Applications of Cutting-Edge Immunoproteomics Technology in Human Immunotherapy

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Abstract Harnessing the ability of the immune system to mount robust and effective responses in the face of pathogenic challenge or cancer development is rapidly developing into frontline treatment for these diseases. This field, called immunotherapy, relies on the activation of antibody mediated B cell and/or cellular mediated T cell responses that directly target diseased cells and tissues. One of the most challenging aspects of developing effective immunotherapeutics, however, is first identifying the target antigens that the immune system should recognize and 'attack'. Among the many methods available today immunoproteomics is ideally suited to identify relevant target antigens. Immunoproteomics combines cutting edge proteomic methodologies to identify physiologically relevant target antigens expressed and/or produced by the diseased cells with standard immunological techniques to validate these targets. In this topic, we explore how immunoproteomics can shape the development of effective immunotherapeutics. We focus primarily on immunotherapies harnessing the cell mediated arm of the adaptive immune system and review promising clinical data on T cell-based immunotherapies in cancer, infectious diseases, and autoimmune disorders.

Keywords Major histocompatibility complex · T cell epitope · Cytotoxic T cells · Active immunotherapy · Epitope prediction · Vaccine development

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1 Introduction

The immune system has a monumental task. In the simplest terms, it must protect the host from cancers and infectious disease while carefully regulating responses so as not to inflict any long-term damage of host tissues. These immune responses are not perfect: cancers do develop even in the face of an initial immune response; infectious diseases do overwhelm the immune system and claim lives; and autoimmune diseases are a cause of significant pathology in those afflicted. Despite these imperfections, a tremendous amount of data indicates harnessing the beneficial responses of the immune system provides innovative possibilities to treat patients suffering from cancers, chronic infections, and autoimmune diseases. This field, known as immunotherapy, has rapidly developed over the past 2 decades and produced a number of effective treatments mainly in the cancer arena. Immunotherapies are based on either antibody mediated (humoral immunity) or cell mediated immunity by activating T cell immune response.

Cell mediated immunity, driven by $CD4^+$ and/or $CD8^+$ T lymphocytes, plays a critical role in in defending the host against cancers and infectious diseases. These responses begin in secondary lymphoid organs (i.e. lymph nodes or spleen) when dendritic cells and/or macrophages present fragments of protein antigens, termed peptide epitopes, to the T cells. These peptide epitopes are generated via a number of antigenic processing pathways. Antigens endocytosed from the extracellular environment are broken down in the endosomal/lysosomal system and loaded onto major histocompatibility class (MHC)-II molecules for presentation to $CD4^+$ T cells. In contrast, antigens biosynthesized inside of the presenting cell are broken down by the proteasome, peptide fragments shipped into the endoplasmic reticulum, trimmed further, and loaded onto MHC-I molecules for presentation to $CD8^+$ T cells. Importantly, there is a great deal of overlap between these pathways; endocytosed antigenic fragments can be processed by the proteasome and load onto MHC-I molecules while biosynthesized antigens can be processed in the endosomal/lysosomal system and loaded onto MHC-II molecules. This "cross-talk" undoubtedly broadens the number of targets important for an efficient cell mediated response. In normal, healthy cells, self-proteins go through these pathways and an array of peptides is displayed on the MHC molecules. These peptides are recognized as 'self' and therefore do not provoke a T cell response. However, changes in this MHC signature of cells alert T cells to changes in the host that may be associated with infection, malignant transformation, or other abnormal cellular processes, resulting in a cascade of events that induce a cell mediated immune response. In this case, when the right "match" is found, the properly matched T cell clone is activated, expands, and migrates to the tumor or site of infection to mediate effector functions.

Currently, one of the major challenges in the development of immunotherapies is the lack of clearly defined peptide epitopes capable of being recognized by T cells. The identification of such antigens in cancers, infectious diseases, and autoimmunity could provide the basis for a therapeutic vaccine, or for the stimulation of more effective T lymphocytes for adoptive immunotherapies (Fig. 1). Among the many

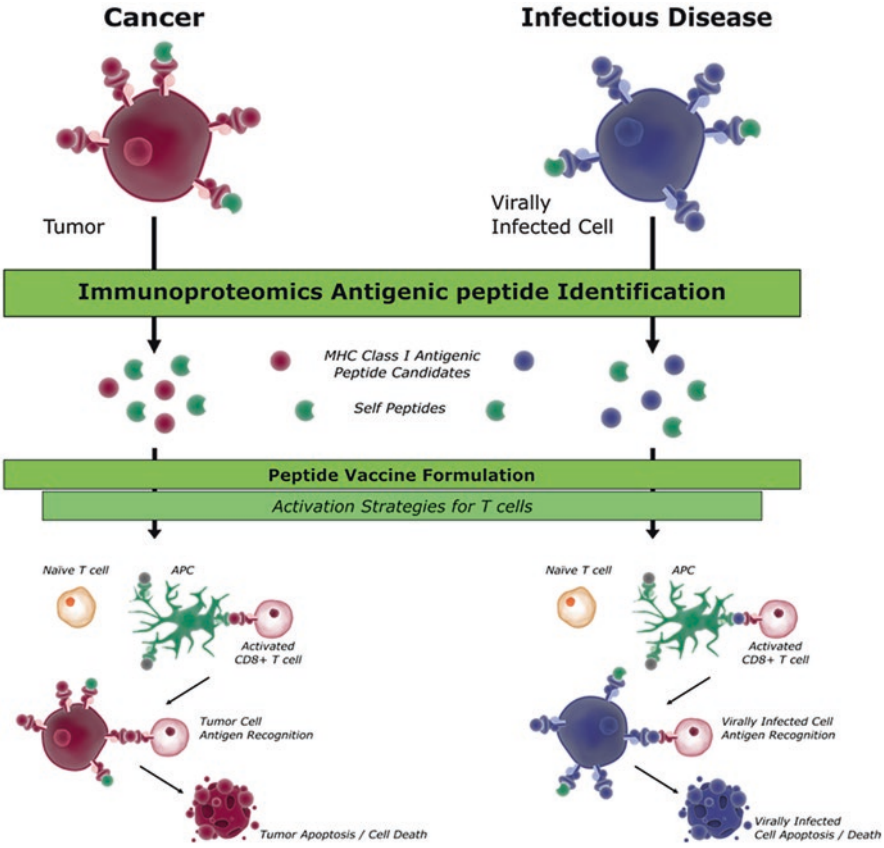

Fig. 1 Immunoproteomic approach for identifying antigens for T cell vaccines

methods available today, immunoproteomics, which is the combination of immunology and the tools of proteomics in particular mass spectrometry, is ideally suited to study these immune responses at a molecular level and identify physiologically relevant peptide epitopes. This topic explores the use of immunoproteomics as a tool for immunotherapy in cancers, infectious diseases, and autoimmune disorders. We focus primarily on immunotherapies harnessing the cell mediated arm of the adaptive immune system and review promising clinical data on T cell-based immunotherapies.

2 Immunoproteomics as a Tool to Identify T Cell Activating Epitopes

Identification of new antigens is limited by certain aspects of the currently available technologies. For example, differential genomic and proteomic approaches identify over- and under-expressed proteins but are unable to identify very low abundant

proteins that are often processed and presented by the MHC molecules as the true recognition targets for T cells. Indeed, the level of protein expression does not always correlate with MHC processing and presentation [1]. Therefore, the most appropriate method for identifying truly relevant antigenic peptides is to identify those naturally presented by the MHC molecules by direct immunoproteomics analysis.

2.1 Genetic Approaches

One of the first methods used to identify specific peptides was a genetic approach in which antigen presenting cells were transfected with cDNA from tumor cells resulting in the expression and subsequent processing and presentation of peptide epitopes. A number of epitopes were identified in melanoma using this methodology: an HLA-A1 restricted epitope from MAGE-1 [2], an HLA-A2 restricted epitope from tyrosinase [3], and an HLA-A2 restricted epitope from MART-1 [4]. However, this methodology has some major limitations including differences in the ability of transfected antigen presenting cells to post-translationally modify proteins, thereby impacting epitope discovery [5]. Perhaps more importantly, transcription and translation of cDNA in different cell types may not generate physiologically relevant epitopes. Antigen presenting cells (APCs), whether professional APCs, infected cells, or malignant cancer cells, have different levels of proteolytic activity [6, 7]. Therefore, epitopes generated in the APC transfected with the cDNA may not be the same as those generated in the infected or malignant cell itself. Although the genetic approach identified a number of cancer peptide epitopes, it was not highly successful in doing so in malignancies other than melanoma.

2.2 Overlapping Peptide Libraries

In this method, proteins (or the entire proteome) of a pathogen or tumor cells are synthesized in 9–20 amino acid stretches and overlapped to an extent that ensures every possible epitope can be presented to cognate T cells [8, 9]. The peptides are assembled into libraries, tested "matrix style" [10], and the libraries that induce T cell responses are teased apart until a number of single peptides that stimulate T cells have been positively identified. Improvements in technology have allowed for this method to be coupled with software to optimize the peptide pools [11]. In this way, the overlapping peptide method allows for the discovery of both MHC class I and class II epitopes in the context of multiple MHC alleles. However, a major disadvantage of this method is it may not identify epitopes that are naturally processed and presented during infection *in vivo*. This is largely due to the processing and presentation necessary to generate epitopes, and the peptides may not reach the appropriate intracellular compartment necessary for processing. Thus, epitopes

identified by this method may not accurately reflect the clinically relevant epitopes for immunotherapeutic formulations.

2.3 Motif Prediction Algorithms

Epitope predicting algorithms are a commonly used method for identifying T cell epitopes, screening the protein sequences for peptide segments predicted to bind to one or more HLA alleles [12, 13]. These prediction algorithms can maximize cross-HLA coverage [12] an important consideration since vaccines formulated with epitopes restricted by HLA "supertypes" might provide the broadest possible coverage for the population [14]. A number of algorithms exist for this purpose including SYFPEITHI [15], RANKPep [16, 17], and the newly developed MetaMHCpan [18]. Computerized predictors have value in identifying epitopes; however, they typically sort potential high binders based on predicted binding scores for the HLA molecule, and usually only the top scoring, or dominant peptides are chosen for further studies. The dominant peptides are then validated by screening circulating CTLs from cancer patients or virus infected individuals to ensure these peptides will activate the T cells. However, there are some significant disadvantages to peptide prediction algorithms. First, selecting only the dominant, or "top scoring" peptides will undoubtedly miss T cell activating epitopes, including those that are subdominant but still clinically relevant as we and others have previously described [19–23]. Secondly, the peptides identified by motif prediction may not be processed and presented at all *in vivo*. As is the case for genetic approaches, because different APC subsets have different processing capabilities, it is likely the epitopes generated *in vivo* may differ substantially from the dominant epitopes predicted from a linear protein sequence by these algorithms. To this end, when Zhong et al. compared motif prediction with mass spectrometry analysis in the identification of naturally processed and presented epitopes derived from influenza virus in a murine model, only 6 of the 16 epitopes that stimulated T cell response were high MHC binders [24]. Reliance only on peptide prediction algorithms is likely to miss a large majority of clinically relevant T cell epitopes.

2.4 Immunoproteomic Method

Within the past decade, direct identification of HLA associated epitopes has emerged as an alternative to the motif and overlapping peptide library methods, a technique termed immunoproteomics. This analysis is based on direct isolation of HLA-peptide complexes from infected or cancer cells and elution of the bound peptides from the HLA molecules. The eluted peptides are then subjected to high-performance liquid chromatography fractionation [25, 26] combined with mass spectrometry [27–29]. The identified peptides can be validated in a number of ways including *in*

vivo using animal models and *in vitro* with cells isolated from actively infected or seropositive individuals [19–21, 23, 30]. There are a number of significant advantages in using this approach to identify T cell activating peptides. First and most importantly, this method allows for the identification of epitopes that are naturally processed and presented during an infection or malignant transformation. As such, these epitopes represent the most physiologically relevant targets and have the potential to be clinically relevant for including in vaccine formulations. Secondly, this method allows for the identification of epitopes that can bind to multiple HLA molecules and with varying affinities (i.e. dominant vs. subdominant) without increasing experimental difficulty. In all, identifying peptides bound to different HLA alleles and/or multiple HLA alleles will be crucial for any vaccine development.

3 Immunoproteomics, Immunotherapy, and Cancer

Transformation of normal cells to malignant cells involves various pathways including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction induced by gene mutations and endogenous and exogenous factors [31]. These transformation pathways usually dysregulate proteins associated with the transformation processes and thereby alter the peptide repertoire associated with MHC molecules on the surface of the cells potentially marking them for detection and destruction by the immune system [32–35]. Thus, the interaction between cancer and the immune system plays a pivotal role in cancer development. However, immune system destruction of cancer cells is not as straight-forward as it seems. Cancer patients are immunosuppressed due to several factors including low frequency of anti-tumor reactive T cells, presence of regulatory T cells and various tumor induced soluble factors [36–38]. Based on these observations, various immunological methods that eliminate antitumor immunosuppression and/or increase antitumor immunity have been successfully developed for the treatment of various cancers.

3.1 Passive Cancer Immunotherapy

Immunotherapy based on the adoptive transfer of tumor-specific lymphocytes dates back several decades [39, 40]. Clinical studies using adoptive transfer of activated T cells, such as lymphokine activated killer (LAK), cytokine-induced killer (CIK) and tumor infiltrating lymphocytes (TIL), are the passive immunotherapy strategies that have been shown to be effective against cancer [41]. The development of adoptive cell therapy started with the generation of interleukin-2 (IL-2) activated LAK cells for cancer treatment [42]. LAK cells have been used to treat tumors such as colon cancer, pancreatic cancer, adrenal gland cancer, esophageal cancer, renal cancer, and

sarcomas in a nonspecific manner as a passive immunotherapy [43]. Although early clinical evaluation of LAK therapy in melanoma showed promising results, clinical efficacy of LAK cell immunotherapy in other cancers appeared to be relatively low and therefore, LAK cell therapy is not currently used in cancer patients. Similar to LAK cell therapy, CIK adoptive cell therapies have been tested in the clinic and showed no sustainable clinical response. The clinical ineffectiveness of these non-specific therapies may be due to the lack of antigen specificities of these T cells. In order to overcome this problem, various antigen specific adoptive cell therapies have been pursued. When antigen pulsed dendritic cells (DC) were used to activate CIK cells, there were significantly increased anti-tumor activities and an increased tumor progression free survival in patients with non-small cell lung cancer [44, 45]. Combination of DC-CIK cell therapy with high-dose chemotherapy also demonstrated progression-free and overall survival in patients with metastatic breast cancer [46]. A number of similar studies are ongoing to confirm the effectiveness of DC-CIK cell therapy. Similarly, tumor infiltrating lymphocytes (TIL) present in many cancers have been shown to play a critical role in tumor development and regression [47–49]. TILs isolated from patients and expanded *in vitro* with IL-2 have been used for clinical application by adoptive immunotherapy in various cancers and induced significant tumor regression, suggesting that adoptive cell therapy with antitumor TIL was an effective method for cancer treatment. However, it is not feasible to obtain TILs from all cancers. Therefore, genetic methods to modify T cells to increase antitumor activities for adoptive cell therapy of cancer patients have recently been developed. Two types of genetically engineered T cells currently being evaluated in clinical studies are (1) gene modified T cell receptors (TCRs) specific to tumor antigens and (2) chimeric antigen receptors (CARs) modified T cells. TCR modified T cells have shown significant anti-tumor activity in various cancers [50–52]. T cells engineered with a CD19- specific CAR induced long term eradication of B cell acute lymphoblastic leukemia (B-ALL) and primary human pre- B-cell acute lymphoblastic leukemia [53, 54]. Recent promising clinical effectiveness of adoptive cell therapy using genetically engineered T cells with antitumor activity seems to be effective in cancer treatment. CAR T cells recognize MHC-non-restricted antigens on the surfaces of target cells, whereas TCR modified T cells recognize antigens that have been processed and presented as peptide complexes with MHC molecules, thus varying clinical efficacy and limitations.

3.2 Active Cancer Immunotherapy and the Importance of Immunoproteomics

As opposed to passive immunotherapy using adoptive transfer of activated or gene modified T cells, active immunotherapy or therapeutic cancer vaccines are strategies aimed to activate a patient's own immune system to generate tumor specific T cells. These active immunotherapies require the knowledge of cancer specific antigens presented by the tumor cells and a vaccine delivery system capable of activating T cells *in*

vivo. Identification of appropriate tumor antigens has been the focus of cancer immunotherapy for many decades. Tumor development and maintenance of malignant phenotypes is driven by a wide range of abnormal cellular events including genetic mutations resulting in changes of protein coding sequences, deletions, insertions, and the abnormal expression of critical genes involved in cancer transformation pathways [55]. Antigens encoded by these dysregulated proteins in a transformed cell are likely to be unique to tumors. Effective therapeutic cancer vaccines must take advantage of these genetic changes by selecting proteins involved in these cancer pathways in order to induce tumor specific T cell responses [56]. Peptides presented by MHC class I molecules reflect the changes that occur in the transforming cell from the normal state, described as "nature's gene chip" by Shastri et al. [1], which could serve as targets for cancer immunotherapy. Therefore, surveying peptides presented by the MHC-I molecules on the diseased cell surface will reveal novel T cell targets for potential immune intervention as tumors have a distinct surface presentation of peptides compared to their normal counterparts [57]. Analysis of the peptide repertoire associated with the MHC class I molecules of cancer cells therefore provides a source for new tumor antigens for development of cancer immunotherapy (reviewed in [58]). Although normal tissues may express the antigen-coding genes, due to the differences in the regulation of expression and proteasomal processing, normal tissues in general do not present these antigenic epitopes in association with MHC-I molecules [57]. Due to the lack of presentation of the epitopes in the context of MHC molecules in normal cells, the CTLs do not recognize normal tissues and therefore are tumor specific and limit the risk of autoimmunity [59]. The large number of peptide/MHC-1 (pMHC-I) complexes expressed at the cell surface combined with multiple pathways to generate epitopes provides a great resource for identifying physiologically and clinically relevant tumor specific or tumor associated antigens. Undoubtedly, an examination of the peptides complexed with MHC-I molecules will reveal novel and immunogenic epitopes capable of inducing effective $CD8^+$ T cell responses. However, despite a growing body of literature indicating that $CD8^+$ T cells are naturally activated during an anti-tumor response [60–62], these anti-tumor T cell responses often fail to eradicate tumors, in part due to suppression in the local tumor environment [63, 64] and/or T cell induced exhaustion from continual antigen stimulation [65, 66]. However, combination therapies incorporating cancer vaccines with various drugs and checkpoint inhibitors to reverse the exhaustion phenotypes of $CD8^+$ T cells are attractive and feasible methods to generate robust anti-tumor responses [65, 67, 68].

3.3 Immunoproteomic Applications in Clinical Cancer Immunology

Cancer vaccines based on MHC class I associated peptides identified by immunoproteomics method are being tested in the clinic with promising results [69] (Table 1). To date, PROVENGE, Sipuleucel-T is the first FDA-approved therapeutic cancer vaccine for patients with metastatic prostate cancer [70]. Most of the peptide-

Table 1 Current and future areas of development for peptide vaccines

Indications	Conditions for which vaccines are in progress *or* may benefit from vaccine development
Cancer	Melanoma, Breast, Ovarian, Lung, Colon, Renal cell, Kidney, Pancreas, Gastric, Glioblastoma, Bladder, hematological malignancies,
Infectious diseases	Malaria, Falciparum Malaria, Anti-Plasmodium vivax, Influenza, HIV, HCV, HBV, CMV, Pneumococcal, genital Herpes—Herpes Simplex Type II, Tuberculosis,
Autoimmunity	Insulin dependent diabetes mellitus, T1D Diabetes Mellitus, Type One, Cat allergy, Allergy, Diabetes, Diabetes Mellitus, Type One, Cat allergy, Ragweed allergy, Grass allergy, Asthma, House dust mites – Rhinoconjunctivitis,

based vaccines tested in the late stage clinical studies include peptides identified by motif prediction methodology with fewer exceptions mainly in melanoma, renal and colon carcinoma. The majority of the peptide vaccine clinical studies were performed pre-realization of existence of regulatory T cells and checkpoint inhibitors that modulated peptide vaccine responses *in vivo*. However, a number of peptide-based vaccines with and without immune modulator combinations have shown success in various cancers.

The majority of the pioneering work was done in melanoma as many well described MHC class I restricted epitopes were identified and tested in the clinic. A clinical study with MAGE-1 peptide vaccine was the first to be tested with limited success [71]. Still, this study was important as it reinforced the idea that $CD8^+$ T cells could be induced to generate an anti-tumor response. Recent studies with peptides identified by immunoproteomic methods utilized a multi-epitope approach in order to induce a broader range of T cell specificities and potentially overcome the problem of antigen loss variants that arise during cancer progression [58, 72, 73]. In these early vaccine studies various adjuvants and cytokines were combined with multiple peptides for vaccination with some clinical efficacy [74]. Data from 115 patients with stage IV melanoma demonstrated functional responses to the peptides (as judged by IFNγ secretion) and were correlated with clinical responses including overall survival and complete and partial remission [74]. The inclusion of cytokines as adjuvants had mixed responses for peptide based vaccines in melanoma [75] with a possibility of accumulation of regulatory T cells (T_{REGs}) [76]. Dendritic cells are considered one of the most important antigen-presenting cells in initiating an immune response and as such have received much attention in designing peptide-based vaccines for cancers. Melanoma peptides, tyrosinase and gp100 pulsed dendritic cell vaccines also induced variable and limited clinical responses in metastatic melanoma patients [77, 78]. Despite these earlier variables promising clinical responses in melanoma, researchers are searching for the most tumor specific peptides and ways to improve the immune responses in patients.

In contrast to melanoma vaccines, peptide vaccines for colorectal cancer have typically relied on a single peptide injected with adjuvant, usually Montanide ISA-51. A single survivin peptide without an adjuvant showed no clinical response in patients with colon cancer [79], although a minor increase in survivin tetramer

positive $CD8^+$ T cells was observed in a few patients. However, survivin peptide with adjuvant and IFNα showed some response including stable disease and a decrease in the CEA (a marker for colon cancer) levels in patients with unresectable colon cancer [80]. Other motif predicted peptide-based vaccines have been tested clinically but these do not induce $CD8^+$ T cell responses. Notably, vaccination of patients with an extended p53 peptide induced sustained $CD4^+$ T cell responses [81] that were enhanced (i.e. higher levels of IFNγ) when administered with IFNα [82]. Early stage clinical trials were conducted using peptide antigen pulsed dendritic cells (DC). DCs were pulsed with peptides derived from CEA, Her2-neu, MAGE-2, and MAGE-3 induced $CD8^+$ T cell responses [83] with no significant clinical benefits. When DCs pulsed with the CEA peptide CAP-1 compared to DCs electroporated with CEA mRNA, $CD8^+$ T cell responses were detectable only in the electroporated group [84]. This latter study reinforces the need to identify naturally processed epitopes presented on tumor cells as it is not clear that the electroporated cells generated the CAP-1 epitope efficiently. On the contrary, 13 rationally selected colon cancer associated peptides (IMA910) identified by immunoproteomics method showed significantly longer overall survival in comparison to a matched-pair analysis of patients from the recently published phase 3 MRC COIN trials [85, 86].

Similar to colon cancer, survivin peptide based vaccine with or without adjuvant was tested in breast cancer with positive T cell responses but no clinical responses [87]. In contrast, prolonged disease free survival was observed in trials with Her2-neu antigenic peptide (E75 or GP2) immunization [88, 89]. A multi-epitope breast cancer vaccine comprised of 12 epitopes, identified by immunoproteomic methods, tested in patients with resected breast cancer generated broader $CD8^+$ T cell responses and objective prolonged diseases free survival [90]. Dendritic cells pulsed with MHC class I and II peptides derived from Her2-neu protein were also tested in breast cancer. Patients with confirmed DCIS treated with peptides pulsed DCs generated detectable $CD4^+$ and $CD8^+$ T cell responses to the vaccine, and a decrease in Her2-neu expression was detected in these patients [91] although a decrease in antigen expression is not necessarily indicative of complete elimination of the cancer. In a second study, majority of patients mounted functional CD4 and CD8 T cell responses against the tumor [92].

Similar to melanoma, renal cell carcinoma (RCC) is one of most common type of cancer [93] that are highly immunogenic. A number of immune based therapies have been tested in RCC including T cell epitope-based vaccines with promising results. Vaccine comprised of peptides derived from VEGFR1 protein generated specific CD8+ T cell responses and several partial regression and stable disease in RCC patients [94]. Antigenic peptides, identified by immunoproteomics approach, were incorporated in a multi-peptide vaccine and tested with and without cyclophosphamide treatment in patients with RCC [69]. $CD8^+$ T cell responses to multiple antigens were associated with control of the disease. Further, inclusion of cyclophosphamide three days before IMA901 injection prolonged survival and reduced the number of regulatory T cells [69]. This latter point is critical: while T_{REGs} are well represented in the tumor microenvironment, peptide-based vaccines may need

a T_{REG} depleting step prior to injection or other modulation of the anti-inflammatory environment by concomitant cytokine treatment. However, not all cytokines are ideal in this application. In trials of DC based vaccines combined with IL-2 administration, T_{REGs} were induced to significantly higher levels than before treatment, albeit transiently [95, 96].

Peptide based vaccines have also been evaluated in patients with stage III-IV non-small cell lung cancer with measurable clinical responses including stable disease and increase in overall survival [97]. Both antigen specific $CD8^+$ T cell responses and clinical responses measured by improvement in overall survival were observed in hepatocellular carcinoma patients treated with peptide derived from glypican-3 based vaccine [98]. A multi-epitope based vaccine demonstrated $CD8^+$ T cell responses and delay in progression of disease in ovarian and breast [90] and prostate cancer [99]. Finally, a multi-epitope vaccination approach was used in a Phase I trial of patients with biliary tract cancer and resulted in a detectable clinical response in 6 of the 9 patients [100]. Peptide vaccines with multiple cancer specificity have undergone clinical studies with promising immunological and clinical results. For example, HER-2/neu immunodominant peptide (lung, breast, or ovarian cancer) [101–103], Mucin-1 (MUC-1, Stimuvax), peptide (breast or colon cancer) [104, 105], Carcinoembryonic antigen (colorectal, gastric, breast, pancreatic and non-small-cell lung cancers) [106, 107], Prostate-specific membrane antigen (prostate cancer) [108–110], HPV-16 E7 peptide (cervical cancer) [111], Ras oncoprotein peptide (colorectal and pancreatic carcinomas) [112–114], and Melanoma antigens (Melanoma) [38, 115–118]. Another vaccine known as GV-1001 is under development, which is an injectable formulation of a promiscuous MHC class II peptide derived from the telomerase reverse transcriptase catalytic subunit (hTERT). GV-1001 is currently undergoing phase II clinical trials for liver cancer and NSCLC (non-small-cell lung cancer) as well as a phase III trial for pancreatic cancer [119].

3.4 Peptide Cancer Vaccines: Current Status and Trends

Tremendous amount of clinical data is currently available attesting to the efficiency of peptide-based cancer vaccines. Combination therapy is emerging as an important strategy to achieve synergistic effects in fighting cancer as a single method alone may not be efficient enough to yield positive results. Combining immunotherapy with conventional therapies such as radiation and chemotherapy or combining an anticancer peptide with a nonpeptidic cytotoxic drug is an example of this emerging field. The peptide vaccines are relatively less expensive, easy to manufacture and manipulate, are of defined structure, and being synthetic in nature do not have a problem of batch-to-batch variation. The major disadvantage of the peptide vaccines is their weak immunogenicity. Several strategies such as epitope enhancement, use of multiple T-cell epitopes, adjuvants, incorporation of costimulatory molecules, and ex vivo loading into professional antigen presenting cells are being explored to enhance the immunogenicity and efficacy of the peptide vaccines. Since the clinical

immunogenicity of the individual peptides is different, it is very hard to conclude which of these strategies was more efficient than the other. Recently, the role of immune checkpoint molecules, such as CTLA-4 and PD-1, programmed cell death-1, on antitumor immunity was clarified, and promising results have been reported in the clinical trials using combination therapies with peptide vaccines and immune checkpoint blockades [120]. Further randomized phase III trials would be essential to prove the clinical benefits of these vaccine therapies, including immune checkpoint blockade combination therapies.

4 Immunoproteomics, Immunotherapy, and Infectious Diseases

Pathogenic organisms are ubiquitous in nature and present a constant challenge for an individual's immune system. Before the development of vaccines polio virus, smallpox, measles, and whooping cough were constant threats. As vaccines were developed and distributed, morbidity and mortality caused by these organisms precipitously dropped; smallpox was completely eradicated, and polio virus may be eradicated 1 day in the near future.

Traditional vaccines offering protection against infectious organisms are prophylactic, designed to stimulate an immune response to a weakened or inactivated version of a pathogen or against macromolecular components of a pathogen (i.e. proteins, carbohydrates, etc.). The goal of prophylactic vaccination is to stimulate the innate and adaptive immune systems *before* exposure to the wild-type or circulating pathogen. This exposure should, ideally, generate memory B and T lymphocytes that can respond rapidly and robustly to a secondary challenge. Although both B and T memory responses are integral for protection against reinfection with an organism, the large majority of prophylactic vaccines in use today are designed to induce a strong B cell mediated response characterized by the secretion of antigen specific neutralizing antibodies. Immunogenicity of a vaccine is often determined by directly measuring the robustness of the B cell response [121]. Although B cell mediated responses are critical for protection, vaccines that predominantly stimulate antibody responses have their shortcomings. First, many strains of pathogens circulate in nature and it is not guaranteed that antibodies induced by one vaccine will protect against all strains. Indeed, a new influenza vaccine formulation is required almost every year due to antigenic drift or shift within circulating viruses [122]. Secondly, antibodies directed against one strain or serotype of a virus might actually enhance infectivity of a second strain/serotype. This antibody dependent enhancement is seen in patients infected with different strains of dengue virus and may lead to Dengue hemorrhagic fever and Dengue shock syndrome (DSS) [123–125].

Despite the drive to develop B cell stimulating vaccines, a large body of literature indicates that T cell responses are equally as important at controlling and eliminating infections. For example, data indicate that the robustness of the $CD4^+$ and $CD8^+$

T cell responses to Hepatitis B virus [126] and Hepatitis C virus [127] is a key determinant of whether these viruses are cleared or establish chronic infection. T cells activated in patients that resolve acute Hepatitis B virus infections recognize a broader range of epitopes and are better able to secrete key effector molecules like IFNγ [126]. Similarly, patients who mount a broad T cell response during a primary influenza virus infection are more likely to have cross-reactive T cells that can be activated during a second influenza infection despite substantial differences in the infecting strain [128]. Together the data make two important points: first, vaccines should be designed to stimulate a broad immune response by activating B cell mediated responses for antibody production as well as both $CD4^+$ and $CD8^+$ T cells for direct targeting of infected cells. Secondly, because multiple, antigenically distinct strains of pathogens circulate in nature these vaccines should stimulate B and T cell responses targeted to antigenic sequences conserved between the many circulating strains. This later point requires a new approach in vaccine development, and such an approach must be flexible enough to be easily and quickly modified if a new strain of virus (or a newly identified virus) emerges.

4.1 Cell Mediated Immunity in Infectious Diseases

B cell mediated responses are necessary during an adaptive response as the first line of response, however antibodies largely recognize antigens that exist extracellularly. Although these molecules can neutralize and eliminate infectious organisms, they cannot directly target infected cells which are often the 'factories' producing new copies of the pathogen. To destroy these factories, the cell mediated immune response consisting of $CD4^+$ and $CD8^+$ T cells is critical. $CD4^+$ and $CD8^+$ T cells are activated after their T cell receptor (TCR) recognizes a peptide epitope derived from a pathogen in complex with major histocompatibility complex (MHC) molecules. $CD4^+$ T cells recognize peptide epitopes ranging from 12–24 amino acids in conjunction with MHC class II molecules, while $CD8^+$ T cells recognize peptides ranging from 8–11 amino acids in conjunction with MHC class I molecules [129]. The generation of peptides for loading onto the appropriate MHC molecule requires degradation of proteins derived from the pathogen by proteases in the endosome or by the major cytosolic protease, the proteasome. Subsequent presentation of these peptides to T cells has the ability to activate a broad response with T cell clones targeting a number of stimulatory pathogen-specific peptide epitopes.

4.2 Influenza Virus Infection

The early experiments implicating T cell responses as critical contributors in controlling influenza virus infections were largely done in mice lacking B cell immunity [130–133]. Graham and Braciale showed adoptive transfer of influenza specific

$CD8^+$ T cells into B cell deficient mice infected with a lethal dose of influenza virus led to their full recovery while transfer of $CD4^+$ T cells lead to only modest recovery [132]. In a similar study, Epstein demonstrated mice unable to mount antibody responses to influenza virus, both $CD4^+$ and $CD8^+$ T cells played a critical role in controlling viral replication with $CD8^+$ T cell responses likely more critical than $CD4^+$ responses [133]. Although the role of $CD8^+$ T cells in controlling influenza virus in murine models is well established, the data for the importance of influenza specific $CD8^+$ T cell responses in human infection is not as abundant. Yet, a number of studies have revealed important roles for these cells during human influenza infection. Sridhar et al. followed individuals during the 2009 H1N1 pandemic in the United Kingdom and demonstrated those with pre-existing T cells directed against conserved, internal proteins of influenza virus (PB1, NP, M1) were better protected against infection. Although these T cell subsets did not protect against complete infection, the subsets limited the severity of infection as infected individuals who did not have symptoms or had minimal symptoms had higher frequencies of influenza specific IFNγ, IL-2 secreting $CD8^+$ T cells [134]. These data are in line with an earlier report from Wilkinson et al. that demonstrated less severe infection in individuals with pre-existing $CD4^+$ and $CD8^+$ T cells directed against conserved epitopes [128]. In a similar study, Wang et al. analyzed PBMCs obtained from individuals infected with a novel H7N9 influenza A virus. Recovery from this infection was associated with more robust IFNγ mediated T cell responses [135]. Interestingly, $CD8^+$ T cells were activated earlier in patients who recovered more quickly (within 18 days) while $CD4^+$ T cells were activated earlier in patients with a delayed (21–27 days) recovery. Together, these data suggest T cells are a key player in the immune response against influenza virus and vaccine formulations should elicit strong cell mediated responses directed against well conserved epitopes of influenza virus.

5 Dengue Virus Infection

Antibodies generated during dengue virus infection play an important role in neutralizing the virus and preventing future infection with the same virus serotype. However, at least four distinct serotypes of dengue virus circulate and antibodies against one serotype do not neutralize others; in fact, these antibodies have been demonstrated to enhance infection by distinct dengue virus serotypes [123–125]. With the potential for enhancing disease using a B cell mediated vaccine, newer vaccine formulations offering protection against dengue virus should stimulate robust T cell responses, ideally against antigens conserved across each of the serotypes. $CD8^+$ T cells play a major role in controlling dengue virus infections *in vivo*. DENV specific $CD8^+$ T cells have been detected after natural infection [23, 136–139], and studies have demonstrated a strong $CD8^+$ T cell response characterized by IFNγ and TNFα secretion in children who were infected but asymptomatic compared to weaker responses in symptomatic and severe infections [139]. $CD8^+$ T cells targeting each of the viral proteins are detectable after infection further suggesting

a broad T cell response is possible. Using an immunoproteomic approach in combination with an HLA-A2 humanized mouse model, our laboratory identified several novel epitopes that induce dengue virus specific, cross-reactive $CD8^+$ T cell responses [21] and of which, two capable of binding to HLA-A24 and two with the unique ability to bind to both HLA-A2 and HLA-A24. Importantly, particularly for designing vaccines for use in humans, we demonstrated these $CD8^+$ T cell subsets were detectable in dengue virus seropositive individuals and these subsets could be activated to produce IFNγ [23].

5.1 Hepatitis B Virus

For the majority of immunocompetent adults, encounter with hepatitis B virus does not lead to chronic infection. However for the remaining 5–10% of adults, neonates, and children infected, hepatitis B establishes a chronic infection that is responsible for approximately 500,000 deaths per year due to complications primarily involving the liver [140]. Individuals who fully recover from a hepatitis B virus infection display strong polyclonal and multi-specific $CD4^+$ and $CD8^+$ T cell responses [127, 141–144] targeting multiple viral proteins. Indeed, the key determinant of whether hepatitis B virus is cleared or becomes a chronic infection is based on the robustness of the immune response- individuals who resolve acute infections have greater numbers of IFNγ producing $CD4^+$ and $CD8^+$ T cells [145, 146] when compared to chronically infected patients [147]. Interestingly, chronically infected patients are also able to mount robust, broad $CD8^+$ T cells responses particularly in response to treatments like IFNα [148]. These data indicate a therapeutic vaccination stimulating a robust adaptive cell mediated immune response may be able to eradicate infected cells in these patients.

5.2 Vaccines Should Establish Protective Immunity to Infection

In order to protect an individual against infection or to stimulate an immune response in a chronically infected individual, i.e. prophylactic and therapeutic vaccines respectively must robustly stimulate the innate and adaptive immune responses. Initial stimulation of the innate immune system, driven by macromolecules derived from the pathogen, activates a relatively non-specific response designed to limit the replication of the pathogen and control its spread. Much of this is done through the secretion of pro-inflammatory cytokines and enhanced phagocytosis at the site of infection. Often, cells of the innate immune system migrate to the lymph nodes or spleen to activate the more specific and robust adaptive immune system. Within 5–7 days (reaching a peak around day 10), antigen specific B and T cells are mobilized and join the fight against the pathogen. After the pathogen is cleared, the immune response dampens and a pool of antigen specific, memory B and T cells persists and,

if the same pathogen is encountered again, they can be activated within 24–48 h. In this secondary challenge, the stronger more specific adaptive immune response is turned on earlier and prevents much of the pathogenesis that would otherwise arise during a primary infection. Any vaccine to any pathogen must induce this long-lasting immune – B cell memory to neutralize infection through antibodies production and T cell memory to perform a variety of tasks including killing of infected cells.

5.3 T Cell Induction Via Vaccines: An Alluring Alternative to Conventional Vaccines

The clearance of many viral infections is dependent upon the robust activation of $CD4^+$ and $CD8^+$ T cell responses, and T cell vaccines have great potential for use to prevent infection or to stimulate responses in chronically infected individuals. An added advantage to T cell-based vaccines is the ability to design these vaccines to induce responses against highly conserved regions of a pathogen stimulating an immune response that potentially protects against multiple strains that may be in circulation. To this end, an ideal vaccine formulation would incorporate multiple conserved targets to stimulate both $CD4^+$ and $CD8^+$ T cells as well as an adjuvant to induce robust innate immune responses. Additionally, vaccine formulations should be flexible with a stream-lined synthesis process in order to respond to emerging infections and to newly identified and potentially more protective T cell targets.

To meet the requirements of the ability to induce cross protection and be readily and quickly modified, peptide-based T cell vaccines are ideal. These vaccines can be formulated to include a variety of $CD4^+$ and $CD8^+$ T cell targets and adjuvants which stimulate a strong innate immune response to enhance processing and presentation of the associated targets to T cells. A number of promising delivery systems are currently in various stages of development including gold nanoparticles, polymeric nanoparticles, liposomes, and virus like particles (VLPs). Other favorable factors for each of these delivery systems are the customization capabilities with regard to size, shape, and antigenic targets/adjuvants. With these delivery systems and the development of more robust immunotherapies, the overall goal of driving T cell mediated immunity for prophylactic vaccine is within reach.

5.4 Peptide Based Vaccines in the Clinical Setting

Peptide based vaccines for infectious diseases are still in their infancy, but these vaccines have been tested in more depth in various cancers (as previously discussed in this chapter) and the results from both settings are promising (Table 1). In general, peptide vaccines are safe and easy to produce and depending on the backbone of the vaccine (i.e. nanoparticle vs. liposome) relatively stable [149–151]. A number of peptide vaccines for various diseases are currently in clinical trials.

A peptide-based vaccine against Hepatitis C virus (HCV) was recently tested in a phase II clinical study. This therapeutic vaccine, IC41, includes five highly conserved HLA-A2 restricted $CD8^+$ T cell epitopes and three $CD4^+$ T cell helper epitopes and is capable of inducing epitope specific IFNγ $CD8^+$ T cells in healthy non-HCV infected patients [152]. In chronically infected individuals, the vaccine stimulated an increase in epitope specific $CD8^+$ T cells in 25% of patients; however, this did not lead to increases in IFNγ production [153]. More recent studies indicate this vaccine is also able to reduce levels of HCV RNA in infected individuals after vaccination, but interestingly this reduction in RNA levels was not correlated with differences in immune responses [154].

Human Papilloma Virus is a sexually transmitted virus causing over 99% of all cervical cancers. Although most individual clear HPV infections roughly 10% are chronically infected. Developing a therapeutic vaccine in hopes to clear the virus from the body and prevent cancer is attractive. One therapeutic vaccine is composed of the E6 and E7 proteins, which are required for transformation, and emulsified with Montanide as an adjuvant. This vaccine induced epitope specific, IFNγ secreting $CD4^+$ and $CD8^+$ T cells that persisted for at least one year after vaccination [155]. A phase I clinical trial of this vaccine formulation in cervical cancer patients demonstrated it was safe, relatively non-toxic, and induced a broad T cell response [156]. Follow up studies with this vaccine (now termed HPV16-SLP) in other cancers offer similar hope. In a study of vulvar neoplasia, this vaccine induced strong T cell responses characterized by IFNγ and IL-5 secretion and resulted in a complete regression in half (10/20) of the patients [157]. Although not all patients experienced regression, in part due to the initial size of the lesion, the heightened T cell response initiated after vaccination suggests therapeutic vaccines based on peptides have promising potential.

Peptide vaccines against HIV are also under development and being tested in clinical trials. Biono Pharama developed a peptide-based vaccine (called Vacc-4x) which is composed of peptide derived from Gag p24, a major core protein of the virus. In clinical trials, this vaccine was immunogenic and decreased viral titers in infected individuals [158, 159] without a detectable impact on the generation of escape mutants [160]. Importantly, Vacc-4x induced an efficient memory response detectable for years after initial vaccination [161]. Other peptide based vaccines have not been as immunogenic [162], perhaps due to adjuvant used or delivery mechanisms. Interestingly, Vacc-4x is being tested with other adjuvants and via other delivery mechanisms; this vaccine candidate is also immunogenic when administered intranasally although the clinical significance has yet to be determined [163]. Overall, the data again indicate that generation of T cell responses are possible and that a peptide-based vaccine could be useful in treatment of HIV infections.

The majority of studies on peptide vaccines for infectious diseases have bene confined to therapeutic vaccines and to studies of pathogens that lead to cancer. A major reason for this is prophylactic vaccines to many pathogens are already licensed, approved and relatively efficacious. However, with the recent emergence of novel strains of influenza virus [164, 165] combined with the length of time it takes to make a strain specific influenza vaccine [166] makes this virus an ideal

target for a cross-reactive peptide based vaccine. To this end, Huber et al. designed a tandem epitope-based vaccine study in mice and ferrets. One vaccine was designed to stimulate B cell responses while the other was one designed to activate T cells, and both vaccine formulations were designed to target conserved regions of the genome. Huber et al. demonstrated these vaccines induced influenza specific antibody and influenza specific T cell responses, reduced viral titers in the lungs of animals, and may improve recovery time [166]. It is clear from the data that peptide-based T cell vaccines have the potential to prevent and treat viral infections, particularly in cases where antibody-based vaccines do not offer protection against all serotypes of a virus. Vaccines that offer cross-subtype efficacy could significantly prevent the spread of an emerging or re-emerging strain.

6 Immunoproteomics, Immunotherapy, and Autoimmunity

The immune system is continually tasked with clearing invading microorganisms and eliminating transformed or malignant cells from the host. The vast majority of the responses required to clear these challenges are pro-inflammatory and are accompanied by the secretion of cytokines and chemokines making the host an uninviting habitat for the pathogen or altered cells. However, these pro-inflammatory responses can be damaging to the host, and in some cases, the pathology seen during an infection is due to the immune response itself rather than the pathogen [167]. As such, these responses must be tightly regulated to avoid overt damage and pathology to host tissues. This regulation is accomplished in a variety of ways including the presence of regulatory cells that function during innate and adaptive responses. These cells release anti-inflammatory cytokines and/or directly modify the activity of responding cells in order to dampen down the immune responses and to prevent long term, systemic inflammation in the host. As pathogens are a constant threat to the immune system there must be a balance between the pro-inflammatory, pathogen clearing responses and the anti-inflammatory regulatory responses. Tipping the scales in either direction can lead to serious immunopathology and the inability to clear an invading organism. In certain disease states like cancer for example the balance is tipped in favor of regulation. Multiple regulatory mechanisms are actively preventing a robust pro-inflammatory response and therefore, an affective immune response against the malignant cells [168, 169]. However, when the balance is tipped in the other direction towards a more inflammatory environment the immune system may begin a robust attack against otherwise normal and healthy tissues, a process called autoimmunity. Undoubtedly both the innate and adaptive arms of the immune system are critical drivers of autoimmune responses [170, 171]. However, for the purpose of this section we will focus on the adaptive immune system, in particular T lymphocyte responses.

Adaptive immune cells begin their development in the bone marrow. Hematopoietic stem cells give rise to a common lymphoid progenitor that, after receiving a number of signals, begins a process of differentiation to form B and T

lymphocytes. B cell development occurs directly in the bone marrow; in contrast, T cell progenitors leave the bone marrow and migrate to the thymus to complete development. For both B and T lymphocytes, the end goal of the developmental process is the same: produce functional cells capable of recognizing and responding to foreign antigens but *not* to self-antigens. Although there are some key differences in the education process between the lymphocyte subsets, we will focus on development of T lymphocytes to illustrate these processes. T lymphocytes display a receptor, called the T cell receptor (TCR), on the surface that recognizes antigens. These antigens are breakdown products produced by an antigen presenting cell and loaded onto MHC molecules to form peptide/MHC complexes (pMHC). In the thymus, developing T cells are first 'educated' to recognize self-pMHC complexes. The affinity of this interaction determines the fate of the developing T cell: too high of an affinity and the T cell undergoes *negative* selection and is most often deleted from the repertoire; too low of an affinity and the developing T cell dies from lack of interaction. However, T cells that recognize p/MHC complexes at an appropriate affinity are *positively* selected and migrate from the thymus to lymphoid organs where these cells will respond to foreign antigens [172]. This developmental process establishes *central tolerance*. However, during development T lymphocytes do not see *all* potential self-antigens. Therefore, some cells reactive against self-pMHC complexes do escape the thymus and migrate to secondary lymphoid organs. In the periphery, a number of *peripheral tolerance* mechanisms exist to prevent fully developed T cells from reacting against these self-pMHC complexes. Peripheral tolerance mechanisms include regulatory T cells dampening or inhibiting adaptive responses, regulatory APC subsets inducing anergy in lymphocytes, and/or the deletion of T cell subsets that continually recognize self-pMHC complexes [173].

Despite central and peripheral tolerance mechanisms preventing T and B lymphocytes from responding against self-antigens, tolerance can be broken resulting in autoimmune diseases and immunopathology. The events leading to breaks in tolerance and subsequent autoimmune responses are not well understood although a number of factors are likely to play a role. First, there is clearly a genetic predisposition to autoimmunity. Expression of certain MHC (HLA) alleles is correlated with a higher likelihood of developing autoimmune disease; for example, expression of the MHC-I allele HLA-B27 is correlated with ankylosing spondylitis while the MHC-II alleles –DR3 and -DR4 are correlated with Hashimotos' thyroiditis [174]. More recently, non-HLA associated genes such as FoxP3, and PTPN22 have also been linked to autoimmune diseases [174–177]. Genetic composition alone however may not be sufficient to drive autoimmune responses, and evidence indicates environmental factors play a role as well, in particular infections. Multiple hypothesis exist as to how infections drive autoimmune responses, but the general idea is that inflammation directed against an invading pathogen leads to responses against self, either through molecular mimicry [178–180], epitope spreading, and/or bystander activation [167, 181]. In all cases, it appears inflammation serves as trigger to overcoming tolerance mechanisms. And because T cell responses are a critical driver of autoimmune diseases, immunoproteomic methodologies have the power to reveal previously unidentified epitopes that may indicate a pathogenic

driver (or enhancer) of the disease process and/or epitopes that can be used in the preparation of a tolerizing vaccine (Fig. 2).

In order to fully understand autoimmune diseases and to develop the proper immunotherapies to alleviate symptoms and, potentially, cure these disorders it is essential to identify the antigenic targets of autoimmune responses. To date, a number of T cell epitopes associated with autoimmune responses have been identified. Below, we summarize a select few of the autoimmune disorders and discovery of T cell epitopes contributing to pathogenesis.

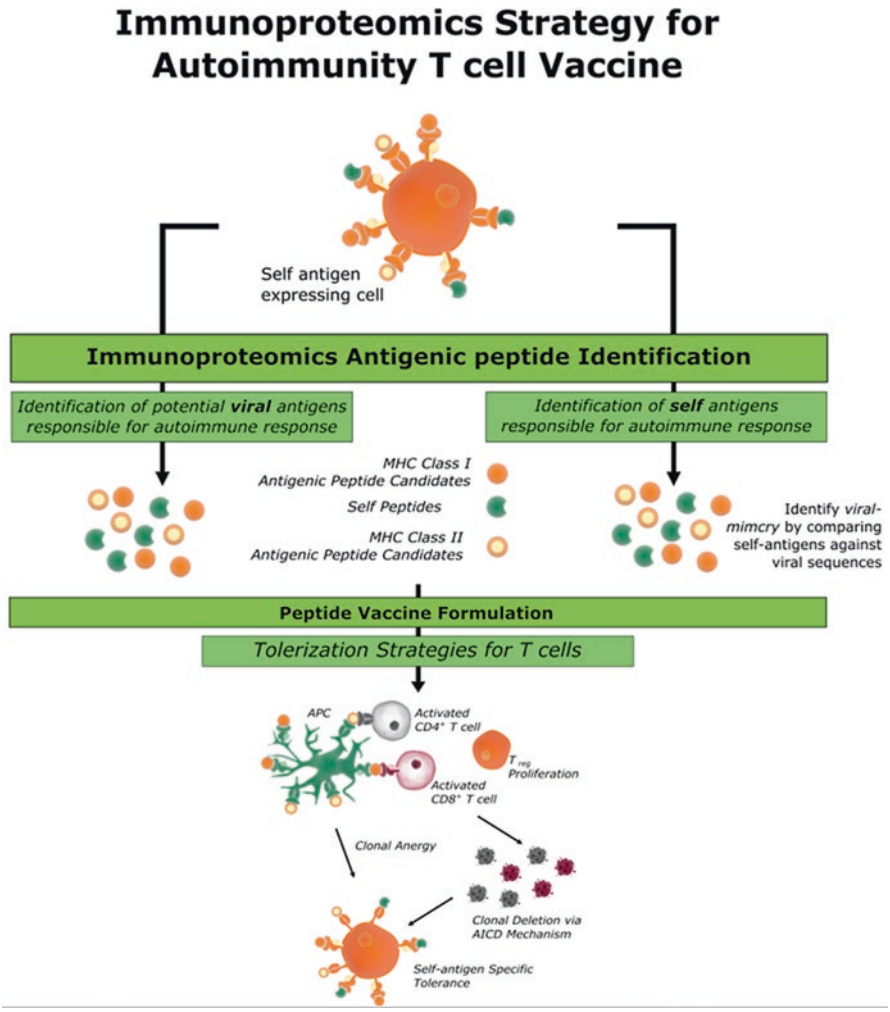

Fig. 2 Identification of novel epitopes presented during autoimmune disease progression

6.1 Type I Diabetes (T1D)

Type I diabetes is an autoimmune disorder characterized by the sequential accumulation of antibodies directed against self-antigens expressed in the pancreas [182] and a robust $CD4^+$ and $CD8^+$ T cell response that contributes to the destruction of pancreatic β cells [183]. Destruction of β cells results in a significant decrease in secretion of insulin and an inability to regulate blood glucose levels. A number of B and T cell epitopes thought to contribute to disease progression have been identified and these drive an inflammatory immune response which might be a trigger for autoimmune responses [183]. Less is understood about the infectious component of T1D; many pathogens have been linked to T1D but the broad nature of these pathogens and the absence of a leading candidate suggests infection may not be a large contributor to T1D [167, 181]. Although the evidence for infections as a trigger for the development of T1D do not point to a specific "culprit", a genetic predisposition is well described: individuals expressing the HLA-DR4 class II molecule are at a greater risk for developing T1D [184] although more recent evidence suggests that the genetic association with T1D is much more complex and involves both HLA and non-HLA related genes [185].

The genetic association with HLA-DR4 class II molecules suggests that $CD4^+$ T cell responses are a major driver of the autoimmune pathology seen in T1D. Using a humanized mouse model restricted to the HLA-DR4 background, Congia et al. identified a number of $CD4^+$ T cell epitopes derived from preproinsulin, proinsulin, and insulin including one epitope (derived from preproinsulin/proinsulin) naturally presented on HLA-DR4 expressing cells [186]. Similarly, Peakman et al. used immunoproteomic techniques to identify six naturally processed and presented MHC class II restricted and immunogenic epitopes derived from the islet antigen IA-2. Such natural processing and presentation allowed the group to identify epitopes that were immunogenic only in a HLA-DR4 restricted setting and did not stimulate non-specific T cell activation [187]. In perhaps the most physiologically relevant study, Kent et al. demonstrated a very small subset (n=3) of T1D patients had clonally expanded $CD4^+$ T cells in pancreatic draining lymph nodes while normal, non-diabetic patients did not [188]. These clonally expanded T cells recognized the insulin derived peptide $A_{1\text{-}15}$. A number of other class II restricted, $CD4^+$ T cell activating epitopes have been verified to various degrees including those derived from proinsulin, insulin, GAD-65, and a number of heat shock proteins [189].

While $CD4^+$ T cells in the pancreas, and draining lymph nodes are relevant for the inflammatory mediators they secrete, it is the $CD8^+$ T cell subsets that mediate direct cytotoxicity. In 1999 Charles Janeway's group made a seminal discovery. Using a pancreatic cDNA library, this group identified an MHC class I epitope derived from proinsulin that was a critical driver of $CD8^+$ T cell responses in T1D in a murine setting. Interestingly, the sequence of this epitope ($B_{15\text{-}23}$) is identical to the human counterpart and overlaps with a previously identified CD4 epitope ($B_{9\text{-}23}$) that contributes to T1D [190]. A few years later Hassainya et al. used a reverse immunology technique to identify ten potential $CD8^+$ T cell epitopes based on pro-

teasomal cleavage patterns and binding scores to HLA-A2 molecules [191]. Of the ten epitopes identified, seven were immunogenic in HLA-A2 transgenic mice suggesting that the T cell response during an autoimmune disease is broad. Using a similar immunoproteomic approach as others, Skowera et al. identified two naturally processed HLA-A2 restricted epitopes localized to the signal peptide region of preproinsulin. Indeed these epitopes appear clinically important as up to 50% of HLA-A2 expression T1D patients have circulating $CD8^+$ T cells directed against these epitopes [192]. Overall, the data suggests there is a broad T cell response directed against a number of protein antigens expressed in the pancreas.

6.2 Celiac Disease

Celiac disease is characterized by T cell responses in the gut directed against antigens contained in grains (wheat, barley etc.). Patients with celiac disease suffer from a number of symptoms including abdominal pain, diarrhea, fatigue, and weight loss. These and other symptoms likely result from the remodeling of the architecture of the small intestine; the continual pro-inflammatory responses lead to flattened villi and an inability to absorb nutrients properly. There is a strong correlation between genetics and the development of celiac disease as more than 95% of patients affected by celiac disease express the class II HLA allele HLA-DQ2 or HLA-DQ8 [193, 194], although it is important to note the expression of these alleles on their own has not been shown to be sufficient to drive celiac disease. Additionally, it is likely that other non-HLA genetic factors play a role in celiac disease development [195].

Due to the strong association with HLA-II alleles, the large majority of epitope identification in celiac disease has focused on $CD4^+$ T cell epitopes. A number of HLA-II restricted epitopes have been identified [196] but the most recent data suggest that a smaller number of epitopes dominate the response. An initial study by Shan et al. identified a long, 33-mer peptide derived from α-gliadin that stimulated T cells isolated from celiac disease patients. Interestingly, this peptide is found in all foods reported to negatively affect celiac suffers [197] suggesting that this peptide (or a derivative) is one of the immunodominant epitopes that drives celiac T cell responses. In an effort to identify immunodominant epitopes in celiac disease patients, Tye-Din et al. evaluated T cell responses in PBMCs obtained from celiac disease patients [194]. Celiac disease or healthy individuals consumed a wheat, barley, or rye grain diet for three days. Subsequently, PBMCs were obtained and tested in a high throughput screen to identify immunodominant epitopes using an overlapping peptide library based on the sequences derived from wheat, rye, and barley. This method led to the identification of 96 peptides capable of stimulating a T cell response. Although an unexpectedly high percentage of these epitopes were cross reactive, three immunodominant epitopes were identified independent of the grain consumed (peptide sequences were similar; derived from wheat (ω-gliadin) and barley (C-hordein)) [194]. Finally, Dorum et al. utilized an interesting HLA-II

capture method to identify novel celiac disease epitopes [198]. In this method, the gliadin protein was digested, incubated with HLA-II molecules (DQ2.5 or DQ2.2), and eluted. The resulting fractions were analyzed by mass spectrometry to identify glutenin and gliadin peptides. Similar to the results obtained by Tye-Din, Dorum's group identified a small number of dominant core peptide sequences (4 out of 86 total) associating with the HLA-DQ2.2 molecule. Two of these epitopes were novel and never before described. Together these latter studies demonstrate that despite the antigenic variation present in grain, the $CD4^+$ T cell response is directed against only a small number of peptides.

Although $CD4^+$ T cells are more strongly associated with celiac disease development, $CD8^+$ T cells also play a role, specifically when inducing lesions in the gut mucosa. Gianfrani et al. (2003) demonstrated an HLA-A2 restricted epitope derived from gliadin ($A_{123–132}$) was capable of inducing T cell responses in PBMCs obtained from celiac disease patients on a gluten free diet [199]. A follow up study using an *in vitro* organ culture system derived from celiac disease patients demonstrated an increase in activated $CD8^+$ T cells ($CD8^+CD25^+$) in the lamina propria when the gliadin $A_{123–132}$ peptide was present [200]. These cells were not detected in cultures including a control peptide or in cultures of HLA-A2 negative patients with the gliadin peptide indicating that the activation of these T cells was epitope and HLA specific. It is likely $CD8^+$ T cells play major roles in remodeling the intestinal architecture through direct cytotoxicity, and there is ample opportunity to identify novel epitopes that may drive the pathology of this disease.

6.3 Multiple Sclerosis

Multiple sclerosis (MS) is a disease in which the myelin covering of nerve fibers is destroyed and replaced by scar tissue build up. The loss of myelin in the nervous system slows down the rate at which impulses are transmitted and has profound effects in cognitive and motor functions. Although full repair to the damaged myelin is unlikely, there may be therapeutic potential to intervene and slow or stop the progression of disease, especially during the early stages. Immunotherapy may be an attractive target, especially considering the recent discovery of lymphatic tissue in the dural sinuses in the CNS [201]. A number of infections are thought to contribute to MS as enhancement of disease is observed after bacterial or viral infections of the upper respiratory tract [202]. Although it is difficult to identify a single pathogen as an environmental contributor to the onset of disease, it seems plausible that the inflammatory response generated to clear the pathogen may contribute to tolerance breakdown and attack of myelin. Like other autoimmune diseases, there is a strong correlation with HLA alleles specifically the class II molecule HLA-DR15 (DR2a and DR2b) [203, 204].

The antigenic targets of T cells in MS patients are hypothesized to be derived from myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein [205]. The first evidence that a peptide segment from myelin basic protein

may be responsible for T cell stimulation was described in 1990 by Ota et al. In this study, the authors mapped two regions of MPB [84–102 and 143–168] that were able to activate T cell lines derived from patients suffering from MS [206]. Subsequent follow up studies mapped the minimal peptide sequences required for T cell stimulation to be residues 85–99 [179] and immunohistochemistry experiments using CNS sections obtained from HLA-DR15 positive patients demonstrated microglial cells and macrophages expressed this MBP peptide in combination with the HLA-DR15 molecule [207]. Most recently Ben-Nun's group used a humanized mouse model to demonstrate that the HLA-DQ6 haplotype may also be a contributing factor to the development of MS like disease, specifically targeting proteolipid protein [208] and myelin oligodendrocyte glycoprotein [209]. Interestingly, this group demonstrated that the DQ6 allele mediated activation of T cells of a Th1/Th17 phenotype marked by secretion of IFNγ, TNFα, and IL-17. However, DR2 haplotype mediated activation of a Th2 phenotype with secretion of IL-4 [209]. Together, the overwhelming evidence suggests there is a strong HLA-II association with MS and the immunopathology is driven, in part, by a proinflammatory Th1/Th17 type CD4 response.

$CD8^+$ T cells also contribute to the pathogenesis of MS [210], and similar to their CD4 counterparts activation of these cells is driven by specific MHC interactions. The class I haplotype HLA-A03 may be a contributing factor to disease especially if a patient is also HLA-DR15$^+$ [211]. Like $CD4^+$ T cells, $CD8^+$ T cells recognize fragments of myelin basic protein, proteolipid protein, and myeling oligodendrocyte glycoprotein [212]. Berthelot et al. investigated the activation of $CD8^+$ T cells in patients with MS by using a library of 188 peptides selected based on class I binding motif algorithms [213]. 69 of the 188 peptides tested activated $CD8^+$ T cells to produce IFNγ in an ELISpot assay; however, there were no activation differences between MS patients and the healthy controls. This result is in line with other observations and reaffirms the idea that the presence of autoreactive T cells is only one part of the autoimmune equation. Interestingly, the data generated by Berthelot et al. indicates binding affinity does not predict the level of T cell activation (as we have previously described [23, 30] and at least one of these 69 activating peptides (MBP200-208) was naturally processed and presented [213]. Although it is clear that $CD8^+$ T cells play a role in MS many of the specific targets have yet to be identified

6.4 Potential Vaccine Immunotherapy for Autoimmune Diseases

To date vaccine formulations that aim to induce tolerance to peptide epitopes or reduce the number of antigen specific T cells have had some success (Table 1). Using a murine model of Type I diabetes (the NOD mouse), Solvason et al. demonstrated that injection of plasmid DNA encoding the preproinsulin II gene resulted in

a reduction of insulin specific pathogenic T cells in hyperglycemic mice [214]. The effect of this DNA based vaccine was enhanced with more injections and higher doses of antigen but was not mediated by regulatory T cell function. Hyporesponsive T cell development in this model is consistent with previous studies that demonstrate T cell anergy developing under conditions of high antigen load [215, 216]. Building upon the success of this initial study, Roep et al. evaluated the $CD8^+$ T cell response in T1D patients after injection of a plasmid containing the proinsulin gene (BHT-3021) [217]. Patients enrolled in the clinical trial were given 12 weekly IM injections and their $CD8^+$ T cell responses against pancreatic and non-specific epitopes were evaluated with flow cytometry. Over the course of the 15-week study, patients receiving BHT-3021 but not the placebo plasmid, had a reduction in $CD8^+$ T cells specific for pancreas antigens. Importantly, there were no non-specific reductions of $CD8^+$ T cells and the overall safety profile was good [217]. In a similar fashion, a plasmid based DNA vaccine encoding myelin basic protein (BHT-3009) or a placebo was administered to MS patients over the course of 44 weeks [218]. Administration of BHT-3009 reduced the occurrence of new lesions appearing in the CNS (as assessed by MRI) and a reduction in autoantibodies specific for myelin antigens. Together the data clearly indicated vaccines designed to induce tolerance are feasible, but more work is to be done.

While traditional vaccine formulations induce effective protection against pathogens, there are significant limitations when designing protective or therapeutic vaccines against other immunological insults like cancer and autoimmunity. For autoimmune vaccines, the candidate vaccine of choice is currently DNA based and while effective there are still major caveats to this approach. First, the coding message must be translated, the resulting protein processed, and the epitopes generated must be loaded onto the appropriate HLA molecule. Despite efficient uptake of DNA based vaccines, there are cellular differences in antigen processing capabilities which may result in a less efficient, but still efficacious vaccine. Further, for autoimmune diseases with multiple gene targets (i.e. MS, T1D) it is not yet clear what DNA sequences are optimal to include. Because of these caveats new generation nanoparticle vaccines may be an innovative step in the right direction. As discussed in preceding sections, these vaccines can be made in various sizes and shapes, have targeting sequences added, and contain multiple epitopes targeting both the CD4 and CD8 pathological responses. Conjugation of adjuvants is also possible including the CpG derived GpG tolerizing adjuvant [219].

7 Advantages and Disadvantages of Peptide Vaccines: Where Do We Go From Here?

Peptide vaccines are gaining momentum in recent years, since they are synthetic, simple to manufacture and cost effective. A number of clinical studies ongoing and in development using peptide vaccines in various disease conditions (Table 1).

7.1 Cancer

Overall, the data discussed above indicate peptide vaccines are capable of inducing robust $CD8^+$ T cell responses that, in some cases, provide clinical benefit to patients. Peptide based vaccines have significant advantages as an immunotherapy option. First, these vaccines are flexible in their design and can accommodate many peptide epitopes in a single dose (Table 1; Fig. 1). This allows for multiple MHC class I epitopes to be included to initiate a T cell response. This is an important feature because not all individuals share the same MHC alleles; peptides that bind to single alleles (i.e. HLA-A2 or HLA-A24) and peptides that bind to multiple alleles (i.e. HLA-A2 and HLA-A24) can be included in the same formulation. Thus, a vaccine derived from naturally processed peptides can be given to individuals with a wide diversity in their MHC alleles and still be effective. Secondly, a multi-epitope vaccine may protect against tumor resistance due to antigen downregulation by inducing a broader, oligoclonal response. Although multiple epitopes from a single antigen have been identified and might overcome HLA-restriction (i.e, MAGE-n [220], survivin [87, 221], and CEA [222, 223]), it is important that the epitopes included in such a vaccine be derived from different parent proteins. This not only will increase the clonality of the T cell response but also prevent tumor cells from downregulating a single protein and escaping the T cell response induced by the vaccine. Finally, peptide-based vaccines can also incorporate MHC class II restricted epitopes to activate $CD4^+$ T cells and/or B cell epitopes to activate T helper and antibody mediated responses. Together, a complete adaptive immune response could prove to be a more effective and robust way by which to eliminate tumors. Despite these advantages, peptide-based vaccine strategies are not without their downfalls. First and foremost, in order for the vaccine to be effective the tumors must be expressing the antigens included in the vaccine formulation. Ideally, the tumors should be *presenting* the epitopes included in the vaccine, which is a major reason for using an immunoproteomic approach for the discovery and selection of antigens in vaccine development. Secondly, peptide based vaccination has been shown to induce the accumulation of immunosuppressive regulatory T cells [76, 95, 96] which would limit vaccine utility *in vivo*. Finally, in some instance peptide vaccines may not be enough to eradicate tumors from patients depending on staging of the disease. Importantly, potential solutions are being evaluated in the clinic to prevent or mitigate each of these limitations. Peptide based vaccines, despite their limited effectiveness to date, have shown promise and progress in the clinic. Identifying novel and perhaps more immunogenic peptides through an immunoproteomics approach combined with a better understanding of adjuvant and cytokine therapy should result in more clinically effective vaccine regimens (Table 1).

7.2 Infectious Diseases

It is clear vaccines of the future will require more than simply inactivating a pathogen strain. Vaccines with built-in cross-subtype efficacy could prevent significant spread of an emerging or re-emerging strain. A cross-subtype vaccine containing immunogenic consensus sequence epitopes could achieve this goal. Fortunately, technology has progressed enough to allow us to identify immunodominant and memory-inducing peptides presented by the MHC class I molecules of virus-infected cells. Armed with these peptides, vaccine formulations will now have to incorporate antigens that activate both humoral and cellular immunity with various adjuvants to drive a strong immune response with high immunogenicity. Additionally, the use of peptides offers a flexible and simple way to synthesize a vaccine. It is therefore highly likely that peptide vaccines will play a large part in overall vaccination strategies and will offer hope to universal prophylactic as well as therapeutic vaccines for protection against infection and therapy for chronic infections respectively. T cell vaccines could play a major role in viral infections such as influenza and dengue viruses where the antibody targeted vaccines have limited clinical efficacy due to significant variations in the envelope protein between various strains. Significant efforts are being directed to find conserved regions of envelope proteins of influenza strains and dengue virus serotypes to generate broad humoral immunity. With the difficulty in finding conserved antigenic regions on the virus surface some efforts are aimed at targeting conserved proteins within the virus. Antibodies cannot reach these proteins to prevent infection, and therefore, peptides derived from intracellular processed protein presented in the context of MHC class I molecules must be utilized. The concept behind this approach is to stimulate T cells to quickly kill virus infected cells before the cells can produce new virions thus limiting disease severity.

7.3 Autoimmunity

Autoimmune diseases are triggered by aberrant B and T cell responses. For a number of reasons these responses have broken tolerance and perpetuate a proinflammatory environment conducive to immune mediated destruction of otherwise normal tissue. In the case of T cells, the responses are driven by specific peptide epitopes associated with HLA molecules. By understanding the specific naturally processed and presented epitopes driving the autoimmune responses, it may be possible to dampen, skew, or completely shut off these responses. Importantly, in order to prevent wide scale immunosuppression, the epitopes specific T cells should be the target of immunotherapeutics and not the HLA alleles. The most attractive mechanism for inhibiting these responses is a vaccine that can induce tolerance and/or anergy in T cells, skew the Th phenotype from Th1/17 to Th2, or induce regulatory T cell development in patients.

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