiotic); rates that are nearly 4 times higher than those observed for other problematic Gram-negative pathogens in these settings, such as *Pseudomonas aeruginosa* (Giammanco et al. 2017). Cases have also emerged of extremely drug-resistant (XDR) *A. baumannii*, isolates that cannot be treated by any antimicrobial agents currently approved by the US Food and Drug Administration (FDA) (Viehman et al. 2014). Cases of XDR *A. baumannii* are no longer confined to densely populated areas, specialist hospital units or patient populations but have been reported in rural health care centres worldwide (Fonseca et al. 2019). The molecular mechanisms and coordinated behaviours (biofilm formation) that allow this pathogen to resist antibiotic therapy within each of these niche areas are a considerable area of research focus. In this chapter, we will detail some of the primary mechanisms *A. baumannii* utilizes to become recalcitrant to antibiotic therapy and discuss some of the new approaches for tackling XDR and MDR *A. baumannii*.

2 Mechanisms of Resistance Acquisition

A. baumannii is particularly adept at developing resistance due to its large repertoire of antibiotic resistance genes, some of which have been critical to the establishment of clonal outbreaks. *A. baumannii* are naturally transformable bacteria with an elevated genomic plasticity. This has allowed the acquisition and accumulation of many antibiotic resistance determinants. *A. baumannii* achieves this through overcoming selective pressures and stresses via the acquisition of foreign material via gene transfer, transformation, conjugation and transduction. Indeed, *A. baumannii* has displayed a high level of natural competency; incorporating exogenous DNA in its genome at high frequencies. Remarkably, this natural competency is increased upon exposure to many host factors such as serum albumin, suggesting that even during infection it is capable of acquiring new genetic elements (Traglia et al. 2016). This ability drives the evolution towards MDR in bacteria. Mobile genetic elements (MGEs) such as plasmids, resistance islands, insertion sequences, transposons and integrons have played a key role in the emergence and success of MDR and XDR in *A. baumannii* (Table 7.1) (Almasaudi 2018; Fournier and Richet 2006; Pagano et al. 2016).

2.1 Resistance Islands

The AbaR type genomic islands are an important group of MGEs that consist of huge clusters of antimicrobial resistance genes involved in MDR in *A. baumannii.* More than 66% of the 3148 publicly available *A. baumannii* genome sequences contain AbaRs with a much lower frequency of occurrence in other species of *Acinetobacter* (Bi et al. 2019). This is likely due to the majority of AbaR islands being localised to the chromosome stabilising resistance in the species but reducing opportunities for interspecies transfer. Most AbaRs are known to site-specifically disrupt the chromosomal *comM* gene. The first AbaR found in *A. baumannii* was an 86 kb resistance island from a MDR strain, AYE, isolated in France (Fournier and Richet 2006). This genomic island, termed AbaR1, like the majority of AbaRs is located on the chromosome rather than on a plasmid and encodes 45 resistance genes conferring resistance to most

Table 7.1 Prevalent mobile genetic elements associated with the transmission of resistance mechanisms

	Mobile genetic element	Resistance profile	References
Resistance Islands	AbaR0, AbaR1, AbaR2, AbaR3, AbaR4, AbaR5, AbaR6, AbaR7, AbaR8, AbaR9, AbaR10, AbaR11, AbaR12, AbaR13, AbaR14, AbaR15, AbaR16, AbaR17, AbaR18, AbaR19, AbaR20, AbaR21, AbaR22, AbaR23, AbaR24, AbaR25, AbaR26, AbaR27, AbaR28, AbaR29, AbaR30, AbGRI1-0, AbGRI1-1, AbGRI1-2, AbGRI1-3, AbGRI1-4, AbGRI1-5	Most antibiotic classes including aminoglycosides, aminocyclitols, sulphonamides, tetracycline, minocycline, chloramphenicol	(Fournier and Richet 2006; Bi et al. 2019; Holt et al. 2016; Hamidian and Hall 2017; Hamidian and Hall 2018b; Nigro et al. 2019)
Insertion sequences	IS*aba1,* IS*aba2,* IS*aba3,* IS*aba4,* IS*aba10,* IS*aba124,* IS*aba125,* IS*aba825*	Carbapenems, Aminoglycosides, Cephalosporins	(Turton et al. 2006; Potron et al. 2019; Lopes et al. 2012; Lopes and Amyes 2012)
Transposons	Tn*2006,* Tn*2007, Tn2008,* Tn*2009*	Carbapenems	(Chen et al. 2017; Zhou et al. 2011; Espinal et al. 2013; Guerrero-Lozano et al. 2015)
Integrons	*Int1, Int2*	Aminoglycosides, Carbapenems	(Pagano et al. 2016; Martins et al. 2015; Turton et al. 2005)

classes of antibiotics (Fournier and Richet 2006; Hamidian and Hall 2018a). Over 40 AbaRs have since been identified in *A. baumannii* and the resistomic consequences of each varies. However, the resistance gene profiles of AbaRs typically follow specific patterns allowing the correlation of resistance gene profiles with specific clonal lineages (Bi et al. 2019).

2.2 Insertion Sequences and Transposons

Acquisition and movement of new or foreign DNA can be determined by analysis of the sequence surrounding the insertion site. Bacterial Insertional Sequences (IS) are usually short in size typically ranging from 0.5 to 2 kb. Two copies of the same IS flanking an antibiotic resistance gene can lead to transposition of resistance genes between strains. IS's also have a role in acting as a promoter sequence, elevating the levels of expression of a resistance gene. It has been demonstrated that many genes encoding β-lactamases in *A. baumannii* are only capable of conferring resistance when they are expressed under a promoter within an upstream IS element (Turton et al. 2006). Relevant IS elements identified in *A. baumannii* so far include IS*Aba*1, IS*Aba2*, IS*Aba3*, ISAba4, ISAba125 and ISAba825 all linked to carbapenem resistance. Of these the most common is ISAba1, which has been identified upstream of *OXA-23-like*, *OXA-51-like, OXA-58-like, eptA* and *ampC* genes (Potron et al. 2019). As well as increasing the expression of resistance genes, IS*Aba*1 has been shown to impact global regulators of virulence such as the TetR type transcriptional regulator AdeN and the histone like nucleoid structuring (H-NS) protein. Disruption of these regulators was shown to lead to an enhanced adherence to human pneumocytes and elevated levels of lethality in a *Caenorhabditis elegans*—*A. baumannii* nematode pathogenicity model (Eijkelkamp et al. 2013; Saranathan et al. 2017; Adams and Brown 2019).

Transposons (Tn) are more complex genetic structures with a role in the spread of resistance

genes that has been extensively documented. Four transposons harbouring the $bla_{OXA\text{-}23}$ gene have been reported: Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009 (*Chen et al. 2017*;* Zhou et al. 2011; Espinal et al. 2013*;* Guerrero-Lozano et al. 2015*)*. Tn*2006*, Tn*2007* and Tn*2008* are globally disseminated, while up until recently Tn*2009* was thought to be confined only to China (Zhou et al. 2011; Kumburu et al. 2019). However, in a study of over 350 South Korean isolates of *A. baumannii,* 88% of which displayed carbapenem resistance, the $bla_{OXA\text{-}23}$ gene was primarily carried either by Tn*2006* (44%) or Tn*2009* (54%), with a few exceptions carried by Tn*2008* (1.6%) (Yoon et al. 2016a).

2.3 Integrons

Integrons are genetic elements that incorporate ORFs by site-specific recombination and express them as functional genes due to the presence of an effective promoter sequence. All known integron cassettes have 3 core components, a gene encoding an integrase (*intI*), a promoter sequence and a primary recombination site. It is now well established that these mobile elements are a major factor in the acquisition of antibiotic resistance in Gram-negative and, to a lesser extent, in Gram-positive bacteria (Pagano et al. 2016). Five different classes of mobile integrons have been defined to date, based on the sequence of the encoded integrase gene. Multidrug resistance in *A. baumannii* has been primarily associated with Class 1 and Class 2 integrons (Martins et al. 2015). Class 1 integrons in particular are commonly associated with clonal outbreaks (Turton et al. 2005). Together these MGEs play a critical role in allowing *A. baumannii* to adapt to, to tolerate and to resist antibiotic exposure by facilitating the transfer of genes capable of conferring resistance, the upregulation of native genes capable of conferring resistance and/or through the disruption of global regulators of virulence, pathogenicity and membrane potential. Recently evidence has emerged that prophages play a role in the transfer antimicrobial resistance genes *in vitro* with intercellular prophages mediating the transfer of chromosomal antibiotic resistance genes between resistant and sensitive strains of *A. baumannii* without the need for direct cell-to-cell contact. This suggests they may play a role in the transfer of acquired resistance mechanisms *in vivo* also (Wachino et al. 2019).

3 Molecular Mechanisms of Resistance

Bacteria possess a wide range of mechanisms that they can utilize to become recalcitrant to antibiotic therapy. These include the expression of antibiotic inactivating enzymes such as β-lactamases, the upregulation of efflux pumps, target modification and changes to the surface of the cell such as the alteration of porins or Lipid A modifications. These mechanisms can often act synergistically to prevent an antibiotic reaching its molecular target (Fig. 7.1, Table 7.1).

4 Molecular Mechanisms of Resistance: β-Lactamases

B-lactams account for 65% of all prescriptions for injectable antibiotics in the United States, they are well tolerated and efficacious (Bush and Bradford 2016). β- Lactams are a class of antibiotic that covalently bind to penicillin-binding proteins (PBPs). PBPs are enzymes responsible for the formation of peptidoglycan in the bacterial cell wall. Inhibition of PBPs by β- lactams disrupts bacterial cell wall biosynthesis, weakening the cell membrane and ultimately causing the cell to burst due to osmotic pressure (Tehrani and Martin 2018; Nowak and Paluchowska 2016). This mode of treatment is however rendered ineffective in many pathogens particularly *A. baumannii*, that possess enzymes that are capable of degrading β- lactams. These enzymes inactivate the antibiotic by hydrolysis of the β-lactam ring (Bush and Bradford 2016). Resistance to a wide range of different groups of β-lactam antibiotics, such as penicillins, cephalosporins, monobactams and carbapenems is mediated by β-lactamases. β-lactamases can be structurally

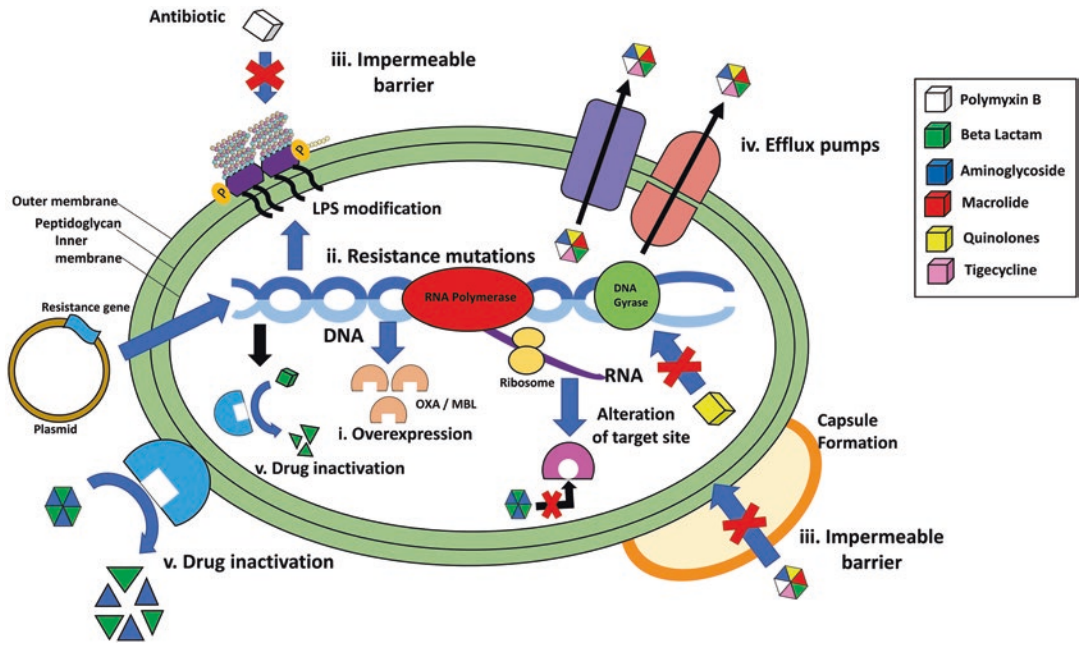

Fig. 7.1 A schematic diagram on various antimicrobial resistance mechanisms. (1) Overexpression. (2) Resistance mutations. (3) Impermeable barrier. (4) Efflux pumps. (5) Drug inactivation

classified into 4 molecular groups. These include class A, C, and D serine-β-lactamases (SBLs) with no significant structural similarities between classes, and the class B metallo-β-lactamases (MBLs) (Ambler 1980; Gordon et al. 2009; Silveira et al. 2018). While some native β-lactamases are encoded in the *A. baumannii* genome such as $bla_{OXA\text{-}51}$ and *ampC,* their capacity to confer resistance is limited except in the case of insertion of upstream promoter sequences. However, the ability of *A. baumannii* to acquire new β-lactamases particularly class B metallo-β-lactamases (MBL) and class D oxacillinases (OXA) has resulted in the widespread dissemination of CRAB (Gordon et al. 2009; Hsu et al. 2017).

Class A β-lactamases are found in many Gram-negative species and mediate resistance to penicillins and narrow spectrum cephalosporins (cephalothin). Extended spectrum β- Lactamases (ESBL) that confer resistance to expanded-spectrum cephalosporins (cefotaxime, ceftazidime) are of greater concern. The first ESBL identified in *A. baumannii* was PER-1 which was initially identified in Turkey but has since been shown to be widespread (Nordmann and Naas 1994; Potron et al. 2015). Numerous other class A ESBLs have since been identified including TEM, SHV, CTX-M, GES, SCO, PER and VEB variants (Gordon et al. 2009; Tada et al. 2017).

Class B MBLs enzymes are capable of hydrolysing all β- lactams antibiotics including carbapenems, with the exception of aztreonam (Almasaudi 2018; Fournier and Richet 2006). Class B enzymes require a metal ion in their active site to be functional, mostly zinc or other heavy metals. This zinc-dependent activity can be inhibited by EDTA, but not by conventional β-lactamase inhibitor molecules such as clavulanic acid, tazobactam, and sulbactam (Jain and Danziger 2004). Verona integron-encoded metallo-β-lactamases (VIM), Imipenem hydrolyzing β-lactamase (IMP), Seoul Imipenemase (SIM-1) and New Delhi metallo-β-lactamase (NDM) have all been identified in *A. baumannii*. IMP was initially identified in a strain of *Pseudomonas aeruginosa* in Japan in the early 1990s but has since been shown to have disseminated globally and has now been acquired by *A. baumannii*. The VIM enzyme has a <40% amino

acid identity to IMP and like VIM was initially described in *P. aeruginosa* before being later identified in *A. baumannii*. SIM-1 was identified first in a Korean University Hospital in 2003 and has been shown to share a high sequence amino acid identity with IMP but a limited prevalence beyond Korea (Kim et al. 2013). NDM-1 was first reported in *Klebsiella pneumonia* in 2009 and identified in Indian clinical strains of *A. baumannii* the following year (Yong et al. 2009; Karthikeyan et al. 2010). In the intervening time since its original identification, NDM-1 has disseminated at an alarming rate with metagenomic sampling recently identifying the NDM-1 sequence in Arctic soil samples (McCann et al. 2019). Twenty-eight further variants of NDM-1 have been identified, typically differing in one to two amino acids however, not all of these variants have been identified in *A. baumannii (*Khan et al. 2017*)*. The spread of the NDM-1 in *A. baumannii* strains has been attributed at least in part to its association with the Tn125 composite transposon that can integrate on the chromosome or on plasmids (Bontron et al. 2016).

Class C and D β-Lactamases have been identified in a large proportion of *A. baumannii* isolates and confer resistance to cephamycins and cephalosporins which include cefoxitin, cefotetan and penicillin type β-Lactams antibiotics. Class C β-Lactamases produce AmpC-type cephalosporinases. At basal levels of expression the poor efficacy of AmpC-type cephalosporinases does not reduce the overall efficiency of β-Lactams such as penicillin and extended spectrum cephalosporins (Almasaudi 2018; Gordon et al. 2009; Corvec et al. 2003). However, in several clinical isolates an IS*Aba1* type insertion has been identified upstream of a number of AmpC type enzymes which enhances their expression (Poirel and Nordmann 2006; Hamidian and Hall 2013). This then enables hydrolysis of β-lactams such as penicillin and extended spectrum cephalosporins at levels high enough to confer resistance (Corvec et al. 2003). Class D (OXA) oxacillinases, are able to efficiently hydrolyze isoxazolyl-type β-lactams like oxacillin. More than 700 different OXA- type enzymes have been identified. The presence of Class D β-Lactamases is considered the leading cause of carbapenem resistance in *A. baumannii*. Four groups of class D carbapenem-hydrolysing enzymes (CHDL) can be found within *A. baumannii*, usually classified into OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like enzymes (Evans and Amyes 2014; Lin and Lan 2014). The $bla_{\text{OXA-23}}$ gene in particular has disseminated worldwide and is considered a major determinant in the emergence of CRAB. IS have been routinely identified upstream of these OXA genes, again leading to increased expression and phenotypic resistance. Studies from both India and China have identified the $bla_{\text{OXA-23}}$ gene in 80–100% of clinical isolates with the IS*Aba1* sequence identified upstream of $bla_{\text{OXA-23}}$ in 80% of cases. In Greece, emerging CRAB strains that produce both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ strains have recently been identified and shown to predominate in paediatric intensive care units (Karampatakis et al. 2019; Huang et al. 2019; Vijayakumar et al. 2016; Hadjadj et al. 2018).

5 Multidrug Efflux Systems

Efflux pumps are transmembrane transporter protein complexes that are crucial to the removal of toxic substances and metabolic end products, regulating the internal environment of the cell (Chitsaz and Brown 2017; Soto 2013; Fernandez-Recio et al. 2004). They have a broad substrate specificity and their acquisition or changes in their expression, has been shown to confer resistance to a wide array of antibiotics (Soto 2013; Sun et al. 2014). Efflux pumps associated with conferring resistance can be categorised into 5 families, the resistance-nodulation-cell division (RND) family, the ATP-binding cassette (ABC) transporters, the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, and the recently identified multidrug and toxic compound extrusion (MATE) family. Among the 5 MFS, RND efflux pumps are most commonly associated with *A. baumannii*. They consist of a tripartite structure composed of an outer-membrane (OM) channel, an inner-membrane

transporter (IM) and a periplasmic membrane fusion protein (MFP) that connects all these components together (Fernandez-Recio et al. 2004; Zgurskaya and Nikaido 2000; Poole 2004). Three specific RND efflux systems AdeABC, AdeFHG and AdeIJK, have been characterised in *A. baumannii* and are associated with a broad range substrate specificity (Coyne et al. 2010a; Magnet et al. 2001; Damier-Piolle et al. 2008) . As well as the presence or absence of these efflux pumps, the levels of expression also plays a significant role in their capacity to confer resistance. Specifically, it has been shown in clinical isolates that the level of resistance is associated with the upregulation of *adeB, adeJ* and *adeG* (D'Souza et al. 2019; Lin et al. 2017a). In naïve conditions, the expression of each pump is tightly regulated. The AdeABC efflux system which confers resistance to aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, some β-lactams, and tigecycline is regulated by two-component regulatory system *adeRS,* with *adeR a* response regulator and *adeS* a sensory kinase. Mutations within *adeRS* can result in the over expression of *adeABC* genes (Marchand et al. 2004; Wieczorek et al. 2008). AdeS IS*Aba1* insertions have also been linked to elevated expression of this efflux pump (Sun et al. 2012). The BaeRS two component system, which is involved in sensing envelope damage, has also been shown to regulate the expression of this efflux pump (Lin et al. 2015). AdeIJK is common among all *A. baumannii* isolates and is capable of expelling the broadest range of substrates. It is constitutively expressed in laboratory conditions but has also been shown to be under the control of the TetR transcriptional regulator AdeN. Mutations and IS*Aba1* insertions have been identified in this regulator in clinical isolates, leading to increased *adeIJK* expression (Saranathan et al. 2017; Rosenfeld et al. 2012; Geisinger et al. 2019). AdeIJK works in accordance with AdeABC to confer tigecycline resistance (Gordon et al. 2009; Sugawara and Nikaido 2014). Compared to AdeIJK and AdeABC, AdeFGH has the narrowest substrate range, however, over expression has been identified in clinical isolates. This over expression is associated with increased resistance to fluoroquinolones and chloramphenicol. AdeL, a lysR type transcriptional regulator can regulate the expression of this efflux pump (Geisinger et al. 2019; Yoon et al. 2015; Coyne et al. 2010b; Gerson et al. 2018). MATE Efflux pump family members AbeM, AbeM2, and AbeM4 have also been associated with drug extrusion. Over expression has been linked to a decreased susceptibility to quinolones, gentamicin, kanamycin, erythromycin, chloramphenicol and trimethoprim (Gordon et al. 2009; Su et al. 2005). SMR family member AbeS was shown to decrease susceptibility to novobiocin and erythromycin but also to a range of disinfectants, dyes and detergents (Srinivasan et al. 2009). A range of MFS family transporters, with a role in antibiotic efflux, have been identified in *A. baumannii* including AmvA, TetAB, CraA, FloR, CmlA, AbaF, EmrAB and AedC. These primarily confer resistance to chloramphenicol. EmrAB in particular is one of the few efflux systems shown to be capable of increasing resistance to the last resort antibiotic, colistin. However, the precise mechanism of action is not known and as colistin exerts its activity extracellularly, this may not be a direct effect (Lin et al. 2017b). AbaQ was recently functionally characterised in *A. baumannii* and shown to be involved in the extrusion of quinolones (Pérez-Varela et al. 2018). The breadth and substrate diversity of efflux pumps encoded within the genome of *A. baumannii* is integral to its emergence as a millennial pathogen of major clinical significance.

6 Porins

Porins are β-barrel proteins that are found in the outer membrane of Gram-negative bacteria and facilitate the transport of molecules across the membrane. They play a central role in membrane permeability and represent a major nutrient entry portal. They also enable the transport of many

antimicrobial molecules into the cell and as such mutations affecting their conduction properties have a major role to play in antibiotic resistance. $OmpA_{ab}$ is a major component of the outer membrane of *A. baumannii* and plays a key role in pathogenesis influencing phenotypes such as twitching motility, desiccation resistance, serum-induced killing and epithelial cell adhesion (Geisinger et al. 2019; Iyer et al. 2018; Skerniškytė et al. 2019). Thus loss or mutation of $OmpA_{ab}$ has a significant impact on fitness but it also reduces susceptibility to chloramphenicol, aztreonam, nalidixic acid and imipenem (Iyer et al. 2018; Smani et al. 2014). $OmpA_{ab}$ is also thought to play a role in the localisation of β- lactamases to the periplasm, with a direct interaction between OXA-23 and $OmpA_{ab}$ demonstrated through cross-linking experiments (Wu et al. 2016). OXA-23 has also been shown to interact with CarO, another porin that has frequently been shown to be downregulated in CRABs (Wu et al. 2016). A disruption in the expression of Omp22-23, OMp33-36, Omp37, Omp43-44 and Omp47 has also been linked to carbapenem resistance. As with $OmpA_{ab}$ the decreased expression of these porins often impacts fitness (Novović et al. 2018; Lee et al. 2017; Rumbo et al. 2014; Smani et al. 2013; Mostachio et al. 2012; Quale et al. 2003).

7 Target Modification: PBPs

As described previously, PBPs are penicillin-binding proteins that are crucial for the synthesis of peptidoglycan, which is a necessary component of the bacterial cell wall (Vashist et al. 2011). β-lactams target PBPs, disrupting their role in peptidoglycan biosynthesis. It has been well established that mutations in PBPs, in particular PBP-2 and PBP-6b, have been directly associated with carbapenem resistance within *A. baumannii (*Cayô et al. 2011*)*. Several studies have also demonstrated alterations in the expression patterns of PBPs among resistant isolates, in particular reduced expression of PBP-2 suggesting that this is a viable mechanism of carbapenem resistance or at the very least can synergistically confer resistance in combination with other mechanisms (Vashist et al. 2011; Gehrlein et al. 1991; Fernández-Cuenca et al. 2003).

8 Target Modification: Ribosomes

Aminoglycosides are bactericidal antibiotics that target ribosomes and bind specifically to the A-site of the 30S ribosome, disrupting the function of the 16s ribosomal subunit and inhibiting protein synthesis (Krause et al. 2016). High levels of resistance to individual aminoglycosides in *A. baumannii* is conferred by aminoglycoside-modifying enzymes (AME). They are typically located on plasmids, transposons or in association with class 1 integrons. This association with MGEs has facilitated the global dissemination of AMEs (Lin and Lan 2014; Lee et al. 2017; Gillings et al. 2008). These enzymes can be categorised according to their mode of action as acetyltransferases, adenyltransferases and phosphotransferases with the latter two categories being the most common in *A. baumannii* (Lin and Lan 2014; Lee et al. 2017). These enzymes modify the aminoglycosides through the addition of amino- or hydroxyl- groups altering binding of the aminoglycoside to the ribosome (Aliakbarzade 2014). Although less frequent, genes encoding 16S rRNA methyltransferases such as *armA* and *rmtA-F*, have also been identified in *A. baumannii* and lead to elevated resistance levels to virtually all aminoglycosides and the neoglycoside plazomycin. In the case of ArmA it has been shown to be exclusively in strains that also produce OXA- type carbapenemases (Hasani et al. 2016; Costello et al. 2019). Aminoglycoside resistance due to amplification of regions containing AMEs has also been reported and shown to be responsible for heteroresistance among subpopulations of an isogenic *A. baumannii* strain (Anderson et al. 2018; McGann et al. 2014). TetM has also been linked to ribosomal protection from tetracyclines, a class of antibiotics that inhibit protein synthesis by preventing attachment of the aminoacyl-tRNA to the ribosome (Ribera et al. 2003).

9 Target Modification: DNA Replication Enzymes

Quinolones such as ciprofloxacin are broad-spectrum antibiotics and are one of the most widely prescribed classes of antibiotic. They target two bacterial type IIA topoisomerases, gyrase and topoisomerase IV, inhibiting their action and resulting in impaired DNA replication (Drlica et al. 2008). Quinolone resistance has been identified in *A. baumannii* and is usually a result of mutations occurring within the *gyrA* and *parC* genes. Mutations in specific regions termed the Quinolone Resistance-Determining Regions in these genes prevent quinolone binding, thus rendering the antibiotic ineffective. A recent study of 140 ciprofloxacin and levofloxacin resistant clinical isolates revealed that >85% of these isolates had mutations in both genes demonstrating the prevalence of this double mutant resistotype (Peleg et al. 2008; Zaki et al. 2018; Aldred et al. 2014).

10 Target Modification: Lipid A

Polymyxins B and polymyxin E (colistin), are cationic antimicrobial peptides that target the lipid A component of lipopolysaccharide (LPS) on the outer membrane of bacteria and are used as antibiotics of last resort for many CRAB infections. LPS plays a key role in the pathogenicity of bacteria and modifications to its structure typically come at a fitness cost to the cell (McCarthy et al. 2017). In Gram-negative bacteria, acquired resistance to polymyxins results mostly from modifications of the drug target, *i.e.* the lipopolysaccharide (LPS). These modifications correspond to addition(s) of cationic groups such as 4-amino-L-arabinose (L-Ara4N) and/or phosphoethanolamine (pETN) on the lipid A, the anchor of the LPS. Unlike *Enterobacteriaceae*, *A. baumannii* lacks all the genes required for L-Ara4N biosynthesis. Accordingly, colistin resistance is caused by the addition of pETN to the lipid A on position 1 or 4′ by an EptA-like phosphoethanolamine transferase chromosomally-encoded by the *pmrC* gene (Jeannot et al. 2017; Cai et al. 2012; Adams et al. 2009). Mutations in the chromosome-encoded *pmrA* and *pmrB* genes result in a constitutive activation of the PmrA/PmrB two-component system, which in turn upregulates the expression of *pmrC*. In polymyxin susceptible strains, the lipid A molecules consist of bis-phosphorylated hexa-acyl and bis-phosphorylated hepta-acyl lipid A, with acyl chain ranging from 12 to 14 carbons in length, respectively (Fig. 7.2a, b). In colistin resistant strains, the lipid A molecules are pETN-modified-bis-phosphorylated hepta-acyl lipid A with acyl chain of 12 carbons in length (Fig. 7.2) (Arroyo et al. 2011; Beceiro et al. 2011; Larrouy-Maumus et al. 2016). Heteroresistance to colistin is readily observed at high frequency in laboratory studies when susceptibility is determined using kinetic or population analysis profiling (Charretier et al. 2018). However, the importance of colistin hetero-

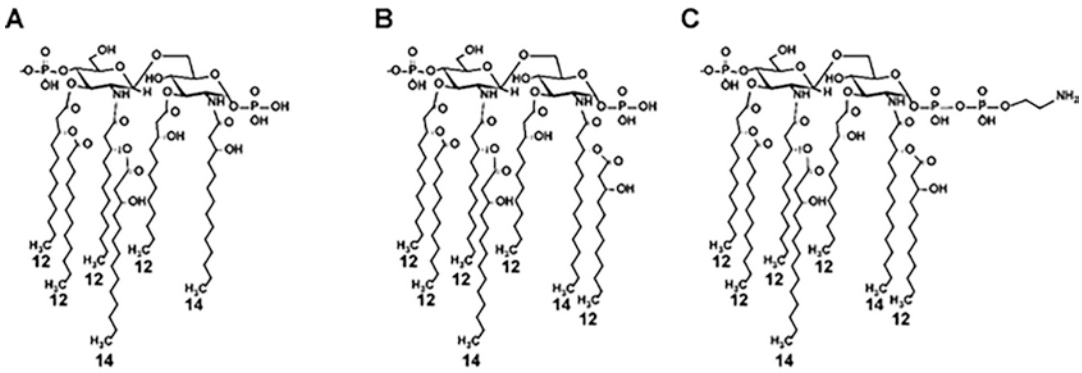

Fig. 7.2 Structures of lipid A from *Acinetobacter baumannii* susceptible to polymyxins (**a** and **b**) and resistant to polymyxins (**c**)

Table 7.2 Summary of *A. baumannii* resistance mechanisms

Resistance mechanism	Class/Group	Associated protein groups	Reference
β-lactamases	Class A Class B Class C Class D	TEM, GES, PER, CTX-M, SCO,VEB, SHV, KPC, CARB IMP, VIM, SIM, NDM AmpC OXA	(Fournier and Richet 2006; Nordmann and Naas 1994; Potron et al. 2015; Tada et al. 2017; Poirel and Nordmann 2006) (Kim et al. 2013; Karthikeyan et al. 2010; Khan et al. 2017; Bontron et al. 2016; Lee et al. 2017) (Corvec et al. 2003; Poirel and Nordmann 2006; Hamidian and Hall 2013; Evans and Amyes 2014) (Karampatakis et al. 2019; Huang et al. 2019; Vijayakumar et al. 2016; Hadjadj et al. 2018)
Efflux Pumps	RND MFS MATE SMR	AdeABC, AdeFGH, AdeIJK TetAB, CraA, FloR, CmlA, AbaF, AedC, EmrAB AbeM AbeS	(Chitsaz and Brown 2017; Soto 2013; Coyne et al. 2010a; Damier-Piolle et al. 2008; Marchand et al. 2004; Wieczorek et al. 2008; Rosenfeld et al. 2012; Sugawara and Nikaido 2014; Yoon et al. 2016b) (Lin et al. 2017b; Coyne et al. 2011; Vilacoba et al. 2013) (Lin et al. 2017a; Su et al. 2005) (Srinivasan et al. 2009; Coyne et al. 2011)
Permeability	Porin	CarO, $OmpA_{ab,}$ Omp22-23, OMp33-36, Omp37, Omp43-44 and Omp47	(Iyer et al. 2018; Smani et al. 2014; Novović et al. 2018; Rumbo et al. 2014; Smani et al. 2013)
Alteration of antibiotic or target sites	PBPs AMEs Ribosomal protection DNA replication enzymes Lipid A modifiers	PBP-2, PBP-6b AAC, ANT, APH ArmA, RmtA-F GyrA, ParC PmrC, PmrA, PmrB, LpxC, LpxD, LpxA	(Cayô et al. 2011; Gehrlein et al. 1991; Fernández-Cuenca et al. 2003) (Nemec et al. 2004; Cho et al. 2009) (Hasani et al. 2016; Costello et al. 2019; McGann et al. 2014) (Drlica et al. 2008; Zaki et al. 2018; Aldred et al. 2014) (Adams et al. 2009; Arroyo et al. 2011; Beceiro et al. 2011)

resistance to clinical outcome and therapeutic failure is uncertain (Li et al. 2006) (Table 7.2).

11 Future Treatment Perspectives: Combination Therapies and Resistance Inhibitors

One emergent strategy to tackle XDR and MDR strains of *A. baumannii* is combination therapies, this strategy attempts to achieve synergy, improve overall efficacy and decrease the probability of resistance (Lutsar et al. 2014). Synergistic combinations typically include at least two different classes of antibiotic. The use of combination therapy to tackle *A. baumannii* has been explored extensively *in vitro* and *in vivo* with mixed results (Shin and Park 2017). Unorthodox combinations which use the cell permeabiliszing properties of colistin have been shown to have very marked synergistic effects *in vitro*. These include polymyxins in combinations with many hydrophobic and Gram-positive agents including glycopeptides (Gordon et al. 2010), macrolides, rifampicin and fusidic acid (Phee et al. 2015) which have little or no activity alone. Focusing on *in vivo* data, a combination of colistin with a carbapenem has been shown to improve clinical responses and survival compared to other therapies such as colistin-tigecycline in solid organ transplant patients (Shields et al. 2012). However, in CRAB isolates that also displayed colistin resistance, the use of a colistin-meropenem combination therapy was associated with a significantly higher mortality compared to colistin monotherapy (Dickstein et al. 2019). The combination of colistin and rifampicin in the treatment of VAP and bacteraemia has also demon-

strated improved clinical outcomes (Bassetti et al. 2008; Aydemir et al. 2013; Motaouakkil et al. 2006). However, neither 30-day mortality nor length of hospitalization was reduced by the addition of rifampicin to colistin despite a decrease in pathogen numbers at the primary site of infection (Durante-Mangoni et al. 2013). A randomised control trial investigating colistin-meropenem combination therapy versus colistin monotherapy did not show a significant improvement in survival, clinical cure, microbiological cure, or development of resistance (Paul et al. 2018). These studies are likely impacted by the primary infection site and the immunological status of the patient cohort. They highlight however, that while combination therapies can be effective *in vitro* this synergy does always translate to the clinic. They also highlight the need to interrogate the effectiveness of combination therapies in randomised trials before clinical use.

11.1 β-Lactam/β-Lactamase Inhibitor Combinations (BL/BLI)

One means to overcome resistance to β-lactams is to combine a β-lactam with a β-lactamase inhibitor (BLI). Although classified as inhibitors, many of these compounds are β-lactams themselves (clavulanic acid, tazobactam, sulbactam) and therefore, act as suicide substrates, preserving the activity of the partner drug within the combination. Those licensed, and most widely used in the United Kingdom, are fixed dose combinations of amoxicillin/clavulanate (2:1), ticarcillin/clavulanate (15:1), piperacillin/tazobactam (4:1), ceftolozane/tazobactam (2:1) and ampicillin/sulbactam (2:1). Non-β-lactam inhibitors include diazabicyclooctanes; avibactam, relebactam, zidebactam, nacubactam, durlobactam and boronic acid based derivatives such as vaborbactam. These are all in the later stages of development as treatments for MDR Gram-negative infections yet none provide functional inhibition of all clinically relevant classes of β-lactamase (Table 7.3). Activity of existing and the novel BL/BLI combinations versus *A. baumannii* are compromised by efflux pumps, porin lesions and their intrinsic ability to induce the production of β-lactamase on a strain by strain basis. Due to a heightened affinity for PBP2, sulbactam retains some intrinsic activity against MDR *A. baumannii*. Enhanced activity of sulbactam / cephalosporin combinations has been shown *in-vitro,* but usually only at high dosing ratios (Lai et al. 2018) which is compromised by the action of OXA-23 (Yang et al. 2019). Sulbactam combined with durlobactam is undergoing evaluation as an *A. baumannii* specific therapy (O'Donnell et al. 2019).Other novel attempts to improve the efficacy of β-lactams have involved the addition of catechol groups to the β-lactam ring. This facilitates uptake of the drug by bacterial iron acquisition systems and vast reductions in MIC and bacterial killing are observed when used in combinations (BAL 30072) (Hornsey et al. 2013). Cefiderocol has broad spectrum activity against Gram-negative bacteria and is highly active against CRAB including strains that produce multiple OXA and metallo-β-lactamases (Zhanel et al. 2019).

Table 7.3 β-lactam/β-lactamase Inhibitor combinations (BL/BLI) (Tehrani and Martin 2018; Karaiskos et al. 2019)

	β-lactamase (Class)			
BLI/BLI	A	B	C	D
Aztreonam/avibactam	+	+	+	+/–
Ceftazidime/avibactam	+	–	+	+/–
Ceftaroline/avibactam	+	–	+	+
Ceftolozane/tazobactam	+/–	–	+	
Cefepime/zidebactam	+	+	+	
Imipenem/relebactam	+	–	+	+/–
Meropenem/vaborbactam	+	–	+	+/–
Meropenem/nacubactam	+	–	+	–

12 Future Treatment Perspectives: Vaccines, Bacteriophage and Phytochemicals

A wide range of alternative strategies to tackle XDR and MDR *A. baumannii* infections have been proposed, such as blue light therapy, aug-

mented nanoparticles, probiotics, phytochemicals, phage therapy and vaccines (Zhang et al. 2014; Mihu et al. 2010; Chan et al. 2018; Asahara et al. 2016). Of these vaccines and phage therapy currently, show the most promising clinical potential. Vaccines have the potential to confer long-term protection while phage therapy has shown success in particularly recalcitrant clinical cases.

A wide range of different vaccine candidates have shown efficacy in mammalian models, these include vaccines using antibodies raised to specific proteins such as the surface autotransporter Ata and OmpA (Bentancor et al. 2012), to polysaccharides such as beta-(1→6)-Poly-N-acetyl-d-glucosamine (PNAG) and K1 (Gening et al. 2010; Russo et al. 2008), inactivated whole cell vaccines (McConnell and Pachón 2010) and outer membrane vesicles based vaccines (McConnell et al. 2011). A recent report of a live attenuated vaccine that utilizes *A. baumannii* lacking the gene (*trxA)* encoding thioredoxin was shown to be protective against *A. baumannii* sepsis in mouse models of infection (Ainsworth et al. 2017). A vaccine based on the hollowed out shell of *A. baumannii* termed a bacterial ghost, has also shown high levels of protection in rat models (Sheweita et al. 2019). Recent strategies have focused on utilizing emerging technologies such as reverse vaccinology, protein network analysis, immunoproteomics and generating multi-epitope vaccines (Mujawar et al. 2019; Ahmad et al. 2019; Chen 2015; Shahid et al. 1946). However, despite the progress made on vaccine development for *A. baumannii*, significant scientific and technical challenges remain before an *A. baumannii* vaccine is ready for clinical use. These include deciphering the underlying mechanisms of protection, profiling antigen-specific antibody responses, understanding temporal protection kinetics and evaluating the level of species and sub-species coverage. There is also the timing of administration as the cohort most at risk of *A. baumannii* infection are typically immunocompromised thus the effectiveness of any vaccine must be determined among patients with varying degrees of immune function.

Phage are bacteria specific viruses that have a lytic life cycle. This life cycle involves the attachment of phage to receptors on the bacterial cell surface, the injection of genetic material into the cytosol, viral replication and the formation of new phage particles. This life cycle culminates in the lysis of the bacterial cell typical through the production of a phage-encoded endolysin. Prior to the emergence of the antibiotic era, phage were extensively explored for their therapeutic potential with remarkable success seen in clinical trials treating cholera in India (Dherelle 1929). Interest in phage and phage therapy waned with the discovery of penicillin and the subsequent golden of antibiotic discovery. However, with the emergent antibiotic resistance crisis and the frequency with which MDR and XDR isolates are being identified, the exploration of phage as potential therapeutics is undergoing a renaissance (Kortright et al. 2019). Numerous studies have isolated bacteriophage active against MDR and XDR *A. baumannii* from sewage, waste water and clinical waste. The phage are primarily from the *Myoviridae, Siphoviridae* and *Podoviridae* families with many showing promising activity in animal models (Hua et al. 2018; Bagińska et al. 2019; Wintachai et al. 2019; Leshkasheli et al. 2019; Regeimbal et al. 2016). Significantly, however, a number of human case reports have shown remarkable success with phage cocktails. In one study, a 68-year-old patient with necrotizing pancreatitis complicated by a MDR *A. baumannii* infected pseudocyst was treated with a bespoke combination of 9 different bacteriophages leading to resolution of the infection and a subsequent return to health (Schooley et al. 2017). A second less successful study administered a bespoke 5-phage cocktail to a patient with a post-craniotomy associated *A. baumannii* infection. This study highlighted some important concerns about the viability of phage in the blood as 10 min after administration, phage were undetectable in the blood. The lack of any significant improvement in this patient ultimately lead to a withdrawal of care (LaVergne et al. 2018). While there is significant potential for phage therapy to stem the tide of MDR and XDR *A. baumannii* infections and act

as a viable alternative to antibiotics many questions remain to be addressed before full clinical uptake can be considered. These include greater insights in the pharmacokinetics and pharmacodynamics of phage, the capacity for resistance and the threat that as mentioned previously phage can be vectors for antibiotic resistance genes. More insights are also required into the capacity of phages to disrupt preformed biofilms, as this mode of growth is one of the primary phenotypes associated with clinical manifestations of *A. baumannii* infection.

Phytochemicals are also being explored in combination with traditional antibiotic therapies with some evidence that specific plant extracts can potentiate antibiotic activity; however the underlying mechanisms of action remains to be identified for many of these phytochemicals. Recently, the plant-derived antimicrobials *trans*-cinnamaldehyde (TC) and eugenol (EG) were shown to increase *A. baumannii* sensitivity to a range of different β-lactams (Karumathil et al. 2018). While extracts from *Eucalyptus camaldulensis* have been shown to increase the efficacy of Polymyxin B against MDR *A. baumannii* (Knezevic et al. 2016). Phytochemicals such as coumarin have also been shown to display antibiofilm activity at levels that are non-bactericidal. This compound is thought to inhibit biofilm formation through the disruption of quorum-sensing pathways in a wide range of pathogens. As biofilm formation plays a key role in antibiotic tolerance, using phytochemicals to disrupt this behaviour may be a viable strategy to augment the efficacy of front line antibiotic therapies (Shin and Park 2017; McCarthy and O'Gara 2015).

As recognised by the WHO, *A. baumannii* represents a significant threat to global health due to its intrinsic antimicrobial factors and its capacity to acquire genetic elements capable of conferring resistance. New insights at a molecular level are needed to better understand the mechanisms through which this pathogen can subvert the host immune system and antimicrobial therapy. These insights will play a significant role in spearheading the next wave of targeted anti-*A. baumannii* therapeutics.

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Pathogenesis and Host Immune Response in Leprosy

8

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1 Introduction

Leprosy or Hansen's disease is a chronic granulomatous infection of skin and peripheral nerves caused by the unique bacterial pathogen, *Mycobacterium leprae*. Leprosy is an ancient, insidious disease characterized by tissue damage and demyelinating lesions in the peripheral nerves (Graham et al. 2010; Britton and Lockwood 2004). The damage to the peripheral nerves results in sensory and motor impairment with characteristic deformities and disability. The degenerative changes associated with infection of the peripheral sensory nerves are crucial events for the establishment of the Hansen's disease (Rambukkana 2010). Depending on the individual's immune response, leprosy can remain a mild disease with no apparent physical changes and can spontaneously cure in rare cases. *M. leprae* bacilli can proliferate in the body causing extensive peripheral nerve damage, which in turn, can result in changes in physical appearance and morbidity (White and Franco-Paredes 2015).

Leprosy, although curable, continues to be a significant health problem in many parts of the world, primarily due to the development of antibiotic resistance in the existing multi-drug therapy (Cambau et al. 2018; Williams and Gills 2012). Leprosy once existed in Europe and Asia, but now disproportionately occurs mainly in developing as well as poor countries in tropical and warm temperate regions. An effective antimicrobial treatment for leprosy, Sulfone, was first introduced in 1943, heralding a major clinical development of the twentieth century (Faget et al. 1943). Sulfone drugs target the dihydropteroate synthase (DHPS), a key enzyme in the folate biosynthesis pathway in bacteria and its inability to synthesize folate leads to depletion of adenosine, guanosine, and thymidine pools (Richey and Brown 1969; Seydel et al. 1980). It has been suggested that dapsone, a 4,4-diaminodiphenylsulfone, may contribute to tuberculoid reactions by enhancing lymphocyte responsiveness by inhibiting production of suppressor prostaglandins (Anderson 1983). As early as the 1950s, it was observed that lepromatous leprosy patients treated with dapsone for several years,

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relapsed if treatment was stopped. The resistance to dapsone was due to mutation in *folp1* gene encoding DHPS1, which resulted in decreased dapsone binding (Williams et al. 2000). Till 1975, dapsone monotherapy remained the only treatment for all forms of leprosy (Gelber and Grosse 2012). In 1982, multi-drug therapy (MDT) was introduced, which overcame problems associated with monotherapy and the development of drug resistance in case of non-compliance by patients, such as interruption in the medication.

MDT involved an effective combination of antibiotics (rifampin, clofazimine and dapsone) in experimental leprosy infection (World Health Organization Study Group 1982; WHO Scientific Working Group, 2002). In 1985, there were an estimated 12 million people with leprosy worldwide; a prevalence of 12 per 10,000 had come down to 1 per 10,000 by 2002 (World Health Organisation 2016). Fifteen endemic countries still have a prevalence of more than 1 per 10,000, mainly in Asia, Africa, and South America, but 107 of the 122 countries endemic for leprosy in 1985 have achieved the elimination target (Britton and Lockwood 2004). Between 2005 and 2015, there was an overall gradual decline from 265,661 to 210,758 of leprosy cases. The prevalence of leprosy is gradually decreasing in many countries; however, rates of new cases being detected remain at almost the same level globally (World Health Organisation 2016), indicating active transmission. The MDT regimens have been very effective, by curing more than 11 million patients. Many patients complete MDT in a year or less. However, there are many experiencing long-term physical and societal complications of the disease, including temporary and permanent disability, deformity and social stigma.

2 Classification and Clinical Features of Leprosy

Leprosy represents a multi-factorial and complex disease in which the bacilli modulate the host immune response that reflects on the clinical presentations.

2.1 Classification Based on Clinical Prognosis

Depending on the clinical prognosis, leprosy is classified into two types: paucibacillary leprosy (PB) and multibacillary leprosy (MB) (Walker and Lockwood 2007; Ridley 1974). Based on the visual symptoms and the presence or absence of bacilli, the World Health Organization (WHO 1998) recommended that patients containing 1 to 5 diagnostic skin patches in the absence of bacilli in slit-skin smears should be considered paucibacillary; for 1 lesion it is often termed as paucibacillary single lesion leprosy. Those with more than 5 skin patches and bacilli visible by microscopic analysis of skin smears were termed as multi-bacillary. It occurs due to sudden alteration in the immunological response of the host against the living or dead bacilli. In the case of paucibacillary leprosy, skin and nerve lesions have characteristics of Th1-mediated immune response, whereas in multibacillary leprosy, Th2-type cellular response predominates (Legendre et al. 2012).

2.2 Five Group Classification System by Ridley and Jopling

Leprosy presents a spectrum of clinical manifestations; at one end, tuberculoid leprosy represents the resistant response restricting the growth of the mycobacteria while at the other end, lepromatous leprosy represents extreme susceptibility to bacilli infection. Ridley and Jopling (Ridley and Jopling 1966) suggested a five-group classification system according to the patient's immune status, highlighting the regions of the poles (tuberculoid and lepromatous) and also the intermediate zone between them (Borderline).

The five forms namely, tuberculoid polar form (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL) and lepromatous polar leprosy (LL), were classified on the basis of bacteriological, immunological, histopathological and clinical features of leprosy (Table 8.1). In the case of TT, the number of lesions is less but tissue and nerve damage

Table 8.1 Overview of Ridley-Jopling classification of leprosy according to host's immunity (Ridley and Jopling 1966)

Types	Criteria	Observations
TT	Clinical	• Large erythematous plaque showing sharply raised outer edge which slopes down toward a flattened center • Dry, hairless, and sometimes scaly lesion with rough surface • A gross and irregular thickened peripheral nerve usually palpable in the vicinity of a lesion
	Histological	• Well-developed epithelioid cells foci surrounded by a zone of dense lymphocyte infiltration • Presence or absence of Langhans giant cells • Granuloma extends up to the epidermis • Absence of acid-fast bacilli • Nerve bundles rarely found within the granuloma • Caseous necrosis is rare (only in granulomas involving nerves)
BT	Clinical	• Resemble tuberculoid leprosy in its sensory loss and appearance but are more numerous • Hair growth is less affected • Presence of numerous thickened nerves (not glossy or irregularly thickened)
	Histological	• Focalization of the epithelioid cells by a peripheral zone of lymphocytes • Langhans giant cells present, sometimes numerous • Nerve bundles if found are generally swollen and infiltrated • Acid fast bacilli range from 0 to 2 in the granuloma and 1 to 3 in nerve bundles
BB	Clinical	• Lesions are intermediate in number • Erythematous plaques found can be of two types- irregular shape with oval hypopigmented center or a raised oval or circular band with well demarcated outer and inner edges
	Histological	• Granuloma shows diffusely spread well developed epithelioid cells • Langhans giant cells absent • Lymphocytes if present are diffusely spread • Acid fast bacilli ranges from 3 to 4
BL	Clinical	• Lesions with superficial impression of lepromatous leprosy with irregular distribution over affected regions • Lesions are not so shiny and succulent in appearance • Thickened peripheral nerves are seen during the onset of the lesion • Patients may exhibit macules (flat skin lesions), plaques, papules (raised skin lesions) and nodules by the time of reporting
	Histological	• Granuloma is composed of histiocytic cells with or without foamy changes, large globi (bacterial micro-colonies) are absent • Lymphocytes occupy a whole segment of granuloma or found near at the perineural cuffs • Acid fast bacilli are usually more than 5
LL	Clinical	• Lesions are multiple, distributed bilaterally and symmetrically and the first manifestations are dermal • Always erythematous irrespective of skin color • Common appearance of edema on feet and lower legs and skin gets shiny and waxy • Symptoms of a pure neuritic phase do not occur • Peripheral nerves undergo hyaline degradation or fibrosis in later stages leading to anesthesia and muscle wasting in hands and feet
	Histological	• Granuloma composed of histiocytes showing varying degree of foamy changes • Multinucleate globi are observed • Lymphocytes few and diffusely arranged • Nerves do not show cellular infiltration or cuffing • Acid fast bacilli are usually more than 5

are frequent, whereas in LL, skin lesions are numerous with many viable *M. lepare* throughout the skin lesions (Modlin 1994). The TT patients may undergo immunological reactions resulting in rapid healing, but certainly the most severe reactions occur in the middle of the leprosy spectrum, the forms nearer the poles remain more stable (Ridley and Radia 1981). The well-characterized epithelioid granulomatous infiltrates in polar tuberculoid (TT) lesions gradually get disorganized during each increment- borderline tuberculoid (BT), mid-borderline (BB), and borderline lepromatous (BL), until they become completely disorganized aggregates of foamy (high lipid content) histiocytes, with only occasional lymphocytes in lepromatous (LL) lesions (Scollard et al. 2006a, b, c).

The immunopathological reactions within the nerves are vital from the prognostic point of view. Inflammation within the nerve sheath causes functional impairment and nerve pain (neuritis). Sensory motor impairment leads to acute paralysis and may progress through fibrosis. The common peroneal (lateral popliteal), the ulnar and the facial nerves are affected mostly leading to foot drop, claw hand and facial palsy, with poor chance of recovery (Klenerman 1987). Sweat and sebaceous glands are destroyed, resulting in dryness of skin and hair follicles destruction leads to loss of hair. Leprosy rarely involves the scalp, because it is reasonably warmer than the optimal growth temperature of *M. leprae* (Scollard et al. 2006a, b, c).

2.3 Classification Based on Leprosy Reactions

The dynamic nature of the immune response to *M. leprae* leads to spontaneous fluctuations in the clinical state, which are termed leprosy reactions. Two types of acute inflammatory reactions occur: **Type 1** (*Reversal reaction*) and **Type 2** (*Erythema Nodosum Leprosum*)**.** These reactions may be a presenting feature of the disease affecting 30 to 50% of patients and often appearing during or after MDT (Legendre et al. 2012). Most of the deformity and disability in leprosy results from these leprosy reactions.

2.3.1 Type 1 Reaction

It is a delayed hypersensitive response (DTH), occurring mainly in the borderline groups (BT, BB and BL). It may also occur with TT and also with pure neural leprosy. Type 1 reaction is a state of immunological instability, in which immune response alters the clinical manifestations to shift towards polar states (TT and LL).

Reversal reactions are considered to be DTH reactions against *M. leprae* antigens due to its marked lymphocyte response towards the bacillus (Sielin and Modlin 1992). These have histological characteristics similar to DTH with an influx of monocytes and lymphocytes, with early disturbance of collagen fibers through edema and subsequent giant cell formation (Dugan et al. 1985). Patients with type 1 reactions show higher T cell reactivity to mycobacterial antigen, and thus, have fewer bacilli (Britton 1998). Type 1 reactions are characterized by a shift to Th 1 type immune responses in the host with elevated levels of IFN-γ, TNF-α, IL-12 and iNOS (Little et al. 2001). CXCL10 is suggested as a potential immunological marker for identifying type 1 reactions (Oliveira et al. 1999).

In type 1 reaction, edema and painful inflammation are due to infiltration of $CD4^+$ lymphocytes with high levels of IFN-γ, IL-12 and TNF-α in skin lesions and nerves (Little et al. 2001; Khanolkar-Young et al. 1995). Increased intraneural pressure resulting from edema and increased cellular infiltration leads to neural pains. In addition, patients may present with edema of hands and feet, sensory or motor nerve impairment, leading to permanent disability (Walker and Lockwood 2007; van Brakel et al. 2005). Skin lesions become larger, more erythematosus and may ulcerate. The presence of dermal edema, plasma cells and giant cells in biopsy specimen is a good indicator of type 1 reaction (Britton and Lockwood 2004). This type of reaction typically establishes within the first 6 months after the start of MDT, although in some cases, they can happen at any stage of the disease (Graham et al. 2010; Franco-Paredes et al. 2009; Kumar et al. 2004). As type 1 reaction subsides, there is a reduction in edema (Massone et al. 2015).

2.3.2 Type 2 Reaction

It is also called Erythema Nodosum Leprosum (ENL). ENL occurs only in BL and LL. Type 2 reactions are distinguished clinically by fever, malaise and rapid appearance of new subcutaneous nodules that are erythromatosus and quite painful (Britton 2010). These reactions exhibit infiltration of polymorphonuclear cells accompanied by vasculitis and/or inflammation of subcutaneous layer of fatty tissue (panniculitis) (Scollard et al. 2006a, b, c; Kahawita and Lockwood 2008). Fragmented bacilli within macrophages, neutrophils and lymphocytes may be apparent in an ENL reaction. Lee et al. showed enhanced expression of E-selectin and IL-1β through microarray, leading to neutrophil recruitment in ENL and its adhesion to endothelial cells (Lee et al. 2010).

Type 2 reactions tend to be more complicated to treat because of its systemic nature and recurrent episodes (Pocaterra et al. 2006; Kumar et al. 2004). During the course of treatment, large numbers of leprosy bacilli are killed. The released antigens combine with the existing antibodies in the tissues and blood, forming immune complexes, activate the complement system, resulting in immune complex mediated type-III hypersensitivity reaction. The deposition of extravascular immune complexes leads to neutrophil infiltration and activation of complement in many locations such as eyes, testes, lymph nodes, kidney, liver, nerve, endocardium and joints (Lockwood 1996). This further can lead to polyarthritis, iridocyclitis (inflammation of iris), orchitis (inflammation of testicle), lymphadentitis (inflammation of lymph node) and glomerulonephritis (Britton 2010).

3 Pathogenesis

3.1 Cellular Morphology and Genome of *M. leprae*

M. leprae was discovered by G.H. Armauer Hansen in Norway in 1873, making it the first bacterium to be identified as causing disease in humans (Hansen 1874; Irgens 2002; Bhat and Prakash 2012). It is a non-motile, non-spore forming microaerophilic, acid-fast-staining rod-shaped bacterium. The cell wall of *M. leprae* has covalently linked peptidoglycan-arabinogalactan-mycolic acid complex similar to other mycobacterial cell walls (Daffe et al. 1993; Draper et al. 1987; Vissa and Brennan 2001).

M. leprae bacilli are slow growing obligate intracellular organisms trophic for macrophages, dendritic cells (DC) and Schwann cells in peripheral nerves. The bacilli show preference of growth in colder regions of the body. *M. leprae* has an evolutionarily minimized genome that constrains its growth to the intracellular niche. The genome size of *M. leprae* is 3,268,203 bp; a total of 2770 genes compared to *M tuberculosis*, which has a genome size of 4411,532 bp. Leprosy bacilli has undergone extensive reductive evolution, resulting in the functional loss of approximately half of its genes, leaving 1614 (revised number from http://genolist.pasteur.fr/Leproma/) genes encoding proteins (*M tuberculosis* has 3993 genes encoding proteins) and 50 genes for stable RNA molecules (Cole et al. 2001). The remaining *M. leprae* genes help to define the minimal genes necessary for its survival, infection and pathogenesis. For instance, the genes essential for the formation of a mycobacterial cell wall, including peptidoglycan biosynthesis and targets for ß-lactam drugs (Mahapatra et al. 2000), have been retained but the genes involved in catabolism are removed (Brennam and Vissa 2001). Downsizing of the genome has resulted in the elimination of several metabolic pathways, leaving this pathogen with very specific growth requirements. This could possibly explain its long generation time and inability to grow in culture. *M. leprae* can survive up to 8 months within cysts of common environmental free-living amoeba (*Acanthamoeba sp*.) These protozoa likely provide an intracellular refuge for *M. leprae* in the environment for which they would otherwise seem ill suited (William et al. 2014).

A notable feature of *M. leprae* genome is an exceptionally large number of pseudogenes, about 1133 compared to *M. tuberculosis,* which has 6 pseudogenes (Cole 1998). These pseudogenes in *M. leprae* are highly expressed as RNA and their expression levels seem to change fol-

lowing macrophage infection. Suzuki and coworkers (Suzuki et al. 2006) have identified 12 highly expressed gene regions in *M. leprae* showing alteration in expression levels upon infection, and among them, 6 were pseudogenes. Three of these pseudogenes were oxidoreductase, which are essential for generating energy needed for metabolic processes. The *M. leprae* genome includes several novel open reading frames, not present in *M tuberculosis* (Cole et al. 2001). These proteins restricted to *M. leprae* might provide the basis for specific skin tests and other diagnostic assays to detect infection (Dockrell et al. 2000; Brennan 2000).

3.2 Incubation Period and Susceptibility

The incubation period between infection and overt disease varies widely from months to 30 years, and the mean is estimated to be 4 years for tuberculoid and 10 years for lepromatous leprosy (Noordeen 1994). This makes epidemiological assessments of incidence and mechanism of transmission difficult. Leprosy shows a male predominance after the age of puberty, with a male to female ratio of 1.5–2.0 to 1 (Peters and Eshiet 2002). As leprosy prevalence falls in a community, the relative importance of household transmission increases; however, it is difficult to measure the rate of infection with *M. leprae* in a community.

It has been found that most people are not genetically susceptible to the disease. Variation among population groups exists that may be related to both genetic factors and ancestral exposure to the bacillus. Studies involving HLAs and proteins encoded by MHC-linked genes have revealed an association of MHC Class II: HLA-DR3 with tuberculoid leprosy, and of HLA-DQ1 with lepromatous leprosy (Abulafia and Vignale 1999). However, most of the clinical phenotypes may be due to genetic variability determined by different biological pathways modulated by *M. leprae*, reprogramming of adult Schwann cells, and interaction of innate and adaptive immunity (Polycarpou et al. 2013; Masaki et al. 2013). The presence of the Parkin gene (PARK2)/Parkin co-regulated gene (PACRG), located on chromosome 6q25-q27, and the presence of the NRAMP1 (Natural-resistance-associated macrophage protein 1) gene on chromosome 2q35 are associated with susceptibility to leprosy (Scollard et al. 2006a, b, c). Single nucleotide polymorphism-association studies showed a low lymphotoxin-α (LTA) allele as a major risk factor for early onset of leprosy (Alter et al. 2008). Transporter associated with antigen processing gene; TAP1 and TAP2, TNF-α and the Vitamin D receptor gene (VDR) have also been found to be associated with susceptibility to the disease (Rajalingam et al. 1997; Dennehy et al. 2008; Shaw et al. 2001; Misch et al. 2008; Zhang et al. 2009).

Innate immunity plays an important role in determining susceptibility to leprosy and its states (Type-1 and Type-2). Genetic variability associated with Toll-like receptors (TLRs) plays a vital role in a dysregulated inflammatory response. A TLR1 polymorphism, T1805G, encodes a non-synonymous SNP in the transmembrane domain of TLR1 that regulates signaling in response to Pam_3Cysk_4, a synthetic ligand of TLR1 (Hawn et al. 2007; Tapping et al. 2007; Wurfel et al. 2008). Polymorphism in TLRs and the extreme variability of its frequency among different populations worldwide suggests its impacts on susceptibility to leprosy. At the same time, 1805G SNP has been found to be significantly associated with protection against type 1 reactions (Barreiro et al. 2009; Dennehy et al. 2008; Polycarpou et al. 2013). A genome wide association study conducted on 706 patients and 1225 controls in China, has identified 6 genes to be associated with leprosy: CCDC122, C13orf31, NOD2, TNFSF15, HLA-DR and RIPK2 (Zhang et al. 2009).

3.3 *M. laprae* Transmission and Interaction with the Host

Humans are the primary reservoirs of *M. leprae.* Besides man, only wild nine banded armadillos (*Dasypus novemcintus*) are known to be natural

host of *M. leprae* (Rojas-Espinosa and Løvik 2001; Scollard 2016). Several cases of suspected zoonotic transmission from armadillos to human have been reported. *M. leprae* can replicate in the mouse footpad (Shepard 1960a, b) and the nine-banded armadillo (Rambukkana et al. 1997) which have provided opportunity to study the pathogenesis.

The principal mode of transmission of *M. leprae* is probably by aerosol spread of nasal secretions and uptake through nasal or respiratory mucosa. Nasal secretions collected through blowing the nose can show up to 10 million viable organisms per day in majority of the patients (Pedley 1973; Davey and Rees 1974). The skin and the upper respiratory tract are most likely the route of entry; however, recent research increasingly favors the respiratory route (Rees and McDougall 1977; Chehl et al. 1985). Two exit routes of *M. leprae* are the skin and nasal mucosa. Large numbers of *M. leprae* were found in the superficial keratin layer of the skin of lepromatous leprosy patients, suggesting the exit along with sebaceous secretions (Job et al. 1999).

M. leprae shows prominent tropism for Schwann cells of the peripheral nervous system (PNS) and uses the regeneration properties of the PNS for expansion of bacterial niche within Schwann cells (Rambukkana 2010; Rambukkana et al. 2002; Tapinos et al. 2006).The cell wall of *M. leprae* is similar to that of most *Mycobacterium* species. Phenol glycolipid-1 (PGL-1) is the dominant lipid in the cell wall which gives immunological specificity to *M. leprae* that binds on the Schwann cells. As shown in Fig. 8.1, the uptake of *M. leprae* into Schwann cells occurs when the PGL-laminin-2 complex interacts with α–dystroglycan (α-DG), the laminin-2 receptor located on the Schwann cell membrane (Ng et al. 2000). α-DG is a component of the DG complex involved in the pathogenesis of muscular dystrophy. *M. leprae* specifically binds to α-DG in the presence of the G domain of the α2 chain of laminin-2 (Rambukkana et al. 1997; Rambukkana et al. 1998). PGL-1 present in the *M. leprae* cell wall interacts with the α2 chain of laminin-2 (LAMA2) and α-dystroglycan on the Schwann cell membrane (Misch et al. 2010). *M. leprae* also makes

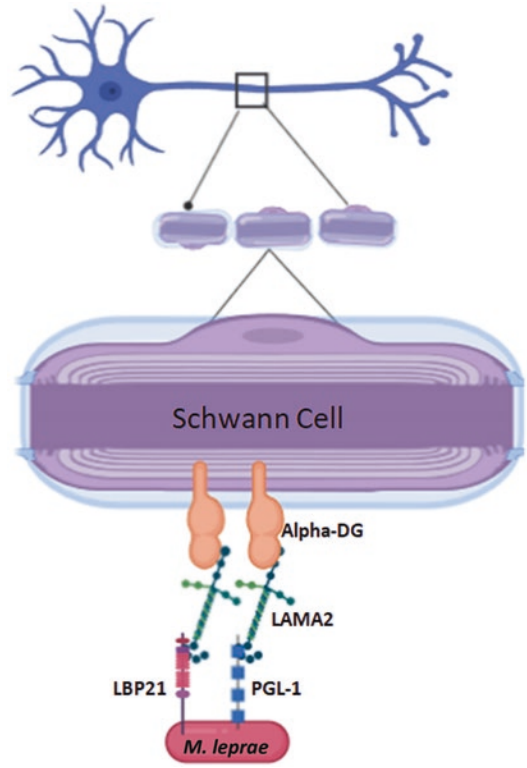

Fig. 8.1 Cellular and molecular interaction of Schwann cell with *M. laprae*

intracellular entry into the Schwann cell through laminin binding protein 21 (LBP21), one of its major surface antigen.

α-DG and LAMA2 interact with PGL-1 and LBP21 of *M. leprae.* These interactions mediate intracellular entry of *M. leprae* into the Schwann cell.

The interaction between *M. leprae* and the host cell is mediated by pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are primarily expressed on macrophages and DCs and recognize PAMPs. TLR2 forms a heterodimer with TLR1 to mediate the recognition of several bacterial motifs. *M. leprae* predominately activates TLR2/1 heterodimer (Bochud et al. 2003; Krutzik et al. 2003). Several other signaling receptors are also involved in *M. leprae* recognition, such as TLR4, TLR6, TLR8, TLR9, NOD2, DC-SIGN (CD90), Dectin-1 and Mincle (Misch et al.

2010). A 19-kDa protein of *M. leprae,* which is recognized by the TLR2/1 heterodimer, elicits a strong pro-inflammatory cytokine response (Krutzik et al. 2003) and induces apoptosis in Schwann cells (Oliveira et al. 2003). Proteasome function has been found to play an important role in this apoptosis; treating *M. leprae* stimulated peripheral blood mononuclear cells (PBMC) with the proteasome inhibitor MG132 revealed modulation of ubiquitin-protease pathway. (Fulco et al. 2007). *M. leprae* induced cell death is also characterized by an increased expression of the pro-apoptotic factors Bax-α and Bak, which are dependent on TNF-α biosynthesis (Hershko and Ciechanover 1998).

DCs are very effective presenters of *M. leprae* antigens (Maeda et al. 2003; Marlowe et al. 2004). DC-specific ICAM-3 grabbing non-myeloid integrin (DC-SIGN) is a C-type lectin expressed by immature myeloid DCs and has been associated with Th2 response (Soilleux et al. 2002). A more prominent expression of DC-SIGN-positive cells was noted in LL lesions and $CD1b^+$ cells in tuberculoid leprosy (Krutzik et al. 2005). It has also been proposed that virulent mycobacteria may downregulate DC function by suppressing DC maturation via DC-SIGN, possibly through the inhibition of IL-12 production (Nigou et al. 2001) and induction of IL-10 (Geijtenbeek et al. 2003).

Neutrophils are commonly found in ENL or type 2 reactions and may contribute to the majority of TNF production that is associated with tissue damage in leprosy. Microarray analysis has demonstrated that the mechanism of neutrophil recruitment in ENL involves the enhanced expression of E-selectin and IL-1β, leading to neutrophil adhesion to endothelial cells (Lee et al. 2010).

4 Host Immune Response to *M. leprae*

Immune response to *M. leprae* operates at two levels. First is the manifestation of innate immune resistance mediated by cells of the monocyte lineage. If innate immune resistance is insufficient and infection becomes established, genetic influence operates at the second level, i.e. the degree of specific cellular immunity and delayed type hypersensitivity generated by the infected individuals (Scollard et al. 2006a, b, c).

Once *M. leprae* invades the body, it migrates towards the neural tissue and enters Schwann cells. Invading *M. leprae* has three main targets: Schwann cells, small endothelial vessels and monocyte-macrophage system. The mycobacterium takes 12–14 days to divide into two within the cells. Once the bacilli reach the interstitium of the fascicle, it may be ingested by the resident macrophages or they may get attached to the basal lamina of Schwann cells.

Schwann cells synthesize myelin sheath around axons and provide external milieu needed for neuronal survival (Pereira et al. 2012). Terminally differentiated Schwann cells show plasticity in switching off the myelin program and attain a dedifferentiated state (Chen et al. 2007; Jessen and Mirsky 2008), contributing to a remarkable regenerative capability of peripheral nerves following injury (Fancy et al. 2011). *M. leprae* causes demyelination to establish the infection and subsequently reprograms them to attain dedifferentitation stage. As PNS blood-brain barrier protects *M. leprae* from host immune attack, Schwann cells also serve as a safe niche (Job 1989, Stoner 1979, Masaki et al. 2013).

M. leprae induced demyelination is a result of direct bacterial ligation to neuregulin receptor, ErbB2 and Erk 1/2 activation and subsequent MAP kinase signaling and proliferation. MEK-dependent Erk1 and Erk2 signaling is a downstream target of *M. leprae* induced ErbB2 activation and mediates demyelination (Tapinos et al. 2006). Matrix metalloproteinases (MMPs) mediating demyelination and breakdown of the blood brain barrier in peripheral neuropathies are also upregulated in leprosy (Teles et al. 2007; Oliveira et al. 2010). *M. leprae* induced upregulation of MMP-2 and MMP-9 in cultured Schwann cell line, derived from a malignant peripheral nerve sheath tumor, ST88-14, has also been found to induce NK-κB-dependent production of TNF-α (Pereira et al. 2005).

Soon after demyelination, *M. leprae* reprograms Schwann cells to dedifferentiate into stem cell-like stage to spread infection, creating a suitable environment for the mycobacterium to proliferate (Rambukkana 2010). This reprogramming allows redifferentiation into mesenchymal cells with the ability to spread infection or attracting macrophages and lymphocytes forming a granuloma (Polycarpou et al. 2013; Masaki et al. 2013). Masaki and collaborators have suggested the role of chemokines, CCL2, CCL7, CXCL10 and receptor tyrosine kinase protein, KIT 1, in this reprogramming (Masaki et al. 2014). As Schwann cells do not have lysosomal enzymes capable of degrading the mycobacterium, bacilli may survive for a long time. The presence of *M. leprae* in the endoneural macrophages and the rupture of Schwann cells due to excessive bacterial load triggers a perineural inflammatory response, liberating the bacilli to infect other cells.

$CD4^+$ and $CD8^+$ T cells play a crucial role in orchestrating the adaptive immune response in leprosy, so do CD1-restricted T cells and γδ T cells (Modlin et al. 1988; Sieling et al. 1999; Modlin et al. 1989a, b). It has been observed that macrophages remain inactivated if *M. leprae* specific T cell immune response is not being generated. Thus, the growth of the bacilli proceeds indefinitely in lepromatous leprosy (Hagge et al. 2004). In paucibacillary leprosy, $CD4^+$ T, cytotoxic $CD8^+$ T or NK cells destroy the infected and incapacitated macrophages or Schwann cells, and release the intracellular bacilli (Chiplunkar et al. 1986; Kaleab et al. 1990; Spierings et al. 2001; Gu and Krahenbuhl 1995; Kimura et al. 2004). Upon killing of infected macrophages by CTLs, the bacilli get released into the extracellular space where they get phagocytosed again by activated macrophages providing them a fresh habitat (Kaufmann 1988). Lysis of target cells by $CD8^+$ CTLs involves perforin and cytotoxic granules such as granzyme B. Granulysin, a defensive antimicrobial protein used by CTLs is found to be over-expressed in leprosy (Ochoa et al. 2001). There is also evidence to suggest that *M. leprae*-specific CTLs, generated at the tuberculoid end of the spectrum, lyse *M. leprae*-infected macrophages and Schwann cells (Chiplunkar et al. 1986: Kaleab et al. 1990; Spierings et al. 2001). Interstitial macrophages may also cause mechanical injury (Abulafia and Vignale 1999).

In the absence of an effective adaptive immune response, over 100 bacilli can multiply within one macrophage (Hagge et al. 2004). In case of resting macrophages, phagosome-lysosome fusion is blocked by live *M. leprae;* in activated macrophages, phagosomes harboring *M. leprae* get fused with secondary lysosomes (Sibley et al. 1987). Monocyte derived macrophages induce phagocytosis of *M. leprae* via complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18), regulated by protein kinase (Prabhakaran et al. 2000). *M. leprae* is well equipped to handle anti-microbial reactive oxygen intermediates generated by the host macrophages, possibly due to downregulation of superoxide generation by PGL-1 (Chan et al. 1989), possessing superoxide dismutase (Thangaraj et al. 1990) and expressing SodC and SodA (Williams et al. 2004).

Downregulation of macrophage function seems to be a characteristic pathogenic mechanism induced by the mycobacteria. In leprosy, both TNF-α and IFN-γ have been shown to bind to the cellular receptors of macrophages, thereby changing the behavior of M0 macrophages, which undergo phenotypic modification to become M1 pro-inflammatory macrophages (de Sousa et al. 2017). The fate of macrophage activation, be it pro-inflammatory (M1) or anti-inflammatory (M2), influences the type of T cell activation and differentiation (Mills 2012). M1 macrophages induce killing of *M. leprae* through nitric oxide release and promote Th1 immunity (Verreck et al. 2004), while IL-10 producing M2 macrophages contribute to immunosuppressive response in lepromatous leprosy lesions (Montoya et al. 2009; Mège et al. 2011). Polarization towards the regulatory M2 phenotype in live *M. leprae* infected macrophages is preferentially primed by Treg cells, which downregulate Th1 and CTL immune responses (Yang et al. 2016). M2 macrophages express the scavenger receptor, CD163, which may contribute to entry of the bacillus, thus playing an important

role in the immunopathology of the lepromatous form of the disease (Sousa et al. 2016; Moura et al. 2012). It is clear that macrophages play a pivotal role in the induction of various pro-inflammatory modulators and activation of specific lymphocyte subpopulations that influence the fate of the disease pathogenesis.

As mentioned earlier, the incubation period, or the lag phase, before the manifestation of specific immunity may be quite prolonged, probably years, following initial exposure in case of leprosy. During this time, innate immunity is presumably active and gradually establishes an immune response that determines a subsequent path into clinical leprosy. One of the hallmarks of clinical leprosy is the formation of *granuloma*, on the basis of which histopathological discrimination between tuberculoid and lepromatous leprosy is based.

4.1 Microenvironment of the Granuloma

A granuloma consists of macrophages turned epithelioid cells that are accumulated in small, nodular entities surrounded by varying numbers of lymphocytes and multinucleated giant cells, usually of the Langhans type.

In case of leprosy, immunopathological spectrum encompasses classical epithelioid cell granuloma formation in *tuberculoid leprosy* and no organized granuloma and epithelioid cells in *lepromatous leprosy*, with a broad, interconnecting spectrum of intermixed inflammatory manifestations in intermediate (borderline) disease (Skinsnes 1970).

In case of Type1 reactions, ganuloma is observed in superficial dermis with appearance of giant cells, epidermal erosion with spongiosis and fibroplasias in the dermis (Massone et al. 2015). Epithelioid cells fuse to form giant cells characterized by large mass of cytoplasm with several nuclei, 20 or more (Chattopadhyay 1994; James 2000). These giant cells can be formed by cell fusion and nuclear division without cytoplasmic separation (Macfarlane et al. 2000). Macrophages also get converted into epithelioid and multinucleated giant cells (MGC) by cell–cell fusion induced by different cytokines, such as IFN-γ, IL-1, IL-3, IL-4, IL-6, and GM-CSF (Hernandez-Pando et al. 2000).

Type 2 reactions or ENL appear as two distinct types histopathologically: *Pink node type* or *Classic ENL* (mild form) and *Necrotizing ENL* (severe form) (Job 1994; Soler & Bernaudin 1993). In Pink node type, as reported by Ridley, the infiltrate is centered around small granulomas in the subcutis with clusters of neutrophils around foamy macrophages. Plasma cells, mast cells and eosinophils are also present. Necrotizing ENL shows neutropilic infiltrate, hemorrhages, and thrombi and may produce degeneration of collagen with necrosis of both dermis and epidermis, even leading to dermal fibrosis. This rare, but potentially life-threatening reaction to *M. leprae,* is also known as Lucio's phenomenon (Ang et al. 2003), characterized by necrotizing vasculitis with diffuse infiltration of Virchow cells (Chan & Smoller 2016).

4.2 Tuberculoid Granuloma

As seen in Fig. 8.2, the tuberculoid form is characterized by a well- demarcated granuloma, infiltrated by $CD4^+$ T lymphocytes (Yamamura et al. 1992), containing epitheloid and multinucleated giant cells and with a small number of bacilli or without bacilli. The decrease in bacillary load is associated with a Th1 response where TNF-α and IFN-γ activate macrophages and induce the production of iNOS that destroys the bacillus due to the release of free radicals (De Sousa et al. 2017; Sibley & Krahenbuhl 1987). Active macrophages continue bacterial killing until epithelioid and Langerhans cells develop. The cytoplasm of the epithelioid cells shows normal lysosomes and numerous swollen, degenerated mitochondria, some of them partially phagocytosed (autophagosomes) (Abulafia and Vignale 1999).

Tuberculoid leprosy shows vigorous cellular immune response to *M. leprae*, which limits the disease to a few well-defined skin patches or nerve trunks (Britton 2004). $CD4^+$ T cells are in abundance and remain scattered inside the granuloma in the form of a ring. The presence of mem-

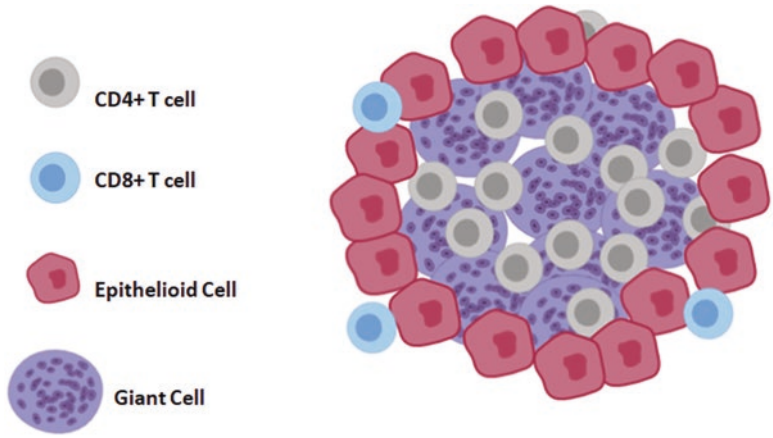

Fig. 8.2 Tuberculoid Granuloma is characterized by a Th1 cell immune response. $CD4^+$T cells predominates and are located centrally along with the giant cells (Langhans cells) and epithelioid cells. $CD8^+$T cells are very few in number and are located at the periphery of the granuloma. Mycobacterium is not found inside the granuloma due to active CMI response

ory $CD4^+$ T cells as well as macrophages in the granuloma shows an active immune interaction, leading to *M. leprae* killing (Narayanan et al. 1983; Modlin et al. 1982). In tuberculoid leprosy, IL-2 and IFN-γ production is evident; antibody responses to *M. leprae* are absent or weak.

4.3 Lepromatous Granuloma

In the lepromatous leprosy (Fig. 8.3), there is no characteristic granuloma but only unstructured accumulation of ineffective macrophages containing engulfed pathogens and degradation debris. Lepromatous leprosy (ENL; subcutaneous nodules), is characterized by Th2 cytokine profile with poorly developed cell-mediated immune response. It is an acute inflammatory condition with high TNF-α levels. $CD8^+$ T cell population is higher than $CD4^+$ T (mostly naïve T subset) (Modlin et al. 1989a, b). It has also been noted that the $CD4^+/CD8^+$ T cell ratio in the lesions are independent of those found in the blood of patients, indicating selective migration, proliferation and homing of these cells in the granuloma (Modlin et al. 1986).

The pathological form of LL skin lesions are characterized by a high number of foamy macrophages containing a very large number of bacilli, and disorganized lymphocyte infiltration (Kumar et al. 1989). Skin biopsies of polar lepromatous patients reveal sheets of foamy macrophages in the dermis containing a large number of bacilli and microcolonies called globi. Infiltration of neutrophils, plasma cells and deposition of antigen-antibody complexes are also observed (Abulafia and Vignale 1999).

During leprosy infection, lipid homeostasis plays a vital role in host–pathogen interaction (Wenk 2006; van der Meer-Janssen et al. 2010), as evident from the heavily infected macrophages with a typically 'foamy' appearance (also referred to as Virchow cells) in LL dermal lesions (Virchow 1863; Scollard et al. 2006a, b, c). *M. leprae* are found to reside and replicate within enlarged, lipid-filled phagosomes (Chatterjee et al. 1959), suggesting an important lipid metabolism alteration during infection. Initially, these lipids were believed to be derived from mycobacterium (Sakurai and Skinsnes 1970; Kaplan et al. 1983; Brennan 1984) but recent reports indicated it to be host-derived (Cruz et al. 2008.; Mattos et al. 2010).

Foamy macrophages found in dermal lesions are highly positive for adipose differentiation-related protein (ADRP), a classical lipid droplet (LD) marker, indicating accumulation of LDs contributing to their foamy

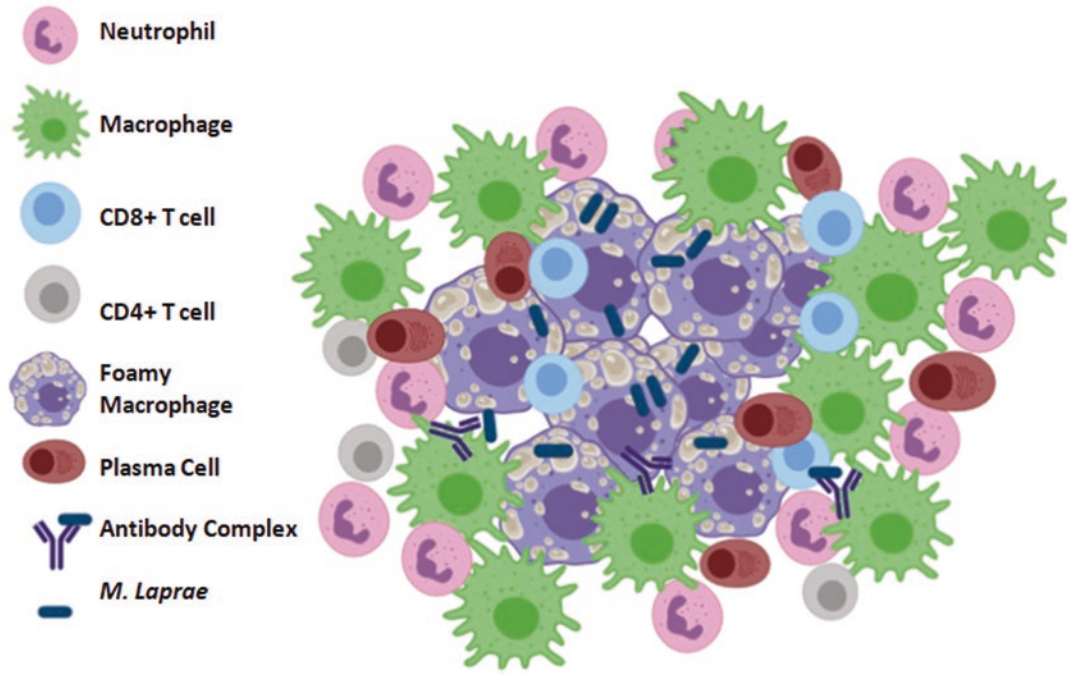

Fig. 8.3 Lepromatous Leprosy Lesion (*Erythema nodosum leprosum*) is characterized by a Th2 -cell immune response. Lymphocytes are diffusely spread throughout the lesion predominated by $CD8^+$T cells, located centrally. The lesion shows large proportion of histiocytes with varying degree of fatty change producing foam cells. Infiltration of macrophages, neutrophils, $CD4^+$T cells, plasma cells and deposition of antigen-antibody complexes (immune complex) are also observed within the non-definite granuloma formation. Large amount of antibody formation occurs which is usually non-protective. *M. leprae* seemed to be thriving well inside the lesion leading to its intense growth inside the foamy macrophages. Presence of large number of $CD8^+$T cells inside the lesion indicates generation of a suppressive function, where CMI has been compromised

nature (Tanigawa et al. 2008; Mattos et al. 2010). *M. leprae* has been shown to suppress lipid degradation through inhibition of hormone sensitive lipase (HSL) expression (Tanigawa et al. 2012). Foamy degeneration of the LL nerves is also related to LD biogenesis induced by *M. leprae* infection and this process plays a central role in bacterial survival (Mattos et al. 2011). Similar to *M. leprae, Mycobacterium bovis* and *M. tuberculosis* also mediate foamy cell formation and depend on host lipid acquisition for survival and successful infection (D'Avila et al. 2006; Pandey and Sassetti 2008; Kim et al. 2010). Cholesterol is one of the host lipid molecules that accumulate in *M. leprae*-infected macrophages by stimulating the *de novo* Cholesterol synthesis pathway (Mattos et al. 2014). Besides increasing endogenous Cholesterol synthesis, ML relies on another mechanism to induce intracellular Cholesterol accumulation by increasing the uptake of exogenous sources of Cholesterol via upregulation of LDL receptors.

4.4 Role of Cytokines in Th1/Th2 Paradigm

The Th1/Th2 paradigm, based on functional discrimination of T-helper cells according to their pattern of cytokine production, asserts that Th1 and Th2 cells promote a cellular and humoral immune response, respectively (Mosmann et al. 1986). This functional differentiation has offered an attractive hypothesis to explain the differences between tuberculoid and lepromatous response to *M. leprae* (Scollard et al. 2006a, b, c). Therefore, it appears that the nature of the host immune

responses dictate the clinical outcome of *M. leprae* infections (Yang et al. 2016).

In leprosy, Th0 lymphocyte differentiates to Th1and Th2, and the main cytokines involved in the process are IL-2, IL-12 (Th1), and IL-4 (Th2). Th1 cells often express CCR5 and CXCR3 chemokine receptors, whereas Th2 lymphocytes express CCR4, CCR8, and CCR3 to a lesser extent (Zhou et al. 2003; Quaresma et al. 2012). Th1 response is marked by IFN-γ and IL-2 production, which activates macrophages in killing intracellular pathogens (Mosmann and Coffman 1989). IL-12 and IL-18 promote resistance to *M. leprae* and are highly expressed in tuberculoid leprosy (Garcia et al. 1999; Mosmann et al. 1986; Sieling and Modlin 1994). Conversely, a Th2 response is characterized by the production of IL-4, IL-5 and IL-13 which helps in antibody production and consequently downregulates the Th1 response (Mosmann and Coffman 1989). A large number of IL-2, IFN-γ, TNF-α, LT and GM-CSF producing T cells were documented using in-situ hybridization in case of tuberculoid leprosy (Arnoldi et al. 1990; Modlin et al. 1986c). In lepromatous form, anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 and growth factors such as TGF-β and FGF-β contribute to the development of immunosuppressive mechanism and tissue repair (Sousa et al. 2016; Fachin et al. 2017; Mège et al. 2011; Oliveira Fulco et al. 2014).

5 Animal Models in Leprosy

One of the major hurdles in leprosy research is the lack of suitable animal models. This hindered the development of our understanding and treatment of the disease for almost a century. Initial attempts to identify suitable animals included, but were not limited to rabbits, saltwater fishes rainbow perch, goldfish, turtles, pigs, cats, tadpoles, guinea chipmunks, dogs, parrots, pigeons, frogs, chickens, toads, snakes (including rattlesnakes), gerbils, lovebirds, black mice, white mice, rats, paddy birds, albino hamsters, golden hamsters, canaries, eels and non-human primates (Johnstone 1987). In addition to the natural resistance against *M. leprae* seen in most animals, a major issue was the poor quality of *M. leprae* inoculum. This was because most of the isolated bacilli were from fresh or frozen homogenates of nodules and lesions from untreated human lepromas (Scollard et al. 2006a, b, c). In 1956, Binford postulated that *M. leprae* preferred less warmer anatomic sites for growth (Binford 1956). This revelation, combined with the knowledge of the prolonged growth cycle of the bacteria, allowed the development of the Shepard mouse footpad (~32 °C) model in 1960 (Shepard 1960a, b), and the subsequent nine-banded armadillo model (32–35 °C) (Kirchheimer and Storrs 1971).

Leprosy research also was severely affected by the bacilli inability to grow via *in vitro* and *in vivo* models. A major breakthrough in leprosy research came in with the ability to cultivate *M. leprae* using the footpads of immunocompetent white mice (Shepard 1960a, b). An inoculation of 5000–50,000 bacteria into the plantar space of the hind footpad yielded up to 1×10^6 bacteria/footpad in 6 months (Rees 1988). It was found that certain strains of mice like BALB/c, CBA and CFW produced a higher level of infection. This enabled study into the immunological mechanisms involved in the infection. It also allowed for the screening of anti-leprosy drugs and identification of drug resistant strains of *M. leprae*. Host pathogen studies revealed that T lymphocytes played a crucial role in host resistance (Colston and Hilson 1976; Katoch 1999; Rees et al. 1967). This led to the development of the athymic nu/nu mice model, which produced 1×10^{10} or more bacilli per footpad. This was considered a major milestone in leprosy research as it allowed the cultivation of a large amount of highly viable *M. leprae* acid-fast bacilli (Scollard et al. 2006a, b, c).

An ideal animal model should present similar bacteriological and histopathological characteristics of humans following infection of a healthy host (Blake et al. 1987). The infection in immunocompetent mice was much more emphatic in the footpad but lacked nerve involvement (de Medeiros Oliveira et al. 2019). Hence, the natural susceptibility of the nine- banded armadillo (*Dasypus novemcinctus*) and its ability to mimic

histopathological changes in tissues and nerves, as seen in a human infection, made it the best experimental model (Sharma et al. 2013). The disease manifests as a systemically disseminated infection, especially in reticulo-endothelial tissues, with intermittent bacteraemia in all organs. The effect is more pronounced in regions with lower temperatures, like the extremities (de Medeiros Oliveira et al. 2019; Sharma et al. 2013). Infection of nine-banded armadillo through the intravenous route produced the best model 18 months post infection (Balamayooran et al. 2015). It was found that armadillos yielded 10^9 to 10^{10} *M. leprae* bacteria per gram of liver, spleen or lymph node with an inoculum of as low as 1000 bacilli (Job 2000; Truman et al. 2008).

Although armadillos can exhibit the full spectrum of the disease, approximately 70% exhibit LL form (Job and Truman 1999). Abrasions around eyes, nose, feet are common signs and typical plantar ulceration is observed at later stages of infection (Sharma et al. 2013). In addition, these armadillos also exhibit severe anemia and compromised liver and renal function (Truman and Sanchez 1993). Unlike the mouse model, armadillos develop extensive neurological involvement and they can be examined for rare neurological events over time (de Medeiros Oliveira et al. 2019; Truman et al. 2014). Infected armadillos are known to exhibit clinical signs of focal anaesthesia, impaired sensation, nerve thickening and motor dysfunction (Sharma et al. 2013). Studies have revealed that the lower extremities of armadillos exhibit several abnormalities observed in human patients. For example, the flexor and lumbrical muscles are found to be atrophied in infected armadillos due to damage to the medial and lateral plantar nerves that innervate these muscles (Truman et al. 2014). Histopathological studies also revealed that the skeletal muscles of infected armadillos resembled muscular pathology as in patients with LL leprosy (Werneck et al. 1999). Electrophysiological analysis, used to assess functional characteristics of the peripheral nerves, demonstrated that 75% of experimentally infected armadillos develop a demonstrable conduction deficiency in the posterior tibial nerve. The manifestation of this reduced conduction coincides with the development of immunological responses. These mostly include detectable IgM antibodies against PGL-1 (Sharma et al. 2013). A heavy infiltration of *M. leprae* loaded nucleated cells in many fascicles of nerve trunks and invasion of progressively demyelinating Schwann cells by *M. leprae* was observed in infected armadillos (Scollard 2008; Sharma et al. 2013). Histopathological examination revealed characteristic interstitial neuritis, with the infiltration of inflammatory cells such as macrophages, and bacilli in the perineurium, epineurium, and endoneurium (Scollard et al. 2006a, b, c). As leprosy can only be clinically diagnosed at the later stages of the disease, the shorter interval between infection and disease development in experimentally infected armadillos provides a unique window to evaluate pathogenesis in preclinical stages and develop therapeutic interventions. Gene expression profiles show a constant state of degeneration in armadillo nerve segments via down-regulation of growth factors such as Delta Like Non-Canonical Notch Ligand 1 (DLK-1) and Nerve Growth Factor Beta (NGF-β). In addition, pro-inflammatory cytokines such as IFN-γ and TNF-α show enhanced expression along with constitutively expressed neural proteins such as Ubiquitin carboxy-terminal hydrolase L1, neurofilament and β-tubulin (Sharma et al. 2013). Development of a viable vaccine against leprosy has also used armadillos extensively as a preclinical model. Unfortunately, the number of armadillos and time required (up to 1140 days) for effective challenge studies limit the use of armadillo as a vaccine model (Scollard et al. 2006a, b, c).

The natural susceptibility of the nine banded armadillos to *M. leprae* infection, their lower body temperature and similarity in clinical manifestation of leprosy, has made the animal the best source of highly pure and viable of *M. leprae* bacilli, a clinical and immunological model to study host pathogen interactions of leprosy like neuropathies, myopathies and gene expres-

sion, and develop vaccines and other therapeutic interventions.

6 Conclusion and Perspectives

The current treatment against leprosy is an MDT regimen using clofazimine, dapsone, rifampicin, ofloxacin and minocycline (Duthie et al. 2011a). While this has been proven to highly effective against the global prevalence of leprosy, multiple indicators have hinted that a larger effort would be required to thwart the re-emergence of leprosy and achieve the goal of total disease eradication. The MDT approach has many complications. The long time period required by the rigorous drug regime is often shortened in patients who are either misdiagnosed on the severity of the disease or due to poor compliance by the patient (Duthie et al. 2011b). This has led to either relapse or re-infection in patients treated with a truncated MDT regimen (Ellard et al. 1988; Roche et al. 2000). Ineffective MDT treatment has created a conducive environment for the development of multidrug resistant *M. leprae* (Emmanuelle et al. 2002; Ji et al. 1997; Maeda et al. 2001; Matsuoka et al. 2000, 2003). In response, the WHO initiated a sentinel surveillance network in 2008. The analysis of 213 relapse cases by the network revealed the existing resistance against dapsone, rifampicin, and ofloxacin (World Health Organization 2011a, b). The emergence of these multidrug resistant strains seriously undermines the efficiency of the MDT and exponentially increases the risk of leprosy incidence. The MDT approach relies on a passive case detection system. This strategy lacks a provision for the prevention of leprosy instead focusing on reducing the number of cases identified as carriers. This relies on self-reporting and early treatment, which are often compromised due to the social stigma present around the disease and the delay between the onset of the first symptom and clinical diagnosis (Chen et al. 2000; De Rojas et al. 1994; Deps et al. 2006). Another risk against the total eradication of the disease is the erosion of leprosy clinics, specialists, and research caused by the success of the MDT program. This shows that the eradication and treatment of leprosy will require a multipronged approach that deals with eradicating the social stigma surrounding the disease, improving the lifestyle of at-risk population, and developing a new vaccine that would provide long lasting immunity.

Currently, vaccine used against leprosy is the same as the one used against tuberculosis, the BCG vaccine. The efficiency of the vaccine has been reported as 26–41% in experimental studies and 61% in observational studies. The recent drastic difference in the degree of protection offered by the vaccination has been attributed to the use of different BCG strains, the innate diversity in the genetic fingerprints of the mycobacterium present in various geographic areas, the immune, nutritional and socio-economic status of the patients, viral or helminthal co-infection and induction of immunity by environmental mycobacteria that masks the effect of BCG vaccination (Abebe and Bjune 2006; Andersen and Doherty 2005; Brosch et al. 2007; Comas et al. 2013; Elias et al. 2008; Hagge et al. 2017; Hoang et al. 2015; Lavania et al. 2015; Moliva et al. 2015). Studies have revealed that, BCG vaccination offers a higher protection efficiency when offered to younger individuals (<15 years of age) (Rodrigues et al. 2007; Zodpey et al. 1999, 2005). The use of heat killed *M. leprae* has been shown to offer protection against subsequent infections in mice (Shepard et al. 1983). The use of other species of mycobacteria such as *M. vaccae, M. haban* and *Mycobacterium indicus pranii,* have been explored and found to offer protection (Duthie et al. 2018*;* Singh et al. 1989, 1991*;* Truoc et al. 2001). Vaccines containing both BCG and heat killed *M. leprae* or other mycobacteria were found to have no significant effect in terms of protection against leprosy when compared against the regular BCG vaccination (Singh et al. 1991; Truoc et al. 2001). One of the drawbacks of the BCG vaccination is that it loses its protective efficiency after a period of 10–20 years. Hence, it was found that a booster of the BCG vaccination afforded better protection against leprosy (Mangtani et al. 2018). Vaccines containing individual components of *M. leprae,* such as

the cell wall, cell membrane, and cytosol when combined with a suitable adjuvant were found to offer protection prior to infection (Gelber et al. 1990; Ngamying et al. 2003). The use of Ag85 proteins, purified from BCG culture filtrate, along with Freund's incomplete adjuvant, was found to inhibit *M. leprae* growth. Immunisation with recombinant BCG strain engineered to overexpress the Ag85 complex was found to significantly reduce the multiplication of the pathogen when compared to non-recombinant BCG (Matsuoka et al. 1997; Naito et al. 1999; Ohara et al. 2000; Roche et al. 2001). Additionally, another recombinant BCG strain engineered to secrete *M. leprae* major membrane protein-II was found to induce a more potent Th1 response when compared to non-recombinant BCG (Maeda et al. 2005). One major drawback of using recombinant live bacteria is its inability to boost antigen-specific responses for individuals who have already been vaccinated with BCG (Duthie et al. 2011a). The use of a defined subunit vaccine would provide long lasting protection. It would also help overcome the issues faced with live whole or recombinant bacteria as a vaccination in the immunocompromised population. LepVax, A subunit vaccine, which comprises a hybrid recombinant protein, linking four *M. leprae* antigens: ML2531, ML2380, ML2055, and ML2028 (LEP-F1), was formulated in a stable emulsion with a synthetic, TLR4 agonist (GLA-SE) as adjuvant; its pre-clinical testing has been completed. In addition to offering protection, the vaccine was found to alleviate and delay neurological damage caused by the infection (Duthie et al. 2018).

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9 Innate Immune Pattern Recognition Receptors of *Mycobacterium tuberculosis*: Nature and Consequences for Pathogenesis of Tuberculosis

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1 Introduction

Tuberculosis (TB), predominately caused by the bacterium *Mycobacterium tuberculosis*, remains one of the world's most significant infectious diseases, with a worldwide yearly burden of approximately 8.7 million new cases of active TB, 1.4 million deaths and a third of the world's population with latent TB infection (LTBI) (WHO 2019). The major burden for TB disease in still bared by developing countries, with Asia (e.g. China and India) having the highest number of cases of the disease. The epidemiological trend in developed countries continues to be that the majority of TB cases have originated from recent immigrants that have come from TB endemic areas of the world (Zumla et al. 2013; Zaheen and Bloom 2020). There are also significant numbers of TB patients co-infected with Human immunodeficiency virus (HIV), particularly in sub-Saharan Africa, resulting in the highest rates of TB cases per capita (WHO 2019). The outbreak of the COVID-19 pandemic may also result in a similar dangerous synergy with TB, although the impact of co-infection of *M. tuberculosis* with the SARS-CoV-2 virus in patients remains to be determined (Ong et al. 2020). The frequency of multi-drug resistant tuberculosis (MDR-TB) to the main drugs used for treatment (e.g. rifampicin and isoniazid) is still worryingly high, particularly in India, Russia, China, Pakistan and South Africa (WHO 2019; Zaheen and Bloom 2020). Of further concern is the rise of extensively drug resistant (XDR-TB) in several countries to all current second and third-line therapies (Zaheen and Bloom 2020).

There continues to be a strong concerted effort to develop new interventions and therapies against TB with a particularly important focus on understanding innate immunity against TB particularly in the early stages of infection and the granuloma. It is well know that *M. tuberculosis* is able to persist as an intracellular parasite for years in the host as LTBI, mainly because of its ability to persist in the host macrophage by manipulating phagolysosome maturation, providing a favourable niche for it to be able to reside (Russell 2001; Gupta et al. 2012). *M. tuberculosis*-infected macrophages in LTBI are predominantly present within the granuloma, which is a complex structure of T cells, B cells,

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and giant epithelioid cells among others and a resectioning of tissue to contain these infected macrophages (Gupta et al. 2012). The formation and maintenance of this immune cordon against TB infection is crucial in preventing disseminated disease and transmission of the infection to other hosts. It is still not fully understood how the granuloma is formed and maintained in TB infection and the full extent of the contribution played by innate immunity. Here, we will discuss the latest advancements in the understanding of innate immune recognition of *M. tuberculosis* and how these contribute to both downstream protection and pathogenesis of TB.

The primary interactions of *M. tuberculosis* with the host upon inhalation remain to be determined fully, particularly the targeting and recognition by the innate immune response. The lungs are the main route of entry to the host for *M. tuberculosis* and is the main anatomical site for infection and pathogenesis, but not entirely as extra-pulmonary TB (EPTB) also relatively commonly occurs in 10–42% of cases (Caws et al. 2008). The establishment and dissemination of *M. tuberculosis* infection is dependent on several host and pathogen factors with the pathogen able to alter and circumvent facets of both the innate and adaptive immune responses. Initially, *M. tuberculosis* bacilli, within aerosol droplet nuclei (on average 4–7μm in size), are inhaled into the pulmonary alveoli where they come into primary contact and are phagocytosed by alveolar macrophages (Fennelly et al. 2004; Fennelly 2020). During this interaction, most of the bacilli are killed, but some can endure within the macrophage (Russell 2001; Gupta et al. 2012). The recognition and uptake of *M. tuberculosis* by the host is govern by several soluble and cell-bound factors such as pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) that are present on the surface of microbes and normally absent on host cells. Examples of PAMPs include lipopolysaccharide (LPS), porins, peptidoglycan, lipoteichoic acid (LTA), mannose-rich glycans, flagellin, bacterial and viral genomes, mycolic acid, and lipoarabinomannan (LAM). PRRs include phagocytic PRRs and signalling PRRs. Examples of phagocytic PRRs include C-type lectins receptors (CTLRs) (e.g. collectins such as surfactant protein A (SP-A), surfactant protein D (SP-D), mannose receptor (MR), Dectin-1), scavenger receptors (e.g. CD-36, CD68, and SRB-1), opsonic receptors (e.g. plasma acute phase proteins like mannose binding lectin (MBL), C-reactive protein (CRP)) and complement proteins (e.g. C3b, iC3b, factor H and properdin). Signalling PRRs are either present on cell surface (e.g. Toll-like receptors (TLRs), CD14, on intracellular membranes (e.g. endosomes, lysosomes) or in the cytoplasm (e.g. nucleotide-binding oligomerization domain (NOD)-like receptors. In microbial infection and in particular *M. tuberculosis* infection the type of interaction of PRRs with PAMPs and innate immune cells (e.g. macrophages) also determine the subsequent cell signalling pathways (leading to production of cytokines/chemokines), which initiates inflammation and tissue modification (e.g. granuloma formation) (Feng et al. 2006; Lockhart et al. 2006; Eum et al. 2010). Furthermore, the formation of the granuloma occurs without the requirement for specific immunity (North and Izzo 1993; Hansch et al. 1996; Smith et al. 1997), with both tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) being the foremost signalling cytokines for cell infiltration, although they are not needed to begin the process of granuloma formation (Flynn et al. 1995; Smith et al. 1997).

After *M. tuberculosis* enters the alveoli space, the bacteria are internalized into alveolar macrophages by phagocytosis, a process triggered by receptor-ligand engagement. *M. tuberculosis* tends to target binding to macrophages in cholesterol-rich regions of the host cell membrane (Gatfield and Pieters 2000). Mycobacteria can be targeted via a wide variety of receptors that recognise opsonised and non-opsonised bacilli. These include collectins (SP-A, SP-D, conglutinin), MR (CD207), dendritic-cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), dectin-1, complement receptors (CR), surfactant protein (SP) receptors, scavenger receptors, and glycosylphosphatidylinositol (GPI)-anchored receptors such as CD14 (Schlesinger et al. 1990;

Schlesinger 1993; Stokes et al. 1993; Hirsch et al. 1994; Zimmerli et al. 1996; Ehlers and Daffe 1998; Ernst 1998; Peyron et al. 2000; Rooyakkers and Stokes 2005). Various TLRs have also been shown to play important roles in *M. tuberculosis* interactions on the surface and within phagocytic cells (Means et al. 1999). In addition, *M. tuberculosis* can also recruit several host cell molecules on its surface that enhance its uptake by phagocytes.

2 Collectins and Mycobacteria

Collectins are a group of soluble C-type (calcium-dependent) lectins, characterised by an N-terminal collagen region, an alpha helical coil neck motif, and a C-terminal carbohydrate recognition domain (CRD). Collectins can recognise and bind to PAMPs on variety of microbes via their CRD region and have a crucial role in their neutralisation and clearance but are also a critical bridge between the innate immunity and adaptive immunity (Kishore et al. 2006). The mammalian collectin family comprises of SP-A and SP-D, MBL, liver collectin (CL-L1), kidney collectin (CL-K1), CL-LK (composed of CL-L1 and Cl-K1) and placenta collectin (CL-P1), conglutinin, CL-43 and CL-46, with the latter three found in Bovidae (Murugaiah et al. 2020; Tsolaki and Kishore 2020). Several of these collectins have a role in mycobacterial infection and pathogenesis.

Both SP-A and SP-D are the most relevant collectins for pulmonary TB as they are important components of pulmonary surfactant which is essential for the physiology of alveoli (Murugaiah et al. 2020). Furthermore, early observations also showed that pulmonary surfactant had anti-microbial properties being able to enhance clearance of *Staphylococcus aureus* by alveolar macrophages (AM) (Laforce et al. 1973). In fact, both SP-A and SP-D can target Gram-negative and Gram-positive bacteria enhancing their clearance through phagocytosis by AM (Pikaar et al. 1995).

Both SP-A and SP-D can bind and agglutinate mycobacteria but seem to have opposing effects on the phagocytosis of *M. tuberculosis*. SP-A targets the putative surface adhesin Apa glycoprotein on *M. tuberculosis* (Ragas et al. 2007), whilst SP-D can also bind to LTA and peptidoglycan and to lipoarabinomannan (LAM) from *M. tuberculosis* and *Mycobacterium avium* (Ferguson et al. 1999; Van De Wetering et al. 2001; Kudo et al. 2004). SP-A can facilitate uptake of *M. tuberculosis* and *M. avium* by enhancing the expression of macrophage MR (Gaynor et al. 1995; Beharka et al. 2002; Kudo et al. 2004) (Fig. 9.1). Similarly, SP-A enhances expression of scavenger receptor A (SR-A), increasing the phagocytosis of *Streptococcus pneumoniae* by AM (Kuronuma et al. 2004). Additionally, bound SP-A can also facilitate uptake of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) by binding to specific 210-kDa SP-A receptor (SPR210) in U937 macrophages and rat AM (Chroneos et al. 1996; Weikert et al. 1997). Furthermore, this interaction led to increased mycobacterial killing and production of TNF-α and nitric oxide (Weikert et al. 2000). In contrast, SP-D inhibits phagocytosis of *M. tuberculosis* by blocking the interaction of LAM with macrophage MR, and is independent of agglutination by SP-D (Ferguson et al. 1999; Ferguson et al. 2002) (Fig. 9.1). Gene knockout mice (SP-A$^{-/-}$, SP-D$^{-/-}$, and SP-A/D$^{-/-}$) infected with *M. tuberculosis*, still processed the ability for phagocytosis and bacterial clearance, suggesting that both SP-A and SP-D are not crucial for protection in this animal model for TB (Lemos et al. 2011). SP-A and SP-D can also influence the intracellular environment post phagocytosis, by stimulating and enhancing reactive oxygen and nitrogen species enabling the killing of intracellular pathogens such as mycobacteria (Fig. 9.1). SP-A enhances the intracellular killing of *M. bovis* BCG by enhancing nitric oxide (NO) levels and releasing TNF-α (Weikert et al. 2000). However, in *M. tuberculosis* and *M. avium*-infected AM primed by IFN-γ, SP-A was able to supress intracellular NO levels by inhibiting TNF-α production and nuclear factor-kappa B (NF-κB) activation (Pasula et al. 1999; Hussain, 2003). Thus, SP-A facilitates the intracellular survival of *M. tuberculosis* (Gaynor et al. 1995; Pasula et al. 1999). Moreover, HIV-1 infected

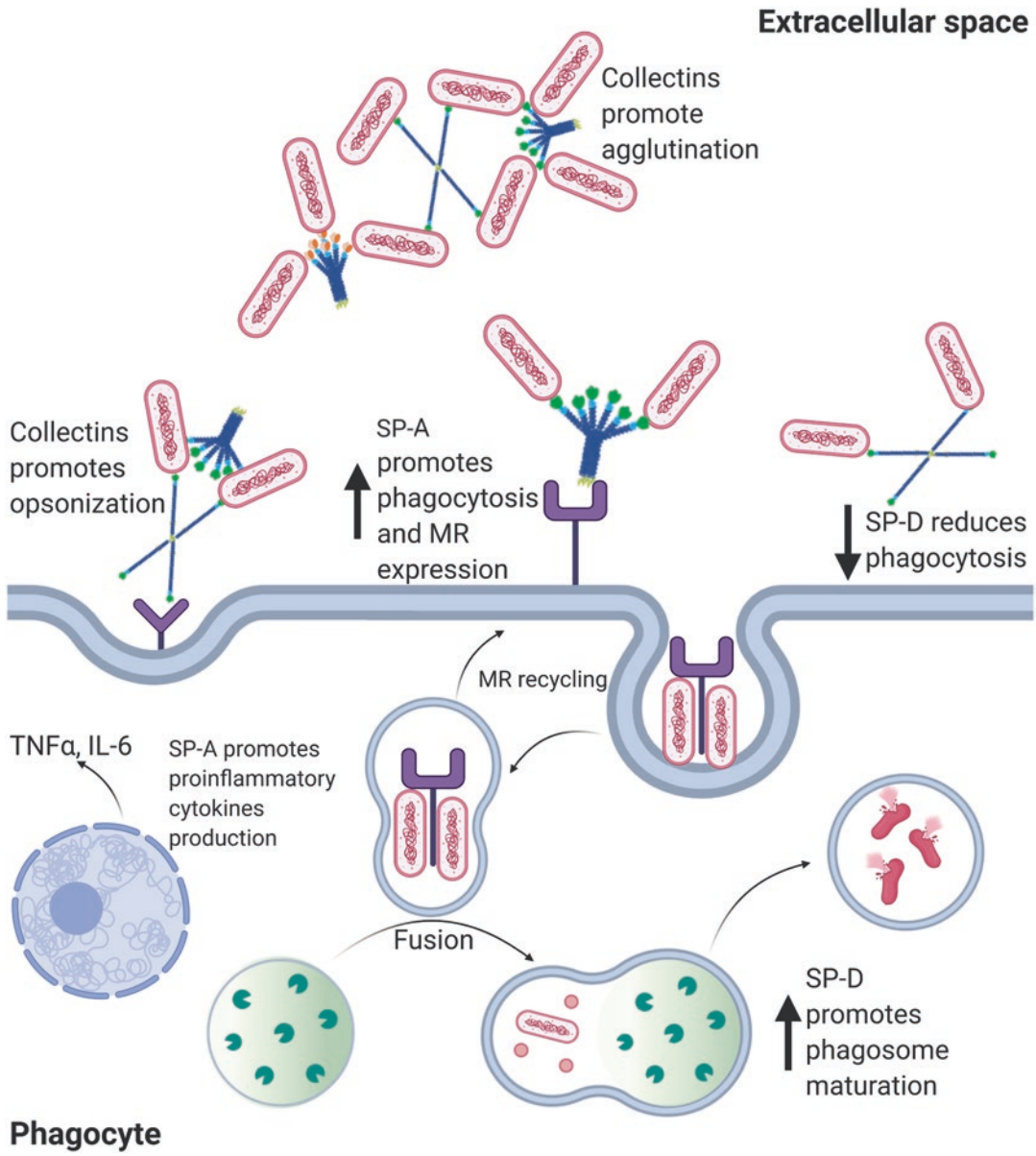

Fig. 9.1 Role of collectins in recognising M. tuberculosis and subsequent consequences. SP-A and SP-D can bind and agglutinate mycobacteria but have opposing effects on phagocytosis. SP-A binds to *Apa* glycoprotein, whilst SP-D binds to lipoteichoic acid, peptidoglycan and to lipoarabinomannan (LAM) on *M. tuberculosis*. SP-A enhances the expression of macrophage mannose receptor (MR) facilitating uptake of *M. tuberculosis*, increasing mycobacterial killing and production of inflammatory components TNF-α and IL-6. SP-D inhibits phagocytosis of *M. tuberculosis* by blocking the interaction of LAM with macrophage MR

patients, who had raised levels of pulmonary SP-A, had a significantly greater susceptibility to *M. tuberculosis* infection (Downing et al. 1995). Thus, SP-A appears to have pleiotropic effects being able to both enhance inflammation in the presence of infected macrophages and inhibit inflammation in uninfected macrophages, thus possibly acting as a protective molecule against lung tissue damage from excessive and non-specific inflammation (Gold et al. 2004).

A number of genetic polymorphisms in the SP-A and SP-D genes are associated with TB susceptibility and protection in humans. SP-A is secreted as two distinct variants (SP-A1 and SP-A2) which are coded for by distinct genes. In individuals from Mexico, Ethiopia, India and China, mutations within and flanking the SP-A1 and SP-A2 genes are linked with protection or susceptibility toward pulmonary TB (Floros et al. 2000; Madan et al. 2002; Malik et al. 2006; Vaid et al. 2006; Yang et al. 2014). A study of Indian individuals identified a single mutation in the SP-D gene that was significantly associated with TB susceptibility (Vaid et al. 2006).

MBL is a serum protein and has a similar overall structure to SP-A and can target PAMPs on the surface of several Gram-positive and Gram-negative bacteria (Ip et al. 2009; Lugo-Villarino et al. 2011). MBL that is bound to microbial surfaces can activate complement via MBL-associated serine proteases (MASPs) of the lectin complement pathway, resulting in deposition of complement components (e.g. C3 and C4) that facilitates microbial phagocytosis and clearance. MBL also possesses complement-independent activity, acting directly as an opsonin, and an inhibitor of bacterial adhesion (Kuhlman et al. 1989; Polotsky et al. 1997; Jack et al. 2005). MBL can also bind to peptidoglycan and LTA from *Staphylococcus aureus* (Polotsky et al. 1996; Nadesalingam et al. 2005). In mycobacteria, MBL can bind to LAM from *M. avium* (Polotsky et al. 1997), antigen 85 (Ag85) of *M. tuberculosis* (Swierzko et al. 2016), and mannosylated lipoarabinomannan (ManLAM) from several mycobacterial species (*M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. gordonae* and *M. smegmatis*) (Bartlomiejczyk et al. 2014). MBL can also enhance the uptake of mycobacteria by macrophages (Polotsky et al. 1997). Both normal and elevated levels in serum MBL have been associated with recurrent infection with *M. tuberculosis* and *M. leprae*, probably driven by enhanced complement-mediated phagocytosis (Garred et al. 1994, 1997). Genetic polymorphisms associated with MBL serum-deficiency are common and some of these are linked to susceptibility to TB and other inflammatory diseases in several ethnicities (Takahashi and Ezekowitz 2005; Thiel et al. 2006; Goyal et al. 2016). Among the minor collectins, CL-L1, CL-K1 and CL-P1 bind to bacteria, with CL-K1 being able to bind *M. tuberculosis* (Troegeler et al. 2015). The heteromeric form CL-LK binds to ManLAM of *M. tuberculosis*, but not *M. smegmatis* because of the absence of capped mannose on its LAM (Troegeler et al. 2015). Furthermore, serum levels of CL-LK in TB patients are almost depleted, compared to normal healthy controls (Troegeler et al. 2015).

Of the bovine collectins, conglutinin has protective activity against several microbes, including mycobacteria. Conglutinin has a similar structure to SP-D (which targets mycobacterial LAM) (Murugaiah et al. 2020) but is predominantly a serum protein synthesised by the liver (Holmskov et al. 1998). Conglutinin has antimicrobial properties; low serum levels of conglutinin are linked with acute infections (e.g. pneumonia, metritis and other respiratory infection (Ingram and Mitchell 1971; Holmskov et al. 1998). Conglutinin is able to bind to Gram-positive bacteria such as mycobacteria (Dec et al. 2012; Mehmood et al. 2019), and uniquely to complement C3 fragment iC3b, via the mannose residues (Laursen et al. 1994). A recombinant truncated form of conglutinin (rfBC), containing the α-helical neck region and the CRD of conglutinin (Wang et al. 1995), is able to bind to *M. bovis* BCG and inhibit phagocytosis of the bacterium both in the presence and absence of complement deposition (Mehmood et al. 2019). Furthermore, there is a modulation of the inflammatory response with the elevation of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-12) and suppression of anti-inflammatory cytokines (TGF-β and IL-10) (Mehmood et al. 2019). Thus, it is probable that conglutinin interferes with the phagocytosis of *M. bovis* BCG by macrophages through two separate mechanisms: firstly, inhibiting binding of mycobacterial LAM (like SP-D) with mannose receptor, and secondly, inhibiting binding of iC3b with complement receptors CR3 and CR4 (Mehmood et al. 2019).

3 Complement and Mycobacteria

The complement system is a major arm of the innate immune response and is crucial for clearing microbial infection. The interactions between complement system and mycobacteria are not fully understood and this is probably more important in EPTB and disseminated disease than pulmonary TB. The complement system is composed of nearly 50 different proteins that are involved in three distinct pathways for activation: Alternative, Classical and Lectin (Carroll and Sim 2011) (Figs. 9.2a, b). Complement can be activated through several target surfaces including pathogens and altered-self cells or indirectly by pathogen-bound antibodies and results in the covalent binding of C3b component to the targeted cell, and subsequent cell lysis via the assembly of the membrane attack complex (MAC) (Carroll and Sim 2011). The classical pathway is activated by C1q binding to a target ligand either directly or to bound IgG/IgM antibodies (Fig. 9.2a). The lectin pathway is activated by the binding of MBL or ficolins to a target ligand (Matsushita and Fujita 1992; Matsushita et al. 2000; Matsushita and Fujita 2001) (Fig. 9.2a). However, the alternative pathway is activated differently and does not require an initiator like C1q or MBL but instead relies on the constant spontaneous hydrolysis of C3 to C3(H_2O). The consequences of complement activation by any pathway is the formation of C3 convertase and the deposition of C3b on target surfaces to prompt opsonisation, the formation of the MAC and several other immunological functions (Carroll and Sim 2011) (Fig. 9.2b). There are also other complement regulatory proteins such and properdin (CFP) and factor H (CFH), where the latter is also a cofactor for factor I that is involved in the cleavage of C3b to iC3b (Whaley and Ruddy 1976a; Sim et al. 1993) (Fig. 9.2b).

In the classical pathway, C1q seems to bind in the presence of IgG and IgM from serum, presumably because of *M. bovis* BCG vaccination (Carroll et al. 2009). Experiments using C1q-deficient serum result in a reduction of C3 binding to mycobacteria (Ferguson et al. 2004). The levels of C1q are significantly elevated in the lungs (determined by bronchoalveolar lavage (BAL) and sera of active TB patients), compared to control patients and those with LTBI, indicating that C1q is an important biomarker for TB (Lubbers et al. 2018). The classical pathway may be more relevant in EPTB where C1q is predominantly a serum protein, however, local pulmonary synthesis occurs in the lungs during active TB accounting for the raised levels of C1q observed. Furthermore, systemic and local C1q levels are raised significantly upon vaccination with BCG in non-human primates (Dijkman et al. 2020). Complement receptor CR3 deficient mice ($CR3^{-/-}$), infected with *M. tuberculosis*, had a lower percentage of infected macrophages at 2 h but not at 4 h post infection, suggesting the opsonisation and uptake via complement and receptors may be key during the early stages of infection (Hu et al. 2000). Genetic polymorphisms in complement receptor CR1 have been reported to increase susceptibility to *Mycobacterium leprae* infection and TB disease (Fitness et al. 2004a, b; Kretzschmar et al. 2018), whilst a congenital deficiency of the classical pathway did not seem to affect susceptibility to TB (Kumararatne 1997). In contrast, a recent study has shown that polymorphisms in the C1q gene cluster are significantly associated with TB susceptibility and differing plasma levels of C1qA in South African TB patients (Bruiners et al. 2020).

The alternative pathway differs from the classical and lectin pathways because it does not need a specific stimulus for activation, since the alternative pathway is constantly active at low levels and able to target pathogens promptly (Kouser et al. 2013). Properdin (CFP) is a key regulatory protein of the alternative pathway and contains thrombospondin (TSR) type 1 repeats (TSR1-TSR6), which are crucial for its function; TSR4 stabilises C3bBb; whilst TSR5 binds to C3b (Higgins et al. 1995; Kouser et al. 2013). Both CFP and recombinant TSR4 + 5 are able to bind to *M. bovis* BCG, inhibiting its uptake by macrophages (Al-Mozaini et al. 2018). In contrast, CFH downregulates complement activation

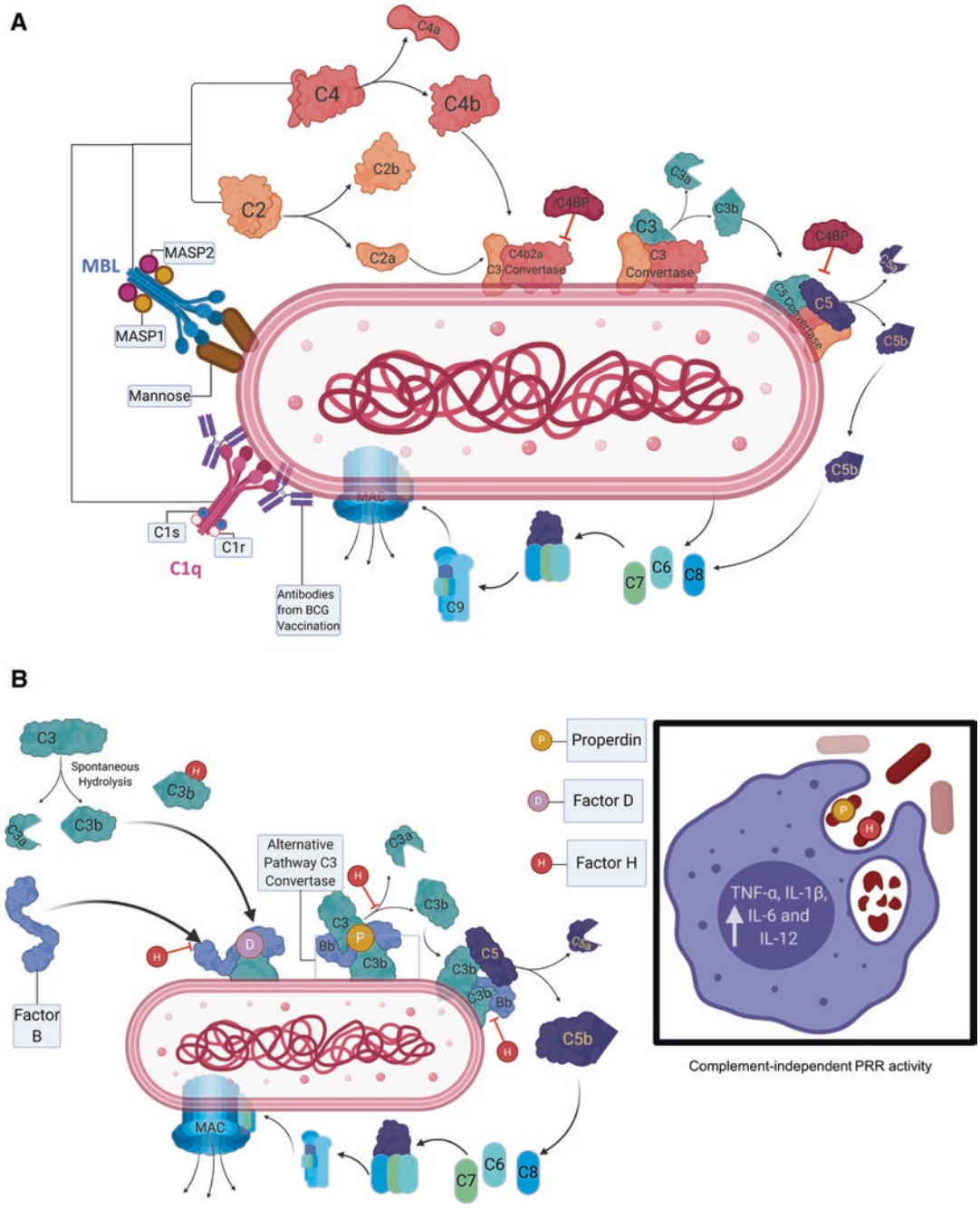

Fig. 9.2 Role of complement activation and complement proteins in recognising M. tuberculosis and subsequent consequences. (**a**) The classical pathway is activated by C1q binding to a target ligand either directly or to bound or anti-mycobacterial antibodies. The lectin pathway is activated by the binding of MBL or ficolins to target mycobacterial ligands. (**b**) The alternative pathway is activated via the constant spontaneous hydrolysis of C3 to C3(H_2O). Properdin and factor H both act as patten recognition receptors (PRR) and have complement independent functions on mycobacteria, being able to inhibit phagocytosis and alter the subsequent inflammatory response

but has also been shown to bind to *M. bovis* BCG and inhibit its uptake by macrophages in a similar manner (Carroll et al. 2009; Abdul-Aziz et al. 2016). Both CFP and CFH are also able to modulate the immune response from the macrophage during *M. bovis* BCG phagocytosis by enhancing the pro-inflammatory response (TNF-α, IL-1β, IL-6 and IL-12) and dampening the anti-inflammatory response (TGF-β and IL-10). This suggests consequences for the adaptive immune response to follow against *M. tuberculosis* infection, particularly in the formation and maintenance of the protective granuloma.

The lectin pathway is primarily triggered by MBL and ficolins that recognise terminal sugar residues on the surface of bacteria (e.g. mannose, fucose and N-acetyl-glucosamine) and subsequently activates MASPs resulting in the cleavage of complement components C4 and C2 to C4bC2a. There are three human ficolins: L-ficolin and H-ficolin, which are synthesised by the liver and predominantly circulate in the serum and M-ficolin which exist in granules of monocytes, neutrophils, and type II alveolar epithelial cells. All three human ficolin can associate with MASPs and activate the complement cascade (Liu et al. 2005). MBL and ficolins can bind to several mycobacteria (*M. tuberculosis*, *M. gordonae*, *M. kansasii* and *M. smegmatis*) (Bartlomiejczyk et al. 2014). Direct binding of L-ficolin from human serum to *M. bovis* BCG and subsequent MASP-2 activation has been reported, but no binding was detected for H-ficolin (Carroll et al. 2009). L-ficolin is also able to bind with higher affinity to *M. tuberculosis* than to non-virulent mycobacteria and inhibit infection of human lung A549 epithelial cells (Luo et al. 2013). In mice, exogenously administered L-ficolin had a significant protective effect against virulent *M. tuberculosis* infection, whilst Ficolin-A (homologous to human L-ficolin in mouse) demonstrated increased susceptibility to *M. tuberculosis* infection (Luo et al. 2013). Furthermore, L-ficolin also modulates the immune response against *M. tuberculosis* infection by partially activating c-Jun N-terminal kinase (JNK) phosphorylation, stimulating the secretion of IFN-γ, IL-17, IL-6, TNF-α, and NO production by macrophages (Luo et al. 2013). Clinically, L-ficolin serum levels in pulmonary TB patients are much lower than compared to healthy controls (Luo et al. 2013), suggesting an important role for L-ficolin in *M. tuberculosis* infection.

The cell wall of mycobacteria is complex and is composed of a thick peptidoglycan layer, which covers the bacteria plasma membrane and is the scaffold to which various components are covalently attached (e.g. LAM, arabinogalactans, arabinomannans, glycolipids and mycolic acids) (Daffe and Draper 1998). Furthermore, there is also a capsule layer surrounding the mycolates made up of additional proteins, polysaccharides and lipids (e.g. phospholipids and glycolipids) (Daffe and Etienne 1999). One of these components is trehalose dimycolate (TDM) (also known as cord factor), which activates complement (Ramanathan et al. 1980). The complex mycobacterial cell wall has evolved to protect the bacterium from immunological attack (particularly intracellularly), but also plays a major role in determining ant-mycobacterial drug efficacy (Besra 1998). Several bacteria have evolved strategies to circumvent the immune response by interfering and inhibiting complement activation by either producing bacterial complement inhibitors, inactivating host complement inhibitors (e.g. CFH), or secreting bacterial proteases that break-down complement proteins (e.g. *Salmonella enterica* and *Porphyromonas gingivalis*) (Wingrove et al. 1992; Jagels et al. 1996; Ramu et al. 2007). *M. tuberculosis* is a highly evolved intracellular pathogen, being able to persistently reside in the phagosome of macrophages. Nevertheless, the interaction of complement and mycobacteria and the implications for pathogenesis and protection against TB are not well understood.

M. bovis BCG can activate the classical, lectin and alternative pathways (Ramanathan et al. 1980; Ferguson et al. 2004; Carroll et al. 2009). Activation via the alternative pathway also occurred in the absence of antibody, but intriguingly CFH was also found to bind to the mycobacterial surface, possibly indicating a means for complement moderation (Carroll et al. 2009).

Although C3b is deposited on the mycobacterial surface (Carroll et al. 2009), it is not clear if MAC formation occurs. The fixation of complement proteins on the mycobacterial surface may enhance the phagocytosis of mycobacteria via complement receptors. Several studies have reported C3b deposition on mycobacteria and its role in phagocytosis via complement receptors CR1, CR3 or CR4 on macrophages (Hetland and Wiker 1994; Schlesinger and Horwitz 1994; Cywes et al. 1996; Hetland et al. 1998; Mueller-Ortiz et al. 2001; Ferguson et al. 2004). However, the specific importance of C3b and iC3b deposition on mycobacteria is not well understood. Complement activation by classical and alternative pathways has been shown on *M. tuberculosis* and *M. bovis* BCG resulting in C3b and iC3b deposition, but the target ligands are not known (Ferguson et al. 2004; Carroll et al. 2009). During the alternative pathway, CFH plays a major role in the cleavage of C3b to iC3b by acting as a cofactor of factor I, whilst also controlling the formation of the C3 and C5 convertases (Whaley and Ruddy 1976b; Whaley et al. 1976). C3b component is essential for the complement cascade to proceed to the terminal MAC, whilst iC3b is unable to facilitate this. Also, both C3b and iC3b have different complement receptors (C3b is a ligand for CR1; iC3b is a ligand for CR3 and CR4) (Ross 1986). iC3b exits as a cleavage product from C3b produced by factor I with cofactors CFH and CR1 (Figueroa and Densen 1991). Thus, both opsonic C3b or iC3b complement components may facilitate phagocytosis of host cells by mycobacteria, either promoting clearance or intracellular persistence. Indeed, a recent study showed enhanced uptake of complement-deposited *M. bovis* BCG by THP-1 macrophages compared to non-deposited *M. bovis* BCG (Mehmood et al. 2019).This same study also observed that phagocytosis of complement-deposited *M. bovis* BCG bacteria are inhibited from phagocytosis by THP-1 macrophages by rfBC (a recombinant truncated form of bovine conglutinin) which uniquely binds to iC3b (Mehmood et al. 2019). These observations suggest that the blocking of iC3b by conglutinin may be indicative of a protective mechanism against mycobacterial infection in the bovine host, by inhibiting phagocytosis via macrophage receptors CR3 and CR4 (Mehmood et al. 2019).

Both properdin (CFP) and factor H (CFH) are complement components that have also been observed to bind to mycobacteria in a in a dose-dependent manner and independently of C3b deposition (Carroll et al. 2009; Abdul-Aziz et al. 2016; Al-Mozaini et al. 2018). Both CFP and CFH have been shown to be PRRs for mycobacteria that have complement-independent functions. *M. bovis* BCG bound with CFP or CFH are inhibited for phagocytosis by THP-1 macrophages compared to *M. bovis* BCG alone (Al-Mozaini et al. 2018; Abdul-Aziz et al. 2016). Moreover, the subsequent macrophage inflammatory response was altered in terms of enhanced secretion of TNF-α, IL-1β, IL-6 and IL-12, whilst simultaneously dampening anti-inflammatory cytokines (TGF-β and IL-10) (Al-Mozaini et al. 2018 Abdul-Aziz et al. 2016). CFH binding has been reported in other bacteria where it serves to circumvent complement activation and thus opsonisation and killing through MAC (e.g. *S. pyogenes*, *Streptococcus pneumoniae*, *Yersinia enterocolitica*, *Haemophilus influenza*, *Neisseria gonorrhoea* and *N. meningitidis* (China et al. 1993; Diaz et al. 1997; Ram et al. 1998a, b; Dave et al. 2001; Meri et al. 2002; Schneider et al. 2006). In the case of mycobacterial infection, the ability to bind CFH may serve its immune evasion by activating C3 and using C3b opsonisation to enhance phagocytosis by macrophages via complement receptors (Schlesinger et al. 1990; Ferguson et al. 2004). These intriguing results describe potentially novel mechanisms in shaping the adaptive immune response against mycobacterial infection. For *M. tuberculosis*, there is a fine balance in activating complement to an optimum limited level to allow for enhanced opsonisation and uptake into macrophages, whilst avoiding being killed. Thus, the complex interactions between *M. tuberculosis* and complement is a major mechanism through which mycobacteria can evade the immune response by persistently intracellularly in the macrophage.

CR3 is an integrin (also known as $\alpha_M\beta_2$; CD11b/CD18), commonly expressed on

neutrophils, macrophages, NK cells, and monocytes and is involved in both opsonic and non-opsonic phagocytosis (Le Cabec et al. 2002; Velasco-Velazquez et al. 2003). CR3 can bind iC3b (particularly on complement-deposited mycobacteria), mycobacterial LAM, Ag85C, PIMs, ICAM-1, several bacterial ligands and other carbohydrate residues (e.g. β-glucan, glucose, N-acetylglucosamine (GlcNAc) (Arnaout 1990; Ehlers and Daffe 1998; Velasco-Velazquez et al. 2003; Villeneuve et al. 2005). Elevated levels of CR3 in tuberculosis patients have been reported in several studies, particularly in phagocytic cells in the peripheral blood and AMs, suggesting a probable role in pathogenesis (Yassin and Hamblin 1994; Kuo et al. 1996; Juffermans et al. 2001). Indeed, complement activation via classical pathway in the lungs may also be a major mechanism for opsonin-mediated uptake of *M. tuberculosis* by AMs (Watford et al. 2000; Ferguson et al. 2004). However, CR3 deficiency in mice does not appear to affect the intracellular killing mechanisms (induction of reactive oxygen and nitrogen intermediates), or on the survival of the mycobacteria inside the cell, but it did result in reduced opsonisation and phagocytosis (Hu et al. 2000; Melo et al. 2000; Rooyakkers and Stokes 2005). CR3 has been found associated with several GPI-anchored proteins localized in cholesterol-rich rafts of the plasma membrane in neutrophils and is involved in the uptake of *Mycobacterium kansasii* (Peyron et al. 2000). Moreover, the existence of host plasma membrane cholesterol appears to be critical for CR3-mediated uptake of *M. tuberculosis* (Gatfield and Pieters 2000; Peyron et al. 2000). *M. tuberculosis* may also use cholesterol as an energy source during intracellular survival in macrophages (Van Der Geize et al. 2007). Furthermore, survival of mycobacteria within the macrophage may depend on the receptor involved in phagocytosis, since pro-inflammatory responses and respiratory burst occurs when mycobacteria are phagocytosed via Fc receptors (Russell 2001), whilst macrophage activation is inhibited when mycobacteria are phagocytosed via CR3 receptors (Caron and Hall 1998).

CR3 mediates the phagocytosis of ~80% of complement-opsonized *M. tuberculosis* (Schlesinger et al. 1990). CR3 is also able to facilitate phagocytosis of non-opsonized mycobacteria (Velasco-Velazquez et al. 2003). CR3 is mainly expressed on the cell surface of macrophages, neutrophils, monocytes, and natural killer cells. In lung alveolar macrophages, expression of CR3 is relatively low, whilst *in vitro*, differentiated macrophages have increased expression of CR3, enhancing their capacity to bind mycobacteria (Stokes et al. 1998). Several mycobacterial ligands are recognised by CR3, including Ag85C and LAM from *M. tuberculosis*, with the latter being the main ligand for CR3 (Velasco-Velazquez et al. 2003). Whilst CR3 plays a major role in facilitating the phagocytosis of *M. tuberculosis*, it does not necessarily result in the intracellular killing of the pathogen (Velasco-Velazquez et al. 2003; Rooyakkers and Stokes 2005). Furthermore, it may also not be essential in protection against *M. tuberculosis* infection, since CR3-deficient and wild-type mice are equally resistant to *M. tuberculosis* infection, suggesting that *M. tuberculosis* phagocytosis may occur efficiently through alternative receptors (Hu et al. 2000). Therefore, the role of CR3 in TB pathogenesis may be redundant. To date, no genetic polymorphisms in the CR3 genes have been associated with susceptibility to TB.

4 Toll-like Receptors (TLRs) and Mycobacteria

TLRs are key signalling PRRs present on several immune and non-immune cells (e.g. monocytes/macrophages, B and T cells, dendritic cells, neutrophils, epithelial and endothelial cells). TLRs recognise a wide variety of microbial ligands (PAMPs) and host danger signals (DAMPs). TLRs have key roles in innate immunity and are an important bridge to adaptive immunity (Fig. 9.3). 13 TLRs have been described in human and mouse so far. TLRs are transmembrane proteins that have ligand sensing N-terminal leucine-rich extracellular domains and a cytoplasmic

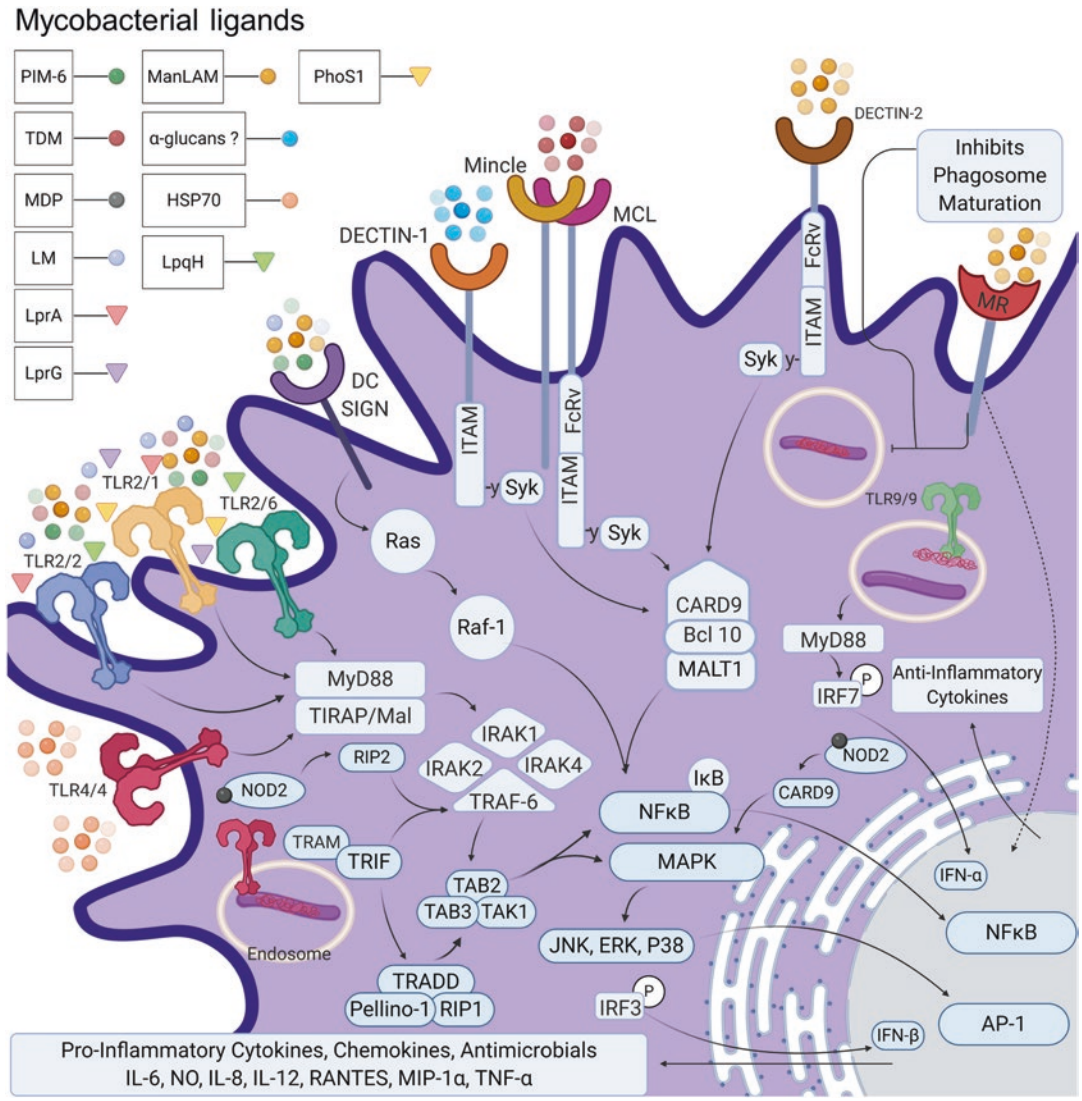

Fig. 9.3 C-type lectin receptors (CTLRs) involved in the recognition of M. tuberculosis and subsequent consequences. Several mycobacterial ligands are recognised by a variety of host CTLR PRRs that can stimulate a multitude of signalling pathways involved in mycobacterial phagocytosis, clearance, and inflammatory responses

Toll/IL-1R (TIR) C- terminal domain. The TIR domain mediates interactions between TLRs and adaptor proteins (e.g. myeloid differentiation primary response protein (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIRAP/MAL, and TRAM) (Lim and Staudt 2013). Several kinases are also activated and involved in signalling, e.g. Interleukin-1 receptor-associated kinases (IRAK4, IRAK1, IRAK2), IκB kinase-ε (IKKε) and TANK-binding kinase-1 (TBK1), and ubiquitin ligases TNF receptor associated factor 6 (TRAF6) and Pellino-1. Upon ligand recognition, TLR signalling progresses via two distinct signalling pathways: either MyD88-dependent or TRIF-dependent pathway. Of the two, MyD88 is the most involved in TLR signalling. The triggering of the MyD88-dependent pathways ultimately results in the translocation of transcription factors NF-κB (RelA/p50) and activator protein 1 (AP1), inducing pro-

inflammatory cytokine production (IL-6, TNF-α, and IL-1β). For the TRIF-dependent pathway (most relevant for TLR3 and TLR4), its signalling involves either 1) interaction with TRAF6 which goes on to activate transforming growth factor-β-activated kinase (TAK1) complex that in turn activates NF-κB and mitogen-activated protein kinases (MAPKs), or 2) interaction of TRAF3 which induces activation of interferon-regulatory factor 3 (IRF3) transcription factor that leads to the production of type I interferon (IFN-α and IFN-β) (Kawai and Akira 2010).

In humans, TLR1, 2, 4, 5, 6, and 10 are found on the host cell surface and mainly target microbial surface components (e.g. membrane of cell wall ligands), whilst TLR3, 7, 8, and 9 are found intracellularly in the endolysosomal membrane compartments and target nucleic acids (Akira et al. 2006; Triantafilou et al. 2006; Seo et al. 2018). TLRs are key downstream signalling molecules which can stimulate the production of pro-inflammatory cytokines, chemokines, and interferons (type I IFN) (Kawai and Akira 2010). These pathways are sometimes over-activated, in an uncontrolled manner, in response to stimuli, generating severe immunopathology (Vijay 2018).

TLRs play several important roles in TB. In blood samples from patients with active pulmonary TB, the expression of several TLRs are upregulated (Chang et al. 2006). TLRs recognise *M. tuberculosis* or a variety of its components and can initiate a set of innate and adaptive immune responses (Jo et al. 2007). The main TLRs involved in host-pathogen interaction in TB are TLR2, TLR4, TLR9 and TLR1/TLR6 (Jo et al. 2007; Kim et al. 2019). The precise nature and consequence of the signalling pathways induced by mycobacteria remain to be fully understood (Berrington and Hawn 2007; Holscher et al. 2008). Although TLRs target *M. tuberculosis*, this does not occur directly, but is triggered intracellularly by TLR signalling via MyD88-dependant pathway (Quesniaux et al. 2004). This also results in the activation of pro- and anti-inflammatory responses via enhanced NF-κB expression and MAPKs activation generating secretion of TNF-α, IL-1β and IL-12, and production of nitric oxide (Yamamoto et al. 2003; Jo et al. 2007; Xu et al. 2007; Jo 2008; Garlanda et al. 2007).

TLR2 plays a key role in recognising mycobacteria PAMPs and is central to activating the intracellular signalling that triggers NF-κB and MAPKs pathways, inducing secretion of pro-inflammatory cytokines and chemokines and initiating phagocytosis, intracellular killing of *M. tuberculosis*, and antigen presentation. TLR2 also works together with TLR4 and TLR9 during *M. tuberculosis* infection (Jung et al. 2006). TLR2 can bind to several mycobacterial ligands, such as LpqH, LprA, LprG, LAM, lipomannan (LM), 38-kDa lipoprotein, 19-kDa lipoprotein, phosphatidylinositol mannoside (PIMs) (Quesniaux et al. 2004; Kawai and Akira 2011; Kleinnijenhuis et al. 2011; Basu et al. 2012; Kim et al. 2019) (Fig. 9.3). However, TLR2 does not seem to be necessary for protection in mice during acute *M. tuberculosis* infection (Reiling et al. 2002; Sugawara et al. 2003; Mcbride et al. 2011). However, TLRs are important for the long-term control of the *M. tuberculosis* infection in mice (Abel et al. 2002; Drennan et al. 2004). TLR2 knockout mice (but not TLR6 knockout mice) have an impaired ability to clear *M. tuberculosis* infection and form granulomas compared to wild-type animals; TLR2-deficient mice have significantly lower pro-inflammatory cytokine production in response to *M. tuberculosis* infection (Reiling et al. 2002; Sugawara et al. 2003; Drennan et al. 2004). TLR2 knockout mice also exhibit increased *M. tuberculosis* bacterial load and impaired neutrophil inflammation via the downregulation of CXCL5 during infection (Gopalakrishnan et al. 2019). During *M. tuberculosis* infection, TLR2 is critical for the expression of TNF-α (Underhill et al. 1999), whilst both TLR2 and TLR6 are key in the expression IL-1β via MyD88 (Kleinnijenhuis et al. 2009). Another key cytokine in TB, IL-12, which is also dependent on TLR2 in macrophages and dendritic cells (Pompei et al. 2007). Indeed, the production of TNF-α and IL-12 is mainly dependent on TLR2 rather than TLR4 signalling during *M. tuberculosis* infection (Means et al. 2001), with TLR2 and TLR9 also being involved in controlling dendritic cell-derived IL-12 secretion in mice infected with

M. tuberculosis (Bafica et al. 2005). Furthermore, in monocytes, reactive oxygen species (ROS) production and the expression of CXCL8 and CCL2 is also dependant on TLR2 during *M. tuberculosis* infection (Lee et al. 2009a). In dendritic cells, TLR2 induces ROS production, facilitating dendritic cell maturation and subsequent lymphocyte proliferation during *M. tuberculosis* infection (Romero et al. 2016). *M. tuberculosis* lipoproteins induce significant signalling of TLR2 which inhibits macrophage major histocompatibility complex (MHC) class II (MHC-II) expression and antigen presentation (Fulton et al. 2004; Pai et al. 2004), resulting in poor activation of $CD4^+$ T cell responses (Noss et al. 2001; Jo 2008). TLRs gene polymorphisms also seem to have an influence on the immune response in TB (Mukherjee et al. 2019; Zhang et al. 2013b; Sun et al. 2015). A single nucleotide polymorphism (SNP) in the TLR2 gene resulting in an amino acid change (T597C) has been reported to be associated with the development of TB meningitis and miliary TB, suggesting that TLR2 may have relevance for the dissemination of *M. tuberculosis* infection (Thuong et al. 2007). Another gene polymorphism (rs5743708) in the TLR2 gene is also associated with higher risk for TB (Guo and Xia 2015).

TLR4 is an important sensor for bacterial endotoxins, particularly those derived from Gram-negative bacteria (e.g. LPS) (Pandey et al. 2014). In mycobacterial infection, the TLR4 signalling pathway plays a central role in immune response (Sepehri et al. 2019). Blocking interaction of *M. tuberculosis* with TLR4, using anti-TLR4 antibody and an endotoxin antagonist, inhibits macrophage-dependent killing of intracellular bacteria as well as the pro-inflammatory response (Means et al. 2001; Lv et al. 2017). TLR4 can target several *M. tuberculosis* ligands, such as heat shock proteins GrpE, Hsp65 and Resuscitation promoting factor (RpfB) (Kim et al. 2019). Additionally, mycobacterial LM can modulate macrophages inflammatory response via the TLR4 signalling (Doz et al. 2007). Both TLR4 and TLR2 expression is significantly upregulated in lymphocytes from patients with active pulmonary TB compared to healthy controls (Chang et al. 2006). Furthermore, increased expression of TLR4, CD14 and MR on monocytes (but not TLR2) was observed in *M. bovis* BCG vaccinated individuals compared to those who were not vaccinated; BCG-vaccinated individuals showed elevated Th1 and Th17 immune responses (Kleinnijenhuis et al. 2014). However, *M. tuberculosis* H37Rv strain was able to significantly enhance the expression of TLR4, TNF-α, and scavenger receptors in neutrophils when compared to mycobacterial vaccine strains (Hilda et al. 2012). The importance of TLR4 in protecting mice from TB infection is controversial. TLR4-mutant mice were observed to be more susceptible to pulmonary TB than wild-type mice, and had a reduced capacity to produce IFN-γ (Branger et al. 2004). After infection with *M. tuberculosis*, TLR4-mutant mice were observed to have lower pulmonary expression of TNF-α, IL-12p40, and monocyte chemoattractant protein 1, compared with the wild-type controls (Abel et al. 2002). In mice, cooperation between TLR4- and TLR2-dependent signalling is critical in macrophage apoptosis induced by *M. tuberculosis* infection, with the absence of TLR4 favouring necrosis instead (Sanchez et al. 2010). However, there are studies that report no significant difference in protection to *M. tuberculosis* infection between wild-type and TLR4-mutant mice (Shim et al. 2003; Gopalakrishnan et al. 2019). Thus, the precise role of TLR4 in TB remains to be fully determined (Reiling et al. 2002; Shim et al. 2003).

It is well known that TLR9 recognizes bacterial DNA, including *M. tuberculosis* DNA, with TLR9 signalling subsequently activating the macrophage pro-inflammatory response and induction of T-cell differentiation (Hemmi et al. 2000; Latz et al. 2004; Jo et al. 2007; Rahman et al. 2009). Cooperation between TLR9 and TLR2 have a protective role against *M. tuberculosis* infection, with TLR2/TLR9 knockout mice showing significantly enhanced susceptibility to infection, coupled together with supressed levels of IL-12p40 and IFN- γ production (Bafica et al. 2005). Interestingly, TLR9 knockout mice have modest susceptibility to *M. tuberculosis* infection compared to the TLR2/TLR9 double knockout

mice (Bafica et al. 2005). Macrophages, pretreated with vitamin D, were able to significantly up-regulate TLR9 expression, which boosted the pro-inflammatory response to DNA from different evolutionary lineages of *M. tuberculosis* (Cervantes et al. 2019). TLR9 genetic polymorphisms in the human population may be linked to susceptibility to TB. In a meta-analysis of 1745 scientific articles, a single TLR9 polymorphism (rs352139) was identified that may be associated with decreased TB risk in Indonesians individuals, whilst increased risk in Mexican individuals (Chen et al. 2015). In a study of Vietnamese individuals, two further polymorphisms were identified, with the first (rs352142) strongly associated with meningeal TB, and the second (rs352143) associated with pulmonary TB (Graustein et al. 2015). Another single-nucleotide polymorphism (rs187084) has been associated with susceptibility to pulmonary TB amongst an Indian tribe (Bharti et al. 2014).

Other TLRs that may have a significant role in TB include TLR7 and TLR8. The upregulation of TLR7 was observed to eliminate intracellular *M. tuberculosis* through autophagy (Bao et al. 2017). TLR7 in *M. tuberculosis* infected macrophages was upregulated and this also increase viability of infected host cells, whilst down-regulation of TLR7 decrease cell viability (Bao et al. 2017). Furthermore, the autophagosome was significantly increased in the *M. tuberculosis*-infected macrophages after upregulation of TLR7, but in contrast, the autophagosome was not observed in macrophages following down-regulation of TLR7 (Bao et al. 2017). Interestingly, TLR8 expression is also upregulated in *M. bovis* BCG infected THP-1 macrophages (Davila et al. 2008), whilst TLR8 expression is significantly upregulated in pulmonary TB patients during the acute phase of disease (Davila et al. 2008). Genetic polymorphisms in TLR7 and TLR8 genes are associated with increased susceptibility to *M. tuberculosis* infection as a result of impaired phagocytosis and TLR signalling (Davila et al. 2008; Lai et al. 2016).

The overall role of TLRs in TB pathogenesis and protection is complex. TLR-mediated signalling in TB results in an inflammatory and protective immune response, instead of a *M. tuberculosis* LAM-(host receptor)-mediated signalling involving C-type lectins such as MR and DC-SIGN, which tends to result in a more anti-inflammatory and suppressive immune response (Kaufmann and Schaible 2003). Furthermore, *M. tuberculosis* ManLAM, which is predominantly recognised by MR and DC-SIGN, results in an anti-inflammatory response and is not recognized by any TLR, suggesting that the type of cap modification on the LAM antigen has an important effect on the downstream immune response against mycobacterial infection (Quesniaux et al. 2004). An optimum IFN-γ secretion in *M. tuberculosis* infection requires crosstalk between TLR2, TLR4 and MR (Mukhopadhyay et al. 2004).

5 Other PRRs and Mycobacteria

5.1 Dendritic Cell-Associated C-Type Lectin (Dectin)

5.1.1 Dectin-1

Dectin-1, coded by the CLEC7 gene, is a non-TLR PRR and a type II transmembrane receptor involved in cellular activation; it is expressed on macrophages, dendritic cells, neutrophils, eosinophils, B cells, and mast cells in the lung (Brown 2006). Dectin-1 tends to target β-glucans on fungal pathogens but can also interact with *M. tuberculosis,* although its specific mycobacterial ligands are not known. During the recognition of fungal ligands, Dectin-1 can induce production of cytokines/chemokines, intracellular killing, phagocytosis, and DC maturation (Brown 2006) (Fig. 9.3). Downstream signalling by Dectin-1 occurs via Spleen tyrosine kinase (Syk)-dependent or -independent mechanisms involving several transcription factors (e.g. NF-κB, MAPK, NFAT, IRF1, IRF5) and the intracellular sensor NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), central to the NLRP3 inflammasome (Kerrigan and Brown 2011; Dambuza and Brown 2015). Dectin-1 can also associate with TLR2 when recognising several mycobacteria facilitating the production of pro-inflammatory cytokines (Yadav and Schorey

2006; Shin et al. 2008; Romero et al. 2016). Dectin-1 is necessary for the TLR2-dependent production of TNF-α, IL-6, RANTES, and GM-CSF by murine macrophages infected with non-pathogenic mycobacteria (*M. tuberculosis* H37Ra, *M. smegmatis* and *M. bovis* BCG), but not for *M. tuberculosis* H37Rv (Yadav and Schorey 2006). In DCs derived from TLR2$^{-/-}$ mice, *M. tuberculosis*-induced IL-12p40 was dampened by inhibition of Dectin-1 by laminarin and by the inhibition of Syk (Rothfuchs et al. 2007). Similarly, enhanced phagocytosis and expression of Dectin-1, Src kinase, and induction of ROS occurs via TLR2 in *M. tuberculosis*-infected human lung epithelial cells (Lee et al. 2009b). *M. tuberculosis*-induced ROS production in human DCs occurs via Dectin-1 associating with TLR2 (Romero et al. 2016). *M. tuberculosis*:Dectin-1 interaction also appears to be the key in inducing Th1/Th17 responses in human monocyte derived DCs, but is inhibited by MR and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) co-expression in the cell (Zenaro et al. 2009). In human PBMCs, *M. tuberculosis* induction of Th17 responses is mediated by Dectin-1 and TLR4, but not TLR2, with IL-17A production requiring the IL-1 pathway (Van De Veerdonk et al. 2010). Thus, Dectin-1 plays a role in the innate immune response against *M. tuberculosis*. However, in knockout (Dectin-1$^{-/-}$) mice, there does not seem to be a difference in survival to *M. tuberculosis* infection compared to wild type animals (Marakalala et al. 2011). Although a genetic deficiency resulting in a truncated Dectin-1 has been associated with susceptibility to several fungal infections (Rosentul et al. 2011; Sainz et al. 2012), no polymorphisms in the Dectin-1 gene have been reported to be involved in TB susceptibility.

5.1.2 Dectin-2

Dectin-2, coded by the Clec4n gene, is also a CTLR similar in structure to Dectin-1, composed of an N-terminal cytoplasmic domain, a transmembrane domain, and a C-terminal extracellular Ca^{2+}- dependant CRD region (Ariizumi et al. 2000; Kanazawa et al. 2004; Sato et al. 2006). Dectin-2 is predominantly expressed in the lungs, but its expression has also been reported in spleen and lymph tissues and on DCs, monocytes, macrophages and B cells (Kanazawa et al. 2004; Sancho et al. 2012). Dectin-2 acts as an adaptor molecule recognising the γ-chain of Fc receptor triggering the activation of cells (Sato et al. 2006). Dectin-2 expression can be influenced by different ligands, with its CRD region targeting mannose residues (Gavino et al. 2005; Taylor et al. 2005; Mcgreal et al. 2006). Moreover, soluble recombinant Dectin-2 has been reported to bind to *M. tuberculosis* (Mcgreal et al. 2006) via ManLAM, although dectin-2 does not bind mycobacteria lacking mannose-capped LAM (Yonekawa et al. 2014; Decout et al. 2018) (Fig. 9.3). Expression of Dectin-2 on macrophages is upregulated by TNF (Decout et al. 2018). Dectin-2 elicits pro- and anti-inflammatory cytokine production (e.g. IL-6, TNF-α, MIP-2, IL-2, and IL-10) in bone marrow-derived DCs and seems to be important for DC maturation and IL-17 secretion (Yonekawa et al. 2014). This effect of ManLAM was completely negated in Clec4n$^{-/-}$ bone marrow-derived DCs, whilst Clec4n$^{-/-}$ mice infected with *M. tuberculosis* showed significantly greater lung pathology than wild-type mice (Yonekawa et al. 2014). To date, no polymorphisms in the human population have been described in the Clec4n gene that are linked to TB susceptibility. Thus, the role of Dectin-2 receptor in TB pathogenesis remains intriguing.

5.2 Macrophage-Inducible C-Type Lectin (Mincle)

The Macrophage-inducible C-type lectin (Mincle), coded by the CLEC4E gene, is a PRR that is found on the surface of macrophages, myeloid DCs, monocytes, neutrophils, and certain B cells and binds to several target PAMPs (e.g. mannose and fucose, among others) (Lee et al. 2011; Kerscher et al. 2013). Mincle is an LPS inducible transcriptional target in macrophages and is able to stimulate pro-inflammatory cytokines via the Syk-CARD9 pathway (Matsumoto et al. 1999; Yamasaki et al. 2008; Schoenen et al. 2010) (Fig. 9.3). Mincle can bind to trehalose dimycolate (cord factor), a key com-

ponent of the mycobacterial cell wall that has also been implicated in lung granuloma formation in mice (Ishikawa et al. 2009). Trehalose dimycolate can inhibit phagosome maturation, promoting intracellular persistence and interfering with antigen presentation (Spargo et al. 1991; Actor et al. 2002; Indrigo et al. 2003; Hunter et al. 2006; Axelrod et al. 2008). Mincle, being a key receptor for the trehalose dimycolate, regulates Th1/Th17 responses in mice (Schoenen et al. 2010). In neutrophils, trehalose dimycolate-induced Mincle signalling increased cell adherence (important in early stages of granuloma formation), CR3 (CD11b/CD18) expression, together with TLR2 activation leading to reactive oxygen species and TNF-α production (Lee et al. 2012). Mincle$^{-/-}$ mice had impaired immune responses when challenged by aerosol *M. tuberculosis*, and exhibited increased inflammation and mycobacterial load than wild-type mice (Lee et al. 2012). Neutrophil depletion (using anti-Ly6G antibody) showed inhibition of IL-6 and MCP-1 (monocyte chemotactic protein-1) following trehalose dimycolate treatment, thus reducing immune cell recruitment (Lee et al. 2012). Therefore, Mincle may modulate neutrophils during the early stage of mycobacterial infection. However, another study concluded that Mincle was not essential for controlling *M. tuberculosis*; Mincle$^{-/-}$ mice could still form granulomas, had Th1 and Th17 responses, and a similar mycobacterial burden after aerosol infection to wild-type mice (Heitmann et al. 2013). Another study using Mincle$^{-/-}$ mice found that inoculation of mycobacteria (*M. bovis* BCG) intravenously, rather than intratracheally, resulted in higher mycobacterial burden in the lungs and other tissues, suggesting Mincle may play a greater role in systemic mycobacterial infection (Behler et al. 2012). Interestingly, in Mincle$^{-/-}$ mice, DCs induced Th1 responses in the spleen, but not in the liver, suggesting a role in systemic mycobacterial infection (Behler et al. 2015). The interaction of Mincle with trehalose dimycolate and *M. bovis* BCG can also promote anti-inflammatory IL-10 but conversely alter pro-inflammatory IL-12p40 secretion from murine bone-derived macrophages *in vitro* (Patin et al. 2016).

Mincle recognises trehalose-6,6-dibehenate (TDB) (a synthetic analogue of trehalose dimycolate), which is involved in NLRP3 inflammasome activation and Myd88-dependent Th1 and Th17 responses through IL-1R-signalling in mice bone-derived DCs (Desel et al. 2013; Schweneker et al. 2013; Shenderov et al. 2013). Mincle appears to be a crucial switch for macrophages to shift from cytokine expression to high nitric oxide (NO) production. Mincle can have dual functions in mycobacterial infection: 1) having a stimulatory role on TLR-mediated transcription, and 2) enhancing the translation of key genes required for NO synthesis, thus in the promotion NO production and subsequent resolution of inflammation and the granuloma (Lee et al. 2016b). In fact, in resting murine macrophages, Mincle is expressed at low levels but is upregulated by LPS (a TLR ligand), leading to Myd88-dependent NO production (Matsumoto et al. 1999; Schoenen et al. 2014; Kerscher et al. 2016a). Together with TLR4, Mincle has been reported to induce autophagy through Myd88, which facilitates *M. tuberculosis* intracellular growth (Pahari et al. 2020).

Much of the above data on Mincle has come from the mouse model of *M. tuberculosis* infection, but there are several studies that show similar immune responses in humans. Human antigen presenting cells have a similar response to trehalose dimycolate/TDB, inducing various cytokines via Syk-signalling (Ostrop et al. 2015), whilst the CRDs of human and mouse Mincle are similar in structure, having comparable affinity to trehalose dimycolate, but not other mycobacterial ligands (Rambaruth et al. 2015; Richardson et al. 2015; Van Der Peet et al. 2015). The downstream signalling resulting from trehalose dimycolate-Mincle interaction seems to be more complex. A recent study used quantitative phosphoproteome analysis and showed substantial reprogramming of macrophages by trehalose dimycolate and revealed both Mincle-dependent and Mincle-independent signalling mechanisms (Hansen et al. 2019).

There have been a few reports of genetic polymorphisms in the CLEC4E gene and susceptibility to TB in the human population. In one study in South African, 4 SNPs (rs10841845, rs10841847, rs10841856 and rs4620776) were described in the CLEC4E gene, but no association was found with TB susceptibility (Bowker et al. 2016). However, two of the SNPs in CLEC4E (rs10841845 and rs10841847) described earlier, were found to be associated with increased individual protection against pulmonary TB in the northern Chinese population (Kabuye et al. 2019). Furthermore, SNP rs10841847 in the CLEC4E gene was also associated with pulmonary TB risk in a study population from Guinea-Bissau (West Africa) (Olvany et al. 2020).

Mincle remains a fascinating PPR and its involvement in tuberculosis pathogenesis remains to be fully elucidated. Further studies are needed on Mincle's involvement with genetically diverse *M. tuberculosis* strains, other mycobacterial ligands and in resolving the complex Mincle-dependent and Mincle-independent intracellular pathways that can be elicited in immune cells.

5.3 Macrophage C-Type Lectin (MCL)

Macrophage C-type lectin (MCL; also known as Clecsf8, Dectin-3 and CD368) is a membrane-bound PRR coded by the CLEC4D gene. First described in mice (Balch et al. 1998), MCL was subsequently characterised in humans as a type II membrane glycoprotein composed of an N-terminal cytoplasmic region lacking the consensus signalling motifs and an extracellular C-terminal region with a single CRD (Arce et al. 2004). MCL is commonly expressed on myeloid cells but it is also found on neutrophils, monocytes and DCs (Graham et al. 2012). MCL expression is downregulated upon DC maturation or monocyte/macrophage differentiation (Graham et al. 2012). The CLEC4D gene is proximal to the CLEC4E gene, and thus, the MCL gene may have originated from Mincle gene duplication. Like Mincle, MCL can also bind to trehalose dimycolate (but with lower affinity) as well as some fungal species (Arce et al. 2004; Miyake et al. 2013; Zhu et al. 2013) (Fig. 9.3).

The expression of MCL and Mincle are co-regulated, induced via Myd88 (Lobato-Pascual et al. 2013; Miyake et al. 2015; Kerscher et al. 2016a). Thus, MCL is closely linked with Mincle function, with the FcRγ region of MCL being essential for inducing Mincle expression upon binding to trehalose dimycolate (Graham et al. 2012). Furthermore, MCL cross-linking can lead to initiation of phagocytosis, intracellular respiratory burst, and cytokine secretion via Syk-signalling (Graham et al. 2012). In contrast, MCL knockout mice (Clec4d$^{-/-}$) have compromised trehalose dimycolate-induced responses, cytokine production and a reduced ability to form granulomas (Miyake et al. 2013; Zhao et al. 2014). An alternative idea is that MCL and Mincle do not co-associate, but instead, MCL's function is to induce initial Mincle expression (Zhao et al. 2014).

MCL appears to be a key, non-redundant PRR in anti-mycobacterial immunity; MCL knockout mice (Clec4d$^{-/-}$) show significantly higher mycobacterial loads and increased mortality after *M. tuberculosis* infection (Wilson et al. 2015), concomitant with enhanced pulmonary inflammation and neutrophil recruitment (Wilson et al. 2015). Phagocytes derived from MCL knockout mice show impaired phagocytosis of mycobacteria, but this defect is restored when MCL-opsonized mycobacteria are challenged (Wilson et al. 2015).

A single genetic polymorphism (rs4304840) in MCL in humans (Indonesian cohort) has been associated with an increased susceptibility to pulmonary TB (Wilson et al. 2015). MCL seems to play a central role, together with Mincle, in the protective anti-mycobacterial immune response.

5.4 Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin (DC-SIGN)

DC-SIGN (encoded by CD209 gene; Geijtenbeek et al. 2000) is a type II transmembrane receptor expressed predominantly on some macrophages

(alveolar), DCs (myeloid) cells and activated B lymphocytes (Rappocciolo et al. 2006; Lugo-Villarino et al. 2011). DC-SIGN recognizes PAMPs such as N-linked high-mannose and branched fucosylated residues. DC-SIGN has a key role in the clearance of microbial infections, but conversely, pathogens can also manipulate DC-SIGN to alter DCs in their favour for their survival. DC-SIGN is made up of four domains: the N-terminal cytoplasmic domain, transmembrane domain, extracellular domain comprising the neck region, and a single C-terminal CRD (Garcia-Vallejo and Van Kooyk 2013).

DC-SIGN is a PRR for several microbes, most notably HIV-1 (Curtis et al. 1992; Geijtenbeek et al. 2002), but can also bind to bacterial and fungal species (Van Kooyk and Geijtenbeek 2003). DC-SIGN recognises and binds the ManLAM from *M. tuberculosis* (Appelmelk et al. 2003; Maeda et al. 2003), and enhances the internalization of both *M. bovis* BCG and *M. tuberculosis* (Geijtenbeek et al. 2003; Tailleux et al. 2003). Interestingly, mycobacteria are able to subvert DC-SIGN function by altering TLR-mediated activation of DCs. Mycobacteria are strong inducers of the Th1 response and can also facilitate the expression of downstream co-stimulatory molecules and cytokines (e.g. IL-12) by DCs via TLR2 and TLR4 PRRs (Nigou et al. 2001). Despite alveolar macrophages being the predominate targets of mycobacteria in the lungs, the role of DCs is becoming increasingly key in understanding the pathogenesis of TB since DCs expressing DC-SIGN are present in the airway mucosa and interstitial sites of the respiratory system (Soilleux et al. 2002; Tailleux et al. 2003).

The importance of DC-SIGN in TB pathogenesis is also shown in several studies involving transgenic mice. In fact, mice have eight different DC-SIGN homologues (SIGNR1-8). Gene knockout studies have shown that SIGNR3 (the most similar to human DC-SIGN) has a key role in resistance to early *M. tuberculosis* infection (Tanne et al. 2009; Tanne and Neyrolles 2010; Lugo-Villarino et al. 2011). Furthermore, transgenic mice expressing human DC-SIGN showed decreased pathology and prolonged survival following mycobacterial infection (Schaefer et al. 2008).

Capped ManLAM is the main PAMP for DC-SIGN (Geijtenbeek et al. 2003; Kaufmann and Schaible 2003; Maeda et al. 2003). DC-SIGN does not bind to non-capped LAM (AraLAM), which is present on fast-growing mycobacterial species (*M. smegmatis*, *M. fortuitum* and *M. chelonae*) (Geijtenbeek et al. 2003; Tailleux et al. 2003). DC-SIGN appears to be the main DC receptor for mycobacteria (Geijtenbeek et al. 2003); competitive inhibition using anti-DC-SIGN antibodies inhibited *M. bovis* BCG and ManLAM binding by 80% (Geijtenbeek et al. 2003). DC-SIGN also binds to other mycobacterial PAMPs (mannosylated and α-glucan cell wall components, and PIMs). However, mycobacteria can be phagocytosed by DCs in a non-DC-SIGN dependent manner, showing a degree of redundancy in the host–pathogen interaction (Gagliardi et al. 2005; Pitarque et al. 2005; Appelmelk et al. 2008; Driessen et al. 2012; Geurtsen 2009 #972).

DC-SIGN-mediated DC responses requires prior activation of NF-κB via TLR signalling (Geijtenbeek and Gringhuis 2009; Gringhuis et al. 2009; Sancho et al. 2012; Garcia-Vallejo and Van Kooyk 2013), whilst several different PAMPs can trigger a variety of intracellular signalling from DC-SIGN (Gringhuis et al. 2009; Sancho et al. 2012) (Fig. 9.3). DC-SIGN-ManLAM interaction results in Raf-1 phosphorylation and then phosphorylation of transcription factor NF-κB, inducing cytokine production (e.g. IL-12, IL-10, IL-6, and CXCL8) and other co-stimulatory molecules (e.g. CD80, CD83 and CD86) (Gringhuis et al. 2007, 2009). Infection of immature monocyte-derived DCs by *M. tuberculosis* facilitated the maturation of DCs, producing TNF-α, IL-1β, IL-6, and IL-23, and stimulated $CD4^+$ T cells to produce IFN-γ and IL-17 (Zenaro et al. 2009). Furthermore, DC-SIGN interferes negatively with the pro-inflammatory responses and control of *M. tuberculosis* intracellular growth in human macrophages mediated by Dectin-1 (Lugo-Villarino et al. 2018).

In immature DCs, internalisation of *M. tuberculosis* via ManLAM-DC-SIGN interaction results in the pathogen being directed to the late endosomes/lysosomes and suppression of LPS-

induced IL-12 secretion (Nigou et al. 2001). ManLAM-DC-SIGN interaction on immature DCs also interferes with TLR4 signalling, since LPS binding and signalling is via TLR4 (Akira et al. 2001). *M. tuberculosis* interferes between the balance of TLR signalling (DC maturation and inflammation) and DC-SIGN signalling (inhibition of DC maturation and immunosuppression) (Nigou et al. 2001; Engering et al. 2002a, b; Geijtenbeek et al. 2003). Both *M. tuberculosis*-infected DCs and macrophages can secrete the ManLAM that can bind to DC-SIGN on other proximal DCs (Sada et al. 1990; Chatterjee and Khoo 1998); this interferes with the TLR-signalling, inhibiting DC maturation and inducing anti-inflammatory IL-10 cytokine production (Tsuji et al. 2000; Geijtenbeek et al. 2003). Thus, *M. tuberculosis* is able to modulate the DC response to immune suppression to facilitate its intracellular survival (Fortsch et al. 2000; Jiao et al. 2002).

Two genetic polymorphisms have been reported in the DC-SIGN promoter region (-336A/G and -871A/G) but it is unclear as to their effect on TB susceptibility. The polymorphism -336G results in reduced expression of DC-SIGN, which also correlates with the severity of dengue disease (Despres et al. 2005). In a meta-analysis study, polymorphisms (-336A/G, -871A/G) were found not to substantially contribute to TB susceptibility, except that the genotype -336G/G might be associated with increased TB susceptibility for the Asians population (Chang et al. 2012). In another meta-analysis study, the -871A/G polymorphism was associated with decreased susceptibility to pulmonary TB, whilst the -336A/G polymorphism was associated with increased susceptibility of pulmonary TB in the Asian population (Yi et al. 2015). However, an additional polymorphism (-139G/A) was not found to be associated with susceptibility to pulmonary TB (Yi et al. 2015). Moreover, two other genotypes (-871G and -336A) seem to be associated with protection against TB and may have an increased frequency in non-African populations, possibly due to host genetic adaptation as a result of longer history of exposure to *M. tuberculosis* (Barreiro et al. 2006). In the Russian population, −336A genotypes were more sensitive to infection with an *M. tuberculosis* lineage 2 (Beijing/W) strain, whilst those with the -336G genotype and *M. tuberculosis* lineage 2 genotype had increased frequency of death due to pulmonary TB (Ogarkov et al. 2012).

DC-SIGN plays a key role in host-pathogen interactions in TB. Whether DC-SIGN plays a protective role for the host, or is manipulated by the *M. tuberculosis* to circumvent immune responses needs further study. Further data is needed from GWAS as to the genetic susceptibility to TB from CD209 polymorphisms in the human population. Further studies are also required that investigate the interaction of DC-SIGN with different phylogeographic lineages of *M. tuberculosis* strains.

5.5 NOD-like Receptors (NLRs)

NOD-Like Receptors (NLRs) are a large family of intracellular PRRs that contain a nucleotide binding oligomerization domain (NOD). Structurally, NLRs have a variable N-terminal interaction domain, a central NACHT domain (NTPase domain that is evolutionarily conserved), and a C-terminal leucine-rich repeat domain (Fritz et al. 2006; Werts et al. 2006; Franchi et al. 2008) (Fig. 9.3). NLRs are cytosolic sensors that tend to target bacterial cell wall components such as peptidoglycan (containing N-acetylglucosamine and N-acetylmuramic acid) and muramyl dipeptide (MDP) (Girardin et al. 2003a, b; Chen et al. 2009; Franchi et al. 2009). Some NLRs have an amino-terminal caspase recruitment domain (CARD), which is critical to initiate NF-κB signalling, resulting in the release of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, and IL-8), antimicrobial peptides (β-defensin 2), other chemokines, NO and upregulation of adhesins (Darcissac et al. 1996; Heinzelmann et al. 2000; Chin et al. 2002; Guo et al. 2006; Kramer et al. 2006; Uehara et al. 2007). Some of the most prominent members involved in innate immune detection of *M. tuberculosis* in the cytosol are NOD1, NOD2, NLRP3 and NLR family CARD domain containing 4

(NLRC4). This stems from the ability of *M. tuberculosis* to escape from phagosomes into macrophage cytosol via the early secretory antigenic target-6 (ESAT-6) secretion system-1 (ESX-1) mechanism (Simeone et al. 2012). NOD2$^{-/-}$ knockout mice have impaired resistance to *M. tuberculosis* infection because of decreased production of type 1 cytokines and reduced recruitment of CD8^{+} and CD4^{+} T cells; there is a higher bacterial burden in the lungs, 6 months after infection than wild-type controls (Divangahi et al. 2008). MDP treatment of AMs also activates NOD2, which enhances the control of intracellular growth of *M. tuberculosis* and the release of TNF-α, IL-6 and bactericidal LL37 (Juarez et al. 2012). Furthermore, an increase in autophagy proteins (e.g. IRGM, LC3 and ATG16L1) was observed in the mycobacteria-containing autophagosome, suggesting a PRR-dependent mechanism for autophagy activation (Juarez et al. 2012). The CARD9 domain plays a central role in NOD2-mediated activation of p38 and JNK signalling during innate immune responses to intracellular pathogens (Hsu et al. 2007). NOD2 can act in synergy with TLR2 to induce inflammatory cytokines during *M. tuberculosis* infection, and this synergism is lost in mononuclear cells defective in either TLR2 or NOD2, suggesting a non-redundant recognition mechanisms (Ferwerda et al. 2005). Similarly, NOD2 and TLR4 also work synergistically in stimulating the activity of DCs, enhancing T cell recruitment by inducing autophagy and bolstering IL-12p40/70, IL-6, IFN-γ and CD40, CD80 and CD86 co-stimulatory molecules (Khan et al. 2016b). Activating DCs through NOD2 and TLR4 restricts *M. tuberculosis* intracellular survival through strong release of cytokines, nitric oxide, autophagy and enhanced DC migration to lymph nodes (Khan et al. 2016a). NOD1 seems to co-operate with NOD2 or TLRs to produce cytokines (IL-6 and IL-1β) in bone-marrow derived macrophages in response to *M. tuberculosis* infection (Lee et al. 2016a). Similarly, NOD1 is involved in AM and MDM innate responses, which include pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, and TNF-α) and autophagy (Juarez et al. 2014). Intriguingly, an approach using adjunct therapy (with ligands of NOD2 and TLR4) to treat *M. tuberculosis*-infected mice in conjunction with isoniazid, improved drug efficacy against *M. tuberculosis* (Khan et al. 2016a). A therapeutic role for NOD-2 has also be suggested in augmenting T cells responses to *M. tuberculosis* infection (Pahari et al. 2017).

ESAT-6 is a potent activator of the NLRP3/ASC inflammasome and NLRs and CARD proteins play a central role in IL-1β secretion during *M. tuberculosis* infection, via an NLRP3, ASC and caspase-1 infection-inducible inflammasome complex (Mishra et al. 2010). Mycobacterial PPE13 triggers the inflammasome-response in macrophages, by binding to the LRR and NATCH domains of NLRP3 via its MPTR domain (Yang et al. 2020). In DCs, PPE60 was observed to activate the NLRP3 inflammasome, followed by caspase-1-dependent IL-1β and IL-18 synthesis (Su et al. 2018). However, NLRP3 may not be essential for survival in the early stages of *M. tuberculosis* infection or in granuloma formation (Allen et al. 2010; Mcelvania Tekippe et al. 2010; Walter et al. 2010).

Mutations in the NLR genes suggest their importance in protection against several microbial infections, granulomatous inflammatory disorders and inflammatory bowel disease (e.g. Crohn's disease) (Hugot et al. 2001; Miceli-Richard et al. 2001; Ogura et al. 2001). Several polymorphisms in NLR genes linked to TB susceptibility have been reported. Two polymorphism in the NOD1 gene (rs751770147 and chr7:30477156(T)) are associated with TB progression in the Ethiopian population (Mekonnen et al. 2018). Three polymorphisms (Pro268Ser, Arg702Trp, and Ala725Gly) in the NOD2 gene are significantly associated with TB disease in African-American subjects in the USA (Austin et al. 2008). Another polymorphism (Arg587Arg) in the NOD2 gene has been associated with TB susceptibility in the Chinese population but not in the Uyghur and Kazak populations (Zhao et al. 2012). In a recent meta-analysis of NOD2 polymorphisms, no significant association was found

between the Arg587Arg polymorphisms and TB risk; however, Arg702Trp polymorphism was found to be likely associated with protection against TB (Wang et al. 2013). For NLRP3, a single polymorphism (rs34298354) was associated with protection against TB (Liu et al. 2020). A single polymorphism (Q705K) in the NLRP3 gene was associated with poor TB treatment outcome in the Ethiopia population (Abate et al. 2019). Interestingly, in TB/HIV patients from Botswana, a NLRP3 polymorphism (rs10754558-G) was associated with an increased risk for early mortality after starting initiating anti-retroviral therapy (ART), suggesting that these patients may benefit from interventions that decrease inflammasome-mediated inflammation (Ravimohan et al. 2018).

NLRs have given significant insight into the innate immune recognition of *M. tuberculosis* in the cytosol. The role of the inflammasome in protection/pathogenesis is unclear and its activation may be triggered by *M. tuberculosis* as a means of latent infection.

5.6 Mannose Receptor (MR)

Mannose receptor (MR; CD206), coded for by the MRC1 gene, is a type I transmembrane glycoprotein of 165 kDa made up of a C-terminal cytoplasmic domain containing a tyrosine-based motif and three types of extracellular domains (an N-terminal cysteine-rich R-type domain, a fibronectin type II repeat (FNII), and eight consecutive CRDs) (Taylor et al. 1990; Stahl and Ezekowitz 1998). MR is mainly expressed on the surface of macrophages (particularly AMs), monocyte-derived DCs and other cells (e.g. non-vascular endothelial cells) (Martinez-Pomares 2012). MR is also commonly found in intracellular membranes; only 10–30% is constitutively expressed at the cell surface, which reflects its role in recycling and internalization (Schweizer et al. 2000). MR is unique in that its multiple CRDs recognise different PAMPs. The R-type domain can bind to glycans (without the need for Ca^{2+}) (Leteux et al. 2000), whilst the FNII domain binds to collagens (Martinez-Pomares et al. 2006). MR is able to bind to mannose via CRDs 4 to 8, with CRD4 having the primary preference for terminal mannose-containing glycoconjugates, fucose, and N-acetylglucosamine, but less well to glucose (Lennartz et al. 1987; Taylor et al. 1990). In contrast, CRD5 and CRD7 are involved in binding to mannose-containing glycans, whilst CRDs 1 to 3 seem to pay less of a role in binding sugars (Kery et al. 1992; Taylor and Drickamer 1993).

MR recognises complex glycoproteins or glycolipids with multiple sugar moieties endogenously and exogenously. MR may interact with an additional receptor, or soluble MR (as a result of proteolytic cleavage) to facilitate phagocytosis (Le Cabec et al. 2005; Martinez-Pomares 2012) (Fig. 9.3). Intriguingly, pulmonary TB patients with poor prognosis show significantly higher levels of serum soluble MR; pathological analysis revealed enhanced levels of soluble MR in the lung and pleural tissues with caseating granulomas (Suzuki et al. 2018).

ManLAM is a major ligand for MR and this interaction on DCs initiates uptake of the mycobacterium, with probable antigen presentation via CD1b and the major histocompatibility complex class II (MHC-II) (Prigozy et al. 1997). In addition to ManLAM, MR can also bind to PIM, lipomannan (LM), and other mannosylated proteins on *M. tuberculosis* (Schlesinger et al. 1994; Diaz-Silvestre et al. 2005; Torrelles et al. 2006). MR is a major macrophage phagocytic receptor for virulent *M. tuberculosis* strains (H37Rv and Erdman) but not the attenuated strain H37Ra (Schlesinger 1993). Additionally, structural differences in LAM from different *M. tuberculosis* strains seem to alter adherence during the initial interactions with macrophage MR (Schlesinger et al. 1996).

Binding and phagocytosis of ManLAM or mannosylated beads via MR can inhibit phagosome-lysosome fusion, facilitating intracellular persistence of *M. tuberculosis* (Astarie-Dequeker et al. 1999, 2002; Kang et al. 2005). In DCs, ManLAM facilitates intracellular persistence of *M. tuberculosis* and *M. bovis* BCG by inhibiting IL-12 responses via interfering

with the LPS-induced signalling from TLR2 (Nigou et al. 2001). This indicates a cross-linking between MR and TLR2 when binding to ManLAM (Nigou et al. 2001). Cross-linking of MR using a specific anti-MR monoclonal antibody during binding of ManLAM inhibited IL-12 production, but also induced the production of anti-inflammatory IL-10, IL-1R antagonist, and IL-1R type II in DCs (Chieppa et al. 2003). A recurring theme during TB host-pathogen interaction is the degree of cross-linking between various PRR in the recognition of *M. tuberculosis*, via its several PAMPs. In addition to TLRs, MR and DC-SIGN co-stimulation inhibits Dectin-1-induced Th17 responses, whilst enhancing the Th1 responses in *M. tuberculosis*-infected DCs (Zenaro et al. 2009). In macrophages, binding of *M. tuberculosis* mannosylated ligands to MR results in receptor-mediated signalling mechanisms (modulation of cytoskeleton, activation of protein kinases, and transcriptional activation by AP-1), leading to production of matrix metalloproteinase-9 (MMP-9) that may contribution to lung tissue pathology during TB *in vivo* (Rivera-Marrero et al. 2002). SP-D is able to bind to *M. tuberculosis* and inhibit its MR-mediated uptake by macrophages (Ferguson et al. 2002), suggesting that SP-D may be masking mycobacterial ligands and inhibiting phagocytosis of mycobacteria by macrophages. MR may benefit *M. tuberculosis* intracellular persistence; however, in mouse models of TB infection, MR does not seem to be implicated in determining survival or disease severity (Court et al. 2010).

The frequency of a polymorphism of the MRC1 gene (rs34039386), allele G1186A, was higher in individuals with pulmonary TB than healthy controls (Zhang et al. 2012), including in the Uygur population (Zhang et al. 2013a). The G1186A polymorphisms (in exon 7 for CRD2 of MR) may affect the affinity of MR binding to mycobacterial ligands (Zhang et al. 2013a). MR is undoubtedly a major phagocytotic receptor for *M. tuberculosis*, but its importance is overshadowed by many other PRRs. However, entry of *M. tuberculosis* via MR may be a key route for the pathogen to manipulate and circumvent the immune response and prolong its intracellular survival.

5.7 CD14

CD14 receptor is a lipid-anchored glycan-linked protein lacking transmembrane and cytoplasmic domains and is mainly expressed on myeloid monocytic cells. CD14 can bind to *M. tuberculosis* LAM, resulting in the macrophage production of IL-8 (Pugin et al. 1994). CD14 binding of bacterial ligands (LPS, lipoteichoic acid and peptidoglycan) requires co-interaction with other host receptor and cell surface components (TLRs) to facilitate phagocytosis, cell activation and cytokine secretion (Dziarski et al. 2000; Kaisho and Akira 2000). CD14 has also been shown to mediate uptake of non-opsonised *M. tuberculosis* by microglia cells, suggesting that this may be important in the pathogenesis of cerebral TB (Peterson et al. 1995). In AMs expressing high levels of CD14, the phagocytosis of *M. bovis* was enhanced (Khanna et al. 1996); however, *M. tuberculosis* merely up-regulates CD14 expression in macrophages without mediating phagocytosis (Shams et al. 2003). *M. tuberculosis* molecular chaperone chaperonin 60.1 protein partially activates human peripheral blood mononuclear cells via a CD14-mediated mechanism (Lewthwaite et al. 2001).

In mice, CD14 deficiency seems to be protective against chronic *M. tuberculosis* infection by supressing inflammatory responses. Mouse bone marrow derived macrophages deficient in CD14 exhibited a significant reduction in TNF-α secretion when infected with *M. avium* compared to controls, but infection of CD14-deficient mice with *M. avium* or *M. tuberculosis* showed no difference in controlling mycobacterial infection compared to controls (Reiling et al. 2001, 2002). However, another study found $CD14^{-/-}$ mice survive chronic *M. tuberculosis* infection, although their wild type counterparts succumbed to infection due to reduced pulmonary inflammation (Wieland et al. 2008).

Soluble CD14, produced from proteolytic cleavage of membrane CD14, seems to be significantly elevated in patients with pulmonary TB (Hoheisel et al. 1995). A SNP in the promoter region (C(−159)T) of the CD14 gene has been found to be associated with high levels of soluble CD14 and increased probably of developing pulmonary TB in the Mexican population (Rosas-Taraco et al. 2007). In another study, the -159TT allele in the CD14 promoter was also significantly associated with TB risk in the Korean population, probably from higher promoter activity resulting in higher level of soluble CD14, but also decreased IFN-γ secretion in individuals with this genotype (Kang et al. 2009).

6 Concluding Remarks

The nature of host-pathogen interaction is complex in tuberculosis. At the very heart of this is the host receptor-mycobacterial ligand interaction, which is the critical molecular dialogue in the early stages of *M. tuberculosis* infection. Understanding this molecular dialogue is profoundly important in determining the infection outcome. In vitro studies have proven an essential first step, but they often only involve one receptor-ligand interaction. In vivo, the host-pathogen communication is undoubtedly more complex involving an array of mycobacteria ligands that interact with several host PRRs, both soluble and membrane bound. There is indeed redundancy in both the mycobacterial ligands and host PRRs. In vivo, internalisation of *M. tuberculosis* involves multiple routes of cellular entry and crosstalk and co-operation between different PRRs. There seems to be a balance between TLR and CTLR entry with TLR often favouring a pro-inflammatory response, whereas CTLR favouring an anti-inflammatory response. Furthermore, the favoured target cell of *M. tuberculosis* (macrophage or dendritic cell) adds an additional layer of complexity. Fully understanding the host-pathogen dialogue in the early stages of infection is the 'holy grail' in preventing tuberculosis, because only then can we devise strategies to fully block mycobacterial interaction and entry into human cells.

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Vaccination Strategies Against *Mycobacterium tuberculosis*: BCG and Beyond

10

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1 Introduction

Tuberculosis (TB) remains the major global health concern where an estimated 1.7 billion people are latently infected with *M tuberculosis (Mtb*) globally, and thus, are at a regular risk of developing the disease (World Health Organisation (WHO) 2019). For almost a century, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is the only licensed vaccine against the world's leading cause of mortality for TB (Kroesen et al. 2019). BCG is an attenuated form of *M. bovis* obtained by 13 years of *in vitro* serial passage before making it capable of providing protective immunity to challenge with virulent *Mtb*. As per WHO report, BCG is administered to over 100 million people every year. Despite the widespread use of BCG, there are an estimated ten million cases of TB and 1.5 million deaths globally in 2018 (World Health Organisation (WHO) 2019). This is due to the inability of BCG to induce long term protection, poor efficacy in adults and in latent TB infection in spite of its booster doses.

Estimates of protection imparted by BCG against pulmonary TB vary between 0 and 80%; this variability is due to strain variation in BCG preparations, host genetic, nutritional and environmental aspects (Fine 1995). The burden of this communicable disease varies enormously among countries; populations and racial groups vary in their resistance to TB. Host genetic factors play an important role in determining inter-individual differences in susceptibility or resistance to TB infection. While considering the susceptibility of *Mtb* and establishment of the disease, the antigenic virulence of the pathogen is also equally relevant acting as a potent immunogen.

BCG often has been found to protect animals against secondary infections with *Candida albicans* and *Schistosoma mansoni* (Quintin et al. 2012; Cheng et al. 2016; Arts et al. 2016). It has been suggested that sometimes re-infections as well as exposure to microorganisms acting as an adjuvant can not only induce specific secondary immune response, but also mount non-specific response against the same antigen as well as another antigen or pathogen. This state of long-term functional reprogramming of innate immune cells due to secondary antigenic challenge is termed as 'trained immunity' and can be a promising tool for vaccine development (Netea et al.

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2020). Innate immune cells were found to respond to secondary non-specific diverse antigenic challenges such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) as if they had a memory of it. Trained immunity induces metabolic reprogramming which further influences the epigenetic reprogramming of the innate immune cells activating cytokine production. A future vaccine if designed with proper formulation of microbial antigen with suitable antigenic virulence factor to evoke, trained immunity can be a promising tool to confer broad protection against TB (Ferluga et al. 2020).

This chapter highlights the important factors related to susceptibility of TB, antigenic virulence and its importance in vaccine development. We also discuss the current status of TB vaccine and the recent developments from the existing traditional BCG vaccine.

2 Host Genetic Makeup in TB Resistance and Susceptibility

Different populations across the globe are at different stages in the pandemic of TB, and thus, the implications of genetic and geographical factors in determining the prevalence of TB overtime are of paramount importance. Host genetic makeup plays an important role in determining the differences in susceptibility among individuals with TB in different populations as revealed by studies involving twins, candidate gene approaches, family-based and genome-wide association studies (GWAS) (Hill 2006; Bellamy 2006; Takiff 2007; Thye et al. 2010; Thye et al. 2012; Mahasirimongkol et al. 2012; van Tong et al. 2017).

Human leukocyte antigen (HLA) alleles and differential HLA allele frequencies in distinct populations were found to be linked with TB susceptibility for HLA–I (A2, B8, B17,B27,B35) and HLA-II {DQ β57, DQA1∗0101, DQB1∗0301, -0303, -0304, DQB1∗04 (-0401, -0402), DQB1∗0503, -0502, DQB1∗0601, -0602, -0603, DR2, DRB1∗04-DQB1∗03, DRB1∗-07,-09,-12,-13, DRB1∗1302, DRB1∗14-DQB1∗05, DRB1∗1501 (DR2), DRB1∗1501-DRB5∗0101 DQA1∗0103-DQB1∗0601, DRB1∗16, DQB1∗05} (Cai et al. 2019). Several HLA class-II alleles, particularly HLA-DRB1, were found to be associated with TB susceptibility in Asian population (Harishankar et al. 2018). Various non- HLA genetic markers were also associated with TB susceptibility such as toll-like receptors (TLR1, TLR2, TLR4, TLR8 and TLR9), killer immunoglobulin-like receptor (KIR), vitamin D receptor (VDR), solute carrier family 11 member 1 (SLC11A1) gene system, mannose receptor (CD206), the nitric oxide synthase 2A (NOS2A) gene, the speckled 110 (SP110) gene, and the P2X7 receptor (P2X7) gene (Schurr 2007; Harishankar et al. 2018; Cai et al. 2019).

Polymorphisms in the cytokine gene coding region are also the host factors affecting susceptibility to TB (Cai et al. 2019). Type I interferon have been implicated in promoting progressive infection with *Mtb* to some extent by suppressing expression of the pro-inflammatory cytokine, IL-1 (Mayer-Barber et al. 2011). Intron polymorphism of IFN-γ +874A/T, "T" allele and "TT" genotype were associated with susceptibility with Pakistani population but rendered protection to TB in Sicilian and South Africa populations (Lio et al. 2002; Rossouw et al. 2003; Ansari et al. 2009). Polymorphism in cytokine genes for IL-2, IL-4, IL-6, IL-10, IL-12 and IL-17 were also associated with TB susceptibility in some populations (Harishankar et al. 2018). TNF-α playing an important role in the recruitment of immune cells during granuloma formation have also been found to be associated with TB risk. Analyses among ethnicity showed that TNF-α -308G/A variant was associated with Asians and -238G/A variant in African individuals with pulmonary TB (Yi et al. 2015); in Sicilian and Colombian populations protective associations were found in TNF-α -308(G/A) and haplotype combination of -308A -238G polymprphisms (Scola et al. 2003; Correa et al. 2005). Consistent with this, in *Mtb*-infected mice in conjunction with IFN-γ, their *Mtb* lung burden was much reduced, suggesting *Mtb* elimination (Brightbill et al. 1999; Aliprantis et al. 1999). IFN-γ produced by T cells or NK cells, also induced in macrophages guanosine triphosphatases (LRG-47), controlling phagosome

maturation and vesicular trafficking of *Mtb* and other microbes, for their disposal (MacMicking et al. 2003).

3 Malnutrition, Immunometabolism and Susceptibility to TB

The host susceptibility to TB is always at a much higher risk in disease prevalent areas, owing to malnutrition, host genetic makeup, and gender. As studied on trained immune memory, generally, there is a link between energy metabolism and epigenetic states which may be perturbed by *Mtb* antigens. Energy metabolism is mainly regulated through aerobic glycolysis, connected to intermediates of tricarboxylic cycle, such as acetyl- coA and NAD^+/NADH, based on oxidative phosphorylation (van der Heijden et al. 2017; Jaenisch and Bird 2003). Acetyl-coA metabolism regulation has been linked to histone acetylation, through ATP-citrate lyase (Wellen et al. 2009). Studies are now correlating cellular metabolism with the functional state of the immune cells (Ganeshan and Chawla 2014). The source of energy used for metabolic activities varied between activated, memory and regulatory T lymphocytes (Netea 2011; Netea et al. 2016a Arts et al. 2016a). Activated T lymphocytes were more dependent on glycolysis and oxidative phosphorylation, metabolising glucose to lactate; memory T cells on lipid synthesis via mitochondrial citrate production and regulatory T lymphocytes were dependent mostly on β-oxidation of fatty acid (Netea 2011; Netea 2016a Wang et al. 2011; Donnelly and Finlay 2015; van der Windt et al. 2013; Michalek et al. 2011; Loftus and Finlay 2015; Gerriets and Rathmell 2012). Netea et al. have shown Akt/mTOR pathway plays an important role in initiating trained immunity corresponding with high rate of glycolysis (Netea et al. 2016b*). In a separate study involving β-glucan-induced trained immunity, a long-term increase in glycolysis was observed which was dependent on mTOR/HIF-1α pathway (Cheng et al. 2014); at the same time, sirtuin 1 expression also decreased in trained monocytes (Netea et al. 2016a; Cheng et al. 2014). BCG trained immunity exhibited enhanced responsiveness of monocytes and macrophages and a shift of glucose metabolism towards glycolysis, found to be crucial for histone modifications and functional changes (Arts et al. 2016).

TB has been associated with undernourishment, especially with a shortage of protein energy. There is a vicious circle, in that malnutrition raises susceptibility to TB in endemic population. Consequently, *Mtb* infection can reduce innate and adaptive immunity effects, and energy metabolism, for a secondary immunodeficiency, fuelling TB patient consumption state. However, such disease spiralling may be reversed in patients, suffering with active lung TB, or preventively, by nutrients supplements. Such treatment may restore their drug sensitivity, and most likely, efficacy of clinical BCG and TB vaccine (Kant et al. 2015). Adipose tissue versatile hormone leptin, regulating energy metabolism, which is low in TB, could also be endogenously boosted, or perhaps added to TB therapy (Kant et al. 2015).

Normally, neonates are protected from pathogens by type 1 innate phagocyte immunity, which overpowers their type 2 tolerance *in utero* against maternal allogeneic antigens. Maternal colostrum contains immune activator proteins which are important, besides antibodies against pathogen. However, infant susceptibility to TB infection and progression is also increased owing to their immunity decline, as a consequence of malnutrition. Paediatric TB defence mechanism on nutrient-dependence, has not been sufficiently studied for a certain prediction, and apparently for efficacy of BCG as well as other vaccines (Jaganath and Mupere 2012).

A historic long population cohort study linked starvation during development stages *in utero*, and post-partum, with metabolic disorders, and a high diabetes type 2 risk in young adulthood, with rural Bangladesh people. This was based on genome wide analysis of whole blood extracted epigenetic DNA methylation-changes. Differences were shown in metastable epi-alleles, prone to peri-conceptual shortage of

food. These findings are in accord with Gambian children studies, identifying epithelial tumour suppressor VTRNA2-1 gene, as contributor to metabolic disease (Finer et al. 2016; Silver et al. 2015; Tobi et al. 2009).

Nutrient deficiency such as of Vitamin D has also been linked with TB susceptibility. Monocytes exposed to *Mtb* show a strong induction of the 1α-hydroxylase (CYP27B1), an enzyme which activates the inactive form 25-hydroxyvitamin D into circulating biologically active compound calcitriol (Liu et al. 2006). Vitamin D receptor (VDR) polymorphisms showed strong relationship with TB in several populations. In a study with Han Taiwanese population restriction endonuclease sites for TaqI, BsmI variants in VDR and polymorphisms rs7041G/T (Asp416Glu) in Vitamin D binding protein (VDBP) were significantly associated with susceptibility to TB infection (Lee et al. 2016). Another study involving Iranian population showed strong relationship between vitamin D deficiency and TB; in contrast, VDR polymorphisms were not associated with susceptibility (Rashedi et al. 2014). Association of VDR FokI polymorphism with susceptibility to TB was found in Asian, but not in African and Caucasian populations (Huang et al. 2015). In some cases, VDR gene variants such as Cdx-2 and 30UTR TaqI which are regulated by vitamin D can possibly modulate the levels of chemokines, suggesting the role of VDR gene variants in inflammatory response during active infection (Harishankar et al. 2018).

4 *Mtb* Antigenic Virulence Factors and Vaccine Strategies

Mtb cell wall is a lipid-rich envelope (about 40%); a large portion of bacterial genome is used for lipid biosynthesis and degradation. The microbial lipids such as lipoarabinomannan (LAM), lipomannan (LM), phosphatidylinositol mannosides (PIMs), trehalose-6,6′-dimycolate (TDM), phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL) are important virulence factors (Daffe and Etienne 1999; Echeverria-Valencia et al. 2018). Differential expression of these cell wall lipids determines the sustenance of the *Mtb* infection. During the earlier stage of *Mtb* infection, PAMPs in the form of different lipoproteins (LAM, LM, PDIM, TDM) are recognized by macrophages and DCs through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin-like receptors (CLRs). TLR2 and TLR4 present on the surface of macrophages can recognize mycobacterial lipoproteins and also whole live *Mtb* to mediate innate immune responses (Harishankar and Selvaraj 2016).

Mtb reduces recruitment of macrophages by masking of bacterial TLR agonist molecules by the lipoglycan and pthiocerol dimycoceroserate (Cambier et al. 2014). *Mtb* can also induce expression of type I interferon, a regulatory cytokine for its own benefit, by involving cyclic GMP-AMP synthase (cGAS) and its downstream signalling molecule, stimulator of interferon genes, STING (Watson et al. 2015; Wiens and Ernst 2016). Mannose receptor (MRC1) expressed on macrophages and dendritic cells is a type I transmembrane C-type lectin, which can recognize lipoglycan and mannose-capped lipoarabinomannan (ManLAM), leading to the stimulation of a nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) that further activates anti-inflammatory immune response (Torrelles et al. 2008). Lack of *O*-mannosylation is associated with increase in LAM production and higher release of LAM/LprG protein, leading to reduction in the virulence of *Mtb* *O*-mannosylated deficient mice (Alonso et al. 2017). Mycolic acid (MA), a major lipid component of *Mtb* cell wall, can also be a potential subunit vaccine candidate for TB as MA-specific CD1b-restricted T cells were found to be cytotoxic, produced Th1 cytokines, and form memory populations (Shang et al. 2018). Several lipid antigens derived from *Mtb* are non-polymorphic to which CD1 molecules may bind. These CD1-restricted *Mtb* lipid antigens are likely to be recognized making them potential vaccine targets (Barral and Brenner 2007). Nucleotide-binding oligomerization domain-containing protein 2

(NOD2), a caspase-recruitment domain (CARD)-containing NLR, recognizes the peptidoglycan muramyl dipeptide (MDP), which is an essential component of mycobacterial cell walls. In a study involving stimulation with *Mtb* of cells of patients with a defective NOD2 showed marked reduction in cytokine production, suggesting NOD2 as a key sensor of *Mtb* (Ferwerda et al, 2005).

Mtb genome exhibits high degree of polymorphism with repetitive DNA sequences and insertion elements that relates to its virulence and infectivity. *Mtb* consists of several virulence-associated genes; a study of transposon mutants of *Mtb* strains in murine infection models showed about 200–500 genes involved that were essential for growth of *Mtb in vivo* (Sassetti and Rubin 2003; Zhang et al. 2013; Kroesen et al. 2019). The important virulent determinants include genes encoding proteins involved in secretion systems and the mycobacterial lipids. There are several specialized protein systems, which control the molecular movement and the secretion of virulence substances, such as ESX/ESAT-6/type VII secretion systems, Twin-arginine transporter (TAT transporter), PE/PPE (proteins whose N-termini contain the characteristic motifs Pro–Glu/Pro–Pro–Glu), protein families and lipoproteins (Echeverria-Valencia et al. 2018; Kroesen et al. 2019).

PE/PPE gene family members represent about 10% of coding capacity of *Mtb* genome and also account for antigenic variability of *Mtb* strains (Bottai and Brosch 2009; Cole et al. 1998a, b; Sampson et al. 2001; Delogu and Brennan 2001; Delogu et al. 2008). Members of PE_PGRS (polymorphic GC-rich repetitive-sequence) family are found to be involved in the regulation of macrophage signalling and in modulating secretion of TNF-α in infected macrophages. ESX-1 system confers its virulence via the secretion of early secreted antigenic target ESAT-6 (6 kDa) and its protein partner, culture filtrate protein CFP-10 (10 kDa). Both of these proteins are not produced by the attenuated BCG as the region of difference 1′ (RD1) genes responsible for their production is deleted from BCG (Bottai and Brosch 2009). *Mtb* utilizes a twin-arginine translocation system (TAT transporter) located in the cytoplasmic membrane for transporting folded proteins (Echeverria-Valencia et al. 2018) and are frequently responsible for exporting virulence factors, and thus, contribute to pathogenesis (De Buck et al. 2008). Mycobacterial phospholipases, virulence-related molecules encoding *plcA, plcB, plcC* and *plcD* genes, are secreted by the TAT transporters (Posey et al. 2006). Among the secretory proteins, the Ag85 complex members (Ag85A, Ag85B and Ag85C) are the most common proteins of *Mtb* secreted into culture fluids transported through the TAT transporter. Although the major component of Ag85 is secreted, a small amount of the antigen remains on the bacterial surface. This Ag85 complex plays a vital role in *Mtb* virulence as it is required for intracellular survival of *Mtb* within macrophages (Karbalaei Zadeh Babaki et al. 2017).

Mtb can withstand quite adverse host environmental conditions including high pH and ROS generation. Thus, it needs constant coordinated regulation of gene expression that mimics the macrophage environment (Timm et al. 2003; Betts et al. 2002). Sulfate-assimilation pathway (SAP) of *Mtb* represents major immunogenic targets of the bacillus, as it mounts strong T-cell recognition by both mice and humans infected with *M. tuberculosis.* Enzymes of SAP were required for the reduction of sulphur in *Mtb.* Sulphur in its reduced form (SO_4^-) is used in biosynthesis of cysteine which is considered as the prime targets of NO intermediates that is being encountered by *Mtb* while inside the macrophage, and disabling biosynthesis of cysteine attenuates bacterial virulence (Buchmeier and Fahey 2006; Newton and Fahey 2002; Sareen et al. 2003). Therefore, availability of cysteine is linked with *Mtb* defence while inside the host, and thus, SAP pathway is very much crucial for its survival (Pinto et al. 2012).

In mycobacteria, there are several virulence genes inhibiting phagocyte apoptosis. Recombinant BCG vaccines are largely devoid of such genes, including urease-*ureC* and *nuoG*, which are able to substantially reduce such anti-apoptosis blockade (Velmurugan et al. 2007; Hinchey et al. 2007; Kaufmann et al. 2017a, b). Another *Mtb* virulence gene is *secA2* coding for bacillus superoxide dismutase. The secA2 deletion mutant strongly

induced in mice apoptosis of infected macrophages and priming of $CD8^+$ T-cells (Hinchey et al. 2007). Apoptosis of macrophages infected with *Mtb* relied on multiplicity of infection (MOI) number. Two to four bacillus per cell induces classical intrinsic apoptosis in infected macrophages. On the contrary, a delayed macrophage infection with 20/cell MOI, over a short time induced apoptosis, which may switch to infected macrophage necrosis, independently from caspases pathway, dissipating TB infection, as in case of vascular granulomas in acute lung TB (Lee et al. 2006). BCG may also induce innate apoptosis of tumours.

Thus, both *Mtb* cell wall antigenic components as well as secreted proteins are important source of immunogenic antigens, which encounter the innate as well as the adaptive immune response (Figs. 10.1 and 10.2). Their immunogenicity, mode of regulated expression and their localization in the bacterial cell envelope make them suitable targets for vaccine design. Targeting these antigens to macrophages, dendritic cells and lymphocytes to tackle the infection can be a promising vaccine strategy, and thus, new strategies using suitable adjuvants, immunodominant antigens involving lipoprotein-based immunization need to be efficiently exploited to come up with a new generation vaccine for TB.

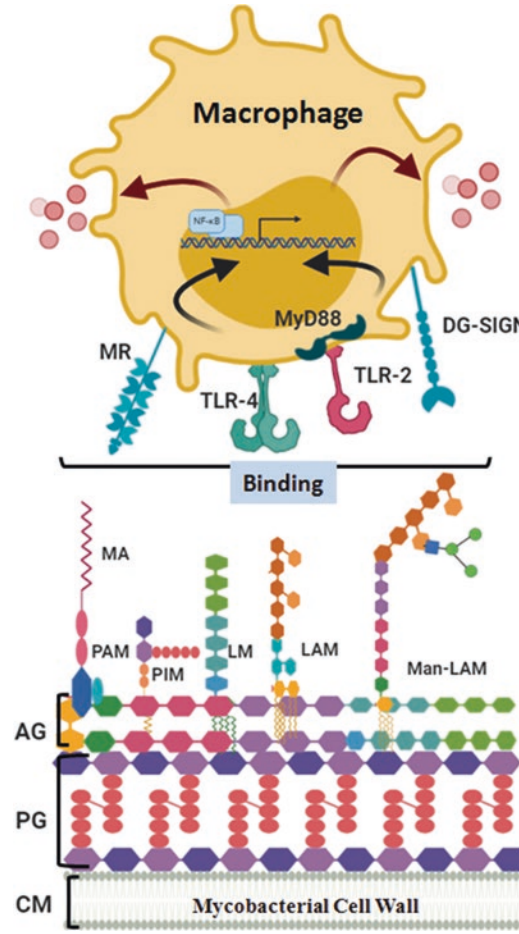

Fig. 10.1 Representation of *Mtb* cell wall lipoproteins that bind with array of receptors expressed on macrophages. Man-LAM can interact with Mannose receptor (MR) and DC-SIGN. TLRs interacts with lipoproteins, peptidoglycan, and cell-wall glycolipids including LAM, LM, PIM and Man-LAM. TLRs on macrophages and dendritic cells stimulates the production of proinflammatory cytokines such as IL-1β, TNF, and IL-6 via activation of the NF-κB and MAPK signalling pathways

4.1 *Mtb* Sulphate Assimilation Pathway Immunogen in Pre-Clinical Vaccines

Mtb antigens include non-secreted microbe sulphate assimilation pathway (SAP) enzymes, containing adenosine-5′-triphosphate (ATP) sulfurylase, guanosine triphosphate (GTP) hydrolase, and adenylyl sulphate-kinase, designated as cysD, cysN and cysC, respectively. SAP proteins are critical for the intra-macrophage phagosome *Mtb* survival, by maintaining an adequate cellular redox potential in conjunction with bacterial Mycothiol. These proteins are sulphate reducing agents, which are able to detoxify IFN-γ mediated iNO-synthase- NO intermediates bactericidal radicals. This function applies also to *Mtb* granuloma redox potential conditioning (Pinto et al. 2013; Buchmeier et al. 2003). *Mtb* SAP vaccine components were also found to be very strongly immunogenic in upregulating $CD4^+$ T helper cells type 1 response involving IFN-γ, TNF-α and IL-2, in protection of mice lungs. SAP vaccination substantially boosted *Mtb* killing in their lungs, afforded by their BCG vaccination, upon their aerosol *Mtb* infection. Similarly, TB patient peripheral blood $CD4^+$Th cells were hyper-activated (Pinto et al. 2013; Fan et al. 2009). In vaccine experiments, C57BL/6 mice were sub-cutaneously BCG vacci-

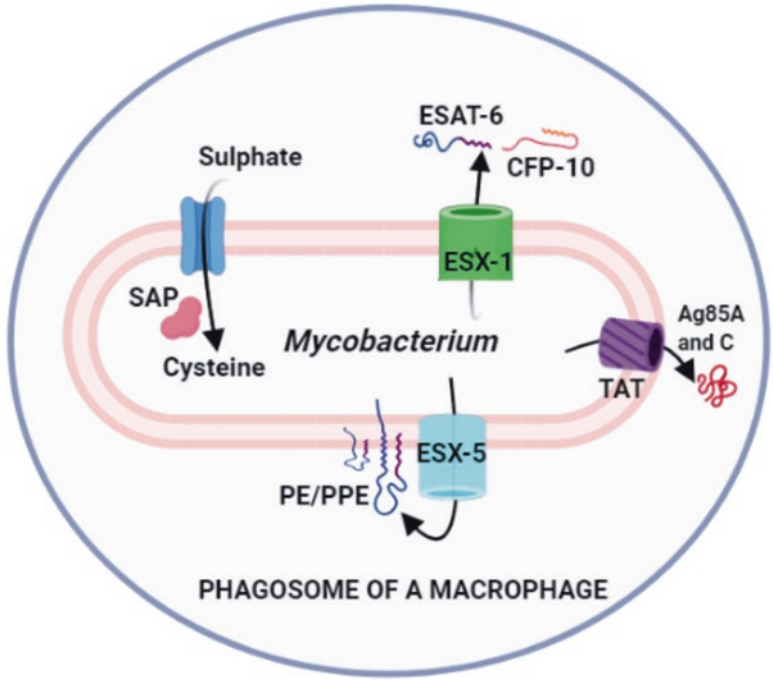

Fig. 10.2 Role of protein secretion and transport system of *Mtb* relating to its virulence and infectivity: ESX1 secrets ESAT-6 and CFP10, which are highly immunogenic proteins encoded by members of the *esx* family of genes and are exported together as a 1:1 complex where each protein depends on the other for its export. ESX5 exports PE/PPE proteins containing N-terminal Pro-Glu or Pro-Pro-Glu repeats, are surface exposed and account for antigenic variability of *Mtb* strains. TAT transporter system present on the bacterial cell wall is responsible for exporting virulence factors such as Ag85 complex. SAP enzymes plays an important role in the survival of *Mtb* by catalysing the synthesis of cysteine required for balancing the ROS generation by the macrophage

nated, and later in intervals immunized intramuscularly with SAP proteins, i.e. cysDNC, as their locus operon complex. These antigens were co-administered with adjuvants dimethyl dioctadecyl ammonium bromide (DDAB), and with monophosphoryl lipid A (MPL), or with mixed locus coded DNA vaccine. They were then challenged with 100 viable *Mtb* infective dose in cytosol/per mouse. Killing of *Mtb* was detected in the lungs and spleen by decline in colony forming units (CFUs), producing IFN-γ. These results are interesting, since in clinical vaccine trials with infants, BCG, even in connection with other subunit vaccines, insufficiently protected their lungs (Pinto et al. 2013). In contrast to early secreted antigenic target ESAT-6, SAP-Cys DNC system operates intracellularly, when the bacillus is resident in macrophages. SAP antigens could be sensed by intracellular NOD-like receptors, and by cytosol TLR5, TLR7and TLR9 proteins (Wang et al. 2009; Pinto et al. 2013; Akira and Hemmi 2003). These preclinical vaccine findings may foretell a better protection in clinical vaccine trials against lung TB in infants, and potentially also in adults (Pinto et al. 2012; Akira and Hemmi 2003). Apparently, the vaccine antigens, together with its adjuvants, could overcome pathogen immune evasion, by polarising the infected macrophage type 2 (M2), into type 1 phenotype (M1), expressing -inflammatory cytokines and the NO reactive radicals (Fig. 10.3).

4.2 *Mtb* Subdominant-Weak Antigens as TB Protective Vaccine Candidates

It has been suggested that immunodominant virulent antigens such as ESAT-6 of *Mtb* may have a co-evolutionary advantage, by evading host

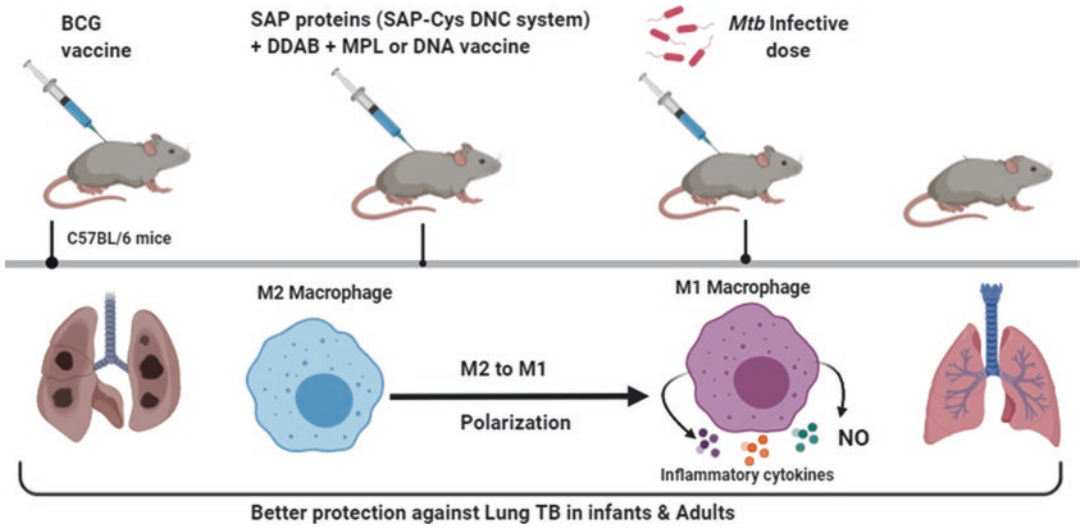

Fig. 10.3 Production of Pre-clinical vaccine with *Mtb* SAP immunogen: C57B/6 mice were first BCG vaccinated, and then in intervals immunized with SAP proteins, co-administered with adjuvants dimethyl dioctadecyl ammonium bromide (DDAB), and with monophosphoryl lipid A (MPL), or with mixed locus coded DNA vaccine. Mice were again challenged with 100 viable *Mtb* infective dose/per mouse (Pinto et al. 2013). ATP sulfurylase conferred significant protection against murine TB and boosted BCG-induced protective immunity in the lungs by polarising the infected M2 into M1 phenotype, expressing pro-inflammatory cytokines and NO

focused immune responses. However, a vaccine against *Mtb* subdominant-weak antigens may boost their immunogenicity, protective against TB, as studied with mice in their prophylactic lung protection (Orr et al. 2014). In vaccine construction, from a large number of *Mtb* antigens as a hierarchy, subdominant antigens were selected for fusion vaccine proteins, in combination with Th1 augmenting glucopyranosyl lipid adjuvant. Such combination proteins were highly immunogenic. They induced Th cell type 1 polarisation with IFN-γ, TNF-α and IL-2 production; in the absence of BCG vaccination, mice were immunized with recombinant vaccines intra-muscularly, one, two or three times, in three weekly intervals. After 4 weeks, they were infected with a low number of 'multiplicity of infection' (MOI) *Mtb* strain M37Rv aerosol. After 3–6 weeks, it was shown that their viable *Mtb* lung burden was substantially reduced, as detected by ELISPOT analysis. It was comparable to that of virulent dominant antigen vaccines. This study also suggested engaging several vaccine combined complexes for vaccine efficacy to cover for immune cell variation by host, and for *Mtb* strain antigen variation. These findings may broaden prospective adaptive anti-TB vaccine repertoire also in humans, instead of being focused on immunodominant specific antigens (Orr et al. 2014). However, the protection against *Mtb* infection in mice also suggests that innate macrophage-unique mycobacterial immunity was also augmented. It is also reminiscent of recombinant BCG vaccines lacking virulence genes, overcoming *Mtb* virulence proteins. Apparently, adaptive immune cells are unable to enhance TB immunity via apoptosis induction (Divangahi and Behr 2018).

4.3 *M. smegmatis* Pre-clinical Vaccine

A recombinant *M. smegmatis* is also a promising preclinical candidate vaccine against TB. *M. smegmatis* is a saprophytic, fast-growing mycobacteria, but can be pathogenic due to its conserved virulence *esx-3* locus, as found with *M. smegmatis* infected C57BL/6 mice strain. The *M. smegmatis esx*-3 deletion mutant, *Δesx-3*,

named IKE strain vaccine, afforded an innate immune protection of mice, in their prolongation and survival after *Mtb* infection. Moreover, IKE strain, transgenic with *Mtb esx-3*-orthologue, designated IKEPLUS strain, upon vaccination, induced in mice a potent adaptive Th1 cytokine bactericidal protection from *Mtb* infection, including IFN-γ, IL-12 p40 and p70. This robust type 1 response was dependent on CD4 memory cells. At the same time, IKEPLUS strain retained its capacity to augment innate immune cell responses in killing *Mtb*, when residing within macrophages. Accessory innate cells may be recruited by type 1 cytokines, including NK-cells, NKT-cells and γδ T-cells. Mice were vaccinated intravenously with IKE, IKEPLUS or subcutaneously with BCG for comparison. They were challenged with a high dose of *Mtb* aerosol and intravenous infection. Mice survival rate, and their *Mtb* lung and other organ burden, were monitored. It was shown that IKEPLUS vaccine strain was superior to BCG and to paternal *M. smegmatis* in clearing *Mtb* infection in lungs and spleen, and in mice survival (Sweeny et al. 2011) (Fig. 10.4).

A prospective clinical vaccine candidate is the *M. smegmatis* derived, recombinant fusion protein Hybrid 1 (H1). H1 contains insertion of two dominant *Mtb* genes, Ag85B and ESAT-6. H1 is strongly immunogenic; it induces in mice Th1 potent cytokine responses, as measured by IFN-γ production. Mice were vaccinated with H1 fused DDA-TDB adjuvant CAF01, a mycobacterial specific adjuvant, subcutaneously at 3 weeks intervals. After 3 weeks, mice were culled and their spleen cells re-stimulated in culture with H1. After 3 days, their IFN-γ released into medium was analysed by ELISA test. For comparison, *E. coli* expression system derived H1, likewise purified by various procedures, was included, which was of similar immunogenic efficacy. *M. smegmatis*-based vaccines seem to have an advantage over that of BCG, perhaps by their faster replication, in addition to their attenuated virulence nature (Tsolaki et al. 2013).

Another prospective TB vaccine candidate target is the *Mtb* mammalian cell entry1A gene (*mce 1A*) expression, which is specific for macrophages. *Mtb* Beijing strain isolate from Indonesia was utilized for *mce1A* vaccine construction, where this strain is prevalent in causing TB. *mce1A* was cloned and expressed in *E. coli* system, for preparation as a subset of TB vaccine, based on BCG. *mce*1A protein facilitates the pathogen's in entry to the macrophage and their intracellular survival. Other family gene members the operon may contribute, suggesting a requirement of multiple sources for *Mtb* evasion of host immunity, targeted by multiple vaccines (Indriarini et al. 2018; Harboe et al. 1999; Srivastava et al. 2007; Saini et al. 2008).

In a murine TB model, three various strains of recombinant BCG vaccines were generated, to assess their protection of mice against *Mtb* infection. These virulent antigen vaccines were rBCG:85A, rBCG:85B and rBCG:85X, which became expressed separately with TB stage progression, but have been tested also as combined complex cocktail ABX. ABX strongly promoted $CD4^+$ Th type 1 responses with a higher cell number secreting IFN-γ, IL -12, of $CD8^+$ T cells proliferation, and protection of mice against *Mtb*. This property of ABX vaccine in mice suggested a promising vaccine candidate for clinical trials against active and latent TB forms (Liang et al. 2015).

4.4 Dendritic Cell Immune Vaccine Candidate

As a basic link of innate immunity with adaptive immunity, are dendritic cells (DCs) are responsible for microbial antigen-specific priming of naïve T cell precursor differentiation in lymph node germinal centres. DCs, via their own migration, are also chaperons for *Mtb* transmission to lymph nodes, where it is able to replicate in endothelial cells, but not in DCs (Lerner et al. 2006).

Previously, a study on human DC specific type-1 lectin receptor, the ICAM3-grabbing-nonintegrin (DC-SIGN), ligating with *Mtb*-cell wall component ManLAM, revealed that serine and threonine Raf- kinase upregulated DC-type-2 (DC2)-IL-10 expression. This effect was mediated by NF-κB subunit p65, inducing epi-

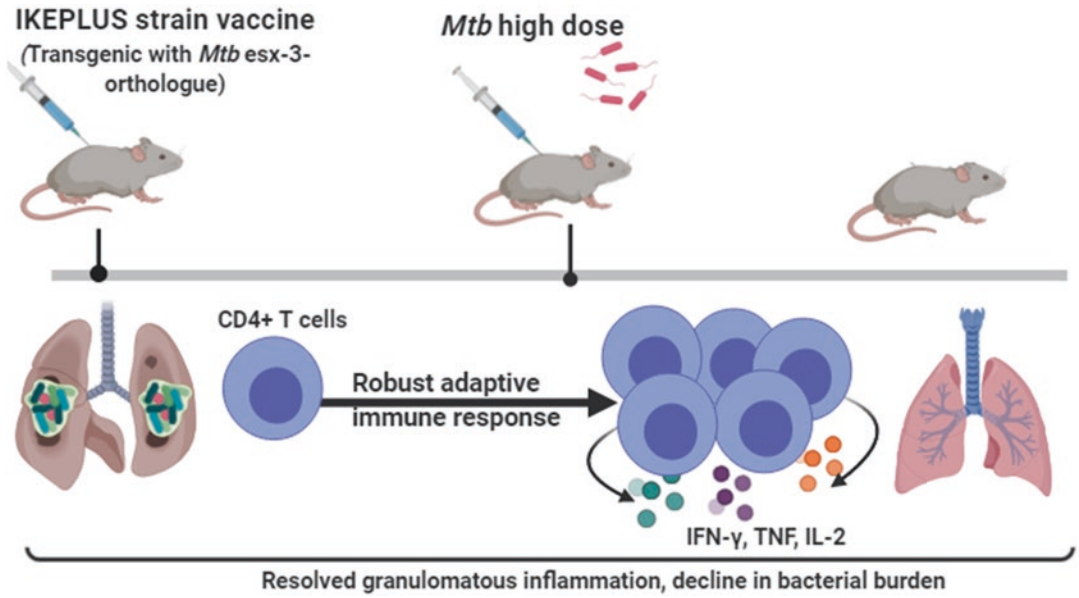

Fig. 10.4 Production of *Mycobacterium smegmatis* pre-clinical vaccine: IKE strain vaccine (*M. smegmatis esx*-3 deletion mutant, *Δesx-3)* transgenic with *Mtb esx-3*–orthologue, is designated as IKEPLUS strain. Mice were vaccinated intravenously with IKEPLUS strain vaccine (efficacy compared with IKE strain and BCG vaccine) and were also challenged with high dose *Mtb* aerosol. IKEPLUS induced better protective bactericidal immunity that was dependent on $CD4^+$ memory T cells and involved a distinct shift in the pattern of cytokine responses by $CD4^+$ T cells

genetic remodelling *via* gene acetylation silencing. On the other hand, p65 could also transmit pro-inflammatory transcription signalling, generated by DCs, via TLR2 and TLR4 upon their activation by ManLAM or other bacterial components such as LPS. DC-TLR pro-inflammatory pathway activation, mediated by MyD88C or TRAF transmitters, is the prerequisite for Raf-1 kinase modulation balance of both pathways at the transcription level. Such *Mtb*-DC interaction may diminish type 1 cytokine polarisation, which is critical for DC maturation and in specific bacterial antigen presentation, MHC II dependently, to naïve Th and B cell differentiation. Immature DCs bactericidal capacity and their antigen processing may be weakened. DC-SIGN interacts, besides *Mtb*, with M. *leprae, M. bovis, Candida albicans*, and measles virus or with HIV, associated with chronic disease. These pathogens exploit different TLRs, which are modulated by DC-SIGN/Raf-1 kinase pathway. Immature DC intracellular bactericidal phagosome/lysosome fusion function may be also affected by this pathway (Akira et al. 2006).

However, human DC-SIGN receptor can be employed as a potent activator of $CD4^+$ Th1 phenotype in association with Th17 cells, in responses to *Mtb* antigens, as shown in the receptor transgenic mice (Velasquez et al. 2018). The vaccine is based on human anti-DC-SIGN receptor antibodies, conjugated with *Mtb* antigen Ag85B protein, i.e. DC-SIGN:Ag85B, or its peptide P25, which is a major Th1 epitope. As such, the vaccine targeted *Mtb* antigens to DC-SIGN for their engulfment to induce in DC a magnified type 1 phenotype to prime the antigen to Th1, secreting IFN-γ, IL-2 and TNF-α. Ag85B is a sub-dominant, but a very potent immunogen. The Th1 phenotype only occurred in conjunction with adjuvants such as CD40, fungal wall zymosan, and/or cholera detoxified fusion protein CTA1-DD, which can apparently override DC and Th2 phenotype. Experiments were also carried out *in vitro*, where these adjuvants were able to induce the activation of DCs and the secretion of IL-6, IL-23, and IL-1β, all important cytokines necessary for the induction of Th17 responses (Fig. 10.5).

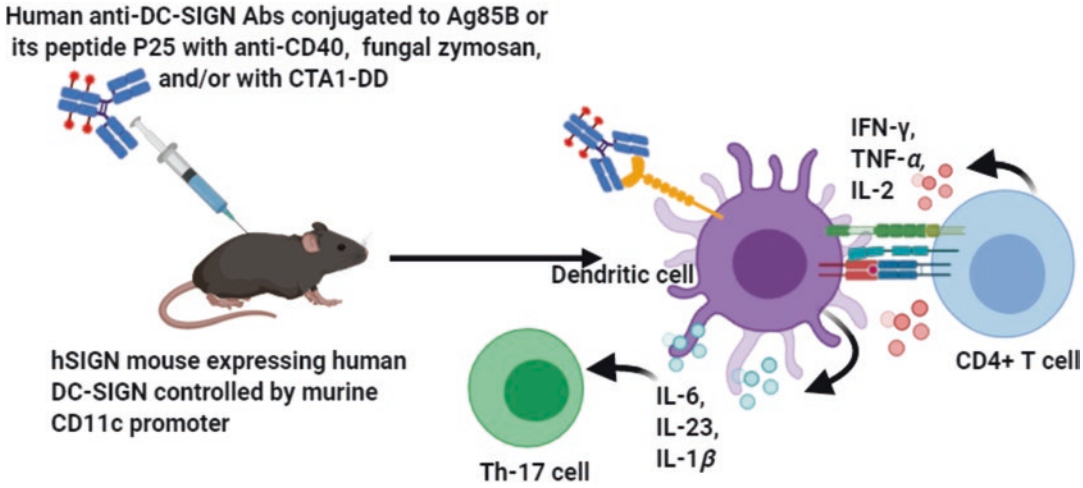

Fig. 10.5 Targeting DC-specific-ICAM3-grabbing-nonintegrin (DC-SIGN) vaccine against TB. Transgenic mouse model that expresses human DC-SIGN under the control of the murine CD11c promoter were used. Both *in vitro* and *in vivo* delivery of anti-DC-SIGN antibodies conjugated to Ag85B and peptide 25 of Ag85B in combination with anti-CD40, fungal zymosan, and CTA1-DD, was able to induce strong Ag-specific $CD4^+$ T-cell responses. Improved anti-mycobacterial immunity was observed with increased frequencies of Ag-specific IFN-γ^+ IL-2^+ TNF-α^+ polyfunctional $CD4^+$ T cells and further activating Th17 cells

Thus, there may be various *Mtb* vaccine possibilities, which can reduce pulmonary load, eradicate the pathogen, or keep *Mtb* at bay in granulomas (McShane and Williams 2014; Kaufmann et al. 2017a).

4.5 TB Vaccine, Innate Immune Adjuvants, and TLR Control of the Adaptive Immunity

Anti-mycobacterial protective innate immunity, augmented by BCG vaccination, is apparently distinct from that by other pathogens, in recognizing through its receptors including TLRs and NOD-like receptors, unique mycobacterial molecular pattern signature (Schnare et al. 2001). Such innate specificity is also valid for targeting TB vaccine adjuvants, augmenting phagocyte's capacity for killing intracellular *Mtb*. This host protective efficacy has been demonstrated in a mouse model (Coffman et al. 2010). For example, mice were immunized three times subcutaneously with recombinant antigen H56 (Ag85B-ESAT6-Rv2660c), in conjunction with adjuvants CAF01, GLA-SE, or $IC31^R$, which are based on mycobacterial Core factor. Such adjuvant vaccinated mice were subsequently challenged with aerosol *Mtb* Erdman. Their *Mtb* lung burden was reduced five-fold, obtained with CAF01 adjuvant, as compared with vaccination without adjuvant, and from Alum vaccine. Their PBMCs in culture upregulated secretion of IFN-γ, IL-6, IL-17 and TNF-α (Knudsen et al. 2016). Hybrid BCG- subset clinical vaccines also utilize these adjuvants. A study on DC responses to other pathogens, including influenza A virus, *Salmonella enteritidis* and *Staphylococcus aureus* unveiled that specific DC subsets for particular pathogen adaptive signature are unique in response to vaccine-adjuvants by employing distinct gene transcription cluster profiling. This strategy may improve an adaptive immune antigen specific vaccine-adjuvant formulation for efficacy, such as of Influenza A vaccine, Fluzone 09-10. In general, adaptive immunity is controlled by TLRs (Schnare et al. 2001; Banchereau et al. 2014). BCG vaccine itself appears to possess adjuvant properties, owing to its Core and other cell wall protein, which is sustained with its growth. It helps innate trained immunity-memory, and in augmenting

adaptive antigen-specific T helper cell immunity in pathogen response. This property may be seen in most BCG vaccinated individuals, on which subset vaccines rely (Kleinnijenhuis et al. 2012; Knudsen et al. 2016; Kaufmann et al. 2017b). Other chronic disease-causing pathogens such as malaria, HIV-1, and hepatitis C virus, based on adjuvant principles are also in clinical trials (Kaufmann et al. 2017b; Quattara et al. 2015; Richert et al. 2015; Pierce et al. 2016).

5 TB Vaccines in Clinical Trials, BCG and Recombinant BCG Neonate Immunity

5.1 Clinical TB Vaccine Development

A vaccine improvement against TB is regarded as the only affordable means to eradicate the disease worldwide. Drug cocktail treatment is beyond the reach of low-income population, living in TB endemic areas. *Mtb* strain drug resistance in various demographic areas hampers the success. There are several promising types of TB vaccines based on BCG, which are ongoing in clinical trials (Parkash 2014; Tang et al. 2016; Kaufmann et al. 2017a). BCG has been in practice since the 1920s, but is still the only licensed TB vaccine. It affords a partial protection of neonates/infants from a severe TB, such as meningitis and *Mtb* disseminated miliary TB, but is less efficient against pulmonary disease. Its prophylactic effects wane towards adulthood (Triccas and Counoupas 2016; Kaufmann et al. 2017a). To improve BCG vaccination, various types of subset *Mtb* antigen-specific vaccines have been included, based on BCG pre-vaccination. There have been successes with infants in boosting adaptive Th-cell anti-bacterial protein secretion, which is critical in *Mtb* safe containment, but were less protective in neonates/infants.

BCG vaccination-dependent subset antigen-specific vaccines have been mostly constructed to target dominant immunogenic *Mtb* virulence antigens including ESAT-6, which prevailed in vaccine clinical candidates, based on adaptive immunity. Some examples of such TB subset vaccines are recombinant viral vectors, fused with *Mtb* antigens, such as Ag85A, incorporated in recombinant modified vaccinia Ankara vector (MVA85A). Ag85A is a mycolyl transferase, involved in formation of mycobacterial cell wall (McShane et al. 2004). Further examples are, Ag85B subset vaccine, which is a fused adenosine viral vector with Ag85B, and the TB-FLU-04K replication-deficient Influenza virus vaccine, also fused with Ag85A. Further subset vaccines are hybrid/BCG vaccines.

5.2 Hybrid/BCG Vaccines for Adult Population

Hybrid fusion subset vaccines are mycobacterial signature distinct commercial adjuvants, fused with various *Mtb* virulent antigens such as ESAT-6 and TB10.4. For example, hybrid H1, H4, and H56 vaccines entail the adjuvant IC31^{R} fused with ESAT-6, or Ag85B; TB10.4 or Ag85B; and H1+ RV2660v- bacillus dormancy antigen, respectively. H1 also covers H1-CAF01 adjuvant system. IC31 is a cationic mycobacterial peptide, an agonist for phagocyte intracellular TLR7 and TLR9, as it is also the cationic liposome-immunoregulatory CAF01. Their effects were apparently independent from specific antigen Th cell responses, which may also activate innate phagocytes for lung protective responses against *Mtb* infection, as indicated by such TB distinctive adjuvants in murine model. These commercial adjuvant effects were based on mycobacterial adjuvants (Knudsen et al. 2016). Other vaccine adjuvants GLA-SE and ASO1E engage TLR4 (Kaufmann et al. 2017a). Thus, BCG subset hybrid vaccines strongly augment adaptive Th cell anti-bacterial cytokine secretion such as IFN-γ, TNF-α and IL-β, which are vital in *Mtb* containment and latency regulation. Concomitantly, the hybrid vaccines may apparently can also strongly activate innate immunity, which may be lung protective in infants and adult, pre-vaccinated with BCG. C31^{R} and CAF01 recombinant antigen vaccination were first tested in volunteers vaccinated previously

with BCG, or without BCG, respectively, or having latent TB, which responded with a robust adaptive cell immunity (van Dissel et al. 2011, 2014). In a separate role, these adjuvants also improved immune function in elderly subjects (Knudsen et al. 2016).

5.3 Next Generation Vaccine Candidates for Neonate Protection

Next generation of TB vaccines have been introduced with the aim to immediately protect neonates and adults against *Mtb* infection *via* innate immunity, in addition to adaptive immunity. Such requisites are apparently met by the BCG- recombinant live vaccines such as BCGΔureC::hly/VPM2001, expressing *Listeria monocytogenes s*pore forming protein, listeriolysin O (LLO), instead of urease, catalysing urea-ammonia conversion, favouring intracellular pathogens (Kaufmann et al. 2017b; Nieuwenhuizen and Kaufmann 2018). VPM1002 is a prominent clinical trial candidate. It has fulfilled phase-I criteria for neonate-infant safety and its immunogenicity, and has now passed phase IIb stage in new-born-infant immune requirements, ahead of BCG subunit vaccines, and that of live *Mtb* attenuated recombinant vaccine MTBVAC. VPM1002 is expected to substantially reduce neonate-infant and adult lung *Mtb* burden, judging by its pre-clinical studies. It may eradicate *Mtb* in some individuals living in TB endemic areas, as it is apparently naturally acquired and maintained in TB resistant infants and adults. Neonate genetic resistance may be influential (Nieuwenhuizen and Kaufmann 2018; Kowalewicz-Kulbat and Locht 2017). In pre-clinical studies, VPM1002 strongly reduced *Mtb* pulmonary burden in mice lung infection over that by BCG. These effects also occurred in adaptive immunity deficient SCID mice, demonstrating an all innate cell macrophage immunity protection, consistent with clinical trial findings (discussed below). There are other pre-clinical-animal BCG-recombinant vaccines, some with TB protective efficacy, but apparently none seem to have been chosen yet for clinical trials (Nieuwenhuizen and Kaufmann 2018).

An inactivated whole cell non-TB mycobacterial protective vaccine (DAR-901) in adults, has also passed phase IIa clinical trial (Kaufmann et al. 2017a; Nieuwenhuizen and Kaufmann 2018). Therapeutic inactivated whole cell vaccines include *M. indicus pranii* (Mw), *M. vaccae* (Vaccae) and *Mtb* (RUTI) (Groschel et al. 2014; Tang et al. 2016).

6 Progress and Challenge of TB Vaccines

In a recent international statement on progress and challenge of TB vaccines, it has been recommended that viable or inactivated whole-cell mycobacterial vaccines remain central in TB vaccine development (Voss et al. 2018). In addition, a search for biomarker profiles is needed, which may detect in people TB infection, its stages, and may predict progression to pulmonary active disease, i.e., evaluate people who are at risk to contract TB infection, or to develop a clinical TB. Knowledge of TB biomarkers may accelerate TB vaccine trials (Petruccioli et al. 2016; Kaufmann et al. 2017b; Voss et al. 2018). In order to detect and evaluate some biomarker profiles, whole blood QuantiFERON analysis has been used in a wide evaluation study with African population as classified for TB disease, and other respiratory disease (ORD). It comprises biomarker signature of un-stimulated levels of 12 biomarkers, including IFN-γ, macrophage inflammatory protein (MIP-1β), and TNF-α, levels of antigen specific TGF-α, and VEGF (Chegou et al. 2018). Some biomarkers can predict in TB latent people risk to their progression into active TB, spreading the disease through cough aerosol inhalation. Between these stages, there is a continuum pathogen/host activity, requiring different markers and treatment. For example, blood RNA sequence profile signature can predict in people at risk for a year ahead of clinical TB (Petruccioli et al. 2016). In a case-control study in South Africa with infants and adolescents, which were pre-BCG vaccinated post-partum, and subse-

quently vaccinated with MVA85A vaccine, it was revealed that Th cells activated by the vaccine were important immune collateral, prognostic biomarkers in predicting the outcome of TB infection. Expression of their *ex-vivo* blood lymphocyte non-specific HLA-DR^{+}CD4^{+} T cells was an increased risk marker for clinical TB. On the contrary, induction of antigen-specific Th cells expressing INF-γ, by MVA85A vaccine, was associated with a decreased risk for active TB. These collateral effects have been recommended for inclusion in TB vaccines (McShane et al. 2014; Fletcher et al. 2016).

Large cohort studies, independently with South African and Gambian healthy adolescents, but infected with latent TB, found that persons at risk to progress into clinical disease can be identified by sequencing whole blood RNA. In this analysis, 16 gene expression signature profile specifically corresponded to "progressors" over a period of 2 years. It has been suggested that such people, known to be at risk of clinical disease, may be then treated with drugs against TB. It was a percentage wise low number of people at clinical TB risk, but with a specificity of 80.6%, preceding in 12-month clinical TB. By closing the circle of TB spreading, such selection may strongly contribute towards decline of TB in population, even in endemic TB regions, together with TB vaccines (Zak et al. 2016). A further recommended prerequisite is the inclusion of *Mtb* challenge, and host protection in preclinical vaccine studies, which is likely to foretell their bacterium killing efficacy (Kaufmann et al. 2017b; Voss et al. 2018).

Apparently, the route of BCG vaccination matters. Current BCG based vaccines are administered by intradermal route, but other vaccination routes may be more host protective. For example, mice vaccinated with BCG intratracheally, induced mucosal infiltration of memory T cells, which strongly protected their lungs against *Mtb* infection. It reduced their *Mtb* burden upon aerosol *Mtb* challenge. This was comparable with their reduced lung pathology, in contrast with subcutaneous vaccination and control. Perhaps mucosal innate immune macrophage-mediated BCG ingestion, and Natural Killer (NK) cell activation were also engaged as effectors. A parallel study indicated that such BCG vaccination-driven host protection depended on IL-17 expression (Aguilo et al. 2016). Finally, intravenous BCG vaccination targeting haematopoietic stem cell system in C57BL/6J wild-type mice seems to be a compelling candidate for clinical trials. This system is connected to primordial protective immunity for its functional persistence, and for its substantial pulmonary protection as well as reduction of *Mtb* burden following *Mtb* aerosol challenge. This protection also occurred in mice with T cell-depleted bone marrow (Kaufmann et al. 2018).

Here, as examples, a hybrid subset vaccine, and two recombinant live TB vaccines, are discussed.

6.1 H4:IC31 Hybrid Subunit Fusion Vaccine for Adults

The fusion subunit vaccine H4:IC31 (AERAS-404) is composed of *Mtb* antigens Ag85B protein fused with TB10.4 protein (a member of virulent ESAT-6) in combination with immunological adjuvant IC31®. Safety and immunogenicity randomised trials were conducted on healthy male and female individuals in Sweden and Finland, which were previously vaccinated with BCG, as compared with persons which did not receive BCG vaccine. H4 vaccine was administered by intramuscular injection in escalated doses for the optimal safety and immunogenicity vaccine-adjuvant dosage. Immunogenicity was assessed by IFN-γ production by participant PBMCs and by antigen-specific Th cell expansion which lasted for 18 weeks. Participants experienced mainly mild adverse reactions. Such an optimized vaccine is needed for the potential TB prevention, or amelioration in clinical trials in TB endemic areas. It has been registered under NCT01861730 (phase I and phase II, infants and adults, respectively; Norrby et al. 2017). Previously, in phase I vaccine trial, the authors showed that the subset Ag85B-ESAT-6 antigen fusion vaccine together

withIC31^{R} adjuvant promoted a powerful long-lived *Mtb* antigen specific T cell responses which in naive volunteers, after they had been vaccinated with BCG, or harbouring a latent TB. H4-IC31^{R} was administered at (O) and in 2- month time. After second vaccination, the immune response was augmented, suggesting immunological T cell antigen-specific memory. Anti-H4 vaccine antibodies increased sharply after the second 2 months vaccination, and declined gradually after 15 weeks, which could agree with a humoral B cell memory (van Dissel et al. 2011). The adjuvant IC31® is composed of a leucine rich peptide KLK, and a synthetic bacterial DNA analogue (Winslow et al. 2008). H4-IC31^{R} promoted Th1 responses by producing IFN-γ, TNF-α and IL-2, which are vital in anti-*Mtb* adaptive immunity. For this effect, H4-IC31^{R} may involve intracellular TLR7 and TLR9 signalling pathways, including NF-κB activator of pro-inflammatory cytokines (Schellack et al. 2006).

6.2 Recombinant MTBVAC

A new promising candidate live vaccine is the recombinant *Mtb*, which is a genetically attenuated *Mtb* lineage-4 clinical isolate, MTBVAC. It has fulfilled in a randomised double blind controlled clinical phase I trial as per Geneva consensus requirements in terms of human safety and immunogenicity comparable to BCG. In pre-clinical studies with mice, MTBVAC exhibited an augmented reactogenicity and protective immunity, improving upon BCG, in killing *Mtb* in their lung infection (Aguilo et al. 2017). It also retains virulence gene region of difference-1 (RD-1), coding for ESAT-6. However, it has two engineered independent, stable gene deletions, lacking antibiotic-resistant markers, i.e., coding for *phoP* and *fadD26* genes. *phoP* is a critical transcription factor for *Mtb* antigen expression. *fadD2*6 regulates synthesis of cell wall complex lipids, phthiocerol dimycocerosates (PDIM), which are major mycobacterial virulence factors (Arbues et al. 2013; Spertini et al. 2015; Triccas and Counoupas 2016).

MTBVAC is based on the *Mtb* strain 4 clinical isolates, since *Mtb* lineage 4 is most widespread globally, apart from geographically restricted sub-lineages. Such distribution may make MTBVAC isolate 4 strain more universal. Lineage 2 (Beijing strains) are also widely distributed, which may add to lineage 4-based vaccines efficacy (Stucki et al. 2016). Similar to BCG, in vaccinated neonates, MTBVAC-lipoproteins may induce M1 macrophage protective response against primary *Mtb* infection (Brightbill et al. 1999). If not cleared, their innate delayed reactogenicity may further reduce their lung burden, together with adaptive Th1 antigen immunity. MTBVAC is expected to be more host protective than the BCG-subset vaccines, by potentially targeting most of the parental *Mtb* virulence strain antigens. MTBVAC is undergoing clinical phase I trial in children, and phase IIa trial in adults (Clinical identifier NCT02729571).

6.3 Recombinant BCG VPM1002 Vaccine

Listeria monocytogenes phagosome-lysosomal escape mechanism into macrophage cytosol is a role model, of which TB vaccines have taken advantage in building a recombinant BCG vaccine candidate against *Mtb*, designated VPM1002 (Farinacci et al. 2012; Grode et al. 2013). VPM1002 (rBCG) vaccine is a BCG recombinant re-construct, expressing listeriolysin protein (LLO; encoded by inserted *hly*) gene, but is devoid of urease C, and contains a hygromycin resistance marker, designated rBCG *ΔureC: hly* HM^{R}. In pre-clinical mice evaluation, rBCG upgraded efficacy of BCG in apoptotic and infected macrophage cross-priming by DCs of CD4 and CD8 Th cells (Farinacci et al. 2012). Similarly, rBCG mice vaccination improved five-fold over that by pBCG in terms of their lung protection against aerosol *Mtb* infection (Saiga et al. 2015). LLO is a cholesterol binding pore forming protein of phagosome membrane, allowing escape of mycobacterium and its constituent, including the bacillus DNA and antigen release into cytosol. rBCG-DNA, in turn, activated the

cytosolic inflammasome, inducing in BCG/*Mtb* infected macrophage expression of caspase 3 and 7 to induce their apoptosis. For these LLO effects, an acidic pH of phagosome is needed, hence, the deletion of urease C (Δ*ureC)* gene of paternal BCG. Urease C catalyses conversion of urea into ammonia, which favours *Mtb* intra-macrophage habitat. rBCG vaccination upregulated *Mtb*-infected macrophage apoptosis, and strongly augmented IL-12 and cofactor expression in DCs for antigen cross-presentation to Th cells, secreting antibacterial cytokines such as IFN-γ, IFN-β and TNF-α. IFNs and TNF-α appear to be preferred effectors against intracellular pathogens. Invariant innate T cells and innate γδ T cells producing IFN-γ were also observed. rBCG also substantially improved in mice their lung protection from TB as compared with paternal BCG, suggesting that innate phagocyte immunity recognising bacterial molecular signature was also potently augmented. Thus, rBCG improved innate and adaptive immune efficacy against TB, making it a compelling candidate for clinical trial (Farinacci et al. 2012; Grode et al. 2013).

VPM1002 was evaluated in randomised clinical trial for safety, immunogenicity and reactogenicity. Healthy male volunteers were enrolled into phase 1 open dose escalation trial (Grode et al. 2013). They were vaccinated with a single intradermal dose of rBCG in arm deltoid area. Like with paternal BCG, intradermal rBCG vaccination may mount an immunogenic skin pustular-blister response in BCG naïve uninfected individuals (Hoft et al. 1999). The participants to be vaccinated with rBCG were divided into BCG naïve group, i.e. non-responders to PPD skin reaction, and into group vaccinated with BCG, PPD-reactive individuals. In each group, there were dose escalated subgroups to optimize their safety and reactogenicity. Participants were assessed for IFN-γ and TNF-α production for their immune cell marker responses, and for serum antigen-specific anti-mycobacterial antibodies. VPM1002 was found to be well-tolerated, with some adverse effect, being more pronounced in BCG-naïve group. rBCG vaccine was substantially immunogenic regarding adaptive cell responses, as assayed on post-vaccination day 29, 57, and 180 by IFN-γ, TNF-α and IL-2 production and blood monocyte ELIspot parameters, including differentiation of oligo-functional Th cells (Grode et al. 2013). At the time, VPM1002 vaccine was undergoing clinical trials with children and adults. Its clinical identifiers are NCT01479972 (Phase I) and NCT02391415 (phase IIa), respectively, available on www.clinicaltrials.gov. Presently, the vaccine has passed phase IIb clinical trial (Nieuwenhuizen and Kaufmann 2018).

In parallel, VPM1002 pre-clinical studies included its host protective efficacy in murine model, even after *Mtb* H37Bv strain lung aerosol infection, in simulated chronic infection latency, reduced by antibiotic treatment to a low basal level. In both models, rBCG subcutaneous vaccination was superior five-fold, over that by paternal BCG vaccination, as measured by their substantial reduction in lung and spleen *Mtb* burden. These findings are seen as encouraging for protection of people in TB latency in vaccination with rBCG (Gengenbacher et al. 2016a).

6.4 rBCGΔureC::hly ΔnuoG rBCG Vaccine

The protective capacity of the rBCG vaccine against TB was further strongly augmented in mice (five-fold over rBCG, and altogether ten-fold over BCG) by deletion of BCG's another virulence gene, the *nuoG* gene, designated rBCG *ΔureC::hly ΔnuoG* vaccine. *NuoG* gene encodes for subunit G in respiratory NADH complex dehydrogenase I, which as a virulence gene, inhibited apoptosis of *Mtb*-infected phagocyte via TNF-α pathway. Its deletion in rBCG further activated AIM2 inflammasome for induction of autophagy and apoptosis for Mtb/rBCG degradation in proteasome (Saiga et al. 2015). A lack of *NuoG* rBCG strongly promoted DC-mediated antigen presentation *via* MHC-1 and CD1 to T cells against TB (Gengenbacher et al. 2016b; Schaible et al. 2003). Expression of autophagy marker, LC3, was upregulated by this rBCG vaccine, which is a new finding of *nuoG* effects, known to be affected by ESAT-6. The vaccine

upgraded clearing of cytosol BCG-*Mtb* apoptotic debris including DNA, may activate together AIM2 inflammasome and GTPases, dependent on INF-γ pathway. Owing to further attenuation, it also improved mice safety. Thus, BCG *ΔureC::hly ΔnuoG* vaccine is considered to be a promising candidate in next generation vaccines for clinical trials, with improved protection of neonates-infants against pulmonary TB (Farinacci et al. 2012; Saiga et al. 2015; Nieuwenhuizen and Kaufman 2018). Attenuated rBCG vaccine may be tolerated by immunocompromised individuals e.g. those who are HIV-1 infected, as simulated by T cell-depleted mice.

6.5 BCG Vaccine as an Adjuvant Model

BCG attenuated vaccine appears to be a general, primordial, potent innate immunity-potentiating adjuvant, through its mycobacterial cell wall core factors, lipoglycans, lipomannans and DNA molecular pattern, recognized by various innate immune PRRs. For example, TLR signalling *via* MyD88 adaptor protein induced in macrophages an immediate M1 polarization, producing inflammatory bactericidal cytokine such as IL-1β, IL-18, IL-6, TNF-α and IFN-γ, in response to microbial infection (Schnare et al. 2001). M1 macrophage polarisation is opposed by intracellular *Mtb* infection shifting to anti-inflammatory M2 phenotype, secreting IL-4, TGF-β and IL-10 for its survival. M2 polarisation is accompanied by expression of *Mtb* ESX-1 and its substrates ESAT-6, CFP-10, and EspB. ESAT-6 disrupts infected phagosome membrane to translocate *Mtb* material to cytosol, a process considered to be a key mechanism in pathogenicity (Wong 2017; Houben et al. 2012). In cytosol, ESAT-6 and EspD proteins interfere with innate immune mechanisms mediated by receptor signalling via TLRs and inflammasome-AIM2-NOD like receptors (Man and Kanneganti 2015). ESAT-6 inhibits TLR signalling via MyD88 and other adaptors, and via NF-κB gene transcription regulator. For the pathogenic effects of ESAT-6 on macrophages, TLR2 and Akt-phosphoinositide 3-kinase pathway are strictly required, making them a pharmacological target (Pathak et al. 2007; Cantley 2002). EspB blocks efferocytosis of infected macrophages and their autophagy, likely through binding phospholipids. Apparently, phagosome membrane disruption also impairs its maturation and lysosome fusion (Wong 2017). Another distortion in *Mtb*-infected macrophage is pathogenic IFN type-1 mediated necrosis, instead of IFN-γ induced apoptosis. Necrosis occurs in active lung TB rupturing granulomas-damaging for host tissue, but sparing *Mtb* dissipation and proliferation. This pathogenic event may depend on a high MOI number (Wong and Jacobs Jr 2013). Most of *Mtb* cytosolic translocations occur on 2nd -3rd day after infection. BCG live vaccines may be able to forestall these pathogenic events in most of susceptible individuals. Nearly a half of new-born and adult population, are already naturally protected against TB, living in endemic community.

As mentioned above, rBCG live vaccines are deleted of one or two virulence genes. These are rBCG *ΔureC::hly* HM^R and rBCG *ΔureC::hly ΔnuoG* vaccines, coding for urease C catalysing urea conversion into ammonia, and coding for an inhibitor for phagocyte apoptosis, respectively. Interestingly, in mice, such vaccines have strongly augmented paternal BCG adjuvant immunity. This property may suggest a vaccine's numerical addition against *Mtb* virulence factors. Both vaccines possess genome inserted gene of LLO. As such they may forestall and outcompete numerically and time-wise the *Mtb* ESAT-6 primary infection or re-infection. They may, in many cases, augment their translocation of various exposed adjuvant molecular pattern to activate cytosol innate immune receptors, and to polarise M1. They may mount an immediate strong conserved immunity, to eradicate the *Mtb*. These effects may be elicited through apoptosis of infected macrophage, their efferocytosis and autophagy by non-*Mtb* infected macrophages and DCs. The intra-macrophage infection level of BCG vaccine is continuous compared to *Mtb,* which may oscillate at very low levels (Wong 2017). Such upgraded BCG vaccines may also non-specifically confer protection against pneu-

monia in infants from various microbes and viral infection in TB endemic areas (also reported in France).

7 Concluding Remarks

TB continues to be a major pandemic worldwide, but its conquest is on the horizon. It has emerged that protective innate immunity against intra-macrophage-dwelling mycobacteria, including *Mtb*, is unique. *Mtb* distinct molecular pattern/signature is recognised by macrophage flexible TLRs in conjunction with IL-1βR, as well as intracellular TLR7, TLR9, NOD-like receptors and AIM2 inflammasome receptors. They transmit signalling through NF-κB for *Mtb* signature to be epigenetically imprinted in chromatin. They may be augmented by innate trained immunity and memory. A deciding battle ground appears to be apoptosis of infected macrophages, to be taken up through efferocytosis by non-infected macrophages for their autophagy, to be degraded in proteasomes together with cellular debris pyrocytosis. These processes are opposed/blocked by phagosome-resident *Mtb*. Next generation of prophylactic TB vaccines, based on recombinant attenuated BCG, have been constructed to overcome the *Mtb* blockade.

There are several recombinant (rBCG) vaccines in pre-clinical murine model stage candidates, but BCG *ΔureC::hly* live vaccine is currently the only candidate vaccine in clinics, which has passed phase IIb criteria in clinical trials with neonates and adults in TB endemic areas in South Africa. It is supported by pre-clinical trials in mice showing strong lung protection, five-fold over paternal BCG, as evaluated by parallel reduction in lung *Mtb* burden upon *Mtb* aerosol infection. It is also safer as compared with paternal BCG vaccine. An advanced rBCG vaccine in pre-clinical trials is regarded to be BCG *ΔureC::hly ΔnuoG* TB vaccine, in which two virulence genes have been deleted. Urease C converts urea into ammonia which is compatible with intercellular microbes. *nuoG* is an anti-apoptotic gene. It is remarkable that such gene deletion in BCG went in steps with reduction of *Mtb* burden in mice lungs, now ten-fold over BCG. Interesting is the insertion into BCG of hly gene, encoding pore-forming LL0. LL0 allows mycobacteria and their proteins, lipids and DNA leakage into cytosol, sensed by activated AIM2 inflammasome receptors, which in turn, may induce apoptosis and autophagy in infected macrophages. In this way, these vaccines may substantially upgrade neonates-infants lung protection, which is only partial in comparison with paternal BCG vaccination. These vaccines also strongly upregulated adaptive Th1 immunity by secreting IFN-γ, TNF-α and IL-2, owing to an improved DC-mediated cross priming of naïve T cell.

It has been recommended in F1000Reseach 2018 report that vaccine candidates should be viable, possessing the *Mtb* killing capacity in pre-clinical tests. Apart from rBCG-VPM1002, several pre-clinical vaccines also have the pathogen killing capacity. These include intravenous BCG vaccination to target bone marrow haematopoietic stem cells. It was tolerated by non-human primates, and is likely to fulfil human safety requirements. Another candidate is saprophytic, fast replicating *M. smegmatis* recombinant live vaccine, providing it is safe for neonates in clinical trials. From such candidate collection, other most effective neonate and adult lung protective vaccines against TB can be selected for clinical trials.

There is a requirement for better TB disease markers in discerning from other lung diseases of people living in TB environments, such as in clinical studies using whole blood QuantiFERON analysis. Further, TB disease stage marker correlates could be employed. This is achieved by selecting a number of immune distinct gene profiling (more than 12), which could correlate as a marker with a particular TB stage. For example, one marker could predict in a minority of people in latency at risk, a year ahead, to develop pulmonary active disease, i.e. in 5–10% of infected people living with non-clinical TB latency. Such people may be cured with antibiotics, thus closing the cycle of disease propagation in a TB endemic community. The recombinant modified vaccinia Ankara vector MVA85A vaccine in clin-

ical trials was able (McShane et al, 2014), as a prognostic marker, to distinguish people at higher risk for TB, from those with lower risk for the disease. Such marker studies may include infants and adults who are naturally resistant to TB infection. Perhaps in such resistant individuals, besides their genetics, such augmented conserved innate immunity owes to their constant challenging by various microbes, including *Mtb*. Various pathogens may act as natural adjuvants to upgrade constantly a certain level of conserved innate immunity, which is non-specific in such individuals (Sanchez-Ramon et al, 2018).

Thus, the likelihood of a protective vaccine against TB for children and adults has sharply increased, owing to a better understanding of the unique innate immunity of phagocytic cells against intracellular mycobacteria. New generation of recombinant BCG vaccines possesses a potent bactericidal capacity for protection of people living in TB endemic areas, as indicated in pre-clinical and clinical trials. TB vaccines, harnessing innate and adaptive immunity, are equipped to accelerate TB decline, so that with help of nutrient complementation to children, WHO projected TB incidence rate and death reduction by 2020–2030 can be met.

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Candida Pathogenicity and Interplay with the Immune System

11

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1 Introduction

Candida species are opportunistic fungal pathogens that are part of the normal human microbiota and usually acquired at birth through direct transmission. *Candida*, as a symbiont, resides on various sites including the skin, gastrointestinal and urogenital tracts as part of the large diverse microflora (Brogden and Guthmiller 2002). However, dysbiosis of microbiome including *Candida* causes infections in immunocompromised patients. *Candida* infections can vary in severity from superficial mucosal candidiasis to life threatening blood stream infection candidemia and invasive candidiasis, affecting multiple organs (Bertolini et al. 2019). Dysbiosis of the gut microbiota has also been associated with inflammatory bowel diseases and neurological disorders (Knox et al. 2019). The army of immune cells and molecules within a host are recruited upon infection, but a better understanding of pathogenesis and virulence factors of *Candida* spp. and the role play of immune system during infection is emerging. Though the host utilises a sophisticated and tightly regulated immune sensing system against *Candida* sp., the pathogen has cleverly adapted and evolved immune evasion strategies that are not yet fully understood. *Candida albicans* is the most prevalent species known to be the leading cause of fungal opportunistic infections. However, emerging *Candida* spp. such as *Candida tropicalis, Candida auris*, *Candida parapsilosis* and *Candida glabrata* are leading to higher morbidity (Gonzalez-Lara and Ostrosky-Zeichner 2020). Higher mortality rates are also seen with *Candida* infections as they are becoming increasingly difficult to treat. Antifungal treatment is available, however, emerging antifungal resistance and a global shift in *Candida* spp. pose challenges for successful treatment (Ksiezopolska and Gabaldón 2018). Therefore, a deeper insight of pathogenesis, virulent factors and mapping out of immune evasion strategies of various *Candida* spp. are required for formulating novel therapeutics to combat *Candida* infections.

1.1 Candida Infections

Candida spp. are the most common cause of fungal opportunistic infections causing candidiasis. They are found as part of the human normal flora, residing on various body sites such as mouth, skin, throat, gastrointestinal (GI) tract and vagina. Overgrowth of *Candida* causes infection, where candidiasis of the mouth and throat is referred to as oropharyngeal candidiasis, vaginal candidiasis

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as vaginal thrush and deep penetration into the internal organs such as heart, kidney, brain and eye is referred to as invasive candidiasis, of which bloodstream infections are known as candidemia (Calderone and Clancy 2012). *C. albicans* is the predominant cause of invasive candidiasis. However, epidemiology of *Candida* infections has changed over the years and a shift in clinical important *Candida* spp. towards non-*albicans* species has been reported by several studies. *C. albicans* still remains the most well-studied *Candida* species. *C. albicans* is a polymorphic fungus that has many virulent factors contributing to its pathogenesis in *Candida* infections. Factors such as adhesion to the host, formation of biofilm and secretion of hydrolytic enzymes allow the pathogenic fungus to penetrate and invade humans as hosts (Mayer et al. 2013).

Candidiasis causes a wide range of symptoms from white/red itchy patches at the site of infection to life threatening and fatal situations depending of the infection severity. Immunocompromised individuals such as HIV patients and patients undergoing chemotherapy for cancer or transplant surgeries are more prone to infections due to *Candida* being an opportunistic pathogen. Invasive candidiasis and candidemia in particular has a high mortality rate. Invasive candidiasis affects more than 250,000 people worldwide and causes more than 50,000 deaths every year (Kullberg and Arendrup 2015) of which Candidemia has mortality rates of 30–40% depending on various factors including the treatment, the types of *Candida* spp. involved and severity of the infection (Arendrup 2010). In the UK, the number of candidemia cases reported were 3.6 per 10,000 from laboratory surveillance carried out by Public Health England in 2017. During this surveillance it was also found that candidemia rates were higher in females and elderly population (Public Health England 2017). *Candida* spp. are also the main cause of nosocomial infections with several studies carried out across the world stating higher incidence rates in intensive care unit wards (Eyre et al. 2018; Chakrabarti et al. 2014; Aldardeer et al. 2020). Currently, there are 3 main antifungal treatments used which are azoles, polyene and echinocandins. They work by destroying the cell membrane, leaking out amino acids, water-soluble substances and preventing chitin and glucan crosslinking, that strengthen the cell wall (Campoy and Adrio 2017). Furthermore, emergence of *Candida* spp., increased rates of infection and antifungal resistance has a huge socio-economic impact due to longer hospital stays and increased health care costs.

Less fatal but more common vulvovaginal candidiasis (VVC) is also a global issue. In healthy females VVC affects between 70 and 75% of women of childbearing age, and it is estimated that 40–50% will experience a recurrence (Rodríguez-Cerdeira et al. 2019). Recurrence of 4 or more episodes a year is defined as recurrent vulvovaginal candidiasis (RVVC) and effects 2–10% females round the world (Cassone and Sobel 2016; Matheson and Mazza 2017). *Candida* found at the vaginal mucosa is a commensal species of the microbiota, living symbiotically with bacterial species including those that commonly cause urinary tract infections (UTI). However, factors such as pregnancy, usage of antibiotics and changes in the host environment could lead to dysbiosis. One reason for such high incident rates in females (Foxman 2002) is the complex microbial colonisation of the lower female genital tract (Larsen and Monif 2001) along with the anatomy and physiology female urinary tracts in comparison to males which also makes females prone to UTIs (Finer and Landau 2004).

1.2 Inflammatory Diseases

Candida spp. are found in the gastrointestinal (GI) tract in a friendly yeast form (Böhm et al. 2017). Studies using animal models suggest colonisation of *Candida* spp. in the gut prolongs inflammatory healing. *Candida* gut commensalism is mediated by multiple factors, either intrinsic such as fungal gene regulation, morphology and host immune status or extrinsic such as host diet, usage of antibiotics, stress and presence of other diseases (Nobile and Johnson 2015). From studies carried out in mice, it is thought that

imbalance in homeostasis of the normal intestinal flora leads to aberrant immune response. There is emerging evidence that such dysbiosis and overgrowth of *Candida* in the intestine can be associated with inflammatory bowel diseases such as Crohn's disease (CD) (Hoarau et al. 2016). There is increasing evidence of genetic similarity between *C. albicans* isolates from the bloodstream and rectal isolates, suggesting gut colonisation increases the risk of disseminated candidiasis in mice (Prieto and Pla 2015; Miranda et al. 2009). Biofilms comprising either *C. albicans* or *C. tropicalis* in association with other microbial species have been observed in patients suffering from CD (Richard et al. 2015). As CD is a multifactorial disorder and exact cause is unknown, with the present evidence, it can be hypothesised that constant activation of the immune system is one of the factors. The pathogenic fungi promote or maintain inflammation, leading to the onset of CD. One way in which the inflammatory response is thought to be maintained is *C. albicans* mimicking epitopes of antiglycan antibodies to generate aberrant immune response (Standaert-Vitse et al. 2006).

Candida also plays a role in neurological disorders, for example, a strong link has been found between the gut microbiota, neurodevelopment and behaviour. Dysbiosis of the gut microflora has been found in patients with neurological disorders such as autism spectrum disorder (ASD), schizophrenia and bipolar disorder. Such disorders cause impairments in social interactions, communication and behaviour. Research evidence reveal the association of fungal overgrowth to such neurological disorders. When probiotics were used to modulate the gut microbiota to reduce the over growth of *Candida*, it was shown to correlate with decreased incidents of neurological disorders (Romeo et al. 2011). The mycobiome gut-brain axis (GBA) model could help explain this but has not yet been well studied. In patients with ASD, increased C*andida* abundance due to alteration in the gut mycobiome has been noticed. *Candida* here plays a major role as alteration in the microbiome and blood brain barrier permeability which triggers an immune response causing systemic inflammation (Enaud et al. 2018). *Candida* species are also associated with increased chances of developing multiple sclerosis (MS) (Benito-León et al. 2010). MS is a chronic inflammatory disease of the central nervous system and has strong correlation with fungal infection (Pisa et al. 2011). Patients with MS are found to have fungal infection and higher titres of anti-*Candida* antibodies (Ramos et al. 2008). Medically important *C. albicans* can cause demyelinated lesions in MS patients, suggesting fungal infections playing a role in immune disorders for development of MS (Amri Saroukolaei et al. 2016). One suggested theory is secretion of fungal proteases that damage the surrounding tissues, causing inflammation (Amri Saroukolaei et al. 2016).

2 *Candida* Species

The genus *Candida* includes about 154 species. Though *C. albicans* is believed to be the most common clinical isolate, there has been a global shift in the *Candida* species. Several non-*albicans* species have recently been found to be emerging *Candida* pathogens. Common non-*albicans* species reported are *C. glabrata*, *C. tropicalis*, *C. auris, C. parapsilosis* and *Candida krusei*, and less frequently reported*; Candida guilliermondii*, *Candida lusitaniae*, *C. kefyr*, *C. inconspicua*, *C. rugosa* and *C. dubliniensis* (Sanguinetti et al. 2015; Vitális et al. 2020; Peremalo et al. 2019; Heaney et al. 2020; Mäkinen et al. 2018). Some of the clinically important *Candida* spp. are discussed here.

2.1 *Candida albicans*

C. albicans is the most common cause of *Candida* infections in immunocompromised patients around the world (Berman 2012). *C. albicans* lacks a complete sexual cycle but exists as yeast, hyphal, and pseudohyphal form. It can go through morphological switching when forming pseudohyphal or true hyphae by cell elongation and budding (Whiteway and Bachewich 2007). This shift is initiated by changes in the

environment such as pH, temperature, CO_2 level and nutrients (Sudbery 2011). The hyphal form is known for penetrating and damaging the host epithelial cells to establish an infection in different organs such as kidney, brain and spleen (Zuza-Alves et al. 2017). Another unique morphological characteristic of *C. albicans* is 'white-to-opaque epigenetic switching' (Ramírez-Zavala et al. 2008; Zordan et al. 2006). The two types of cells, 'white' and 'opaque' vary in morphology, mating behaviour, metabolic state and most interestingly interactions with host immune system. The switching happens stochastically and allows *C. albicans* to escape from specific host defence mechanisms (Sasse et al. 2013; Lohse and Johnson 2009).

Phenotypic switching causes changes in the cell wall of the fungi which allow the hyphal form of *C. albicans* to express numerous hydrolases for adhesion onto the host such as Secreted aspartyl proteases (Saps), lipases and phospholipases (*discussed in Sect. 3.3*) (Moyes et al. 2015; Jain et al. 2008). These proteins contribute to adhesion and colonisation to multiple types of host tissue to cause an infection. The most commonly used diagnostic method to detect *Candida* is by growing culture from patients' samples along with microscopic examination. However, this is not suitable when accurate rapid diagnosis is required for serious infections such as systemic or bloodstream candidemia. As *C. albicans* is a commensal coloniser, diagnosis using antibody detection is complex in such cases due to the presence of pre-existing antibodies, the levels of which can vary between individuals. Also, antigens derived from *C. albicans* are rapidly removed by the host and thus minimising the scope of detection. Molecular techniques such as polymerase chain reaction (PCR) to diagnose a larger number of patients with invasive candidiasis have been useful. Development of novel diagnostic method is still needed as even molecular techniques with high sensitivity and specificity sometimes lack reproducibility and have not yet proven to be ideal for large scale clinical implementation. Standardisation and reliability of molecular methods for identification of fungal pathogens requires further improvement for large scale clinical diagnostic use (Arvanitis et al. 2014).

2.2 *Candida tropicalis*

C. tropicalis in known as one of the commensal non-*albicans* emerging opportunistic pathogen. It is commonly isolated in tropical countries and with India having a tropical climate, *C. tropicalis* is the most common cause of nosocomial candidemia (Kothari and Sagar 2009; Kothavade et al. 2010; Mathews et al. 2001). *C. tropicalis* is found as part of various candidiasis including vaginal, mucosal and invasive. Some studies show *C. tropicalis* is more invasive than *C. albicans* in the human intestines of some individuals, especially oncology patients (Yesudhason and Mohanram 2015; Walsh and Merz 1986). Previously, *C. neoformans* was the only *Candida species* found associated with chronic mucocutaneous candidiasis but now *C. tropicalis* isolates have also been reported (Dixon et al. 2004). Bone marrow and acute leukaemia immunocompromised patients with GI infections are more prone to invasive candidiasis and many clinical isolates from these patients were found to have high abundance of *C. tropicalis (*Sandford et al. 1980*). C. tropicalis* is a diploid dimorphic yeast, often found as budding cells or in short chains and clusters as it is very rarely found as true hyphae. As it is difficult to distinguish between colonies of different C*andida* species by macroscopic morphology or growth rate, commercial chromogenic agars are available that allow *Candida* spp. to be distinguish based on colour. Currently, in-house PCR is the diagnostic method of identification. Antifungal drugs such as amphotericin B, fluconazole and echinocandins are used as treatment option. However, treating *C. tropicalis* abundant infections are becoming more challenging due to resistance of some strains to these antifungal drugs (De Barros et al. 2018). Many factors are contributing to *C. tropicalis* emergence and increase in antifungal resistance. For example, immunocompromised patients at risk for invasive fungal infections are also at risk for serious

bacterial infections for which, antifungal drugs and antibiotics are used as treatment for synergistic effects. In addition, long term catheter users are prone to bacterial and *Candida* infections, to whom antibiotics and antifungals are frequently prescribed. Such overexposure can lead to genetic changes in Candida resulting in development of antifungal resistance (Kojic and Darouiche 2004).

2.3 *Candida auris*

C. auris was first found in 2009 from a suspected ear infection in Japan. Since then, outbreaks have been reported in numerous hospitals around the world (Schelenz et al. 2016; Rhodes and Fisher 2019). Unlike *C. albicans*, *C. auris* can colonise on the skin as normal flora and can cause invasive candidiasis, in which the blood stream, the central nervous system, kidneys, liver, spleen, bones, muscles, joints, or eyes are invaded (Yue et al. 2018). *C. auris* demonstrates thermotolerance by growing optimally at 37 °C, but can maintain viability up to 42 °C allowing it to tolerate the fever response. Salt tolerant property of *C. auris* suggests hypersaline rivers and oceans as possible reservoirs but, so far, no reservoir of *C. auris* has been identified and hence further studies are required (Chowdhary et al. 2017). The thermotolerance is believed to be acquired through effects of global temperature changes. Increase in the global temperature narrows the temperature difference between the environment and mammalian body temperature, allowing the emergence of new fungal pathogens such as *C. auris (*Jackson et al. 2019*)*. It also forms cell aggregation into large clusters (Rossato and Colombo 2018), which is an advantageous factor for some strains to persist in the hospital environment (Cortegiani et al. 2018). *C. auris* isolates do not produce hyphae, they only produce pseudo-hyphae. Collectively, multiple evidence suggests transmission of *C. auris* in healthcare settings and multiple body sites are likely to be high due to persistent colonization, leading to possible outbreaks (Schelenz et al. 2016). Although different biochemical systems are used in microbiology laboratories, it is evident from several published studies that *C. auris* in routine microbiology laboratories remains an unnoticed pathogen, as 90% of the isolates characterized by commercial biochemical identification systems are misidentified primarily due to lack of the yeast in their databases (Mizusawa et al. 2016). *C. auris* is the only species in which several isolates have been identified with resistance to all 3 classes of human antifungal drug and hence becoming more of a clinically important pathogen (Ben-Ami et al. 2017; Sarma and Upadhyay 2017). Multidrug resistant characteristic of *C. auris* is the main reason for the large number of nosocomial infections across the globe.

2.4 *Candida parapsilosis*

C. parapsilosis is also a commensal coloniser on the human skin, usually isolated from subungual areas. Studies carried out on *C. parapsilosis* reveal that it proliferates and forms biofilm in response to medium containing high glucose level. *C. parapsilosis* can also adhere to medical devices and colonise on human hands, which allows nosocomial spread of disease through the hands of health care workers (Rossignol et al. 2009). However, it is less adhesive and invasive to the vascular and mucosal epithelium when compared to *C. albicans (*Levy et al. 1998*).* It only exists in two forms, yeast and pseudohyphal as it cannot form true-hyphal state (Laffey 2005). Low birth weight infants are at greatest risk of *C. parapsilosis* infection. Neonates are more prone to *C. parapsilosis* infections as they have compromised skin integrity, susceptible to GI infections and prolonged endotracheal intubation allows easier invasion of the pathogen (Trofa et al. 2008). Although various methods of molecular diagnostics are available to identify clinical isolates, species identification through microbiological phenotyping is still a commonly applied method. Azoles and echinocandins are used as treatment as they are still found susceptible, though several clinical isolates of *C. parapsilosis* have been reported to be less susceptible

to echinocandins, which limits treatment options (Tóth et al. 2019).

2.5 *Candida glabrata*

C. glabrata is frequently co-isolated with *C. albicans* from patients with oropharyngeal candidiasis. Both the *Candida* spp. work together causing tissue damage and pathogenesis but lack virulence factors of hyphal growth and protease secretion (Silva et al. 2010; Kaur et al. 2005). *C. glabrata* has invasive properties on gastric epithelium (Westwater et al. 2007). However, it lacks the ability to switch from yeast cell to true hyphae which is a disadvantage at the invasion stage, but still has the ability to form pseudohyphae and biofilm (Rodrigues et al. 2013). Expression of adhesins are known to overcome this disadvantage for *C. glabrata* colonisation and invasion into the host tissue (Timmermans et al. 2018). Thus, microscopic diagnosis alone is not sufficient since *C. glabrata* can only form pseudo-hyphae but not true-hyphae. Hence, both fungal culture and microscopy are required. On CHROMagar, *C. glabrata* colonies appear white, pink or purple in contrast to *C. albicans* that form blue-green colonies (Fidel et al. 1999). Azoles and echinocandins are used as treatment, however, *C. glabrata* is intrinsically resistance to some azoles such as fluconazole (Chew et al. 2019). Also, decreased susceptibility to more than one echinocandins is seen in clinical isolates of *C. glabrata (*Pristov and Ghannoum 2019*)*.

3 *Candida* Infections: Pathogenesis and Virulence Factors

3.1 Morphological Changes and Host Invasion

As *C. albicans* is the most well-studied organism of *Candida* spp. this chapter is based primarily on *C. albicans* unless stated. The pathogenic yeast *C. albicans* is a polymorphic fungus found in yeast, hyphal and pseudo-hyphae forms. The hyphal form is the most clinically relevant morphological state (Desai 2018). The morphology affects pathogenicity in multiple ways; host recognition, adhesion, invasion, tissue damage, biofilm formation and immune evasion (Jacobsen et al. 2012). The hyphal form is of anisotropic growth where non-uniform cellular expansion occurs over polarised axis. Following this, development of the germ tube occurs for hyphal growth initiation, the germ tube extends, and growth is enhanced by the cell wall and the tip that is forcefully expanded by hydrostatic pressure driven by cytoplasmic forces. During hyphal invasion, distribution of hypostatic pressure in all directions causes hyphal tip swelling. Hyphal form is strongly associated with invasion (Yang et al. 2014).

C. albicans interacts with human host epithelial and endothelial layers. Invasion occurs via 2 routes, induced endocytosis and active penetration through the epithelial layer. During endocytosis, *C. albicans* expresses multiple invasins such as Agglutinin-like sequence 3 (Als3) and heat shock protein Ssa1 (Liu and Filler 2010). These cause binding of hyphal surface proteins to ligands such as E-cadherins and N-cadherins on endothelial cells, to induce endocytosis by mimicking host cell cadherins (Phan et al. 2007). Cadherins normally regulate the formation of cell-to-cell junctions in a calcium dependant manner (Gumbiner 2000). Als3 gene encodes for glycosylphosphatidylinositol (GPI) anchored cell surface proteins that express only in the hyphal form of *C. albicans*. Ssa1 are members of the heat shock proteins expressed on the cell surface of *C. albicans* to act as receptors for antimicrobial peptides such as histatins (Sun et al. 2010). These interactions lead to activation of endocytosis of hyphal *C. albicans* to enter the host using epithelial cells. When the endocytosis route is blocked, *C. albicans* takes the route of active penetration through oral epithelial cells and is known as the more dominant route of *C. albicans* invasion (Wächtler et al. 2012). Several fungal factors that play a role in active penetration and elongation of hyphae form, such as Als3 and secretory proteins such as Saps, phospholipases and lipase proteins (Nikou et al.

2019; Wächtler et al. 2011). There are many different isoforms of Saps that have been identified in *Candida* and are encoded by 10 Sap genes in *C. albicans,* which exhibit different virulent and invasive properties (Li et al. 2014). Sap5 has been identified to degrade E-cadherin in epithelial junctions (Villar et al. 2007), Sap2 to degrade host protection proteins (Colina et al. 1996) and Saps4–6 for hyphae formation (Staib et al. 2000). Phospholipases also play a similar role by degrading the major cell component, phospholipids and proteins, forming blastopores and causing hyphae elongation (Ghannoum 2000).

The switch between yeast and hyphal growth affect the morphology-dependent cell wall adhesins, proteases and other phenotypic and biochemical properties, affecting penetration through the host epithelial layer. Mutants trapped in either the yeast or hyphal form are avirulent, suggesting the switch to be virulent. The morphological switch is not independently regulated by single fungi species, various *Candida* species in the presence of bacterial cells play a role (Sudbery et al. 2004). Pseudo-hyphae are a distinct growth form that differs from both yeast cells and hyphae and are characterized by dividing elongated yeast cells at the same time, however, very little is known about the immune response to pseudo-hyphae (Gow et al. 2002).

3.2 Biofilm Formation

It is now widely accepted that biofilm formation is one of the main virulence traits associated with *Candida* pathogenesis (Hasan et al. 2009). The process of biofilm formation is divided into 4 stages: adherence, proliferation, maturation and dispersal (Crouzet et al. 2014). During the adherence stage, the fungal cells form a basal layer by attaching to the host surface to anchor the biofilm. During the proliferation phase the filamentation is initiated by hyphal formation and anchor the basal layer. During the maturation phase, the hyphal scaffold becomes enclosed in a blanket of self-produced extracellular polymeric substances (EPS). The EPS acts as a glue to hold together the biofilm structure (Cavalheiro and Teixeira 2018). The biofilm continuously releases elongated yeast cells that infect new sites to ensure that in the dispersal stage the biofilm life cycle can be repeated. The process of biofilm formation is known to take 24–48 h under experimental conditions and can be several hundred micrometres thick (Gulati and Nobile 2016). Building a network in the form of a biofilm allows the cells to have the optimised space and nutrients along with efficient removal of waste products (Rajendran et al. 2016). A special characteristic possessed by *Candida* biofilm that allows protection against host immunity and antifungal agents is formation of extracellular matrix (ECM) (Silva et al. 2012). However, composition of ECM varies between species of *Candida* and very little is known about ECM of non-*albicans Candida* species (Al-Fattani 2006). Another characteristic is emerging dormant variant cells called persister cells, which are located deep within the biofilm and have been shown to be related to multiple antifungal drug resistance, though the mechanisms of resistance are yet to be investigated (LaFleur et al. 2006).

C. albicans cells within a biofilm display high levels of resistance to two out of the three main classes for antifungal drugs, azoles and polyenes. Biofilms are intrinsically resistant to fluconazole and other azole derivatives. Also, anti-biofilm activity of polyenes occurs at such a high concentration which is considered toxic and unsafe to be used as treatment (Pierce et al. 2013). On the other hand, echinocandins, the newest class of antifungal agents targeting the cell wall component β (1,3)-glucan, display excellent activity against *C. albicans* biofilms at therapeutic concentrations (Kuhn et al. 2002), and are used as a first line therapy against these infections (Sobel and Revankar 2007). However, due to the emergence of anti-fungal resistance, further insight into novel therapeutics is needed.

Studies through chemical screening to assess the inhibition of biofilm formation have identified several molecules belonging to a series of diazaspiro-decane structural analogs (Wu et al. 2017a). These, unlike conventional antifungal drugs, exhibit anti-biofilm characteristics and inhibit the ability of *C. albicans* to

form biofilms. These anti-biofilm compounds were effective against both oral and systemic candidiasis using animal models of infection, demonstrating the role of biofilms in the pathogenesis of *C. albicans* infections (Romo et al. 2017).

3.3 Virulent Molecules of *C. albicans*

Secreted and cell surface molecules are known to be another key virulence factor in *Candida* infections (Deena et al. 2015). One of the main groups of hydrolytic enzymes known to facilitate invasion and colonization of mucosal membrane and degrade immune components, are Saps (Pietrella et al. 2012). Other cell surface and secreted proteins expressed by *C. albicans* that interact with human immune system are pH related antigen 1 (Pra1), High affinity glucose transporter (HGT1), Phosphoglycerate mutase 1 (Gpm1) and lipases (Hernández-Chávez et al. 2017).

Proteolytic activity of *C. albicans* enables the pathogen to utilise proteins as the sole nitrogen source, such activity is known to be carried out by Saps (Morschhäuser 2011). Expression of Sap antigens has been detected within cell wall of yeast and hyphal cells in multiple organs of immunocompromised patients with disseminated candidiasis (Calderone and San-Blas 2004). Gene expression of Sap is known to be a major virulence factor of *Candida* adhesion to host epithelial as its activity is associated with tissue invasion (Yang 2003; Monod et al. 2002). The *C. albicans* Sap isoenzymes take advantage of varying pH (2.0 to 7.0) at multiple locations relevant to the site of infection, such as the vagina having low pH and neutral pH at the oral cavity which regulates enzymatic activity (Li et al. 2014). All 10 Sap proteases contain a propeptide and a signal sequence at the N-terminal of the mature sequence which is required for protein folding and secretion (Carvalho-Pereira et al. 2015). The mature enzyme ranges from 35 to 50 kDa in size and both signal and pro-peptide sequences are cleaved upon expression. Saps are known to have two active sites conserved of highly reactive aspartic residues. Though named within the family of secreted proteases, Sap9 and Sap10 are membrane-bound depending on the type of infection and the stage. Each Sap protein has its own distinct role to play (Hornbach et al. 2009). Expression is induced upon infection and also varies. Sap1 and Sap3 are expressed upon phenotype switching and are found on yeast and hyphal cells while Sap9 and Sap10 express in all growth forms, Sap4 and Sap6 are exclusively expressed in the hyphal form at neutral pH (Schild et al. 2010). Saps1–3 contribute more significantly to *C. albicans* infections in comparison to Saps4–6 suggesting their importance in the pathogenesis (Dabiri et al. 2016; Borg-von Zepelin et al. 1998). *In-vivo* study of Sap2 shows it was the most abundant and overexpressed antigen in the presence of protein as the sole source of nitrogen.

pH related antigen 1 (Pra1), a surface and secreted protein of *C. albicans,* is a multifunctional virulence factor of *C. albicans* that interacts with several human immune regulators. Pra1 is involved in the pathogen-host interaction as it sequesters zinc from human tissue to mediate leukocyte adhesion and migration. *In-vitro* studies show Pra1 expression at alkaline pH, regulated by the Rim101 transcription factor. Has been detected on hyphal and yeast cells of *Candida*, and depending on the *Candida* strain, The expression is found to be induced upon hyphal induction, which is initially favoured by alkaline pH (Losse et al. 2011). Expression analysis via Western blotting resulted in intense bands at 68 kDa and 130 kDa, higher than the predicted molecular mass of 31 kDa due to N-glycosylation properties of Pra1 (Luo et al. 2009). Glycosylation moieties on fungal cell wall are essential to trigger epithelium innate response and induce both cell cycle arrest and apoptosis mechanisms in mucosal epithelial cells (Wagener et al. 2012). Pra1 localisation is primarily at the tip of the cell highlighting role of Pra1 upon contact with host tissue and surface during infection (Marcil et al. 2008). As a cell surface and secreted protein Pra1 plays many roles in immune evasion (*discussed in Sect. 5*).

High Affinity Glucose Transporter (HGT1) is a transmembrane protein that spans the membrane 12 times, where it carries out its function of

transporting glucose for metabolism. The predicted molecular mass of the protein is 60.67 kDa (Varma et al. 2000). The highest expression level has been found in the presence of low glucose concentration, similar to human's physiological glucose level. This could assist the formation of hyphal form and promote expression other invasion facilitating proteins such as Sap (Buu and Chen 2014). Twenty genes of *C. albicans* HGT have been identified (HGT1-HGT20). They share 10–93% of sequence identity and the expression of the HGTs genes is related to the glucose concentration in the growth medium (Fan et al. 2002). Hgt1 protein was initially identified from the cDNA library expression as the Factor H (FH) binding protein which allows the pathogen to escape the immune system by preventing complement regulation and inhibiting complement system on *C. albicans.* HGT1 is also found in non-*albicans* species such as *C. glabrata,* and *C. parapsilosis (*Kenno et al. 2019*;* Kotyk and Michaljaničová 1978*;* Ng et al. 2015*).*

Phosphoglycerate Mutase 1 (Gpm1) was the first fungal complement binding protein of *C. albicans* to be identified. It is a 27 kDa cytoplasmic and cell surface protein, found at the tip on the cell wall of both yeast and hyphal form initially known to be part of the glycolysis and gluconeogenesis process (Poltermann et al. 2007; Sharif et al. 2019). Cytoplasmic Gpm1 converts 3-phosphoglycerate to 2-phosphoglycerate but during infection, it helps fungal to attach human endothelial cells and keratinocytes via complement regulator exposed on host cell surface (Karkowska-Kuleta and Kozik 2014). Gpm1 is a vital protein for fungal adherence as knock-out mutants have shown to lower the binding affinity to endothelial cells (Lopez et al. 2014). The ability of fungi to adhere to host cells leads to increased chances of tissue invasion and therefore infection.

Phospholipases are a group of enzymes that hydrolyse ester bonds of glycolipids. *C. albicans* phospholipases are found in both the yeast and hyphal form (Vakhlu and Kour 2006). *C. albicans* phospholipases have both intracellular and extracellular activity at optimal pH of ~3.6 to 5.0 in 5 different subclasses, PLA1, PLA2, PLB, PLC and PLD with molecular mass varying from ~33 to ~65 kDa (Vakhlu and Kour 2006; Niewerth and Korting 2001; Park et al. 2013). The differentiation of the subclasses is dependent on its mode of action and target within the phospholipid bilayer. PLA, hydrolyse carboxylic esters, PLA2 cleaves fatty acids and PLB cleaves the acyl chains of the phospholipid. PLC and PLD are phosphodiesterase where PLC hydrolysis the bonds between the glycerol backbone and PLD acts in a similar manner to hydrolyse the head group. Phospholipases contribute to *C. albicans* infections by host cell penetration, adhesion to epithelial cells and interrupting host signal transduction pathways (Ghannoum 2000). Non-*albicans* species known to also secrete phospholipases are *C. tropicalis*, *C. parapsilosis, C. glabrata, C. krusei and C. lusitanie,* though secreted at a much lower level in comparison to *C. albicans (*Haynes 2001*).*

Lipases are a group of secreted hydrolytic enzymes that catalyse hydrolysis of triglycerides into fatty acids and glycerol (Roustan et al. 2005). They differ from phospholipases as they have the ability to act on soluble substrates. Similar to the family of Sap proteins, 10 lipase genes are found *in C. albicans* (LIP1 to LIP10) and each lipase sequence contains four conserved cysteine residues and N-glycosylation sites, which are known to be lipase motifs (Hube et al. 2000). These lipases digest lipids which then facilitate *C. albicans* for nutrients acquisition, along with playing a role in C*andida* pathogenesis by releasing hydrophobic free fatty acids that are essential for adhesion of the fungus to the host (Gacser et al. 2007). The lipase gene expression is dependent on the stage of infection rather than infection site on the host (Stehr et al. 2004). Lipase expression has also been observed in non-*albicans species* including *C. tropicalis (*Jiang et al. 2016*), C, krusei, C. glabrata (*Barros et al. 2016*), and C. parapsilosis (*Tóth et al. 2015*).*

4 Immune Sensing of Candida

The symbiotic environment is tightly controlled and regulated by the complex host defence system. This involves innate, adaptive (humoral and

cell mediated) and nutritional immunity to prevent or eliminate *Candida* infection. As different *Candida* spp. affect different locations, the immune response is tailored dependant on the infection. For example, antigen presenting cells (macrophages, neutrophils and dendritic cells) dominate protection against candidemia, whereas adaptive immunity T-lymphocytes and cytokines dominate at the mucosal level for protection. The involvement of humoral immunity in *Candida* infections is still controversial (Fidel 2002).

Activation of an immune response leads to phagocytosis of *C. albicans* during innate immunity for production of pro-inflammatory cytokines, which signal and recruit immune cells to prevent or combat against candidiasis (*overviewed in* Fig. 11.1). Pattern recognition receptors (PRR) are part of the first line defence in antifungal immunity. PRR—pathogen-associated molecular pattern molecules (PAMPs) interaction initiates an immune response. The response is driven by phagocytotic cells to produce signalling molecules for T-cell expansion and neutrophil recruitment (Nur et al. 2019). Failure in such mechanisms leads to life threatening infections by opportunistic pathogens in immunocompromised patients.

4.1 Innate Immunity

Interaction between the fungus and immune cells occurs via PRR and PAMPs to activate an immune response. The cell wall of *C. albicans* is comprised of inner and outer layer made of chitin β (1, 3 glucans) and mannans to which PAMPS are found covalently bound. The fungal cell wall components are recognised by complementary PRR expressed on various immune cells. The main groups of PRR involved in *C. albicans* sensing are C-type lectins (dectin-1, dectin-2, galectin-3, DC-SIGN), Toll like receptors (TLR), mannose receptors, complement receptors (CR1, CR2 and CR3) and Fc receptors (FcγR) as seen in Fig. 11.2. Recognition of pathogen by these

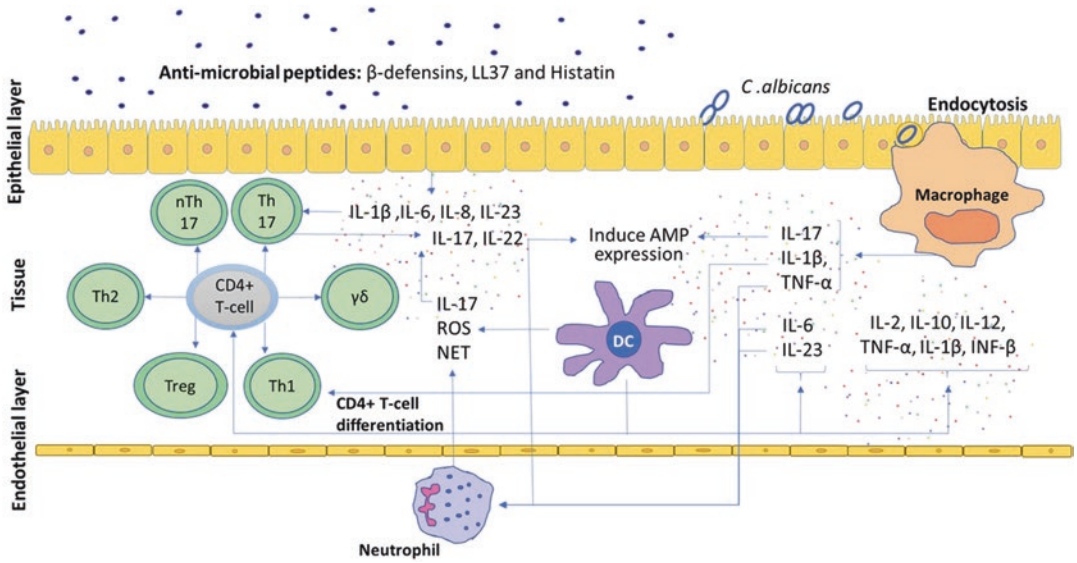

Fig. 11.1 A schematic diagram of Candida immune sensing mechanism in the gastrointestinal system. Epithelial cells act as first line of defence to produce pro-inflammatory cytokines, but pathogenic yeast can invade host tissue via endocytosis and active penetration. Upon invasion, patrolling macrophages phagocytose the cells and produce signalling molecules. This recruits neutrophils to produce reactive oxygen species (ROS) and neutrophil extracellular trap formation (NET) for clearance of the pathogens. Cytokines also activate adaptive immunity for differentiation of T-lymphocytes into their subsets Th1, Th2, Th17, Treg and nTh17

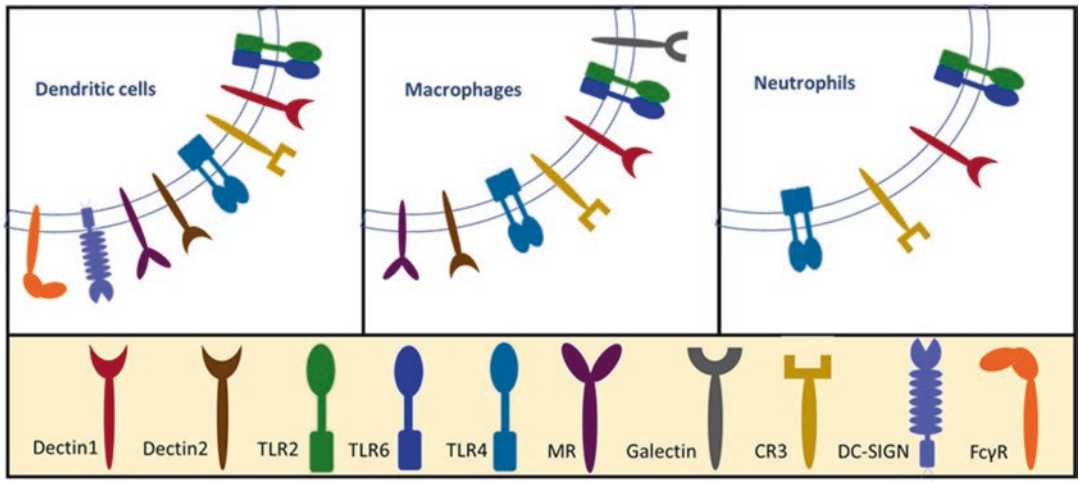

Fig. 11.2 An overview of the main immune receptors expressed by dendritic cells, macrophages and neutrophils

receptor leads to activation of an immune response; phagocytosis, cytokine production, chemokine production, reactive oxygen species (ROS) and neutrophil extracellular trap formation (NET) (Urban et al. 2006).

4.1.1 Anti-Microbial Peptides (AMPs)

Upon *C. albicans* colonisation, epithelial cells lead to the production of proinflammatory cytokines such as IL-6, IL-8 and IL-1β. PAMP-PRR interactions between O-mannan on *C. albicans* cell wall and TLRs 2 and 4 on epithelial cells, activate TLR signalling pathways for transcription of AMP coding genes (Lai and Gallo 2009). AMPs are peptides or protein molecules that are part of the first line defence. In healthy individuals, expression of AMPs is regulated at low levels to maintain homeostasis. However, during infection, the secretion of IL-6, IL-8 and IL-1β by epithelial cells, induces Th17 differentiation. Th17 cells produce IL-17, IL-22 and TNF-α which signal immune cells to increase AMPs expression. AMPs gene expression is drastically increased through activation of signalling pathways such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Signal transducer and activator of transcription 3 (STAT3). Once expressed, AMPs are stored and released from immune cells such as neutrophils and paneth cells of the epidermis. The three main AMPs against *C. albicans* are cathelicidin LL-37, β-defensins and histatins. Cathelicidin LL-37 is secreted by epithelial cells of oral and urogenital tract, and neutrophils (Tsai et al. 2011). They can bind fungal mannan and alter the membrane morphology of *C. albicans* by preventing adhesion to host surface and disintegrating the cell membrane, causing content and proteins up to 40 kDa to leak out and breakdown the cell membrane (Den Hertog et al. 2005). Defensins are small proteins (29–35 amino acids) that have antimicrobial activity against bacteria viruses and fungi. The 140 different defensins found in mammalian species are classified into 3 mains groups; α−, β−, and θ− defensins depending on their structural differences (Yacoub et al. 2015). Six α-defensins and three β-defensins have been identified in humans (Schneider et al. 2005). α-defensins are found in immune cells of the intestine such as neutrophils, macrophages and paneth cells, whereas β-defensins are secreted by leukocytes and epithelial cells of the gut, lung, kidney, oral cavity, and also found in body fluids that play a role in mucosal and surface defence (Kastin 2013; Diamond et al. 2001). Histatins are histidine-rich human proteins produced in the human parotid and salivary gland (Oppenheirn et al. 1988). They exhibit antifungal activity and histatin-5 in particular, can cause disruption of membrane morphology by binding to ATPase

and other cell wall transporters for histatin uptake by *C. albicans*. They accumulate within the cell to produce ROS and create pores, inducing ATP and nucleotide efflux for *C. albicans* cell death (Den Hertog et al. 2005).

4.1.2 Dendritic Cells (DC)

Another way in which the host recognises *C. albicans* is via DC, which are a form of antigen presenting cells that patrol the peripheral tissues under the mucosal surface (Cutler and Jotwani 2006). These immune cells are recruited at the site of infection by interleukins, chemokines and AMPs secreted by epithelial cells in response to microbial infection (Medzhitov and Janeway 2000). DCs express TLR, lectin receptors, FcγR and CR3 (Romani 2004a), of which TLR and CLR are predominantly expressed (Diebold 2009). DCs are important for sensing microbes and signalling for differentiation of lymphocytes leading to adaptive immunity.

Dectin-1 is one of the extensively studied lectin receptor that recognises β-glucans on many fungal species including *C. albicans*. On the surface of *C. albicans*, β-glucans are shielded by outer wall components but yeast budding and cell separations causes deformation of the cell wall in which enough β-glucans is exposed to trigger an immune response (Chen et al. 2019). Therefore, dectin-1 recognises β (1, 3 glucans) present on yeast cells only, as cell separation does not occur during filament growth (Gantner et al. 2005). Dectin-1 induces intracellular signals and collaborates with TLRs for inflammatory activation (Goodridge et al. 2007). Dectin- 1 is a tyrosine based immunoreceptor that multimerises upon extracellular interactions and results in phosphorylation of the cytoplasmic domain of the receptor for binding of spleen tyrosine kinase (SYK). The recruited SYK activates caspase activation recruitment domain-containing protein 9 (CARD9) to initiate the NF-κB pathway (Takano et al. 2017). Through the SYK-CARD9 and NF-κB signalling pathways produces; IL-2. IL-6, IL-10, IL-23, IL-1β TNF and INF-β (Sancho, and Reis e Sousa, C. 2012; Del Fresno et al. 2013). Overall role of dectin-1 leads to phagocytosis, respiratory burst and production of ROS (Drummond and Brown 2011). Dectin-2 differs from Dectin-1 as it recognises α-mannan on the surface of hyphal filaments (Alt et al. 2011). It associates with FcγR for phosphorylation of tyrosine residues to activate the SYK-CARD9 signalling pathway and stimulate production of ROS (Saijo and Iwakura 2011). Dectin-2 present on DC also assist fungal phagocytosis and signal production of proinflammatory cytokines such as IL-1β, IL-6, IL-10 and TNF-α (Ifrim et al. 2016).

Interaction of host receptors and *C. albicans* is a complex process as TLR can function as both homo- and heterodimers as well as interacting with other host receptors such as C-type lectins to recognise or initiate intracellular signalling (Bourgeois and Kuchler 2012). Collaboration of both CLRs and TLRs are required for best protection against candidiasis. Ten functional TLRs identified in humans, the transmembrane receptors found on the cell surface membrane or intracellular membrane; TLR1, -6 and 10 are expressed on the surface and TLR3, 7-9 are expressed on the intracellular membranes (Chaturvedi and Pierce 2009). They recognise lipopolysaccharides and peptidoglycans to initiate an immune response via myeloid differentiation factor 88 (MyD88) signalling pathway (Kawai and Akira 2007). Upon ligand recognition, the signalling pathway is induced by intracellular domain MyD88 which interacts with the cytoplasmic domains of all TLR except TLR3 (Brown et al. 2010). MyD88 signalling pathways is crucial for optimal Th1 response to *C. albicans (*De Luca et al. 2007*)*. It activates the NF-κB signalling pathway for production of pro-inflammatory cytokines. Activation of TLR induces expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 (Zheng et al. 2015). The two main TLRs involved in *C. albicans* infections are TLR2 and TLR4. They recognise O-linked mannans on fungal cell wall which activates the NF-κB signalling pathway for production of IL-8 and TNF-α. However, the role of TLR4 in candidiasis remains controversial suggesting the role of TLR4 only contributing to minor protection (Gil and Gozalbo 2009)or having no role in host protection (Gil and Gozalbo 2006). Though TLRs are not the central dominant PRRs in humans, polymorphism of TLR gene in

immunocompromised patients results in predisposition of fungal infection (Plato et al. 2014).

Other receptors such as DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and mannose receptor (MR) are also expressed on DCs. They recognise N-linked mannan on *C. albicans* cell wall to induce expression of proinflammatory cytokines such as IL-17, TNF, IL-1β, IL-10 which in turn play a role in signalling Th17 response (Gringhuis et al. 2007; Poulain and Jouault 2004). DC-SIGN contain conserved cytoplasmic tail motifs that include tyrosine-based, dileucine, and triacidic cluster, which are believed to regulate ligand binding, uptake, and trafficking (Azad et al. 2008). DCs also have the capability of sensing and responding to different morphological forms of *Candida* morphology to initiate an immune response. DCs can phagocytose both yeast and hyphal forms. The yeast form is detected within the phagosome, whereas the hyphal form escapes to the cytoplasm (d'Ostiani et al. 2000).

4.1.3 Macrophages

Macrophages cause direct killing of *C. albicans* and trigger an immune response. *C. albicans* cell wall glycosylation is important for macrophage TLR to recognise and phagocytose β -glucans, leading to activation of ROS (Brown 2011). TLRs of macrophages recognise O-linked mannans and β-glucans on the fungal cell wall, except TLR9 which is an endosomal receptor and mediates sensing of unmethylated DNA of *C. albicans (*Ramirez-Ortiz and Means 2012*;* Miyazato et al. 2009*)*. Mannose receptors recognise N-linked mannan present on *C. albicans* surface to initiate pro-inflammatory cytokine response (Cheng et al. 2012). MR are C-type lectin receptors found on macrophages that interact with pathogen cell wall for IL-17, TNF-α and IL-1β production (Van de Veerdonk et al. 2009; Geraldino et al. 2010; Goyal et al. 2018). Interleukins such as IL-17 is essential in antifungal activity through the IL-17 receptors. IL-17 activates signalling cascade to induce other proinflammatory cytokines, AMPs and recruitment of neutrophils, especially in disseminated candidiasis (Mengesha and Conti 2017). Cytokine TNF-α stimulates leukocyte adhesion molecules that lead to recruitment of polymorphonuclear leukocytes, and enhanced phagocytosis for fungus killing. Importance of IL-1β in candidiasis is for polymorphonuclear leukocytes recruitment and generation of superoxide production along with bridging into adaptive immunity by stimulating Th1 response (Le et al. 2019).

In addition to expression by DCs, dectin-1 is also expressed by macrophages and neutrophils (Brown et al. 2003). Dectin-1 and galectin-3 of macrophages are required to recognise and produce proinflammatory cytokine TNF-α (Esteban et al. 2011). Galectin binds to β -galactoside to induce chemokines, cytokines and ROS and essential for *C. albicans* recognition in the gut (Wu et al. 2017b). Galectin is important for phagocytosis of *C. albicans* hyphae but not the yeast form (Linden et al. 2013). Dectin-2 interacts with α-glucans but cannot recognise β-glucans to carry out a pro-inflammatory response. It is mainly involved in the recognition of C. *albicans* hyphae and uses FcγR to induce intracellular signal (Kottom et al. 2018). Macrophage-inducible C-type lectin (Mincle) is another C-type lectin receptor predominantly expressed on macrophages and its expression is upregulated upon *C. albicans* stimulus. It is a transmembrane receptor containing short intracellular tail and extracellular domain. Cell signalling via Mincle is induced upon α-mannan binding in association with FcγR to initiate an immune response via SYK and NF-kB mediated cytokines (Yamasaki et al. 2008). Mincle is required for TNF-α production by macrophages against *C. albicans* infection (Wells et al. 2008).

4.1.4 Neutrophils

Neutrophils are form of antigen presenting cells that are important for both mucosal and systemic *C. albicans* infections (Archambault et al. 2019) and primarily the most potent for fungus killing (Zhang et al. 2017). They are recruited at the site of infection by chemokines released by epithelial cells and macrophages. Along with ROS oxidative *C. albicans* killing, they can induce non-oxidative killing by producing AMPs such as lactoferrin and β-defensins. Neutrophils aggressively ingest and

destroy fungal particles through phagocytosis, initiated by cell surface receptors. Neutrophils express CLRs, phagocytotic receptors such as CR3 and less commonly TLRs. Killing of unopsonised *C. albicans* by neutrophils relies on the CR3 ligation as it recognises inactive form of opsonin C3b (iC3b) for CARD9 recruitment. CR3 is a widely expressed β-intergrin, and mediates recognition of both yeast and hyphal form of *C. albicans* for phagocytosis by polymorphonuclear cells. FcγR differs from CR3 as it clears opsonised *C. albicans* using proteins kinase C and this mechanism is ROS dependant, unlike CR3 which is ROS independent (Verma et al. 2017). Smaller fungal forms such as yeast form can be destroyed via phagocytotic but larger yeast require NETosis. NET traps and kills microbial pathogens in the presence of ROS (Small et al. 2018) and has been associated with only larger forms of *Candida,* such as the hyphae form (Branzk et al. 2014). NETosis also allows exposure of fungal PAMPs i.e. exposure of β-glucans to allow recognition by immune cells via dectin-1. Also, like DCs, neutrophils can also discriminate between yeast and hyphal morphology of *C. albicans* as IL-12 (Romani et al. 1997) is produced in response to yeast form and IL-10 in response to hyphal form (Brown 2011).

Neutrophils produce autocrine IL-17 that induces innate immunity in response to IL-6 and IL-23 production. IL-17 is important as it is needed for activation of cytokines required for neutrophils in antifungal activity such as ROS production and controlling systemic *Candida* infections (Taylor et al. 2013). Neutrophils are also required to maintain immunity during mucosal *C. albicans* infections and furthermore prevent disseminated infection in the gut. Individuals with low neutrophil count have a higher risk of all types of *Candida* infections and strong evidence suggests disruption of GI mucosal in neutropenia patients leads to disseminated candidiasis with 100% mortality (Koh et al. 2008).

4.1.5 The Complement System

The complement system is a central part of the innate immunity which consists of 40 different plasma and cell membrane surface proteins (Mayilyan et al. 2008). Complement proteins are abundant in the serum, an ideal location for the host as fungus can live and survive in the bloodstream and can lead to disseminated infections. Opsonic serum proteins bind to the pathogen cell surface and activate the complement system for recruiting immune cells to the site of infection, facilitate phagocytosis by macrophages and neutrophils and activate cascade of proteins for formation of membrane attack complex (MAC)—pore like structure. Fungus cell walls consists of thick carbohydrates making complement mediated killing difficult. However, opsonisation and stimulation of inflammatory response still allows host resistance against pathogens.

Complement component 3 (C3) convertase (C3bBb or C4bC2a) cleaves C3, central component of the complement system, to form C3a and C3b. C3b is in turn, part of C5 convertase (C4b2b3b or C3bBbC3b) that cleaves C5 leading to MAC formation. MAC produces a pore in the cell membrane and causes disbalance in osmolality for pathogen cell lysis and can enhance inflammation in aim to destroy pathogens (Tegla et al. 2011). The complement system is well regulated, and cascade of events occur for MAC formation. Complement system can be mediated by three different routes, classical, lectin and alternative pathways which differ at the initiation stage. Classical pathway is initiated by C1 complex recognising antibodies bound to fungal surface. In *Candida* infected individuals, it is activated by anti-mannan IgG and anti-mannan IgM antibodies, which are also present in the human serum of most adults (Merle et al. 2015a). Whereas the lectin pathway is initiated by mannose binding lectin (MBL) binding to mannose residues on the pathogen surface. It is activated by MBL recognising and binding to mannose residues. *C. albicans* contains 40% of mannan in its cell wall (López-Ribot et al. 2004). Evidence from studies highlights the importance of MBL, as MBL deficient patients are more prone to fungal infections (Fidel and Huffnagle 2005). The alternative pathway is activated upon spontaneous hydrolysis of complement protein (Merle et al. 2015b). Alternative pathway is activated by C3b directly depositing on *C. albicans* surface.

C. albicans has the ability to activate all three complement pathways (Blom et al. 2009; Meri et al. 2004).

4.2 Adaptive Immunity

4.2.1 T-Lymphocytes

Following innate immunity, differentiation of T-lymphocytes is an important part of the adaptive immune response to *C. albicans* infections. Differentiation into $CD4^+$ (Naive T-cells) and $CD8^+$ (cytotoxic T-cells) is activated by DC that drive T-cell response in anti-fungal immunity. The degraded products produced by antigen presenting cells releasing exogenous proteins are processed into antigenic peptides within acidified vesicles and assembled onto major histocompatibility complex (MHC) presented to $CD4^+$ and $CD8^+$. Interaction to MHC II causes differentiation of $CD4^+$ into Th1, Th2, Th17, Treg, nTh17 and γδ T-cells, driven by different interleukins such as IL-10 and IL-12 produced during innate immunity (Zhou et al. 2009). Adaptive Th1 and Th17 cellular response are considered as most successful immune defence against *C. albicans* infections and allows maintenance of tissue homeostasis (Richardson and Moyes 2015). Antigenic peptides are also presented by MHC I to $CD8^+$ cells, but direct killing by cytotoxic T-cells has not yet been widely explored as part of anti-fungal immune therapy (Kumaresan et al. 2018). T-cells mediate immunity, especially Th1 is required against *C. albicans*. TLRs of innate immune system have a critical role in mediating the signalling for development on Th1 response. Phagocytosis of yeast form produces IL-12 and activate Th1 whereas hyphae form is found in the cytoplasm of the cell and produces IL-4 which initiates Th2 response (Brown 2011). On the other hand, Treg cells prevent excess proinflammatory response and tissue damage by maintaining the peripheral immune tolerance. They have the ability to suppress both $CD4^+$ and $CD8^+$ cells.

Differentiation of $CD4^+$ into Th17 cells secrete numerous cytokines including family of 6 different IL-17 (IL-17A to IL-17F) and IL-22 which are critically important for immune protection by causing pro-inflammatory response against *C. albicans* on mucosal sites of human host (Pietrella et al. 2011). They do this by binding to IL-17 receptor for producing proinflammatory cytokines that recruit neutrophils and macrophages to the site of infection. Th17 response is maintained in the presence of IL-23, induced upon IL-17, IL-23, IL-6 and inhibited by IL-12. Here, CLRs dectin-1 and 2 play a central role by producing cytokines that induced Th17 polarisation. IL-6 and IL-23 are produced by epithelial cells in response to *C. albicans* mannan recognition via antigen presenting cells (Conti and Gaffen 2015). Natural Th17 (nTh17) cells are patrolling guards of the innate system that protect the oral mucosa together with Gamma delta T-cells (γδ T-cells) which also secrete IL-17 in response to *C. albicans* (Conti et al. 2014). Th17 provides protection against cutaneous, whereas Th1 provides protection against systemic candidiasis (Kashem et al. 2015). Th17 are important antifungal cells as defect in Th17 cells leads to recurring fungal infections and autoimmune diseases (Hernández-Santos and Gaffen 2012). Importance of T-cells in *C. albicans* infection have been shown by studies carried out in patients with inherited disorders in Th17-mediated anti-fungal immunity, frequently present with chronic mucocutaneous candidiasis (CMC), which manifests as severe infection of the nails, skin and upper GI tract (McDonald 2012). Th17 immunity is also associated with vaccine response and protection against oral (Conti et al. 2009) and systemic infection (Kumar et al. 2013). The importance of this cellular response is observed in immunocompromised HIV/AIDS patients that are known to have depleted T-cell count are more prevalent to oropharyngeal candidiasis (Patil et al. 2018).

4.2.2 B-Lymphocytes

Antibody response plays a minor role in immunity against candidiasis (Richardson and Moyes 2015). Antibodies produced target the cell wall of fungi due to the accessibility of cell wall molecules. The main target for antibodies is O-linked and N-linked mannan on the *C. albicans* cell wall. Interaction of antibody to cell wall mannan would prevent *C. albicans* carrying out functions

to cause infection such as adhesion to the host and germ tube formation (Sendid et al. 2008). Antibody against 58 kDa fibrinogen binding mannoproteins on the fungus cell surface has been shown to selectively cause an immune response to systemic candidiasis, whereas patients with superficial infection did not react, suggesting this could be a possible diagnostic marker (Martínez et al. 1998). Als3 binding antibody found on the surface of *C. albicans* germ tube interfered with adhesion to host epithelial surfaces. Antibodies against *C. albicans* secreted molecules are also produced, for example, against SAP which was shown to increase protection against vaginal candidiasis in mice (De Bernardis et al. 2002). Antibody against heat shock protein also showed protection against systemic candidiasis in mice (Kumar et al. 2013). In spite of observations found so far, B-cell deficient mice did not show increased susceptibility to *C. albicans* infections, suggesting its insignificance in immune sensing (Wagner et al. 1996).

4.3 Nutritional Immunity

Biological systems are complicated with multiple complex networks and pathways. These need to be well controlled and regulated in a logical manner. Transitional metals are one of many factors required for operating biological system. Properties such as stabilising substrate at the active sites of enzymes and acting as cofactors make transitional metals a critical component. However, the presence of high levels of metal ions can prove to be toxic and hence is tightly regulated. Pathogenic yeast has to regulate essential metals to fulfil their physiological needs and during host-pathogen interactions, but the host exploits this during infections. This is referred to as 'nutritional immunity' where host depletes micronutrients such as iron, zinc, copper and manganese away from pathogens (Lopez and Skaar 2018). The role of iron and zinc are briefly discussed here.

Iron is the most abundant transition metal of the human body, commonly found in ferric (Fe^{2+}) or ferrous (Fe^{3+}) form. It is a valuable component for cytochromes and oxygen binding molecules but circulating levels are found to be low due to the counter activity of converting hydrogen peroxide into free radical ions which damages tissues (Andrews 1999). Low limits are maintained by increasing the level of ferritin and haemoglobin. This has been shown on renal cortex lesions caused by candidiasis, having increased ferritin and haemoglobin as a mechanism of systemic nutritional immunity against fungal pathogens (Potrykus et al. 2013). In order to prevent infection, the host maintains low circulating levels and limits availability of metal ions from pathogenic microbes. Limitation of such nutrients from *C. albicans* starves the cells to prevent prolonged survival and colonisation to establish an infection. Pathogenic yeast would have to lyse erythrocytes to gain access to iron, as it is bound and locked away within haemoglobin and erythrocytes (Fourie et al. 2018).

Zinc is an important co-factor in many enzymes and thus a requirement for yeast growth (North et al. 2012). The known mechanism of limiting zinc is via antimicrobial peptide calprotectin. Calprotectin is a calcium (Ca^{2+}) and zinc binding protein. The high binding affinity property to zinc can limit the amount available to pathogens by trapping the zinc from the local environment (Besold et al. 2017). Calprotectin is present in large amounts in human neutrophils for which neutrophil recruitment to the site of *Candida* infections helps limiting nutrients to fungal pathogens such as *Candida*. Calprotectin is released as part of NET during NETosis upon neutrophils recognition of *C. albicans* hyphae for depleting zinc from the fungus and inhibiting its growth (Urban et al. 2009).

5 Immune Evasion by Candida

Immune evasion mechanisms adapted by pathogens include acquiring human complement, expression of endogenous complement inhibitors and secretion of proteases such as Saps that degrade host complement proteins (Meiller et al. 2009). *Candida* species express virulence factors

such as hydrolytic extracellular enzyme, heat shock proteins and formation of biofilm (Cauchie et al. 2017). These mechanisms are used in order to strategies host immune evasion and cause life threatening diseases. The well evolved microorganisms cleverly find ways to dysfunction the immune system, particularly the complement system (Fig. 11.3). As the complement system is such a complex system, tight regulation is required to ensure the system is activated and act upon pathogenic organisms rather than self. To prevent this, complement regulators are in place to control the system. Most of the regulators act as inhibitors by cleaving the complement convertases or accelerating their decay. Examples of some regulators are Decay regulator -accelerating factor (DAF), Membrane cofactor protein (MCP), C1 inhibitor, Factor H (FH) and C4b-binding protein (C4BP) of which, FH and C4BP mainly are associated with *Candida* evasion strategies.

5.1 Proteolytic Activity on Immune Molecules

Complement protein C3 plays a central in activation of the complement pathways. Cleavage of C3 by C3 convertase produces anaphylatoxin C3a. C3a required for binding to complement receptor 3 (C3aR) for consequent migration of neutrophils to the site of infection and adhesion to the filamentous *C. albicans* for phagocytosis. Immune evasion molecule Pra1 cleaves C3 at a unique site, 6 C-terminal residues upstream C3 convertase site, producing α-chain like fragment (C3α'L). Pra1 cleaving C3 to produce a C3a like residue which causes inhibition of proinflammatory activity. Analysis of Pra1 protein sequence displays the presence of metalloprotease motif, however studies using protease inhibitors suggests Pra1 is not a typical metalloprotease (Loboda and Rowińska-Żyrek 2017).

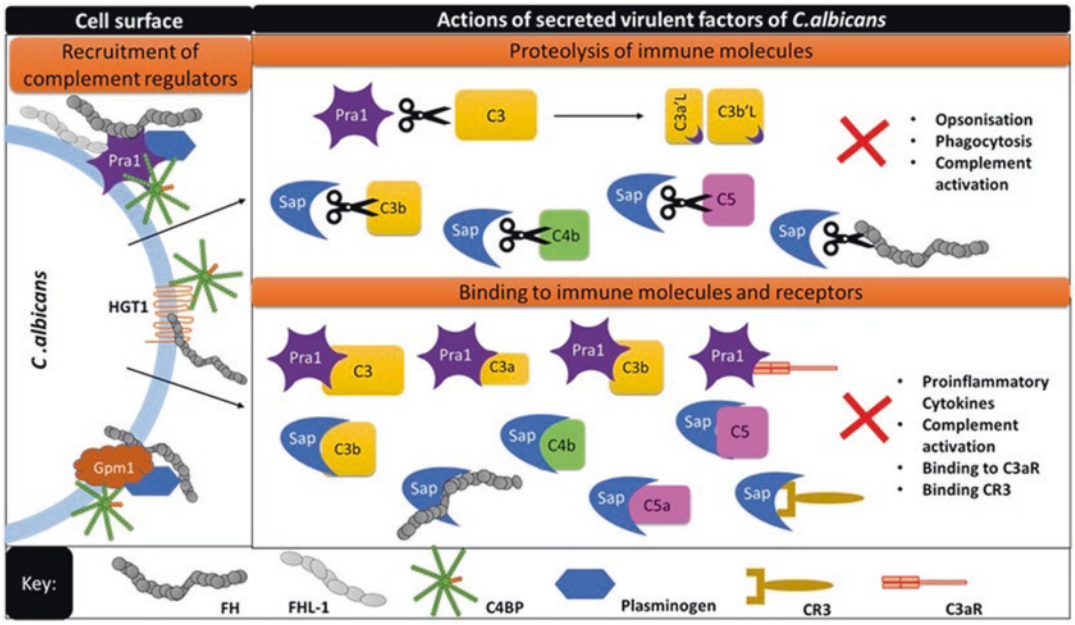

Fig. 11.3 Proteins expressed by C. albicans (Pra1, Gpm1 and Saps) help to evade the host immune system. Pra1 binds to C3, C3a and C3b. It also has the ability to cleave C3 and produce C3a like and C3b like molecules, which are inactive. Sap proteins can bind and cleave C3b, C4b and C5. This inhibits proinflammatory cytokines, signalling via C3aR, opsonisation and phagocytosis. Pra1 and Gpm1 binds to the alternative pathway regulator Factor H and classical pathway regulator C4BP and with cofactor I, inactivate complement C3b to iC3b and C4b to iC4b, inhibiting the complement pathways. Pra1 also binds to plasminogen to activate plasmin for initiating tissue invasion

Saps1–3 of *C. albicans* have been shown to have both binding properties and proteolytic activity on crucial complement cascade proteins; C3b, C4b and C5, to suppress the activation of complement pathways. As a result, these proteases have shown to inhibit both, alternative and classical pathway. Saps play a significant role in immune evasion as they act on many other immune molecules such as AMPs, lactoferrin of the saliva and immunoglobulins such as IgA, which is cleaved to manipulate mucosal and systemic immune response (Naglik et al. 2004). Sap2 in particular has been well studied as an evasion molecule and is known to cleave FH, which is a vital fluid phase regulator of the alternative pathway and functions to facilitate Factor-I to cleave C3b into inactive form iC3b (Svoboda et al. 2015). By doing so, it blocks the formation the of C3 convertase and such hindrance in this mechanism leads to upregulation of C3b opsonisation and activation of the alternative pathways (Pangburn 2000).

5.2 Hijacking Complement Regulators

The complement system is a crucial part of immunity required for clearing pathogens and is highly regulated with fluid phase and cell bound complement regulators. Pathogens have learnt to hinder with the complement pathways by hijacking complement regulators to prevent any damage by trying to protect from host complement killing and prolong survival (Hovingh et al. 2016). This approach is used by *C. albicans* which recruits host regulators FH and FHL-1 to surface to carry out regulatory activity, by doing so, pathogenic yeast control and regulate the complement pathway on the pathogen surface (Meri et al. 2002). The interaction enhances cofactor activity of FH for Factor I mediated cleavage of C3b in fluid phase and enhances C3b inactivation in solution. FH and FHL-1 serve as cofactors of C3b cleavage into iC3b, therefore inhibiting activation of the alternative pathway on the cell surface. This provides additional protective layer and limits and prevents complement attack at the yeast surface. The intermediate molecules of *C. albicans* binding to immune regulators are: Pra1, Gpm1, HGT, Sap2. All 4 proteins expressed by *C. albicans* have been shown to bind FH (Gropp et al. 2009).

Pra1 and HGT1 of *C. albicans* are also C4BP binding proteins. C4BP is a 570 kDa multimeric protein found in the plasma. It binds to cofactor, Factor I and degrades C4b to prevent the formation of C3 convertase of the classical pathway to inhibit the activation of the complement pathway. C4BP also binds C3b to prevent the activation of the alternative pathway (Okrój and Blom 2018). C4BP acts as a cofactor in Factor I mediated cleavage for C4b and C3b. On the surface of *C. albicans*, the complement inhibitory activity is maintained to control the activation of classical pathway (Luo et al. 2011). Pra1 and Gpm1 binds serine proteases plasminogen present in the blood. The interaction mediated by lysine residues converts plasminogen into active plasmin which breaks down ECM components such as fibrin to initiate invasion and degrades complement molecule C3b (Luo et al. 2015). Victronectin is multifunctional protein found to be a serum regulator of terminal complement pathway and in the ECM, it plays a part in cell proliferation, adhesions and angiogenesis (Preissner and Reuning 2011). It is present on endothelial cells and keratinocytes, and acts as a surface ligand for Gpm1. *C. albicans* Gpm1 binds to vitronectin to evade the host immunity and attaches to the human endothelial cells for tissue invasion (Lopez et al. 2014).

5.3 Blocking Immune Receptors and Chemotactic Effects

C3a and C5a are chemotactic factors that attract immune cells such as neutrophils to the site of infection. Pra1 binds to C3, anaphylatoxins C3a and C3b, as well as iC3b and C3d, blocking the direct anti-fungal effect. Generally, C3a induces intracellular Ca^{2+} release for recruiting neutrophils via Ca^{2+} signalling. However, Pra1 complexing with C3a, inhibits binding to C3aR on myeloid cells and consequently prevents C3aR

mediated calcium signalling (Krause et al. 1990). Fortunately, Pra1 suppresses C3a mediated but not C5a induced IL-8 secretion that recruit and activate phagocytes (Luo et al. 2018). On the other hand, *C. albicans* Saps have been shown to block activation of complement effector C5a generation, as a result, obstructing recruitment of inflammatory cells (Gropp et al. 2009).

Pattern recognition receptor CR3, also known as integrin $\alpha_M\beta_2$, Macrophage-1 antigen and Mac-1, is found on polymorphonuclear cells that bind to the surface of pathogens. The main CR3 ligand is iC3b, but many others such as C3b can also bind (Bennett et al. 2015). HGT of *C. albicans* has been found to be an analog of CR3. As CR3 plays a major role in adhesion to human phagocytotic cells, HGT1 and CR3 interaction prevents recognition and phagocytosis by neutrophils (Lesiak-Markowicz et al. 2011). Another immune evasion molecule of *C. albicans* is Sap2 that also has the ability to inactivate CR3.

Another strategy used by fungal pathogens is by masking cell wall components that are recognised by PAMPs. Immune receptor dectin-1 binds to β (1, 3 glucans) on the fungal cell wall to initiate strong inflammatory response. Yeasts such as C*andida* have evolved to shield away β (1, 3 glucans) under mannoproteins and to prevent recognition by dectin-1 on immune cells such as macrophages to initiate an innate immune response. This usually happens at the hyphal form where the glucans are masked by the mannans and β-glucans are exposed during yeast budding.

5.4 Candida Mediated Lysis of Macrophages

Macrophages are important for limiting *Candida* burden during infection and recruiting other immune effector cells. They produce multiple pro- and anti- inflammatory cytokines upon *C. albicans* recognition. They have ability to readily digest round yeast form of *C. albicans* and short filaments (Keppler-Ross et al. 2010). Herein, some *C. albicans* are destroyed and some go through fungal dimorphism induced upon change in the environmental condition of CO_2 production within the cell to switch from yeast to hyphal form within the macrophage (Wartenberg et al. 2014). A reverse cytotoxic effect has been found where engulfed filament and hyphal formation of *C. albicans* can lyse the macrophage. However, this is based on the fungal morphology as only hyphal growth allows the *C. albicans* to escape macrophages. While this process would destroy some macrophages, other macrophages would withstand the pressure (Krysan et al. 2014).

5.5 Host Immune Evasion by *Candida* Biofilms

Morphology of *C. albicans* plays a significant role as a commensal and pathogenic organism. Biofilms are associated with invasive infections rather than yeast cells as healthy individuals are capable of clearing yeast cell, but formation of biofilms adapt to evade host immunity and establish an infection (Nett and Andes 2020). During infections, to prevent activation of host immunity, *C. albicans* illustrate immune silencing by preventing immune sensing. The biofilm matrix prevents the release of PAMPs to prevent penetration of leukocytes. Biofilm also evades clearance as they are ineffective in eliciting a strong immune response (Dühring et al. 2015). *Candida* biofilm also induces the expression of IL-10 which signals the function of Th2 response, resulting in activation of B-cells, rather than activation of Th17 cells for an inflammatory response and activation of neutrophils. Deviation into Th2 response allows biofilm to evade direct clearance due to ineffective Th17 immune response. The changes in morphology also allow prolonged survival of C*andida* within the host, this itself is a mechanism of evasion as many factors of biofilm are resistant to toxic immune mediators for prolonged survival (Garcia-Perez et al. 2018). Asteroid bodies, crown like structure, are formed around the central yeast to provide protection. This plays a part in evasion as antigen-antibody complexes and parts of antibodies IgG and IgM can be trapped in the external crown, interfering with the immune system. The asteroid is resistant to phagocytosis and allows proliferation of yeast cells without any interruptions (Hernández-Chávez et al. 2017).

5.6 Modulation of Nutritional Immunity

C. albicans uses multiple ways in attempt to acquire micronutrient from the host. As mentioned above, iron is one of the essential nutrient requirements for *C. albicans* and is usually found locked away in haemoglobin and within erythrocytes. However, *C. albicans* has evolved to obtain these micronutrients for cellular function. To obtain access, the fungus lysis erythrocytes using receptors present on the hyphae surface, binds to iron binding molecules and uses siderophores to obtain the iron (Hernández-Chávez et al. 2017). Micronutrients transporters such as Als3 are expressed by *C. albicans* to attain micronutrients.

Pra1, Zrt1 and Zrt2 are zinc micronutrient transporters of *C. albicans.* Zinc is one of the most abundant metals in humans (Tapiero and Tew 2003) and *C. albicans* secreted protein Pra1 has been recognised as a zinc sequester during infections as zinc levels are tightly regulated by the host, approximating iron. Secreted Pra1 hunts and binds up to 3 atoms of host zinc to then re-associate to a co-expressed zinc receptor (Zrt1) on the fungal cell wall. *C. albicans* use this route to assimilate zinc from human endothelial cells (Citiulo et al. 2012). *C. albicans* expresses 2 zinc transports Zrt1 and Zrt2 of which Zrt2 if found to be overexpressed during invasive candidiasis (Crawford et al. 2018).

6 Treatment and Antifungal Resistance

There are relatively few classes of antifungal drugs (Cannon et al. 2007). Main three antifungal treatment used are azoles, polyene and echinocandins. Azoles are inhibitors of Lanosterol 14-α-Demethylase and used for treating both topical and invasive candidiasis. Azoles allow accumulation of toxic sterols (14α-methyl 3,6 diol) within a cell causing disruption of fungal cell growth. Many azoles-resistant *Candida* species have been reported, in particular, *C. auris (*Kanafani and Perfect 2008*).* One of the major reasons for azole resistance is overexpression of efflux pump on the cell membrane of *Candida* species, that prevent azoles staying inside the cell. The clinical consequences of antifungal resistance can be seen in treatment failures in patients and changes in the prevalence of *Candida* spp. causing disease (Sanglard and Odds 2002). Another reason for azole resistance by *Candida* is mutation and overexpression of ERG11 gene coding for membrane protein that provides cell integrity. Studies carried on *C. albicans* have reported multiple mutations of this gene (Sohaib Shahzan et al. 2019; Fan et al. 2019). Echinocandins are glycan inhibitors and used for oesophageal and invasive candidiasis. Echinocandins are also fungicidal, as they inhibit β (1,3)-glucan synthesis required for cross linking chitin and β (1,3)-glucan that provides strength and rigidity to the fungi cell wall (Aruanno et al. 2019). Echinocandins are the first line antifungal therapeutic agents against *C. glabrata* strains due to their low susceptibility to azole drugs (Rivero-Menendez et al. 2019). Emerging resistance to echinocandins has been reported with *C. glabrata*, *C. tropicalis* and *C. auris* isolates (Kordalewska et al. 2018; Khan et al. 2018; Dellière et al. 2016). Echinocandin resistance has been attributed to the mutation of Fks subunits in the glucan synthase enzyme, preventing the formation of a strong cell wall (Patil and Majumdar 2017). Polyene have high binding affinity to ergosterol in the plasma membrane to create a pore and interfere with proton gradient across the membrane. They form a micropore through the membrane from which amino acids, potassium and other water- soluble components of the cell outflow from the cytoplasm. Minimal resistance has been reported for common polyene and amphotericin B antifungal drugs. *C. albicans* is the most resistant to polyenes followed by amphotericin B. Emerging *Candida* species such as *C. krusei* and *C. glabrata* show more resistance to amphotericin B compared to *C. albicans* which makes infections caused by emerging non-*albicans* species more challenging to treat (Rodrigues et al. 2018). Apart from the 3 main groups of antifungal drugs mentioned above, nucleoside analogues are another group which are RNA/DNA inhibitors such as 5-flurocytosine, which inhibits fungal

protein and nucleic acid synthesis. However, 10% of *C. albicans* clinical isolates show resistance to this drug (Scorzoni et al. 2017). Another novel and recent peptide-nucleoside drug produced against *C. albicans* is Nikkomycin Z (NIK) which works by being a competitor inhibitor of chitin-synthase and disrupts the fungal cell wall. Targeting the cell wall of the pathogen is an ideal strategy for therapeutic purposes as human cells do not have a cell wall, making fungal cell an ideal target (Shields et al. 2011).

Overtime, increase in antifungal resistance has been widely reported. One possible reason for the emergence of antifungal resistance could be tackling *Candida* infections without specific species confirmation. Majority of *C. albicans* strains are susceptible to fluconazole, but invasive candidiasis is currently treated without species confirmation, for which the current international guidelines recommends echinocandins as treatment. Echinocandins are more effective anti-fungal drugs than fluconazole (De Rosa et al. 2015). Echinocandins resistance is uncommon, they exhibit fungicidal activity and display a better clinical outcome in comparison to fluconazole and amphotericin B (Ou et al. 2017). Early recognition of *Candida* spp. could switch the treatment to fluconazole which would impact treatment costs and reserve drugs for the future. However, *Candida* Spp. such as *C. glabrata* is widely resistant to fluconazole for which echinocandins treatment is recommended. Similarly, *C. auris* isolates are resistant to both fluconazole and amphotericin B for which information such as species specificity is required for changes in the treatment (Sears and Schwartz 2017). *Candida* spp. developing resistance to fluconazole is very likely as a common and consistent trend has been seen in clinical isolates over the decades. It is already an inactive drug against biofilm formation which makes it a common antifungal drug failing to treat *Candida* infections (Pappas et al. 2015). On the other hand, echinocandins have so far had low resistance rate and higher success in treating *Candida* infections in comparison to fluconazole, for which it remains as the first line treatment (Reboli et al. 2007).

7 Vaccines and Immunotherapy for Candida

Emergence of antifungal treatment require novel therapeutic strategy to combat candidiasis of which vaginal candidiasis is of particular interest due to recurrent episodes. Seventy-five percent of females suffer from vaginal candidiasis at least once in their lifetime, followed by many suffering from recurrent vulvovaginal candidiasis. *C. albicans* causes 85% of VC. Closely related species, C. *glabrata,* C. *tropicalis* and *C. parapsilosis* cause 5% or less of the infection (Sobel 2002). Initiation of candidiasis by *C. albicans* is due to defective host cellular response and disruption in the balance between the fungal virulence at the vaginal mucosa and host immunity (Höfs et al. 2016). Saps of *C. albicans* exhibit enzymatic activity at the mucosal site and adhere to host cells for fungal cell evasion. From the family of proteins, recombinant Sap2 tested in a rat model show increase in anti-Sap2 immunoglobulins IgA and IgG shielded from challenged intravaginal *C. albicans* upon immunisation (De Bernardis et al. 2012). Collection of other similar studies lead onto construction of r-Sap2 virosome vaccine named PEV7. Vaccine PEV7 by Pevion Biotech is in clinical trials for recurrent vulvovaginal candidiasis against *C. albicans*. Injecting high-dose PEV7 has shown strong immune response with no adverse effect with 100% mucosal immune response shown during clinical trials (Sandini et al. 2011). The vaccine works using virosomes which are envelopes of the Influenza-virus carrying the antigen, they work by displaying specific antigen on the surface and act as antigen presenting molecule (Mak et al. 2014). In this case Sap2 is an ideal candidate as it is known to be one of the virulent factors of *C. albicans (*De Bernardis et al. 2018*)*. Independent to the enzymatic activity, Sap2 also possesses pro-inflammatory activity (Pietrella et al. 2010). Immunisation of mice with Sap2 also provides immunity against systemic infection by *C. albicans (*Vilanova et al. 2004*)*. Antibodies produced by the PEV7 vaccine also cross-react with Sap1 and Sap3 along with Sap2, which have been

shown as highly expressive proteases of *C. albicans* during *Candida* infection in both humans and animal models (Pericolini et al. 2015). Another possible vaccine candidate called NDV-3 contains the N-terminal portion of C. *albicans* Agglutinin-like sequence 3 (Als3) protein (Segal 2017; Brena et al. 2007). Als3 expressed on *Candida* cell surface plays a key role in many processes, such as attachment to the host surface, biofilm formation and iron acquisition during infection (Richardson et al. 2018). Als3 so far is the best studied candidate for vaccines against *C. albicans (*Lin et al. 2009*)*. Studies carried out on both mice and humans reveal that NDV-3 exhibits antifungal activity and induces a Th17 response (Bär et al. 2012).The NDV-3 vaccine has successfully proved protection for different types of candidiasis. Observations show protective efficacy of the vaccine in preclinical animal models for oral, vaginal and hematogenous candidiasis (Schmidt et al. 2012; ClinicalTrials.gov 2018). Despite this evidence is present, the involvement of antibody response remains controversial as B-cell deficiency in mice did not have increased susceptibility to *C. albicans* infection (Carrow et al. 1984). This shows that antibody mediated protection is not the dominant adaptive cellular response against *C. albicans*. At present, the choice of anti-fungal drugs are limited as they can induce toxicity, acquire resistance and target unaffected organs. Also, treatment to fungal infections has been challenging due to the cell wall of pathogens that consist of complex polysaccharides (Romani 2004b). New antifungal treatment is required as immunocompromised patients fail to restore their immune system to combat fungal infections and hence high mortality rates are associated with these patients.

8 Conclusion

Candidiasis is a global issue. Though antifungal drugs are available, candidiasis is still one a common infection in immunocompromised patients. The elderly population, neonates HIV/AIDS and immune suppressed patient i.e. those undergoing chemotherapy and under a broad-spectrum of antibiotics are the most susceptible. Outbreaks and high incidence rates are often reported in hospitals and especially ICU wards, placing a burden on hospitals, longer stays and health care costs. Emerging non-*albicans Candida* spp. is another factor making treatment challenging as the organisms have adapted and evolved to evade the host immunity and prolong survival to initiate an infection. More studies are required on emerging pathogens due to their adapted behaviour of immune evasion and antifungal drug resistance. Development of new therapeutics and better understanding of pathogenesis, virulence factors and evasion strategies are required. This would then lead onto molecular and structural studies for the discovery of novel therapeutics.

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Immune Responses in Malaria and Vaccine Strategies

12

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1 Introduction

Plasmodium species include deadly *P. falciparum*, and less pathogenic *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is mainly restricted to sub-Saharan countries, whereas the others are more globally spread in temperate Africa, in Asian countries, and Latin America (WHO report, summary 2016). Ever since *P. falciparum* co-evolved, people have been under natural pressure for selection of sickle cell anaemia trait for their protection and survival (Killing and Rayner 2015). It is a recessive disease trait, with which heterozygous people are afforded with a strong protection from malaria, e.g. adults of Gabon are naturally immunised in endemic environment (Lell et al. 2018). Unfortunately, children, who inherit both disease haplotypes, i.e. to be homozygous to the mutant gene, at a haemoglobin chain single amino acid residue exchange, are not protected from either anaemia or malaria. Hence, the sickle cell trait is predominant in sub-Saharan countries (Allison 1954). An organisation has been founded to detect such young children for help (Hsu et al. 2018).

WHO 2016 Malaria Report quotes a reduction of endemic marginal malaria countries, down to 91 from 108, since 2000. In 2015, there were registered 212 million new malaria cases, and 429,000 people mortality, largely among children in sub-Saharan African region (McCall et al. 2018) (Geneva WHO Report 2017). By 2020, 40% reduction in malaria incident and morbidity is projected, and by 2030, 40% more, towards malaria eradication. *P. falciparum* infection is the cause of more than 90% of global malaria deaths, *P. vivax* malaria is largely excluded in Sub-Sahara countries, because of the lack of Duffy antigen expression on blood stage red cells, on which *P. vivax* infection depends. *P. ovale* is independent of Duffy red cell antigen and is also found in Africa.

P. falciparum life-cycle consists of female Anopheles mosquito vector-based sporogony stage, human host pre-erythrocytic liver stage, and asexual blood cell stage (Fig. 12.1). Sexual cycle gametocytes are generated at mature

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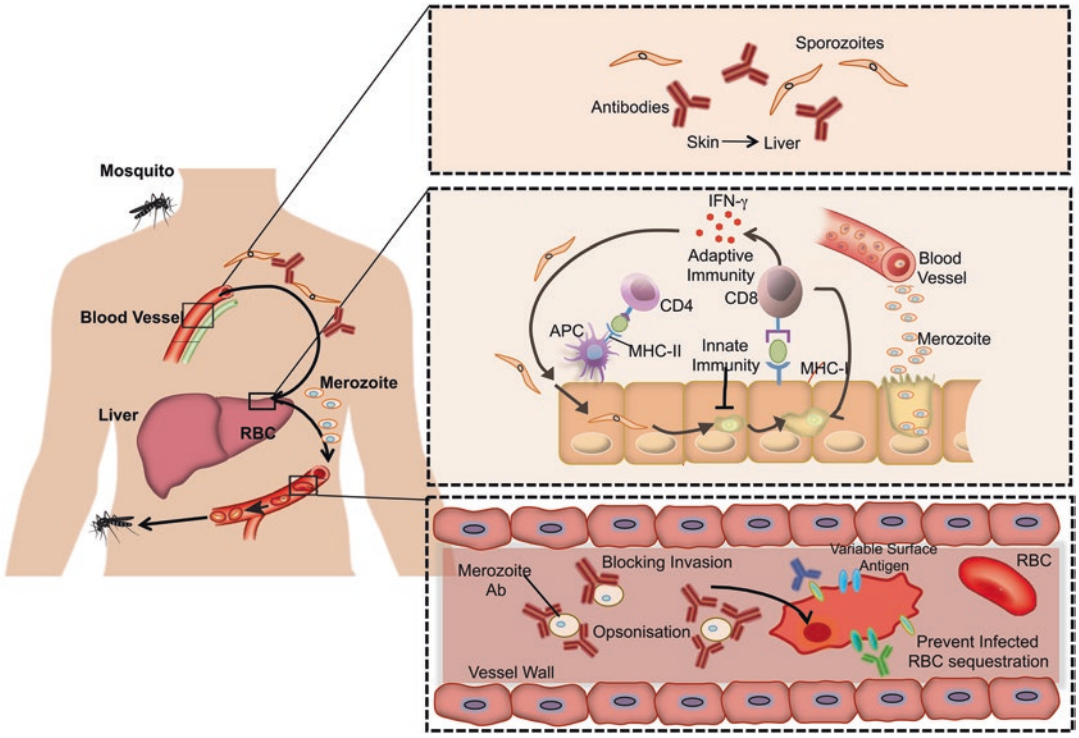

Fig. 12.1 Life cycle of the malaria parasite and its effect on host immune response. In skin, antibodies trap sporozoites, which are injected by mosquito bite, preventing invasion of liver cells. Inside liver, $CD4^+$ and $CD8^+$ T cells produce IFN-γ inhibiting development of parasite into merozoites. Frequently, this immune response is inadequate and merozoites emerge from liver and attack red blood cells followed by replication, burst out of infected erythrocytes and invasion of new erythrocytes. Antibodies specific for merozoites opsonize the parasite by blocking the receptor, which inhibits the RBC invasion. Furthermore, antibodies to surface variant proteins also agglutinate and opsonize the infected red blood cells and prevent their entry into the blood vessels.

trophozoite stage of blood cycle. These are engulfed with blood meal by female mosquitos. In their gut, male microgametocytes and female macrogametocytes undergo fusion, becoming zygotes. These are transformed into motile elongated ookinetes, which in midgut develop into oocysts. Oocysts mature into a number of sporozoites, which on rupture reach the vector salivary gland, producing anti-coagulants. On taking a meal, mosquito releases a number of sporozoites onto host skin and venous blood (Fig. 12.2). By being motile, sporozoites migrate to liver in minutes. There they actively traverse hepatic parenchymal cells *via* transient non-replicative vacuole to invade obligatory hepatocytes, in which they can replicate (Risco-Castillo et al. 2017). Further, the sporozoites transform into very large number of hepatic stage merozoites which are packed into schizonts. Some sporozoites become hypnocytes for storage, to be evoked in long time, especially in *P. vivax* and *P. ovale*, perpetuating malaria. Liver schizonts on rupture, discharge numerous merozoites, which infect red blood cells, in starting immature trophozoite ring form cycle, ending with merozoites. Daughter merozoites rupture and kill red cells to discharge pathogenic and symptomatic parasite debris and parasitaemia, and to invade further uninfected erythrocytes (Fig. 12.3). This febrile infection episode is repeated every 2 days in tertian malaria. *P. malariae* is unique in having quartan 3-day cycle. It can cause chronic disease (Centre for Disease Control and Prevention, 2018). *P. falciparum* possesses more than 5400 genes, including those involved in its stage variability, as well

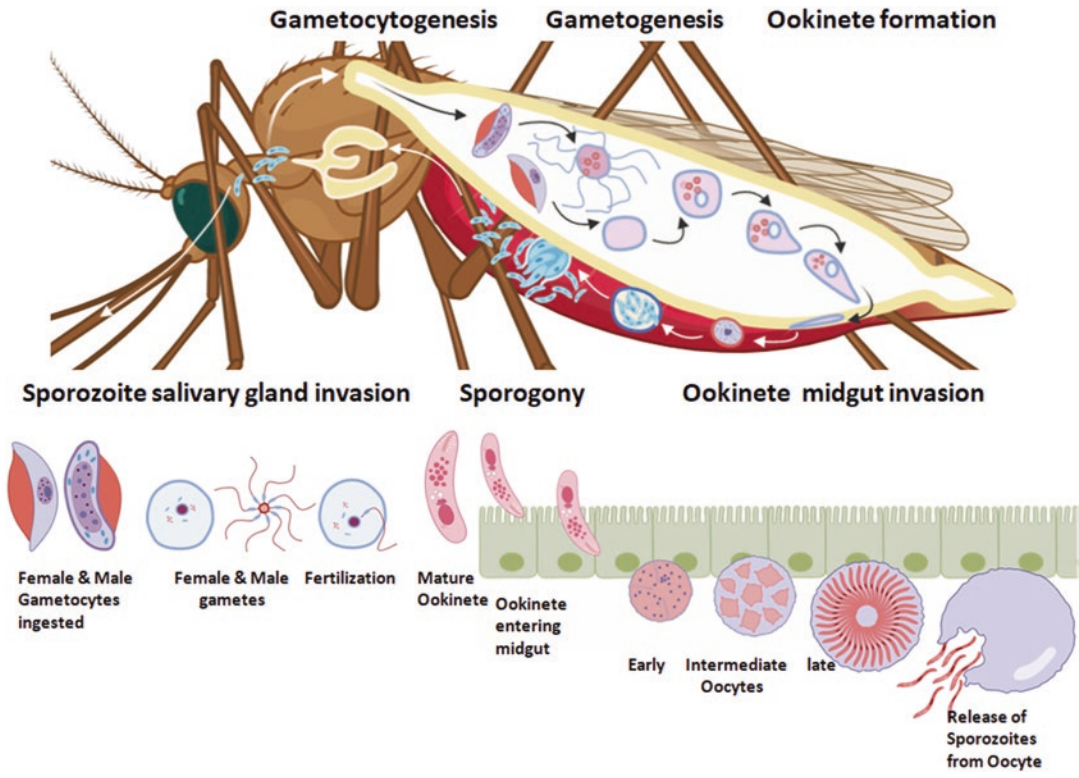

Fig. 12.2 The non-pathogenic sexual stages. Gametocytes fuse in the *Anopheles* mid gut to form the diploid zygote; this transforms into a motile ookinete and commences the mosquito phase of the *Plasmodium* life cycle. Antibody-mediated immunity to gametocytes and ookinetes reduces transmission to the mosquito vector. The approximate timing of malaria parasite development in the mosquito, from the ingestion of parasites by the mosquito to the end of sporogony, when sporozoites reach the salivary glands can be over 2 weeks. The changes in parasite density in the mosquito that occur within the time scale of complete sporogonic development can dictate parasite densities that are variable. Sporozoites are the most versatile invasive stages of *Plasmodium* life-cycle in their passage from mosquito vector to human host. They invade hepatocytes and transform into exo-erythrocytic stages. Sporozoites can be deposited into the skin by a probing mosquito, or they can be deposited into a blood pool created when the mosquito damages the blood capillaries during probing. Sporozoites deposited into the tissue transmigrate via its gliding motility

as escape host immune system (Hoffman et al. 2015).

2 Malaria Immunity and Vaccines

Malaria vaccine-driven immunity relies mainly on innate phagocytes, antigen-specific $CD8^+$ and, $CD4^+$ T cells, antibody-producing B cells, and their specific memory phenotypes. Two leading vaccine candidates have emerged after long clinical trials to protect young children and adults, both based on *P. falciparum* circumsporozoite protein specific antigen (CS) at liver stage. The RTS, S recombinant CS safety and efficacy was evaluated in clinical trials mainly with children for their protection against malaria in endemic regions (White et al. 2015). The Pf sporozoite Sanaria^R live, radiation-attenuated (motile, non-replicative and metabolically active) vaccine was tested in clinical trials predominantly with volunteers in malaria endemic and non-malaria countries (Hoffman et al. 2015). Both vaccines induce specific antibodies against liver stage PfSPZ antigens, preventing the parasite migration to liver hepatocyte, their obligatory habitat, and draining lymph nodes. The live vaccine can also activate

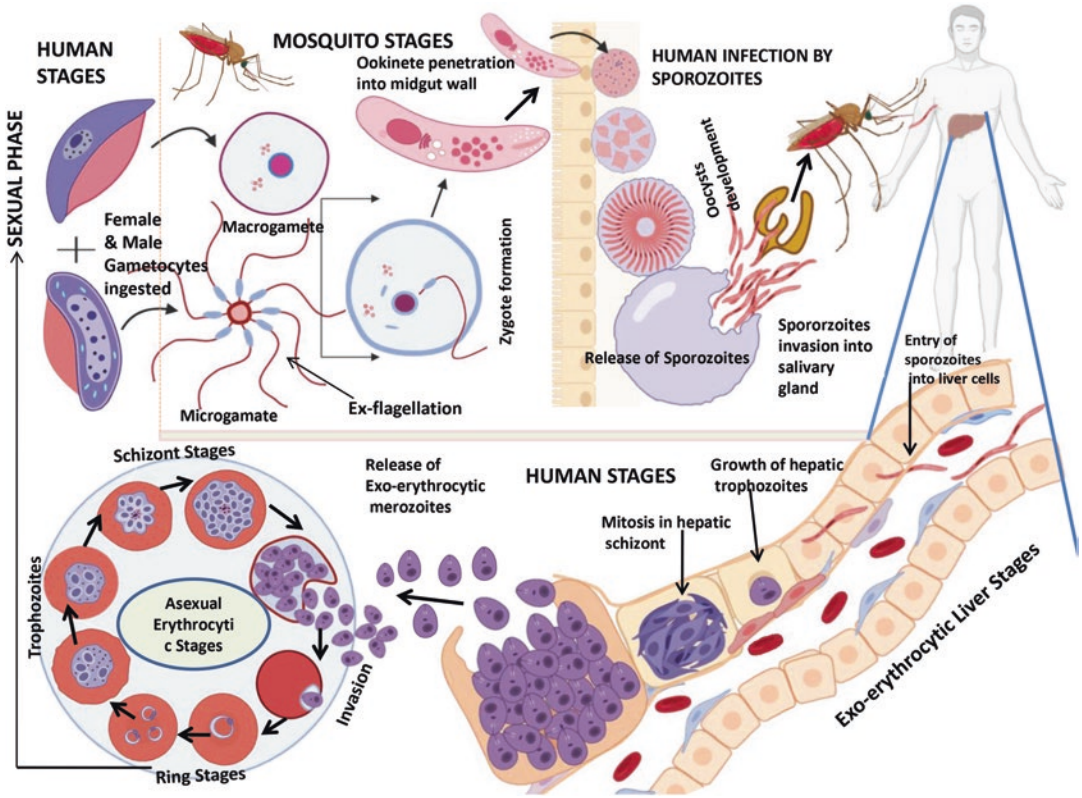

Fig. 12.3 Parasite invasion and differentiation in the two hosts. Infected mosquito bite injects sporozoites into bloodstream, which migrate to liver and infect hepatocytes. The hepatocytes display microvilli and are separated from the sinusoids by a layer of endothelium and Kupffer cells. Heparan sulfate Proteoglycans on the hepatocyte membrane bind sporozoites, leading through to endothelial cells, invading hepatocytes and commencing exo-erythrocytic forms. Sporozoites could move along the endothelial cells by gliding motility and invade Kupffer cells. They then exit the Kupffer cell and invade hepatocytes. In next 5–10 days, parasites differentiate and multiply within hepatocytes. Between 20,000 and 40,000 merozoites are released into blood that invade RBC. During intraerythrocytic stage, parasites develop and multiply over 48 h. When iRBC burst, 15–32 merozoites per iRBC are released, which invade RBC to begin a new cycle. A small proportion of iRBCs have parasites undergoing differentiation into either male or female gametocytes (which can be taken up by blood meal by mosquitoes). In insect mid gut, male and female gametes are released leading to fusion (zygote), which then undergoes a series of complicated differentiation and growth stages, resulting in the production of infective sporozoites in the salivary gland of mosquitoes

antigen-specific effector cytotoxic $CD8^+$ T cells to recognise and induce apoptosis of parasite-infected hepatocytes.

In most malarial vaccines (Fig. 12.4), antigenic breadth is required, such as in attenuated live vaccines involving PfSPZ, around hundred antigens on their surface are required for a potent protection of children and adults. In addition, vaccine dose, number and timing of vaccination is the deciding factor (Weiss et al. 1988; Weiss and Jiang 2012; Hoffman et al. 2002; Lyke et al. 2017; Cockburn and Seder 2018). This is in contrast to most bacterial and viral vaccines, targeting successfully one, or few antigens with the aid of adjuvants. Adjusted adjuvants such as in RTS, S vaccine are required for elevated antigen-specific antibody titre, in safety and efficacy trials in children, as indicated in randomised clinical trial (White et al. 2015). The Sanaria^R live vaccine also underwent years of clinical trials with volunteers, including young children. For strong Sanaria^R-induced immune responses, intravenous vaccination is needed, whereas RTS, S

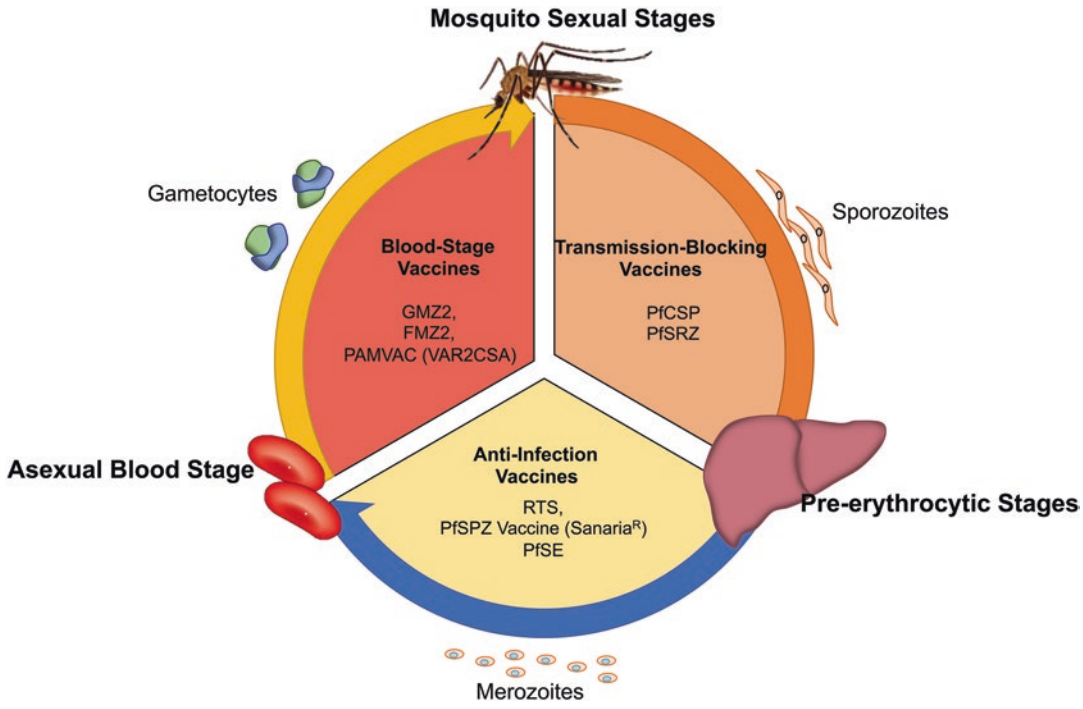

Fig. 12.4 Options for Vaccines strategies for infected stage of malarial infection. Vaccines and antigens discussed in this chapter are presented according to their targeted stage of the parasitic life cycle. Sporozoites specifically invade a hepatocyte and transform into clinically silent liver stages that generate tens of thousands of pathogenic merozoites. Intracellular liver stages reside in a replication-competent compartment-the parasitophorous vacuole. Secretory proteins might be processed and presented in association with MHC I molecules on infected hepatocytes where they are recognized by $CD8^+$ T cells. Attenuation of liver stage development by irradiation or targeted gene deletion confers sterile protection against natural malaria transmission. Following female *Anopheles* mosquito, sporozoites are injected into the skin, actively enter a nearby blood vessel and reach the liver. Successful transmission can be reduced by anti-sporozoite antibodies, which inhibit parasite motility and transmigration. Inhibition needs to be completed without allowing a single living sporozoite. At this stage, sporozoites might also interact with local regulatory T cells in the skin or in draining lymph nodes to induce tolerance. Merozoites specifically invade RBCs and transform into trophozoites. After several rounds of replication merozoites are formed, that invade new RBCs. Invasion, intracellular growth, replication and egress are synchronised (fever–chill cycles). During the few sec. of erythrocyte rupture and re-invasion, merozoites can be blocked by antibodies to result in overall reduction of the parasite load. In addition, toxic components that elicit inflammation can be targeted by anti-disease vaccines. During first-time pregnancies, parasites target a unique adhesin of the PfEMP1 family, termed VAR2CSA, to the RBC surface; this results in massive sequestration of infected RBCs in the placenta. Antibodies from multigravid women protect against pregnancy-associated malaria, suggesting that development of an exclusive vaccine against placenta-associated malaria is realistic

vaccine is intra-muscularly administered. RTS,S trials were conducted mainly with children (as described below).

2.1 Blood Stage Malaria Vaccines

A blood stage malaria vaccine GMZ2 large randomised trial with children in Burkina-Faso, Gabon, Ghana and Uganda was conducted. The vaccine was well tolerated. The risk of clinical malaria decreased with IgG antibody level augmentation, making the vaccine a good candidate for improvement. The antigens are *P. falciparum* glutamate rich protein and merozoite surface protein 3 (Sirima et al. 2016). A later FMZ2 vaccine trial with Gabonese healthy volunteers was performed. Base-line antigen specific antibody level was protective against symptomatic malaria, which is regarded a strong tool against endemic

malaria (Dejon-Agobe et al. 2019). An asexual blood stage vaccine approach involves chemically attenuated parasite with tafuramycin. Naïve Australian volunteers were engaged in a pilot clinical study. They were immunized via a single vaccination. In response, their antigen-specific T cells produced potent bactericidal cytokines including IFN-γ and TNF-α, and induction of $CD3^+CD45RO^+$ memory cells, but specific antibodies were not detected. It is the first whole blood stage vaccine, which warrants further development. Trial registration: ACTRN12614000228604. 4 March 2014 (Stanisic et al. 2018).

Pioneering research by Nussenzweig et al. in 1967 discovered that radiation-attenuated sporozoite vaccine of *P. berghei* protected mice against this malaria species, not only at hepatic cell stage, but also by inhibiting development of sporozoite at oocysts form, at mosquito vector stage (Menard et al. 1997). This vaccine property is also valid in human *P. falciparum* attenuated live vaccine Sanaria^R, and in recombinant RTS,S vaccine in children and adult. They are both targeting major Pf circumsporozoite protein specific antigen (Clyde 1990; Hoffman et al. 2002; Epstein et al. 2011, 2017; Triller et al. 2017).

2.2 RTS,S Malaria Vaccine

RTS,S/AS01 adjuvant vaccine (RTS, S) is a recombinant Pf CSP protein vaccine, which can induce in infants, and toddlers and adults protective titres of specific anti-CSP antibodies, at hepatic malaria stage. It is also able to reduce in malaria-susceptible children, clinical symptoms and severity of malaria disease at blood stage. The vaccine did not interfere with children vaccination (like DTP- diphtheria, tetanus and pertussis, and *Haemophilus influenza* type b virus) (Casares et al. 2012; White et al. 2015). There have been several clinical randomised trials with children and infants in Africa, and with adults naïve volunteers, which established antibody mediated reduction of clinical disease (Casares et al. 2012). The last major phase III RTS,S trial was conducted between 2009 and 2014, 8922 toddlers, and with 6537 infants in 11 sub Saharan countries (White et al. 2015). The trial data have been analysed in terms of safety, immunogenicity and efficacy.

RTS,S vaccination was administered by intramuscular injection in 3 intervals. Children antibody titres against CSP antigen were very elevated; higher in 5–17 month old toddlers than in 6 to 12-week old infants (White et al. 2015). However, the antibody levels waned until after 1 year, and further towards 5-year duration. An additional boosting dose vaccination at 18 months strongly prolonged a higher titre of anti-CSP antibodies, and that of specific memory B cell-plasmablast clones. RTS,S vaccine has passed phase 3 in trials for safety and efficacy. It has been estimated that the vaccine will prevent 50% malaria infection in children and adults, living in malaria endemic countries (White et al. 2015; Cockburn and Seder 2018). Presently, the RTS.S vaccination is administered 4 times at appropriate intervals for a higher and prolonged antibody titre RTS,S has recently been authorized, but not yet licensed, by Government of Malawi and WHO to begin a routine vaccination pilot program in children aged up to 2 years. Ghana and Kenya are to follow. This is the first vaccine against malaria and parasites generally (WHO report 2019).

Recently, additional data from the same randomised RTS,S vaccine phase III trials with children and infants in Africa are focused on patients. Children/infant safety from serious adverse events (SAE) and vaccine reactogenicity are estimated in detail. These include children mortality, anaemia, febrile convulsions, pneumonia, gastro-enteritis, meningitis, cerebral malaria, preterm low weight infants, and gender-specific mortality. Some may be co- morbidities with other infections, including acquired immunodeficiency syndrome (AIDS) and hepatitis virus B type, which was inhibited by the vaccine. On the whole, RTS,S vaccine contributed to reduction of malaria clinical burden in children. There remain some uncertainties about the immune mechanisms, reducing clinical malaria at blood stage malaria, and potential cerebral malaria, which was not clinically examined during the trials. These issues may be clarified in

the next phase IV trial of RTS,S vaccine, and in WHO authorized vaccine pilot program with Malawian children (Mendoza et al. 2019).

The specific anti-CSP antibodies target the parasite during its migration from skin following mosquito bites to draining lymphatics, and on a short way in venous blood to liver (Flores-Garcia et al. 2018). PfSPZs may be also for a moment exposed to specific antibodies, during its journey, and on leaving apoptotic hepatocytes, to infect uninfected hepatocytes. This process occurs after just at a low multiplication number. Coated with specific IgG, they are arrested and opsonised for phagocytes such as macrophages and liver resident Kupffer cells for their engulfment. The Pf SPZ proliferation and form changes at liver stage and are of short duration, usually 5–6 days in humans.

For an explanation of reduction, in malaria cases, RTS,S vaccine induced antibodies may possibly also target merozoites, which may still express some dominant Pf CSP antigens which may inhibit their transition to blood for red cell stage invasion. The Pf CSP protein, a major antigen targeted by RTS,S vaccine, was found to be expressed also on liver stage schizonts. These features are regarded a legacy of Ruth Nussenzweig discovery (Cohen et al. 2010; Casares et al. 2012).

CSP protein regions were analysed for their dominant epitope as RTS,S targets, which appear to be of several antigenic breadth at the epitope level. CSP is of 58 KDa, and embodies central 41 repeats of sequence NANA $(Asn\text{-}Pro\text{-}Asn\text{-}Ala)_6$ amino acid residues, and 2 flanking regions (Good et al. 1988). The N-terminus has a 5 amino-acid motif ^{93}KLKQP^{97} common with other Plasmodium species to invade hepatocytes and mosquito salivary gland (Dame et al. 1984; Enea et al. 1984). Central repeat region of CSP molecule contains dominant B cell epitopes, which might be amplified by their number. C- flanking region is highly polymorphic, perhaps providing a bigger ground for epitope-antibody target diversity. It harbours immunodominant parasite epitopes, targeted by B cells as well as T cell recognition, named Th2R and Th3R, e.g., as shown in an Iranian population study (Good et al. 1988; Zakeri et al. 2007; Casares et al. 2012). Such strong immune responses may be achieved through appropriate adjuvants, serving for cross-priming, via dendritic cells, a specific antigen in naive T or B cell differentiation. This includes their specific antigens and memory B cells against malaria (Casares et al. 2012).

The reduced clinical malaria at asexual blood stage is regarded as a remarkable achievement in children and adults, who are not naturally resistant to malaria burden, living in endemic countries. The clinical case reduction by vaccine may be more important for public, than protection against malaria infection in lucky individuals (Casares et al. 2012). It may help people who are unable to eradicate malaria at pre-blood stage. Putatively, the vaccine may reduce parasitaemia, lower than the threshold for clinical disease. It may thus also help natural immunity for children and adults to fortify such under-threshold parasitaemia, and for those children who could not resist pathogenic malaria infection naturally (Casares et al. 2012; White et al. 2015). Pf CSP is essential for mosquito stage development at oocyst form (Menard et al. 1997). It has been a long road from early 1980s in generating a safe and efficacious recombinant vaccine, with adequate adjuvants, especially for infants and children against malaria. It has been suggested that the efficacy of RTS, S may be augmented by implementing PfSPZ live X-ray attenuated, non-replicating, and metabolically functional vaccination developed by Steven L Hoffman and colleagues. Hepatocyte infection is obligatory for parasite replication. *Pf*CSP promotes sporozoite attachment to hepatocytes for their invasion (Menard et al. 1997; Hoffman et al. 2015; Casares et al. 2012; White et al. 2015).

3 PfSPZ Sanaria^{R} Vaccine

Clinical trials have been undertaken on naïve male volunteers for assessing safety, tolerance, immunogenicity, and protective efficacy of attenuated live *Pf*SPZ vaccine. They were vaccinated by intravenous route, and then challenged intravenously by controlled human malaria infection (CHMI), with virulent live non-attenuated PfSPZ,

which were cryo-preserved. They are both produced by Sanaria^{M} Inc., based on NF54 strain (Hoffman et al. 2002; Epstein et al. 2017).

Randomised double-blind trials with Sanaria^{R} PfSPZ attenuated live vaccine were conducted with U.S.A., Tanzanian, and Malian young men as volunteers (Jongo et al. 2018). Various groups of volunteers were vaccinated intravenously with increasing radiation-attenuated PfSPZ vaccine doses (highest dose of 2.7×10^5 at 4-week interval). Other groups for immunogenicity and efficacy utilised this attenuated PfSPZ vaccine dose at 0, 4, 8, 12, and 20-week intervals. After 24 weeks of the last vaccination, men were challenged i.v. with CHMI 3.2×10^3 of virulent Sanaria^{R} PfSPZ infection, for protective efficacy of the vaccine against malaria, as compared with placebo controls, as monitored by reduction of parasitemia, and by thick blood smears (TBS). In a group, parasitaemia was not detected, suggesting a sterile protection. Antibody sera were assessed by ELISA, Immunofluorescence, and Inhibition of sporozoite-mediated hepatocyte invasion assay.

PfSPZ vaccine of 2.7×10^5 bites was optimal to induce vaccine efficacy of 65% against CHMI challenge in American men, of 52% in Tanzanians, and of 29% in Malians, which are naturally infected during the intense malaria transmission season. *Pf* CSP antibody titre induction against PfSPZ antigens were of similar proportions, the highest in naïve immunized American men. On the whole, the vaccine was found to be safe, well tolerated, immunogenic, and protective against Pf malaria infections up to 90% in Africa and up to 100% in USA. An explanation of a lower cellular and humoral response to PfSPZ vaccination in Malian volunteers was due to heterogeneity of parasite antigenic variation, or to natural immune equilibration in dense malaria endemic exposure. The trial is registered at Clinical Trials.gov (NCT02132299) (Jongo et al. 2018). Previously, it was important to know whether the NF54 vaccine strain would be effective against various PfSPZ strains in different countries.

3.1 PfSPZ Vaccine Against Heterogeneous Malaria

In a randomised trial with people of various ethnicity and of both genders, it was established that the PfSPZ vaccine was protective, not only against homologous strain of PfSPZ-CHMI, but also against heterologous strains, that is, people who have been vaccinated i.v. with the Sanaria^{R} PfSPZ-strain NF54 vaccine, and challenged against homologous (NF54 strain) PfSPZ-CHMI infection. The vaccine was well-tolerated, safe, immunogenic and protective against malaria. In comparison, genetically different (heterologous) PfSPZ strains in CHMI infection were included in the clinical trial, challenging the Sanaria^{R} PfSPZ vaccine. These included Pf7G8 polymorphs selected from genome search, and 19 clinical isolates from Africa. In a group of naïve subjects, the PfSPZ vaccine yielded protective efficacy against homologous CHMI of 87%, and that of heterologous CHMI of 57%. The Vaccine strain cross-protection against heterologous PfSPZ infection is regarded of paramount importance for a global vaccine (Lyke et al. 2017).

3.2 PfSPZ Vaccine Mediated Protection Against Malaria during Transmission Seasons in Endemic Regions

A preceding randomised trial with Sanaria vaccine in malaria was conducted with Malian healthy volunteers for the first time in an endemic malaria region. It was found to be safe, and protective via cellular immunity at a low, but significant, level against malaria during the transmission seasons. The five-dose vaccination schedule took place during dry season from January to July. Antibody production against several malaria antigens was monitored before and after vaccination, but it was at a low level nearly down to- controls. Trial registration is: ClinicalTrials.gov, NCT01988636. (Sissoko et al. 2017). The PfSPZ vaccine, and het-

erologous CHMI challenge against Pf malaria, may serve as a model towards eradication program of malaria in other counties. It requires clinical trial partnerships on a large scale. A parallel randomised placebo, doubled blind controlled clinical trial has been initiated with Equatorial Guinean (EG) Authorities on Bioko Island, where malaria is endemic. There they never had any clinical anti-malaria vaccination trials (Olotu et al. 2018). After the vaccine ethics requirements, men and children have been selected for clinical trials, 135 in number, ranging from 6-month infants to 55-year individual. The same vaccine of 2.7×10^5 PfSPZ schedule, and surface Pf-CSP protein (CVac) was utilized, except children receiving only 3 vaccine doses. The vaccine tolerability and protective efficacy were adequate. However, IgG antibody titre again was insignificant (Olotu et al. 2018). The vaccine is registered: Clinical Trials.gov identifier: NCT02418962. However, while clinical trials with Sanaria PfSPZ vaccine were usually analysing IgG antibodies, in a recent trial with volunteers, the IgM assessment was included. Antigen-specific high titre IgM was found for long-duration, apparently preventing sporozoite entry into hepatocytes *via* complement fixation on their membrane.

3.3 IgM-Mediated Inhibition of PfSPZ Hepatocyte Invasion

Recently, sera of pre-exposed volunteers in a previous PfSPZ vaccine trial in Tanzania have been evaluated for IgG and IgM antibody titres against CSP (Zenklusen et al. 2018; Richie et al. 2015). These antibody classes were found in significant amounts, after they were vaccinated with PfSPZ vaccination schedule, and challenged with CHMI-PfSPZ. The IgG and IgM antibody titres against CSP antigen were detected by ELISA, and antibody binding to the whole sporozoite surface also visualized via immunofluorescence microscopy. Inhibition of Pf sporozoite via hepatocyte invasion assay was included. It was shown that IgM fraction significantly inhibited hepatocyte invasion *in vitro,* by binding to the parasite surface membrane antigens. These are of various specificity, expressed all along the parasite. Such humoral immunity was long-lasting. Moreover, IgM antibody fixed complement on the parasite surface, leading to generation of C5a anaphilatoxin and C5b down to membrane attack complex (MAC) mediating parasite lysis. Clinical Trials Registration is: NCT02132299. It was recommended that these tests should be included in future trials in the liver stage anti-malarial vaccines. Perhaps, a significant inhibition of PfSPZ hepatocyte invasion in clinical malaria is itself a good indication that IgM was involved. It has been reported that on mosquito biting site, some sporozoites are retained in the host dermis, and are immobilized by antibodies in mice model (Flores-Garcia et al. 2018). A very promising candidate is the chemo-attenuated live vaccine in host protection (PfSPZ-CVac).

3.4 A Sterile Protective Chemo-Attenuated PfSPZ-Based Malaria Vaccine

A study with naïve volunteers revealed that they were protected against malaria, only when they were vaccinated with non-attenuated PfSPZ, while on prophylactic chloroquine treatment. Production of their antibody titres against PfSPZ CSP and other liver stage parasite forms, and that of blood stage, were evaluated. Antibodies against sporozoite and pre-erythrocyte stage were mostly correlated with protection, in spite of the fact that chloroquine only targets red blood stages. This includes inhibition of merozoite development to repeat the red blood cell cycle, hence the transient increase of parasitaemia. This event may in malaria-infected individuals give rise to a broad range of antibodies against CSP protein, liver stage of which some may be shared or be cross-reactive (Bijker et al. 2013; Mordmuller et al. 2017).

In later-randomised trials with naïve volunteers undergoing chloroquine prophylaxis, it was demonstrated that PfSPZ-CHMI (Sanaria non-irradiated), i.e. acting as a vaccine in this combination with the drug, was obligatory for a long-lasting protection against malaria, beyond that of drug protection alone. This was evident in

comparison with the placebo that was given i.v. normal saline. The vaccine was named PfSPZ-CVac. Its host protective efficacy is at par with that of Sanaria PfSPZ vaccine, capable of eradicating PfSPZ infection in naïve volunteers. Mefloquine was also similarly effective. The vaccine-chemo treatment of individuals was found to be safe, well tolerated, and protective. Out of 3 dose volunteer groups in the trials, group III was most protected, reaching 100%. It consisted of 5.12×10^4 live PfSPZ number per dose (i.v.), 3 times at 28-day intervals, while receiving chloroquine treatment. Ten weeks after the last vaccination, they were challenged with controlled CHMI once or twice. Lower dose in volunteers I and II had protective efficacy of 33% and 67%, respectively. The vaccine was found safe, well tolerated, and host protective, even in the case of severe malaria. It also augmented $CD4^+$ Th cells and cytotoxic $CD8^+$ T cells, stimulating IFN-γ, TNF-α and IL-4 secretion which are parasiticidal. Memory CD38 positive γδ T cells were also augmented. Antibody titres were analysed in volunteers at pre- and post-vaccination stage against several sporozoite epitopes, mainly for IgG isotypes. All vaccine groups induced antibodies, but only in vaccinees, antibodies correlated with host protection. This correlation also applies to inhibition of PfSPZ hepatocyte invasion. Blood stage was not targeted. 100% protected vaccines did not experience transient parasitaemia of blood cycle, in contrast to non-fully protected volunteers, caused by chloroquine. Presumably, this was because they eradicated the parasite at liver stage. These results may suggest a close interdependence of the vaccine with chloroquine prophylaxis. PfSPZ-CVac, in combination with chloroquine, is projected to be able to eradicate malaria on large scale in endemic regions. It is also open to improvement such as in vaccine dose regime, drugs, and inclusion of heterogeneous Pf strains in the vaccine (Mordmuller et al. 2017; Sissoko et al. 2017). Such vaccination regime may not be suitable for children against malaria protection. Putatively, eradication of adult malaria as a reservoir may reduce malaria incidence in dense malaria endemic areas.

3.5 Vaccine Dose and Origin of PfSPZ Vaccine

In a separate trial, naïve adult volunteers were vaccinated with a higher dose of radiation attenuated (9.0×10^5) Sanaria-PfSPZ-NF54 vaccine, which is of African origin. Efficacy of PfSPZ host protection and durability between homologous and heterologous strains of Brazil were comparable, as measured by their parasitaemia (Lyke et al. 2017). The US army and traveller volunteers received 3 doses of PfSPZ-NF54 vaccine i.v. in 6-week interval. Upon their challenge with CHMI infection, more than 60% of volunteers showed sterile protection against homologous Sanaria CHMI challenge, but equally against heterologous PfSPZ 7G8 strain CHMI of Brazilian origin. All non-vaccinated individuals developed parasitaemia. PfSPZ specific antibodies increased in non-parasitaemia individuals over that of controls in 49 weeks. However, memory $CD8^+$ and $CD4^+$ T cells peaked at 4 weeks, much more in control groups, and levelling later. Parasiticidal cytokine secretion by these T cells, including γδ T cells, such as of IFN-γ, TNF-α, and IL-2, was also much elevated in PfSPZ vaccinated volunteer group (Zaidi et al. 2017). This study showed that Pf SPZ live vaccine of various origins is capable of substantially eradicating malaria in volunteers at liver stage, and prospectively also in people living in malaria endemic countries (Lyke et al. 2017). The trial is registered at ClinicalTrials.gov (NCT02015091). Such individuals might also be protected by macrophage type 2 innate immune tolerance and regulatory T cells, in balance with TLRs, recognizing malarial glycosylphosphatidylinositol molecular pattern in mediating pro-inflammatory cytokine responses, which may damage host tissue (Gowda and Wu 2018).

3.6 *P. vivax* Malaria Vaccine

P. vivax is associated with blood stage malaria, and with natural humoral immunity for protection. However, a naturally modified cellular immunity has been introduced in recent studies. *P.*

vivax sporozoites prefer engaging a distinct subset of circulatory, *P. vivax* primed, antigen specific cytotoxic $CD8^+$ T cells that recognize HLA-1, together with *P. vivax* CSP on infected reticulocytes, for their granzyme-mediated apoptosis. This subset of $CD8^+$ T cells expand upon CSP priming. Reticulocyte red cell progenitors still possess nucleus and immune gene transcription apparatus for responding to *P. vivax* infection (Burel et al. 2016; Junqueira et al. 2018; Anstey et al. 2009; Bassat and Alonso 2011). Similar to liver stage malaria, *P. vivax* granulocyte infection antigen is recognized by specific cytotoxic CD8 T cell receptor to release pro-apoptotic granzyme E, A and B. Granzymes are delivered into the infected target cells by cell membrane pore-forming proteins. These include granulolysin, which normally makes pores in the parasite membrane. After apoptotic cell death, the freed parasite is also subject to killing by granulolysin against reinfection. The clinical trials with malaria-infected volunteers and patients were conducted in Australia and Brazil (Burel et al. 2016; Junqueira et al. 2018). The malaria patients and un-infected subjects were analysed as donors of their circulating activated cytotoxic T cell and of target cell apoptosis *ex-vivo*, who were recruited from the same malaria region in Brazil. For instance, their circulating activated cytotoxic T cell activation markers CD69, HLA-DR and Ki57 associated with cell proliferation were elevated in malaria patients, and they decreased after 30–40 days to normal level upon their cure with chloroquine and primaquine therapy. It has been suggested that reticulocyte malaria infection, targeted by cytocidal $CD8^+$ T cells, may be incorporated in *P. vivax* vaccines, like *P. falciparum* liver stage (Weiss et al. 1988, 2012; Schofield et al. 1987). Perhaps, antibodies against merozoite Duffy binding protein vaccine may help in such combination (Junqueira et al. 2018).

P. vivax causes a major pandemic worldwide, with approximately 100 million clinical cases per year, although in large majority of people, the disease remains asymptomatic for years, but is prone to relapses. *P. vivax* can cause serious disease and fatality in some people, such as in children cerebral malaria, acute respiratory distress syndrome, hepatitis, intravascular disseminated thrombosis, foetal low weight birth, infant mortality, severe anaemia and thrombocytopenia. The parasite biomass and fragile uninfected red blood cells may make *P. vivax* pyrogenic (Anstey et al. 2009). However, in contrast with *P. falciparum*, *P. vivax* infects up to 2% of young erythrocytes in circulation, but is not related to severity of the disease. *P. vivax* malaria is evolutionarily better co-equilibrated than *P. falciparum*, so as to spare the host for its own survival. On the other hand, it may be more difficult to obtain a protective *P. vivax* vaccine (Burel et al. 2016; Bassat and Alonso 2011).

Recently, a cohort study was conducted with Papua New Guinean children aged 1–3 years, with their naturally acquired *P. vivax* IgG antibodies against malaria in endemic regions. The aim was to boost their natural humoral immunity, to identify *P. vivax* antigen combination for their highly protective IgG antibodies from clinical malaria, as prospective vaccine candidates. Thirty-eight *P. vivax* antigens were selected, of which combination of EBP, DBPII, RBP1a, CyRPA and PVX 081550 antigens were most frequently found. Their antibodies protected children with more than 90% efficacy. The antibodies were mainly against late-schizont stage, preventing their erythrocyte adhesion and invasion. Here, in young children, breadth of *P. vivax* antigen-IgG antibody was appreciated. During their 16-month life, some children experienced elevated risk for febrile episode, or a higher parasitaemia in combination with other antigens. Synergistic and additive effects were observed in the five-antigen combination and with other of 38 proteins. Included are also thresholds for IgG level with different antigen dose responses to reduce risk of clinical malaria, and cross-reactivity of antibodies to different antigens (Franca et al. 2017).

However, natural protective immunity against *P. vivax* can be acquired, which is specific for a particular endemic community. People upon migration to another endemic region are not protected. Such immunity was first demonstrated by bacteriologist Robert Koch, researching malaria in Papua New Guinea more than 100 years ago,

where *P. vivax* is predominant. Later studies indicated that the number of malaria exposure in close endemic community is optimal to develop and sustain a balanced natural immunity (Barry and Hansen 2016; Offeddu et al. 2012). Interestingly, there is a decline of *P. vivax* malaria transmission across south-west Pacific due to inbreeding of the parasite (Waltmann et al. 2018).

4 Cytotoxic CD8⁺ T Cellular Immunity and Malaria Vaccine

CD8⁺ T cells are considered to be pivotal in immunity against intracellular *P. falciparum* parasite in humans, aided by IFN-γ also from CD4⁺ T cells. Such unique CD8⁺ T cell property has been demonstrated in mice and primate studies, in which their sporozoite immunity was reversed by monoclonal antibodies against CD8⁺ T cells, but not by monoclonal antibody that depleted CD4⁺ T cells (Weiss et al. 1988, 2012; Schofield et al. 1987; Good and Engwerda 2011; Cockburn et al, 2008). Live attenuated malaria vaccine was designed to promote CD8⁺ T cell immunity (Epstein et al. 2011). Activated cytotoxic CD8⁺ T cells induce apoptosis of parasite-infected cells.

In malaria, PfSPZ specific antigen/MHC-antigen complex on infected hepatocyte surface may be likewise restricted by antigen specific CD8⁺ T cells for apoptosis in individuals. Those naturally exposed individuals may have been previously cross primed by dendritic cells with PfSPZ specific epitope, elicited through PfSPZ vaccination. Putatively, a low immune response to recombinant subset vaccines may be ascribed to a diminished MHC class 1 specific antigen restriction, while dealing also with unrelated bacterial antigens. Apoptosis of infected hepatocytes may have an advantage for the host protection. It induces in local phagocytes anti-inflammatory cytokines such as IL-10 via phosphatidylserine ligation, exposed on outer cell membrane layer. This may be a reason why hepatic malaria stage is not a clinically pathogenic event in infected people (Weiss et al. 1988, 2012; Schofield et al. 1987; Good and Engwerda 2011).

A detailed analysis of liver stage malaria revealed that disease outcome depended on the number of sporozoite-infected hepatocytes, versus that of local liver infiltration of antigen- specific CD8⁺ T cells. For host protection, cytotoxic T cells may outweigh the infection. The study involved mice, infected with *Plasmodium berghei*. It took 3 days for replicative antigen-specific cytotoxic T cell liver migration, which have been produced by infected donor mice, as a titration model (Spencer et al. 2017).

5 Malaria Vaccination in Pregnancy

Cellular cytotoxic T cell specific immunity is also subject to down-regulation of serine proteases by non-classic human leukocyte antigen G (HLA-G) for the maintenance of placental maternal-foetal tolerance, as a semi-allograft. HLA-G is a dimer of class-Ib protein, which inhibits Granzyme B expression. HLA-G soluble level was increased in patients tolerating their kidney transplant, whereas in patients rejecting renal transplant, the level was significantly lower. HLA-G expression is found distinctly on cells/tissues such as blood reticulocytes, placenta trophoblasts, thymus medulla, cornea and pancreatic islet cells, in protection from auto-immune type 1 diabetes in children (Ajith et al. 2019). In placenta, foetal placenta produces HLA- G, which protects foetus from maternal uterus NK cell cytolysis. Such NK cells also produce granzymes. NK cells express receptor for HLA-G protein ligation (Rouas-Freiss et al. 1997, 2007). There has been a delay with candidate vaccines in pregnancy trials. There are, however, studies on malaria impact in pregnant women, foetal life and infants in natural malaria infection in endemic condition.

5.1 Pf Malaria Vaccine Candidate in Pregnancy

It is imperative to protect expecting mothers from malaria. Clinical trials are being conducted with

pregnant women in Mali, utilizing Sanaria Inc. vaccine which is in phase 2 trial (Healy et al. 2019). A strong promising vaccine candidate, first of its kind, which is pregnancy-associated malaria vaccine (PAMVAC) to protect expecting mothers and foetuses against malaria, has been introduced in clinical trials. PAMVAC has passed, in adult volunteer randomised double blind trial for safety, tolerogenicity, immunogenicity, and efficacy requirement, and is now preparing for clinical trials with gravid mothers (Mordmuller et al. 2019; McCall et al. 2018). PAMVAC is a recombinant protein vaccine (VAR2CSA), composed of *P. falciparum* blood stage conserved VAR2 antigen, and of distinct type of placental chondroitin sulphate A (CSA) antigen, which binds infected erythrocytes for their virulent inflammatory placental sequestration (Pereira et al. 2016). They are both potent antigens to induce IgG response in vaccination of naïve volunteers, and are very likely to be also in expecting mothers in malaria regions. PAMVAC-induced antibodies inhibited infected red blood cell ligation to placental CSA, thus strongly preventing their placental sequestration. In healthy volunteer trial, adjuvants employed were glucopyranosyl lipid adjuvant in stable emulsion (GLA-SE), GLA-LSQ, or alhydrogel, of which GLA-SE was most helpful towards the vaccine efficacy. This feature was measured by specific antibody titres by ELISA, in preventing live malaria asexual stage-infected red cell ligation to CSA. CSA was on adherent plates, a test, simulating their sequestration. Naïve individuals were immunized by 3 intramuscular injections, every 4 weeks apart. After last vaccination, such protective immunity was followed for 6 months. Registration: ClinicalTrials.gov NCT02647489 (Mordmuller et al. 2019).

5.2 Embryonic Effector T Cells Primed with Placenta Malaria

Recently, in Ugandan endemic area, a clinical trial with 182 neonates/infants, born to mothers with prenatal placental malaria, has been studied for their protection, as compared with those of non-infected mothers (Odorizzi et al. 2018). It was discovered that neonates and infants could be primed *in utero* with malaria-specific antigen already in their foetal life. This neonate property was assessed with their umbilical cord blood mononuclear cell responses to *P. falciparum* schizont extract (PfSE), and for their memory. Specific $CD8^+$ T memory cells were stimulated to a high frequency expansion. Effector $CD8^+$ as well as $CD4^+$ T cells also showed proliferative response, and pro-inflammatory cytokine secretion. Malaria antigens were found in foetal placenta of active malaria infected expecting mothers. Memory $CD8^+$ and $CD4^+$ T cells were shown to be from foetal origin. Parasite antigen specific- activated/differentiated memory and effector $CD4^+$ T cells were detected *ex vivo* in neonate cord blood. Regulatory tolerogenic T cells (Tregs) and γδ T cells were not noticed to affect $CD4^+$ and $CD8^+$ proliferation.

In Ugandan cohort clinical trial, all infants underwent intermittent preventative treatment with dihydroartemisinin-piperaquine (DP) every 12 weeks, and monitored for their parasitemia and clinical symptoms. High level of antigen-specific $CD4^+$ T cell proliferation correlated with new-born/infant protective immunity against malaria. With a group with low PfSE, more than 70% of infants were protected from clinical malaria. Such a robust protection was only induced *in utero* in expecting mothers, suffering from active placental malaria. Apparently, such robust CD4 Th1 cell proliferation outbalanced T_{reg} tolerance in mother and infant. It provides at birth a continuum transition of protective adaptive cell immunity to specific malaria antigens, while most of the T cells are at a naïve stage. There may be a collaboration with innate immunity, including dendritic cell antigen cross presentation, constantly needed through childhood years together with malaria-specific memory T and B cells (Odorizzi et al. 2018).

Knowledge of such mother-infant placenta malaria immunity and history, which grossly changes their innate immunity profile against malaria, are considered to be very important for individual infants, for their mothers help with medication and nutrition. Some mothers may not exhibit the parasite in peripheral blood. It may

contribute to more efficacy in vaccine construction. TLRs signalling mediate, via NF-κB regulator, anti-parasite cytokine gene transcription. There is a counter balance which may be explored for host safety (Natama et al. 2018; Jagannathan 2018).

5.3 Prenatal Malaria Exposure and Infant Malaria Risk

Other studies with gravid women, exposed to malaria in endemic regions, demonstrated the malaria impact of prenatal exposure on new born and infants, in first year of their life. It has negative consequences for infant immunity against malaria infection. Some malaria-exposed infants have an elevated risk of developing clinical malaria. At perinatal stage, infants rely solely on innate immune protection against microbes while adaptive immunity is still naïve. Clinical trials with 303 cluster cohort of gravid mothers and paired infants have been conducted in African Burkina Faso malaria endemic region (Natama et al. 2018; Jagannathan 2018). It is registered under COSMIC trial (NCT01941264).

Umbilical cord whole blood baseline cytokines, and those of TLR stimulation- dependent mononuclear cell cytokine, chemokines and growth factors as biomarker, have been analysed in unexposed malaria infants, as compared with malaria-exposed infants. Baseline immune factors included intracellular agonists TLR 3, 7/8 and 9, and cord blood levels of IFN-α, IL-1β, IL-1RA, TNF-α, IFN-γ and tolerogenic IL-10, chemokine RANTES, and growth-factors G-CSF, GM-CSF and FGF as biomarkers. Their levels were significantly lower in mother with placental malaria, as compared with unexposed mothers. In such situation, TLRs may differentially skew monocytes and dendritic cells in infants towards acute clinical malaria. Infants of mothers with malaria history were over-responsive to TLR 7/8 activation, and prone to chronic malaria pathology. A marker of such infection is pro-inflammatory placental malaria pigment, hemozoin deposit. Several immune factor combinations may modulate an overt malaria risk in infancy. TLR 7/8 stimulation augmented GM-CSF and eotaxin, and TLR9 activation, which induced higher level of IL-1 β, and stimulation of IL-3 induced IL-7, which rose the likelihood of infant malaria. On the other hand, an elevation of IL-10, mediated by TLR3 or TLR9, reduced risk for infant malaria. There are differences in immune factors for susceptibility of mothers and infants to develop clinical malaria, between African malaria endemic states (Natama et al. 2018).

6 Trained Innate Immunity and Malaria Vaccine

TLR activation apparently extends into innate memory (trained) immunity (Schrum et al. 2018). In healthy volunteers primed with malaria infected red blood cells, or with hemozoin crystal, adherent to PBMC on re-challenge through TLR2 stimulation, induced in mononuclear cells an elevated pro-inflammatory cytokine response. Alternatively, malaria parasite molecular pattern may, through TLR9, induce in humans, tolerance response to malaria infection, engaging innate cell memory. This switch may depend on parasitemia burden. Both phenotypes are imprinted via nuclear chromatin epigenetic changes, which can be recalled by TLR signalling. Such imprints were also found in children of Mali, living in endemic region. Between Malian healthy individuals, 50% had parasitemia, but were lacking symptoms. This may mean that their parasitemia level was kept below the pyrogenic threshold, associated with fever and pathology. Pyrogenic threshold was higher in individuals upon re-infection. Such innate tolerance memory may occur months after primary malaria infection. However, children are at risk for cerebral malaria showing enhanced susceptibility to febrile malaria, where tolerance pathway may not be matured. Such children may need medication (Schrum et al. 2018). A murine model demonstrated that the deadly cerebral malaria is mediated by pathogenic $CD8^+$ T cell responses, which can be suppressed by antigen-specific IgG antibodies. However, resistance to the cerebral condition can be acquired gradually after 3-round of malaria infection, stim-

ulation of anti-CD8$^+$ T cell IgG antibodies, produced and maintained by memory and effector B cells, at a high level (Shaw et al. 2019). Cerebral malaria pathology in children is sequestration of *P. falciparum* infected red blood cells in microvasculature endothelia, through their protein C receptor and ICAM1, differentially binding *P. falciparum* red blood cell var protein 1. Here also, a recombinant vaccine may induce strong antibodies to both antigens, in prevention of children against cerebral malaria (Storm et al. 2019). Prenatal malaria exposure of embryo is also associated with low-birth weight (Jagannathan 2018).

7 Unconventional Adaptive Cellular and Humoral Immunity Against Endemic Malaria in Children

Interestingly, children exposed to repeated high *P. falciparum* burden can develop a natural atypical innate-like cytokine expression profile by effector cytotoxic CD3$^+$ CD8$^+$ T cells (dim), and their memory. This phenotype tends to be tolerogenic, allowing with time, some more resistant children to become malaria asymptomatic, although on initial encounters they respond with an acute clinical malaria. Hence, such immunity is heterogeneous in a population. In low malaria burden countries, children may overcome an acute malaria response and develop a CD3$^+$ CD8$^+$ T cells (bright) memory. However, atypical T cell response is associated with chronic diseases such as hepatitis C and HIV-1 infections (Falanga et al. 2017). Two paediatric longitudinal studies with toddlers and school children in Kenyan western endemic region of Kosumi compared them with low burden area of Nandi. Their *ex-vivo* PfCSZ antigen stimulation of PBMCs, and that of peripheral T cells, as well as their plasma cytokines, chemokines, and PfCPZ specific antibodies, were analysed. The atypical CD3$^+$ CD8^{+dim} T cells expressed Granzyme B^{hi}, IFN-γ^{low}, TNF-α^{low}, PLFZ^{hi}, ID2^{hi}, and IKZF2^{hi}, similar to that of NK cells. In comparison, children living in low malaria infection region, their CD3$^+$CD8^{bright} T cells expressed effector pro-inflammatory factors including IFN-γ, TNF-α, CCL4, and INF-α, NF-κB, I and L-6. IgG titres increased against PfSRZ antigen. Cytotoxic CD8$^+$ T cell cytokine profile was correlated with school children, and not that with toddlers. This difference might suggest prevalence of an innate protective immunity over an immature adaptive immunity. A repeated exposure of children and adults expand anti-parasite adaptive immune memory through unconventional cytotoxic CD8$^+$ T cells. Such stage-immunity changes did not occur with CD4$^+$ T cell subset (Falanga et al. 2017). CD8$^+$ T cell immunity appears to contrast that of CD4$^+$ T helper cell long lived memory, against malaria antigens, in tandem with that of *Schistosoma haematobium* co-infection, in Malian children (Lyke et al. 2018).

Unconventional CD8$^+$ T cell's innate immune feature is regarded as a distinct dynamic adaptation towards protection against repetitive pathogen infection, rather than a fixed cell subset. Asymptomatic low malaria infection of such individuals may result from liver tissue sinusoid resident CD8$^+$ T cells, mediating apoptotic death of infected hepatocytes, which is a silent event. Freed sporozoites are targets of specific PfCSP antibodies, and of liver phagocytic Kupffer cells, which line sinusoid wall cells. Liver tissue residing CD3$^+$CD8$^+$ T cells are pivotal for antigen-specific memory (Falanga et al. 2017; Cockburn and Seder 2018). A study with malaria asymptomatic children, their immune regulation in response to malaria endemic condition has been conducted.

In a school children cohort studies in West African Mbita, a malaria endemic region, more subdued CD8$^+$ T cell effects for children protection against *P. falciparum*, combined with *Schistosoma mansoni* infection, have been reported (Kijogi et al. 2018). In *P. falciparum* positive asymptomatic children, their *ex-vivo* PBMC-CD8$^+$ T cells, CD4$^+$ T helper cell, NK cells, NKT cells, and B cells were stimulated by PfCSP antigens. Their number, their cytokine in humoral functional responses to parasite specific antigens and unrelated pathogen antigen, have been analysed, as compared with Pf negative children. On the whole, in Pf positive children,

their immune cells upon Pf antigen stimulation, downregulated acute immune responses including of IFN-γ, TNF-α and IL-6, by inducing naturally acquired opposing regulatory cells and cytokines. These include IL-2, IL-10, IL-27, TGF-β, and regulatory T cell Foxp3. This tolerance was dependent on $CD8^+$ T cell receptors, but independent from children's measles vaccination, except for chronicity of viral disease such as hepatitis C virus and AIDS, including cancer. In Pf positive children, the immune cell number was reduced, except of B cell memory cytoblasts and NKT cells. Such immunity seems to be a balance against clinical acute malaria. Although unavoidably instructive, *ex vivo* PBMC studies are difficult to completely mirror tissue resident T cell effector function and memory in humans, such as liver $CD3^+CD8^+$ T memory cells and B cells, apart from tissue allografts. Such memory is paramount in malaria for protection (Falanga et al. 2017; Kijogi et al. 2018; Gebhardt et al. 2018).

Natural acquired immunity can foster a rare, potent and specific long-lasting memory B cell clonal accumulation in response to mosquito vector/PfCSP infection (Murugan et al. 2018). This event was revealed in a study with 80 healthy volunteers in Gabon, a malaria endemic country (Triller et al. 2017). Such specific B cell immunity involved two distinct dominant conformational epitopes, located on mid CSP repetitive NANP sequence of the parasite. The epitope specificity was generated by conformational changes of germline-encoded amino acid residues. Here, somatic permutation gene specific antibodies served as stabilizers of germline conformation. This natural anti-malaria B cell memory, confirmed *in vivo*, may serve in the design of next generation malaria vaccine (Triller et al. 2017). Presumably, in some children, this natural immune response to malaria may be more frequent, who are naturally resistant to malaria.

8 Concluding Remarks

Malaria is still with us as a major pandemic, endangering almost half of the population in the world. It is reassuring that safe and effective vaccines in clinical trials against deadly *Plasmodium falciparum* malaria have been developed to protect young children, who are most vulnerable. This includes vaccine protection during their foetal life, in placental malaria infected expecting mothers, living in endemic malaria African sub-Saharan countries. The vaccines also potently protect adult population. For protection from *Plasmodium vivax* malaria which is predominantly globally spread, clinical studies with children have selected highly protective antigenic combinations, to be utilised in recombinant vaccine construction. The WHO authorised malaria recombinant vaccine RTS, S, inducing specific antibodies and B cell memory, are substantially protective for children against clinical severe malaria. Likewise, live attenuated Pf sporozoite vaccine at liver malaria stage affords a strong cellular immune protection for people living in malaria endemic countries. Together with drug, the vaccines may substantially contribute to WHO projection of malaria reduction globally, supported by mosquito nets and other measures.

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