Published in final edited form as:
Int Rev Immunol. 2011 ; 30(5-6): 328–340. doi:10.3109/08830185.2011.604880.

TCR-Like Biomolecules Target Peptide/MHC Class I Complexes on the Surface of Infected and Cancerous Cells

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Summary

The human leukocyte antigen (HLA; also called major histocompatibility, or MHC) class I system presents peptides that distinguish healthy from diseased cells. Therefore, the discovery of peptide/MHC class I markers can provide highly specific targets for immunotherapy. Over the course of almost two decades, various strategies have been used, with mixed success, to produce antibodies that have recognition specificity for unique peptide/MHC class I complexes that mark infected and cancerous cells. Using these antibody reagents, novel peptide/MHC class I targets have been directly validated on diseased cells and new insight has been gained into the mechanisms of antigen presentation. More recently, these antibodies have shown promise for clinical applications such as therapeutic targeting of cancerous and infected cells and diagnosis and imaging of diseased cells. In this review, we comprehensively describe the methods used to identify disease-specific peptide/MHC class I epitopes and generate antibodies to these markers. Finally, we offer several examples that illustrate the promise of using these antibodies as anti-cancer agents.

Introduction

The possibility of directing adaptive immune responses, for example through delivery of vaccines or adoptive transfer of T cells, holds great promise for the treatment of infections and cancer (1, 2). The development of vaccines and immune-based therapeutics requires discovery and targeting of markers that distinguish infected or cancerous cells from normal cells. The immune system distinguishes infected or cancerous cells from normal cells through the human leukocyte antigen (HLA; also known as major histocompatibility complex, or MHC) system, and the HLA system offers an array of disease-specific epitopes that enable potent and highly selective T cell recognition and activation (3–8). Here, we examine the methods that have traditionally been used, with varying degrees of success, to discover and target HLA-based markers. Our approaches to marker discovery and targeting are described in the accompanying review.

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Conflicts of Interest: Jon A. Weidanz is the Chief Scientist and Founder of Receptor Logic, Inc.

The HLA/MHC System as a Source of Novel Markers and Therapeutic Targets

The MHC system presents markers that alert the immune system to intracellular and extracellular threats, including cancer and infections. MHC class I molecules, which are constitutively expressed on the surface of all nucleated cells, provide a highly effective method of immune surveillance for cancer and bacterial or viral infections. The tri-molecular MHC class I complex consists of a non-covalently associated alpha chain, β_2-microglobulin light chain, and a peptide ligand of 8–12 amino acids (9). Proteins from every cellular compartment are processed into peptides by the proteasome, and these peptides are delivered to the endoplasmic reticulum (ER). Stable peptide/class I MHC complexes are assembled in the ER and transported to the cell surface where they are inspected by the T cell receptors (TCRs) of cytotoxic T lymphocytes (CTLs) (10, 11). Viral infection or cancer changes the repertoire of peptide/MHC class I complexes displayed on the cell surface; specific recognition of a disease-associated peptide/MHC class I complex by the TCR alerts the immune system to the presence of diseased or infected cells and activates CTLs to eliminate those cells. By presenting an extracelluar snapshot of the inner workings of every cell in the body, the MHC system enables the immune system to target diseased cells with incredible sensitivity and specificity. Thus peptide/MHC complexes offer a rich source of novel tumor-specific markers and potential targets for therapeutic mAbs.

Discovering Peptide/MHC Class I Targets

Peptides presented by class I MHC molecules can be identified by A) indirect or B) direct approaches. A variety of indirect methods infer those peptides that are likely to be presented by a particular MHC molecule (12). Candidate peptides are then tested for reactivity with peripheral blood mononuclear cells (PBMCs) isolated from patient blood (13–15). Therefore, the peptide/MHC complexes identified through indirect methods are those recognized by activated T cells. However, because indirect methods are guided by predictions, and because activated T cells differ markedly from patient to patient, many relevant peptide/MHC targets are overlooked. In contrast, the entire peptide/MHC repertoire can be catalogued by direct methods, which discover peptide/MHC complexes by identifying endogenously loaded peptides eluted from MHC molecules. However, direct discovery is a technically challenging approach that is further limited by a dependence on cell lines, which may not recapitulate *in vivo* peptide presentation by different primary cells. Therefore, in both indirect and direct epitope discovery, careful validation is of utmost importance.

A) Indirect Discovery

Strategies for the indirect discovery of target peptide/MHC complexes use genomic, proteomic, or immunologic data to infer peptides that may be presented by a particular MHC molecule during cancer, infection, or other disease states. Peptide/MHC complexes are not directly characterized. Based on the assumption that highly expressed proteins are more likely to be represented in peptide/MHC class I complexes, some indirect approaches use genomic or proteomic techniques to identify proteins that are uniquely expressed or over-expressed in a disease state. Gene-centric approaches may use real-time PCR, gene mutation analysis, differential display analysis, microarray experiments, and other genomic expression profiling methods (16). Protein-centric approaches may use 2-D electrophoresis, mass spectrometric analysis of cell fractions, and other proteomic techniques to identify disease-associated proteins (17). After disease-associated genes and proteins are identified, algorithms or experimental peptide binding assays select candidate peptides based on the assumption that peptides with high binding affinity are more likely to be presented by that

MHC molecule. Expression profiling therefore identifies candidate proteins from which representative peptides are synthesized and tested *in vitro* for binding to MHC.

While expression assays are positioned upstream of MHC/peptide presentation, immune-centric assays provide a downstream approach for testing immune recognition of putative MHC/peptide complexes. Immune-centric approaches test candidate antigens for their presentation to CTLs. First, cellular fractions from diseased cells are fed to dendritic cells (DC) for antigen processing and presentation. These DCs are then used to stimulate CTLs. Antigens that DC utilize to invoke a CTL response are considered candidates for further development (18).

Immune-centric assays are utilized to assess the immunologic potential of candidate peptide/MHC complexes as well. Here, bulk PBMC populations isolated from patients or healthy individuals (following *in vitro* stimulation) are assessed for CTL reactivity to pools of peptide/MHC complexes containing either synthetic overlapping peptide libraries or synthetic candidate peptides. Once the minimal epitope is identified, CTL clones specific to the peptide/MHC complex are generated from bulk PBMCs. Reactivity of T cell clones to diseased cells or cell lines is confirmed by target cell lysis via ^{51}Cr release, interferon gamma release as detected by an enzyme-linked immunosorbent spot assay, or by intracellular cytokine staining (13, 15).

Indirect discovery methods have identified many class I MHC restricted epitopes (19), but upstream expression-based methods are limited by their dependence on assumptions that may be incorrect. First, our present understanding of peptide processing and presentation during pathological states is incomplete. Several processes central to MHC presentation, including translation initiation, proteolytic processing, and MHC surface expression, are dysregulated by viral infections and cancer. It is therefore difficult to predict how and whether highly expressed viral or tumor associated proteins will be processed and presented by MHC class I molecules. Second, several studies have shown that protein expression levels do not necessarily correlate with the presentation of peptides from that protein. Thus approaches that prioritize peptides from highly expressed proteins are likely to overlook many relevant peptide/MHC complexes.

Positioned downstream of MHC/peptide presentation, immune-centric assays utilize immune effector cells to assess the reactivity of peptide/MHC complexes. The activation of CTL clones, CTL lines, or bulk PBMC indicates that a particular MHC/peptide complex is immunogenic. One drawback of these methods is that billions of T cell responses are possible yet only a tiny fraction of these T cells are utilized for MHC/peptide screening; there is a strong T cell bias to the peptide/MHC complexes discovered. First, it is now recognized that dendritic cells (DC) process antigens differently than diseased cells do and therefore stimulate CTL to recognize peptides that may not be presented by diseased cells. Indeed, multiple studies show that the epitopes to which CTL respond are not necessarily the epitopes found on diseased cells (20–23). Second, regulatory T cells and holes in the T cell repertoire may keep key MHC/peptide targets from being immunogenic, and these MHC/peptide targets may be the key targets in elusive infections and tumors

Because of these limitations, indirect discovery approaches may not uncover all of the peptide/MHC complexes associated with a disease state and could even implicate peptide/MHC complexes that are not relevant. For example, an immune-centric approach to indirect discovery identified a melanoma specific peptide/HLA-A*02:01 complex containing the human telomerase peptide $hTERT_{540–548}$ (ILAKFLHWL), while other approaches have not replicated this result. Vaccination or *in vitro* stimulation of CTLs resulted in CTL clones that specifically recognized $hTERT_{540–548}$/ HLA-A*02:01, but there are conflicting reports on

the ability of these CTL clones to lyse tumor cell lines that express hTERT (14, 24, 25). In addition, *in vitro* proteasome processing studies did not generate the $hTERT_{540–548}$ peptide, and mass spectrometric analysis of peptides directly eluted from the HLA-A*02:01 molecules of various hTERT positive tumor cell lines failed to reveal the $hTERT_{540–548}$ peptide (unpublished data). The controversy raised over the $hTERT_{540–548}$/HLA-A2 ligand underscores the importance of validating epitopes discovered by indirect means.

B) Traditional Direct Discovery

While indirect discovery methods attempt to infer which peptides are likely to be presented by MHC molecules, direct approaches elute peptides directly from peptide/MHC complexes and identify the peptides specific to diseased cells. Thus direct epitope discovery is conceptually straightforward, but this method faces as many challenges as indirect methods. Several factors contribute to the empiric challenges associated with the use of direct discovery approaches.

Traditionally, direct discovery approaches identify peptides that are presented by the MHC molecules of cell lines (26). The dependence on cell lines can limit the power of direct discovery approaches, since available cell lines might not express the MHC molecule of interest. To identify presented peptides, peptide/MHC complexes are first affinity purified from cell lysates. In addition to using detergents that may interfere with downstream analyses such as mass spectrometry; this purification technique produces low MHC protein yields, typically in the range of a few nanograms. This amount may not allow for adequate analyses, particularly for the overwhelmingly complex mixture of peptide/MHC complexes isolated from cell lysates. Most cells express six different MHC class I proteins, and it is difficult to purify any single MHC class I protein from detergent cell lysates. In addition, the 50,000–250,000 copies of each MHC class I protein on the cell surface present more than 5,000 different peptides. With a total of more than 300,000 unique peptide/MHC complexes on a single cell, only the most abundant peptides can be detected, leaving rare and possibly crucial peptides unresolved. After peptide/MHC complexes are purified, direct discovery methods typically elute the peptides and identify disease-specific peptides by mass spectrometry. Although this approach allows the direct identification of peptides that are presented by MHC molecules, the nature and number of peptides isolated is affected by their solubility and ionization potential. As a result, direct discovery approaches may fail to identify peptides that are highly hydrophobic and/or ionize poorly. Therefore, although direct discovery is a logical starting point for the discovery of disease-associated peptide/MHC complexes, traditional approaches are complicated by several technical challenges.

Making Antibodies to Peptide/MHC Class I Complexes

Many approaches have attempted to discover and validate disease-specific targets by taking advantage of the fine specificity of TCRs in differentiating peptide/MHC class I molecules. T cells have been used to indirectly assess the presentation of peptide/MHC complexes through CTL proliferation, cell lysis, or cytokine production assays. However, the development and maintenance of antigen-specific T cells is labor intensive and expensive, and it is difficult to assure the quality of these reagents for reliable assays. Because of these inherent challenges in using T cells directly for target discovery and validation, many groups have sought to generate antibodies (Abs) with T cell receptor specificity. With potentially high affinity and binding specificity, and relatively stable structure, Abs to peptide/MHC complexes could greatly benefit both immunologic and clinical research. Such Abs have been difficult to generate, for reasons that are still largely unknown. However in the past two decades, Abs to peptide/MHC complexes have been produced by A) classical hybridoma fusion technology after immunization and B) phage display, although these methods tend to either have low efficiency or produce Abs with relatively low binding affinity. These limited

successes have both demonstrated the promise of TCR-like Abs as research reagents and clinical therapeutics, and highlighted the need for innovative and effective methods of producing TCR-like Abs.

A) Immunization and Classical Hybridoma Technology

In this approach to making Abs against target peptide/MHC antigens, B cells are isolated from animals that have been immunized with the antigen of interest, and then fused with myeloma cells to create an immortalized, antibody-producing hybridoma. Most groups that have attempted to generate Abs to peptide/MHC complexes by conventional hybridoma techniques have failed. Thus, it has been proposed that Abs with the MHC-restricted specificity of T cells are rare, perhaps because B cells may not be educated to recognize self-MHC-restricted epitopes. However, some groups, including ours, have used immunization and classical hybridoma technology to produce antibodies that specifically recognize peptide/MHC complexes containing either self or non-self MHC molecules. Furthermore, extensive data indicate that the existing B cell repertoire contains antigen receptors that specifically recognize self MHC molecules loaded with foreign peptide. Therefore, the proposed rarity of Abs that recognize self-MHC-restricted epitopes is not a plausible explanation for the widespread inability to produce TCR-like antibodies. Because the efficient induction of a specific B cell response to immunization is essential to classical hybridoma technology, it is likely that the success of these methods depends strongly on the formulation of the immunogen. The ideal peptide/MHC immunogen should be stable, homogeneous and should induce production of antibodies that recognize the MHC-restricted peptide rather than the peptide or MHC molecule alone.

Over 20 years ago, animals were immunized with syngeneic, SV40-transformed H-2 fibroblast cells pulsed with a peptide of interest (27). These cells tended to present a heterogeneous population of peptides, with relatively low density of the target peptide/MHC antigen, and thus produced ineffective immune responses against the target antigen and difficulty in screening the hybridomas. To address these problems, RMA-S cells were used as immunogens. RMA-S cells are endogenously loaded with low-affinity peptides that can easily be replaced by pulsing the cells with an exogenous high-affinity peptide, thereby producing cells that express a high density of peptide/MHC class I complexes with a homogeneous peptide population (28). In 1997, the Germain group immunized allogeneic mice with RMA-S cells that expressed the MHC class I molecule H-2K^b and had been pulsed with the ovalbumin-derived peptide SIINFEKL. This immunization approach, combined with classical hybridoma technology, produced four murine Abs that specifically recognized RMA-S cells presenting the SIINFEKL/H-2K^b complex; two of these Abs showed moderate binding affinity (~7×10^{-8} M). The low efficiency of approaches that used cells as immunogens might result from a high turnover of peptide/MHC complexes on the cell surface, or perhaps the cells used were unable to recruit sufficient T cell help or provide adequate direct stimulation of antigen-specific B cells. Although these early attempts indicated that cells are inefficient immunogens for the generation of high-affinity Abs against specific peptide/MHC complexes, their limited successes established the usefulness of immunization and classical hybridoma technology.

Later, the use of purified peptide/MHC class I complexes as immunogens yielded hybridomas that produced mAbs with affinities at least 10-fold higher than TCRs, demonstrating that purified peptide/MHC complexes could be immunogenic and leading to several improved strategies for the production of Abs against peptide/MHC complexes. Although immunization with a purified peptide/non-self MHC complex produces Abs that specifically recognize the peptide/MHC complex, this strategy also generates an abundance of Abs that recognize the MHC molecule itself. Thus, in an attempt to reduce the production of non-specifically binding Abs and to produce Abs with increased specificity for non-self-

peptide/MHC complexes, Polakova and colleagues tolerized the B cell repertoire to the MHC class I molecule H-2D^d and self-peptide/H-2D^d complexes by developing C57BL/6 mice transgenic for a soluble, secreted analog of H-2D^d (29). After these mice were immunized with a purified complex of the HIV-derived peptide P18-I10 and H-2D^d, lymph node cells, rather than spleen cells, were used to generate hybridomas. Of the 400 screened hybridomas, two produced Abs with moderate affinity for the P18-I10/H-2D^d complex (K_D of ~7 to 8 × 10^{-7} M; compared to a typical TCR K_D of 10^{-5} to 10^{-6} M). Thus, although immunization with purified peptide/MHC complexes offers many advantages over the use of cells, the 0.5% efficiency of this method and the moderate affinity of resulting Abs are not adequate to provide a ready source of Abs against peptide/MHC complexes.

Such low efficiency suggests that purified monomers of peptide/MHC class I complexes are poor immunogens, but the reasons for this are not immediately clear. The Weidanz group found that monomeric peptide/MHC class I complexes are highly unstable, particularly in blood, which would hinder their effectiveness as immunogens. Multi-valent constructs of peptide/MHC class I complexes showed greater stability, suggesting that they may promote more efficient B cell receptor cross-linking and B cell activation than monomers. Based on these unpublished observations, the Weidanz group developed an innovative strategy that uses tetramer complexes of peptide/HLA class I molecules for immunization of allogeneic or syngeneic mice, followed by classical hybridoma technology and a unique high throughput screening process. With this strategy, the Weidanz group has produced mAbs with high affinity (K_D = ~1.5 × 10^{-9} M) and exquisite specificity for >30 target peptide/MHC class I complexes. The ~10% efficiency of this strategy is several fold higher than that of other immunization-based approaches, making this the first feasible strategy for the rapid and reproducible generation of TCR-like mAbs.

B) Antibody phage display libraries

Prior to the Weidanz protocol, phage display techniques were markedly more successful than immunization-based approaches in generating antibodies to self-peptide/MHC complexes. A widely accepted dogma held that specificity for self-peptide/MHC class I complexes did not exist in the B cell repertoire. Therefore it was thought that conventional B cell hybridoma techniques had a very low probability of success, and phage display became the preferred method of producing antibodies that recognized self-peptide/MHC class I complexes. In this method a combinatorial antibody library is cloned into bacteriophage DNA and transformed into bacteria. Thus when the phage replicates, each of the antibodies encoded in the library is expressed on the surface of a virion, making it possible to screen and select for antibodies that recognize the peptide/MHC complex of interest. Through many rounds of transformation and selection, or panning, phage display mimics the immune system's antibody selection strategy *in vitro* (30).

In 1996, Andersen *et al.* described the first use of an immunized murine phage-Fab library to generate an Ab to a peptide/MHC class I complex (31). To construct the phage library, mice were primed with live attenuated *M. tuberculosis bovis* (bacille Calmette-Guérin, BCG) and then immunized with an aluminum hydroxide emulsion containing purified protein-derived peptide of tuberculin (PPD) covalently coupled with a complex of the $Ha_{255–262}$ peptide derived from the influenza hemagglutinin protein and the MHC class I molecule K^K. After several rounds of panning, a Fab fragment was identified that recognized the $Ha_{255–262}$/K complex (31, 32). Similarly, single-chain Ab (scFV) libraries have been generated by immunizing HLA-A2 transgenic mice with recombinant peptide/HLA complexes and used to produce recombinant Abs with high specificity and binding affinity for peptide/HLA-A2 complexes (33, 34). The Hoogenboom team used a non-immunized phage-Fab library to generate a human Fab fragment specific for a peptide/HLA class I complex containing a peptide from the melanoma antigen MAGE-A1 presented by HLA-A*01:01(34).

Importantly, the isolated Fab could directly detect endogenous MAGE-A1/HLA-A*01:01 complexes on human melanoma cells. Reiter's group also used a non-immunized phage library, and a screening process similar to that used by Chames *et al*, to isolate human Fab fragments specific for the $hTERT_{865–873}$ peptide derived from the telomerase catalytic subunit (hTERT) in complex with HLA-A*02:01. The human Fab fragments directly bound $hTERT_{865–873}$/HLA-A*02:01 on the surface of tumor cell lines and specifically inhibited $hTERT_{865–873}$/HLA-A2-restricted T cell activation and the release of interferon-gamma into culture medium (33). Although phage display generally produces Fab fragments and scFV with relatively low affinity, these advances helped establish phage display of murine or human Ab libraries as a suitable strategy for generating Abs against peptide/MHC complexes (35–37).

Applications for Antibodies Specific for Peptide/MHC Complexes

Antibodies that recognize peptide/MHC complexes are valuable reagents for immunological research and possess tremendous potential as therapeutic agents. Because of their specificity, sensitivity, and suitability for direct quantitative assays, TCR-like Abs have enabled studies that illuminated antigen processing and presentation in healthy and diseased cells, clarified the impact of peptide/MHC presentation on T cell stimulation, and validated model systems for future immunology research. In addition, multiple studies have shown that TCR-like Abs can be used to deliver drugs specifically to diseased cells or to directly induce cancer cell death. The demonstrated applications of TCR-like Abs suggest that novel approaches to the discovery of peptide/MHC markers and the generation of TCR-like Abs could have a tremendous impact on immunology research and immunotherapy.

A) Antigen processing and presentation in infected and cancerous cells

Recent work shows that TCR-like Abs may have distinct advantages over CTLs both for assessing antigen presentation and for clinical applications. Sastry et al used CTLs and a TCR-like mAb against the HLA-A2-restricted, immunodominant Hepatitis B virus (HBV) peptide $Env_{183–191}$ to detect the endogenously processed $Env_{183–191}$/HLA-A2 complex on infected cells. The mAb showed high sensitivity compared to CTLs and detected the $Env_{183–191}$/HLA-A2 complex on HBV-infected hepatocytes despite poor antigen processing and low expression of HLA class I molecules. Comparing the ability of CTL and mAb to recognize cells pulsed with $Env_{183–191}$ peptides containing substitutions at various positions revealed that the TCR-like mAb employed a different mode of target recognition than CTLs and more effectively recognized peptide/HLA complexes that contain mutated HBV peptides. The fluorochrome-conjugated mAb underwent receptor-mediated internalization within 16 hours of binding the $Env_{183–191}$/HLA-A2 complex. These observations suggest that TCR-like mAbs may be useful for targeted drug delivery to infected cells in addition to the study of antigen processing and presentation (38).

The use of Abs that specifically recognize peptide/MHC complexes has greatly improved our understanding of MHC-based immunity. For example, TCR-like antibodies have been used to elucidate the intracellular trafficking of processed peptides and peptide/MHC complexes. In 1997, the Germain group developed the 25-D1.16 antibody, which recognized the SIINFEKL/K^bMHC class I complex with sensitivity similar to the TCR. Flow cytometry confirmed that 25-D1.16 could detect the SIINFEKL/K^b complex on RMA-S cells pulsed with peptide at concentrations similar to those needed to activate a T cell hybridoma or CTL line to cytokine secretion and within a few fold of the minimum concentration required for target cell lysis. The 25-D1.16 Ab was used to determine the intracellular localization of peptide-MHC complexes, to assess the roles of various components of the peptide-loading machinery in MHC presentation, and to quantify the SIINFEKL/K^b complexes presented by cells that had been infected with recombinant Vaccinia virus engineered to express the

SIINFEKL source protein ovalbumin. Further increasing our understanding of the fate, distribution, localization, and surface expression of peptide/MHC complexes, Makler and colleagues developed a recombinant TCR-like Ab against the HLA-A2-restriced cytomegalovirus (CMV) peptide pp65 and used this Ab to study the pp65/HLA-A2 complex in human cells as a function of time after viral infection. After infection, a large pool of pp65/HLA-A2 complexes was observed in the Golgi, but only a small fraction of these complexes were displayed on the cell surface. Curiously, the intracellular pool was not affected by downregulation of the HLA class I protein, while a proteasome inhibitor increased the expression of pp65/HLA-A2 complexes on the cell surface. These results implicated a role for the proteasome in the release of peptide/HLA complexes from the Golgi, suggested that the presentation of viral antigens is intricately regulated, and clarified the mechanisms involved in MHC presentation.

Our improved understanding of the intracellular events involved in antigen processing and presentation has been complemented by several studies that used TCR-like Abs to examine the levels of peptide/MHC class I presentation under various conditions. Cohen et al used Fab mAb fragments specific to $Tax_{11–19}$/HLA-A2 complexes to study the presentation of the $Tax_{11–19}$ peptide derived from human T cell lymphotrophic virus type I (HTLV-1). The presentation of $Tax_{11–19}$/HLA-A2 complexes was quantified by staining EBV-transformed B cells that were transfected with the HTLV-1 Tax gene and HTLV-1 infected RSCD4 T cells with the Fab. In the infected cells, the intensity of Fab staining correlated with the intensity of staining with anti-Tax Ab, suggesting that levels of $Tax_{11–19}$/HLA-A2 presentation were dependent on expression of the Tax protein. Simultaneous staining with the Fab and specific anti-CD markers revealed high expression of the Tax/HLA-A2 complex by regulatory T cells, which could be correlated to their viral load. Thus, TCR-like Abs could be useful for studying the pathology of infections with HTLV-1 and other viruses.

However, other reports indicate that the relationship between protein expression and peptide presentation is not simple. Using Abs specific to peptide/MHC class I complexes, the Reiter group showed that peptide presentation can be influenced by cell type (39). Dendritic cells (DCs) retain peptide/MHC complexes on the cell surface significantly longer than non-DCs do, pointing to potentially important differences in peptide presentation between antigen presenting cells and non-presenting cells. Furthermore, at least two reports have shown that in cancer cells, protein expression levels are not associated with peptide presentation levels. Using TCR-like Abs specific for HLA-restricted peptides derived from gp100, Melan-A/Mart-1, and tyrosinase, the three major differentiation antigens expressed by human melanoma cells, the Reiter group showed that the quantity of peptide/HLA complexes on the cell surface was not correlated with gene expression profiles. Tyrosinase/HLA-A2 complexes were presented at unexpectedly high levels relative to gp100/HLA-A2 and Melan-A/Mart-1/HLA-A2 complexes in human melanoma cell lines. It was proposed that protein stability could play an important role in determining the copy number of a peptide/HLA class I complex on the cell surface. Likewise, using a TCR-like mAb specific for $Her2_{(369–377)}$/HLA-A2, the Weidanz group found that the density of $Her2_{(369–377)}$/HLA-A2 complexes on the cell surface did not correlate with protein expression of Her2. Using the same mAb, they showed that the density of peptide/HLA class I complexes on the cell surface directly correlated with the CTL-mediated killing of interferon gamma treated tumor cells (40). Thus, studies using TCR-like Abs have made it clear that the presentation of peptide/HLA complexes is influenced by multiple factors, and that the density of peptide/HLA complexes on the cell surface affects the immune response to diseased cells.

In addition to increasing our understanding of MHC-based immunity, the use of TCR-like Abs has contributed to the characterization of novel approaches to immune therapies. For example, the Weidanz group made TCR mimic antibodies (TCRms) to overlapping peptides

TMTRVLQGV {residues 40–48}, VLQGVLPAL {residues 44–52} and GVLPALPQV {residues 47–55, designated as GVL} from human chorionic gonadotropin-β (hCGβ) presented in the context of HLA-A2. After human DCs were treated with an hCGβ vaccine construct designed to promote the presentation of hCGβ-derived epitopes, staining with TCRms showed that only two of three possible hCGβ peptides were presented. The presentation of these peptide/HLA-A2 complexes was confirmed using antigen-specific CTL lines. The same TCRms were also used to show that expression of the GVL/HLA-A2 construct was highest at 72 hrs post-treatment (41), suggesting a delay between vaccine uptake and presentation of specific peptide/HLA complexes. In addition, the Weidanz group generated TCRms that recognized the immunodominant peptide/HLA-A2 complexes presented during WNV infection. Kim et al. used these TCRms to confirm that an HLA-A2 transgenic mouse presented the same immunodominant epitopes as human patients during WNV infection, thus validating a mouse model for studying the efficacy of a DNA vaccine for WNV (42). These examples strongly support the ability of TCR-like Abs to provide insight into the specific peptide/HLA complexes expressed on diseased cells and to validate new approaches to immunotherapy.

B) Anti-tumor activity

TCR-like Abs bind selectively to cancer cells that express a particular peptide/MHC complex, making them promising candidates for the delivery of cytotoxic agents to cancer cells. Indeed, recent work has shown that these Abs can be fused to cytotoxic agents, such as immunotoxins, cytokines, or drugs, to kill cancer cells *in vitro*. For example, Reiter *et al*. (43) constructed a recombinant Ab-toxin fusion based on Fab 13.4.1-PE38, which recognizes the Ha $_{255-62}$/K^k complex. Treatment with this Ab induced specific cytotoxicity with an IC_{50} of 5–9 ng/mL in $Ha_{255–262}$ peptide-pulsed RMA-S cells expressing K^k, in a peptide-dependent manner. In addition, Denkberg *et al*. (44) used a murine phage display library to generate a recombinant single-chain Ab against a gp100-derived peptide in complex with HLA-A2 and then fused the gene for this Ab to a truncated form of the gene for pseudomonas exotoxin A (G1scFv-PE38). With a binding affinity in the low nanomolar range (5nM), G1scFv-PE38 bound target complexes expressed on FM3D melanoma cells pulsed with gp100-derived peptides, and on gp100-transfected, HLA-A2-positive JY cells, a B lymphoblastoid cell line. Furthermore, G1scFv-PE38 induced specific cytotoxicity observed of multiple HLA-A2-positive, gp100-positive melanoma cell lines, but not of HLA-A2-negative, gp100-positive cells. Klechevsky *et al*. demonstrated *in vivo* tumor killing by a TCR-like Ab-toxin fusion protein. Two Fab Abs that recognized the MART-$1_{26–37}$/HLA-A2 complex (Fab CAG10, Fab CLA12) were generated from a human phage display library, and the genes for each Ab were fused to a truncated form of the gene for pseudomonas exotoxin (PE38KDEL). NOD-SCID β2M-deficient mice were inoculated with Mel526 melanoma cells and treated with varying concentrations of the two Fab constructs after the tumors grew to 55 mm^2. The Fab constructs produced dose-dependent tumor growth inhibition, suggesting that they selectively bound the MART-$1_{26–37}$/HLA-A2 complexes on tumor cells and induced cytotoxicity (45). Indeed, additional work has confirmed that TCR-like Abs can directly induce cytotoxicity of cancer cells. Sergeeva *et al*. generated an Ab specific for the HLA-A2-restricted PR1 peptide VLQELNVTV, which is derived from leukemia associated antigen proteinase-3, an endogenous protein over-expressed on acute myeloid leukemia (AML) cells. This Ab, 8F4, specifically bound to the PR1/HLA-A2 epitope and induced dose-dependent, complement-mediated cytotoxicity of AML leukemia stem cells but not normal hematopoietic cells. Further the 8F4 Ab selectively inhibited growth of leukemia progenitor cells but not normal progenitor cells (46). The Weidanz group has shown TCRms have anti-tumor effects that are due in part to direct signaling induced by TCRm binding to peptide/MHC (47–49). In a preclinical study, a F(ab)'2 fragment of the RL4B TCRm with specificity for hCG$β_{47–55}$/HLA-A2 demonstrated

growth inhibition of a human breast xenograft. Thus signaling through peptide/MHC complexes may represent a novel cytototoxic mechanism in tumors. Collectively, these results demonstrate the ability of TCR-like Abs to selectively target and kill human tumor cells.

These recent studies indicate that Abs specific for peptide/MHC complexes represent a novel class of agents with a promising array of potential applications for the treatment of cancer and perhaps infectious diseases. Furthermore, since TCR-like Abs can specifically recognize peptide/HLA complexes that are either exclusively expressed or over-expressed on tumor cell surfaces during different stages of tumorigenesis and cancer, these Abs could be developed as a tool for clinically monitoring cancer progression and as stage-specific cancer therapeutics.

Conclusion

Peptide/MHC complexes offer a promising new array of potential markers for cancer and viral infections. Several strategies exist for the discovery and validation of peptide/MHC epitopes, but challenges associated with traditional methods have until recently slowed the development of peptide/MHC complexes as therapeutic targets. The ability to generate antibodies specific for peptide/MHC complexes has enabled several studies that revealed key aspects of antigen processing and presentation in cancerous and infected cells and validated new reagents and model systems for immunological research. The high specificity and strong binding affinities of these antibodies make them useful targeting agents against infectious diseases and cancer; used alone or in combination with cytokines or drugs, TCR-like antibodies are attractive as therapeutic anti-cancer agents. The use of TCR-like antibodies to target disease-associated peptide/HLA complexes therefore holds tremendous potential to advance our understanding of MHC-based immunity and transform our approach to immunotherapy.

Acknowledgments

We thank Bourn Biomedical Communications for writing assistance and proofreading by Dr. Michelle Jones.

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