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Expanding the Targets Available to Therapeutic Antibodies via Novel Disease-Specific Markers

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Summary

The development of immunotherapies offers significant promise for clinical applications in cancer and infectious diseases. Here we describe a novel, integrated approach to immunotherapy that combines novel technologies to discover and target disease-specific peptide/HLA class I complexes. This unique class of markers makes the entire proteome accessible to antibody reagents and offers unsurpassed specificity for targeting cancerous and infected cells. Arm one of our three-armed approach uses an innovative technology for the efficient, direct discovery of new peptide/HLA class I markers. Arm two applies a powerful and inventive strategy to generate T cell receptor mimics (TCRms), which are antibodies with exquisite binding specificity for peptide/HLA class I markers, and uses TCRms to validate the specific expression of markers on cancerous and infected cells. The third arm of our approach uses TCRms to target and kill diseased cells with high sensitivity and specificity. In summary, the combination of two pioneering technologies expands the repertoire of disease-specific markers that can be targeted by therapeutic antibodies and enables a powerful, integrated approach to HLA-based immunotherapy.

The Need for New Selective Therapeutic Targets

Despite recent advances in technologies for target identification, such as genomic, transcriptomic, and proteomic profiling, the discovery of novel, pharmaceutically useful targets has slowed. As a result, the pipeline of potential drug targets is not adequate to support the development of new therapeutic strategies, and many diseases lack effective treatment options. To be pharmaceutically useful, a target must be accessible to drug molecules and must bring about a therapeutic effect upon binding the drug. For example, many therapeutic targets are cell-surface receptors or signaling molecules whose functions can be modified by small molecule and biological drugs. In addition, the therapeutic target should be abundantly expressed by most diseased cells or tissues and absent from healthy tissues. Likewise, new drugs should act on the intended therapeutic target, with minimal effects on other biomolecules. Therapeutic monoclonal antibodies (mAbs) show high affinity and extraordinary specificity for their targets. However, mAbs can only access targets on the cell surface or in the extracellular space, while most disease-specific proteins

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are intracellular and thus inaccessible to mAbs. Therefore, new strategies are needed both to identify novel therapeutic targets and to develop new immunotherapeutic agents.

The paucity of disease-specific targets limits the ability to treat many diseases, but this is especially true for cancer. The broad, undesirable side effects of common treatments for cancer, such as chemotherapy, radiation and adjuvant therapies, highlight the need for new, cancer-specific therapeutic targets. Several mAbs currently on the market target cancer-specific markers and have powerful anti-tumor effects. However, only a minor fraction of the proteome is expressed on the cell surface, so only a handful of cell-surface proteins have been validated as cancer-specific targets. As a result, current therapeutic mAbs can only be used to treat a fraction of all cancers. Product development portfolios and clinical trials show that most ongoing efforts are focused on molecular targets validated over a decade ago, leading most efforts to cluster around a small number of science discovery opportunities. This article provides an overview of our work in improving the technologies available for discovery of disease-specific markers, establishing peptide/HLA class I molecules as suitable therapeutic targets, and developing a new class of TCR-like antibodies that show great promise for the diagnosis and treatment of cancer and infections.

An Integrated Approach to HLA-Based Immunotherapy

An integrated approach to immunotherapy can be used to take full advantage of the remarkable targeting power available through peptide/HLA markers. The first arm of the integrated immunotherapy approach described here focuses on the discovery of peptide/HLA complexes that distinguish cancerous or infected cells. The HLA system and several methods for identifying peptide/HLA markers that might be useful as therapeutic targets are briefly described in the accompanying editorial review. Our method gathers soluble peptide/HLA markers from cancerous or infected cells and normal cells and identifies those that are unique to the cancerous or infected cells. The discovery of novel peptide/HLA markers serves as the foundation for the second and third arms of our approach to immunotherapy.

In the second arm of our systematic approach to immunotherapy, we generate reagents for validating and targeting disease-specific peptide/HLA markers. Despite extensive efforts by many groups, it has been difficult to generate antibodies (Abs) specific for these markers. However, our novel approach enables the reliable and efficient production of Abs that recognize peptide/HLA markers with high affinity and specificity. T cells and mAbs both provide incredible specificity for targeting cell surface markers, and each of these has additional unique qualities that make them strong candidates for immune-based therapies. Via the TCR, T cells show high affinity, exquisite specificity, and unparalleled sensitivity for their cognate peptide/HLA markers. Illustrating their specificity and sensitivity, TCRs distinguish peptide/HLA complexes that contain the same peptide presented by different HLA class I molecules and can detect fewer than 50 copies of their cognate peptide/HLA marker on the surface of a cell. However, T cells cannot be used for direct detection, validation, or quantification of specific peptide/MHC markers, nor to study their presentation in real time. Monoclonal antibodies are ideal reagents for these applications because they are archivable, stable, and maintain their clonality. In addition, mAbs can be abundantly produced *in vitro*, and they are a proven class of clinical therapeutics for cancer and infectious diseases. Recognizing the unique strengths of T cells and mAbs, we combined the power of these two immunologic reagents in a new class of mAbs that replicates the functions of the TCR: T cell receptor mimic mAbs, or TCRms. As the second arm of our integrated approach to immunotherapy, the generation of TCRms links the efficient discovery of disease-specific markers with the precise immune targeting necessary for the diagnosis and treatment of cancer and infectious diseases.

In the third arm of the integrated approach, we use TCRms for targeting and killing tumors and infected cells. Our recently published studies show that TCRms exert potent anti-tumor effects, including *in vivo* and *in vitro* tumor killing via complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC), and apoptosis[1]. It is well established that mAbs that recognize cancer-specific markers can have direct anti-tumor effects *in vitro* and *in vivo* [2–7]. For example, upon binding their cognate antigens on the surface of cancer cells, cetuximab (anti-epidermal growth factor receptor) kills cancer cells by CDC and ADCC [8], and rituximab (anti-CD20) induces apoptosis as well as CDC and ADCC [9]. The anti-tumor activity of TCRms may be enhanced by their ability to specifically target the immune system's natural marker for cancerous cells. Indeed, emerging cancer vaccines illustrate the therapeutic value of targeting tumor-specific peptide/HLA markers. For example Sipuleucel-T, the first FDA-approved therapeutic T cell vaccine, treats prostate cancer by eliciting a T cell response specific for HLA-presented peptide fragments of prostatic acid phosphatase, a marker for metastatic, asymptomatic, hormone-refractor prostate cancer[10]. In addition, targeting tumor-specific peptide/HLA class I markers on human melanoma cells [11–13] with marker-specific T cells results in cancer regression in melanoma patients [14–16].

Because of the unique nature of peptide/HLA markers, the efficient discovery of disease-specific peptide/HLA markers is central to our integrated approach to the development of immunotherapies. Building on this foundation, we generate TCRms that specifically recognize target peptide/HLA markers and use these TCRms to directly validate marker expression on cancer cells and tissues. Finally, we use TCRms to target and kill diseased cells. Here, we describe each of these steps and provide several examples of the profound impact that TCRms have on tumor growth, raising the possibility that this integrated approach to immunotherapy could markedly expand the repertoire of therapeutic mAbs for the diagnosis and treatment of cancer and infections [17].

Direct Discovery of Disease-Specific Peptide/HLA Class I Markers Using Soluble HLA Molecules

To address many of the challenges inherent to traditional direct discovery approaches, we identify peptides presented by soluble HLA (sHLA) molecules that can be easily purified from culture supernatants (see Figure 1 for an overview of this approach). The expression construct for an sHLA protein contains the normal coding sequence for the HLA protein of interest, except that it is truncated immediately N-terminal to the transmembrane domain to produce an HLA molecule that is secreted rather than membrane-bound. Extensive data show that sHLA proteins are loaded with peptides through the normal HLA pathway, indicating that the peptides eluted from sHLA molecules mirror those normally presented by membrane-associated HLA proteins. Cell lines are stably transfected with the sHLA expression construct and grown at a high density. This method allows >25 mg of endogenously loaded peptide/sHLA complexes to be rapidly produced and easily purified from the culture medium.

Next, peptides are eluted and purified from the peptide/sHLA complexes, resulting in a peptide pool that contains up to 10,000 unique peptides. To reduce the complexity of the sample and enable more effective downstream analysis, the purified peptides are separated by size and hydrophobicity into fractions that each contain ~200 distinct peptides. Each fraction is then mapped by mass spectrometric (MS) analysis. To identify disease-specific peptide/HLA complexes, normal cell lines and tumorigenic or infected cells are transfected with the same sHLA expression construct, and the MS/MS ion maps are compared to identify peaks that are unique to the diseased cells. Finally, the unique ions are sequenced, and the computed sequence for each disease-associated peptide is confirmed by comparing

the MS/MS fragmentation pattern of the eluted peptide to that of a synthetic peptide of the computed sequence.

The use of sHLA for the discovery of disease-associated peptide/HLA complexes offers several advantages over traditional direct and indirect approaches to peptide discovery. For example, sHLA molecules are readily purified from the cellular supernatants while the cell's own HLA molecules remain bound to the cell membrane, so all identified peptides are presented by the HLA protein of interest. We have generated sHLA expression constructs for >60 of the most common HLA class I molecules, enabling specific identification of the peptides presented by a particular HLA class I protein. In addition to eliminating the diversity of HLA molecules that complicates traditional direct discovery approaches, our strategy greatly reduces the complexity of the peptide pool that must be analyzed. Because of the high yield of affinity-purified peptide/sHLA complexes, we are able to analyze peptide fractions of ~200 peptides each rather than the complex pools of ~300,000 peptides that are analyzed in traditional direct discovery approaches. Therefore, the use of sHLA makes it possible to detect disease-specific peptides that are presented in low abundance. Finally, the ability to stably transfect virtually any mammalian cell line with sHLA expression constructs enables the detection of relevant markers for nearly any disease or tissue type for which cell lines are available. Because these markers are derived from proteins expressed in every cellular compartment, the discovery of peptide/HLA markers dramatically expands the portion of the proteome that can be targeted by immunotherapies. Thus, an sHLA-based strategy for discovering disease-specific peptide/HLA markers offers the potential to efficiently identify novel markers for a wide range of cancers and infectious diseases.

For example, we have used this approach to discover peptide/HLA markers for breast cancer, West Nile virus infection, and influenza virus infection. To identify breast-cancer-specific peptide/HLA-A*0201 (HLA-A2) markers, we transfected three tumorigenic breast epithelial lines (MCF-7, MDA-MB-231, and BT-20) and a non-tumorigenic breast epithelial line (MCF10A) with an expression construct for the sHLA-A2 protein[18]. Following the method described above, we identified four peptides that were presented by the HLA-A2 molecules of all three tumorigenic cell lines, but not the non-tumorigenic cell line. We also identified a fifth peptide, MIF₁₉₋₂₇, that was only observed in two of the three non-tumorigenic cell lines. Except for one peptide derived from exosome component 6 (EXOSC6), all of the peptides specific to the tumorigenic cell lines were derived from proteins with well defined roles in cancer: ornithine decarboxylase I (ODC1), cyclin dependent kinase 2 (Cdk2), kinetochore associated 2 (KNTC2 or HEC1), and macrophage migration inhibitory factor (MIF). Finally, we assessed the immunological relevance of these markers by testing whether peripheral blood mononuclear cells (PBMCs) recognized and responded to the peptides or the peptide/HLA-A2 complexes. PBMCs isolated from 11 subjects with or without a history of breast cancer were stained with tetramers of the peptide/HLA-A2 markers that were specific to tumorigenic breast epithelial cell lines, or a tetramer of an Epstein-Barr virus peptide/HLA-A2 complex as a positive control. PBMCs from one subject, who had a history of breast cancer, showed staining with the tetramers containing Cdk2 and EXOSC6, as well as the EBV control. Next, PBMCs from 6 of the 11 subjects, including one subject with no history of breast cancer, were stimulated with peptide for one week, and the release of interferon- γ (IFN- γ) was assessed by enzyme linked immunosorbent spot assay. Each of the potential cancer-specific peptides elicited a robust IFN- γ response in the PBMCs from at least one subject with a history of breast cancer. As expected, PBMCs from the subject with no history of breast cancer showed no IFN- γ response to any of the potential cancer-specific peptides. These results indicate that the sHLA-based approach can identify immunologically relevant cancer-specific peptide/HLA markers.

In addition, recent data show that the HLA system presents virus-derived peptides that may provide valuable targets for the diagnosis, prevention, and treatment of viral infections. For example, we compared the peptides eluted from sHLA-A2 molecules of WNV-infected and uninfected HeLa cells and identified six peptides of viral origin that were uniquely presented by the sHLA-A2 of infected cells. Three of the six peptides elicited a stronger IFN- γ response in PBMCs isolated from subjects ~1 month or ~1 year after recovery from WNV infection, compared to PBMCs from WNV-negative healthy controls. Nearly all PBMCs from subjects with a history of WNV infection showed the strongest response to the SVG9 peptide (SVGGVFTSV), which was highly conserved across numerous strains of WNV. These results revealed several WNV-specific peptide/HLA-A2 markers, elucidated the immune response to WNV, and established SVG9 as an immunodominant peptide relevant for multiple strains of WNV. As described below, this work provided the foundation for the development of a DNA vaccine that protected mice from WNV infection. Similarly, the sHLA-based approach to marker discovery revealed that influenza A infection typically results in the presentation of 3–6 viral peptides, which are primarily derived from internal proteins. We identified three peptide/HLA markers that were consistently presented by the HLA-B*0702 molecule during infection with any of the three influenza A strains tested: the highly variable NP_{418–426} peptide and moderately conserved NP_{473–481} peptide derived from the influenza virus nucleoprotein, and the moderately conserved PB1_{329–337} peptide derived from polymerase basic protein 1. The NP_{418–429} and NP_{473–481} peptides were also presented by the HLA-B*3501 molecule during infection with each of the strains tested. In addition, the NP_{418–426} and PB1_{339–347} markers were highly immunogenic; after inoculation with a laboratory strain of influenza A, HLA-B*0702 transgenic mice showed a strong IFN- γ response to the HLA-B*0702-restricted NP_{418–426} and PB1_{329–337} peptides. Together, these published studies demonstrate the ability of sHLA-based marker discovery to identify disease-specific, immunologically relevant peptide/HLA markers and provide insight into the immune responses against cancer and viral infections.

Despite the power of this strategy, the candidate peptide/HLA markers identified using cell lines must be validated in primary tissues. Although cell lines are routinely used to identify candidate proteins for therapeutic targeting or marker development, they may not fully recapitulate the presentation of peptides *in vivo*. Furthermore, while mass spectrometry is a sensitive tool for peptide identification, it does not provide information on the immunologic potential of markers. Therefore, in the second arm of our approach to cancer immunotherapy, we develop antibodies that specifically recognize candidate peptide/HLA markers to enable the validation of these target markers in primary tissues.

Development of T Cell Receptor Mimics that Recognize Target Peptide/HLA Markers

The discovery of disease-specific peptide/HLA markers is an important first step in developing new immunotherapies, but novel markers are only useful if they can be validated and effectively targeted. Therefore, we complement our innovative approach to marker discovery with the production of mAbs called T cell receptor mimics (TCRms) that recognize specific peptide/HLA markers. Several groups have developed Abs against specific peptide/HLA complexes using a variety of methods, including bacteriophage display and classical immunization approaches [2–7]. However, only our technique efficiently and consistently produces mAbs that show high affinity and specificity for the target peptide/HLA complex.

As described in the accompanying editorial review, common methods for producing Abs showed limited value for producing TCR-like Abs. Phage display has been used to produce recombinant Abs that specifically recognize peptide/MHC complexes, but these Abs

typically have relatively low binding affinities. Another common approach, classical immunization and hybridoma technology, uses peptide-pulsed cells or purified monomers of peptide/HLA complexes as immunogens. The extremely low efficiency of immunization-based methods led many researchers to postulate that the B cell repertoire lacked cells with specificity for peptide/MHC class I complexes, and so immunization was inherently unable to induce the production of antibodies that recognize peptide/MHC markers with high specificity and affinity. However, we induced polyclonal Ab responses specific to p68 self-peptide/HLA-A2 complexes in HLA-A2 transgenic mice. Thus, these claims were refuted by our ability to successfully generate high-affinity mAbs that recognize self-peptide/MHC complexes, using a novel immunization-based approach.

We found that the peptide/MHC complex monomers used as immunogens in previous approaches were highly unstable, particularly *in vivo*, while multimers of peptide/MHC complexes were orders of magnitude more stable than monomers of the same complex. In addition, multimers of peptide/MHC complexes were extremely potent stimulators of antigen-specific B cell responses, while monomers were poor immunogens. In addition to their greater stability, we propose that higher order peptide/MHC complexes stimulate efficient B cell receptor cross-linking and activation of B cells, even with little or no T cell help. Based on this hypothesis and our original unpublished observations, we developed synthetic tetramers or higher order complexes of specific peptide/MHC complexes for immunization of allogeneic or syngeneic mice. This novel immunization strategy resulted in the potent expansion of antigen-specific B cell responses. Recently, we have further modified the synthetic multimeric immunogen to induce responses that are confined to the peptide/MHC interface, thus improving the specificity of antigen recognition. Following immunization, we use classical hybridoma technology coupled with an innovative high throughput screening process to generate and positively identify hybridoma clones that produce TCRms, while eliminating clones that produce cross-reactive Abs.

This strategy shows remarkable efficiency in generating Abs that recognize the target peptide/MHC marker specifically and with high affinity. Using flow cytometry to directly quantify marker-specific B cell responses and antibody titers to indirectly assess the B cell response to immunization, we have demonstrated that ~10% of the Abs produced by this strategy are specific for the target marker. We have successfully used this strategy to produce >70 TCRms that are stable and show high binding specificity and affinity for > 30 peptide/MHC class I targets. Like TCRs of cytotoxic lymphocytes (CTL), TCRms show fine binding specificity for peptide/HLA complexes because the epitope consists of not only the peptide, but also part of the HLA molecule. The specificity of TCRms has been established through several different assays in which TCRms bound specific peptide/MHC complexes and tumor cells, but showed minimal or no binding to irrelevant peptide/MHC complexes, relevant MHC molecules without peptide, peptide without MHC molecules, or cells that did not display the peptide/MHC complex. In addition to their TCR-like specificity, TCRms display Ab-like binding affinity for the cognate peptide/HLA marker. Each TCRm has high affinity binding, with many having an affinity constant (K_D) of $\sim 1.5 \times 10^{-9}$ M and a half life of dissociation of $> 8.5 \times 10^3$ seconds.[19, 20]. Thus, by combining novel strategies of immunogen formation and hybridoma screening with newly available methods for the production and purification of Abs, we can now efficiently and reliably produce high quantities of TCRms that specifically recognize many peptide/MHC markers.

A) Marker Validation by TCRms

Although our strategy for discovering peptide/MHC class I markers offers several advantages over traditional methods, the careful and thorough validation of novel disease-specific markers is essential. Traditional CTL-based approaches to marker analysis are cumbersome, indirect, and qualitative rather than quantitative. In contrast, TCRms offer a

robust, efficient and direct approach to validating marker expression in cells or tissues. TCRms enable the real-time quantification of changes in the specificity and timing of peptide/HLA presentation following viral infection or malignant transformation. In addition, unlike CTL-based approaches, TCRms can be used to detect and study peptide/HLA complexes that are present on normal cells. Therefore, TCRms are an ideal reagent for the direct and quantitative characterization of peptide/HLA complexes on normal, infected, and malignant cells.

We first used a TCRm to validate an HIV-specific peptide/HLA marker. Using our sHLA-based marker discovery method, we identified an HLA-A2-restricted peptide derived from eukaryotic initiation factor 4 gamma peptide (eIF4G₇₂₀₋₇₂₈) that was presented approximately three-fold more abundantly by HIV-infected cells than by non-infected cells. To validate the HIV specificity, tissue specificity, and timing of eIF4G₇₂₀₋₇₂₈/HLA-A2 presentation, we used our novel immunization strategy to generate 4F7, a TCRm that recognized the eIF4G₇₂₀₋₇₂₈/HLA-A2 complex, and confirmed the specificity of 4F7 by competitive tetramer binding assays. We stained HLA-A2 positive HIV-infected (p24+) cells and control cells with 4F7 and used flow cytometry to analyze expression of the eIF4G₇₂₀₋₇₂₈/HLA-A2 complex. 4F7 showed strong staining of HLA-A2 positive HIV-infected cells, weak staining of HLA-A2 positive mock-infected or influenza-infected cells, and no staining of HLA-A2 negative cells, thus validating the eIF4G₇₂₀₋₇₂₈/HLA-A2 complex as a marker for HIV-infected cells. Since it had been previously reported that malignant cells overexpress eIF4G, we tested whether malignant cells also presented the eIF4G₇₂₀₋₇₂₈/HLA-A2 complex. A human breast carcinoma cell line (MDA-MB-231) showed 4F7 staining, but a normal human mammary epithelial cell line did not, as expected based on previous reports. These results indicated that the eIF4G₇₂₀₋₇₂₈/HLA-A2 marker is presented by cancer cells and HIV-infected cells, but not normal cells. Finally, we used 4F7 to study the kinetics of eIF4G₇₂₀₋₇₂₈/HLA-A2 presentation during HIV infection. Within three days post-infection, HIV-infected primary CD4⁺ cells showed two-fold higher expression of the eIF4G₇₂₀₋₇₂₈/HLA-A2 marker than mock-infected cells. By the seventh day post-infection, infected cells showed four-fold higher eIF4G₇₂₀₋₇₂₈/HLA-A2 expression than mock-infected cells. Thus, the 4F7 TCRm enabled the validation and quantitative characterization of a novel peptide/HLA marker for HIV-infected cells [3].

In addition to characterizing the disease association and tissue specificity of peptide/HLA markers, TCRms are useful for validating experimental approaches. For example, in 2010, Kim and colleagues used a TCRm to confirm the presentation of SVG9, a West Nile virus (WNV)-derived peptide, by the HLA-A2 molecules of infected cells and to validate a single chain trimer (SCT) expression construct designed to simulate presentation of the SVG9/HLA-A2 marker [21]. After confirming that the RL15A TCRm was specific for the SVG9/HLA-A2 marker, RL15A was used to stain HLA-A2-positive WNV-infected dendritic cells (DCs), uninfected DCs as a negative control, and uninfected DCs pulsed with SVG9 peptide as a positive control. RL15A stained the WNV-infected DCs and uninfected, SVG9-pulsed DCs, but not uninfected DCs that had not been pulsed with SVG9, thus confirming that the SVG9 peptide is processed and presented by HLA-A2 during WNV infection. Next, an SCT construct was designed to encode the SVG9 peptide and the HLA-A2 molecule (consisting of the B₂ microglobulin and α heavy chain) separated by short amino acid linkers in a single open reading frame. HeLa cells transfected with the construct showed RL15A staining, indicating that the SCT construct produced stable cell-surface expression of a properly folded SVG9/HLA-A2 complex. Next, this construct was used as a DNA vaccine in HLA-A2 transgenic mice and successfully elicited a protective immune response against WNV. By enabling the efficient and reliable validation of a WNV-specific marker and a new reagent, the RL15A TCRm was instrumental to the development of a new and promising approach to DNA vaccination. Finally, as described below, TCRms have been used to

validate and characterize several breast cancer-specific peptide/HLA markers. Together, these examples demonstrate that the combination of sHLA-based peptide/HLA marker discovery and TCRm technology is a powerful tool for the advance of immunological research and the development of new immunotherapies.

Potential Clinical Applications of T Cell Receptor Mimics

The final step in our integrated approach to immunotherapy is to develop therapeutic strategies that target the peptide/HLA markers discovered with the use of sHLA and characterized with TCRms. Because of their exquisite specificity, outstanding binding affinity, and because of the success of currently available therapeutic mAbs, TCRms are strong candidates for safe and efficacious immunotherapy. Our data suggest several possible clinical applications for TCRms, including cancer detection and diagnosis, direct anti-tumor therapy, and targeted drug delivery.

A) Diagnostic potential of TCRms

Because TCRms can recognize tumor-specific peptide/HLA class I complexes with high specificity and affinity, they are ideal candidates for use in cancer diagnostics. Using the epitope discovery method described above, we found that the YLL₁₂₈₋₁₃₆ peptide derived from the p68 RNA helicase protein and the MIF₁₉₋₂₇ peptide derived from the macrophage migration inhibitory factor protein are presented by the HLA-A2 class I molecule on tumorigenic breast cancer cell lines.

We developed two TCRm, RL6A and RL21A, which specifically recognize the YLL₁₂₈₋₁₃₆/HLA-A2 complex and the MIF₁₉₋₂₇/HLA-A2 complex, respectively. For these complexes to serve as diagnostic or therapeutic targets, they must distinguish cancerous cells from benign tissues. Therefore, RL6A and RL21A were used to directly determine the levels of the YLL₁₂₈₋₁₃₆/HLA-A2 complex and MIF₁₉₋₂₇/HLA-A2 complex in various primary tissues [19, 22]. Both peptide/HLA-A2 complexes were detected on breast cancer cell lines using our epitope discovery strategy, and immunohistochemical staining of primary tumors confirmed that both complexes were present on neoplastic cells and tumor-associated stroma in breast cancer tissue, but were either absent or expressed at minimally detectable levels on normal adjacent tissue. RL6A staining of various normal, primary tissues indicated that expression of the YLL₁₂₈₋₁₃₆/HLA-A2 epitope was not exclusive to breast tumor cells, but was markedly increased in tumor cells [19]. Therefore, by comparing suspected malignancies with normal tissue, RL6A staining of biopsied tissues could provide valuable information regarding malignant cell transformation. Moreover, the YLL peptide has been independently isolated from transformed B cells [23], and our data show strong RL6A staining of neoplastic cells and tumor-associated stroma from metastatic ovarian tissue. These observations suggest that expression of the YLL₁₂₈₋₁₃₆/HLA-A2 target may be broadly upregulated by cancer cells of different histological origins, independently of tumor stage. Future studies will therefore test the possibility that the YLL₁₂₈₋₁₃₆/HLA-A2 complex could serve as a diagnostic indicator for a wide variety of malignant conditions.

Unlike the YLL₁₂₈₋₁₃₆/HLA-A2 complex, expression of the MIF₁₉₋₂₇/HLA-A2 complex is uniquely tumor-specific. Immunohistochemical staining of primary tumors showed that RL21A did not stain fibroadenoma or ductal carcinoma in situ (DCIS), two non-invasive conditions [22]. In contrast, in invasive ductal carcinoma (IDC) tissues, both the tumor tissue and tumor-associated stroma showed strong RL21A staining, while normal adjacent tissues were not stained by RL21A (Figure 2; Table 1). Because the MIF protein is expressed by multiple cell types, it is possible that the MIF₁₉₋₂₇/HLA-A2 complex could be present on a variety of cell types in healthy individuals. However, RL21A showed little to no staining of normal tissues from healthy HLA-A2 positive donors, including eighteen total

white blood cell samples and a panel of twenty cryopreserved tissues each from a male and a female donor (Table 1). Therefore, RL21A distinguishes invasive ductal carcinoma from normal and diseased non-invasive breast tissues and from other normal tissues.

To further assess the diagnostic potential of the MIF₁₉₋₂₇/HLA-A2 complex, the prevalence of RL21A staining was assessed in 30 invasive breast tumors from different donors. We found widespread expression of the MIF₁₉₋₂₇/HLA-A2 complex in invasive human breast tumors. Regardless of Her2 status, the average score for RL21A staining of invasive tissues was 4- to 5-fold higher ($P=0.033$) than the score for adjacent normal tissues (Table 1). More recently RL21A was shown to stain metastatic ovarian tumors but not normal ovarian tissue, indicating that expression of the MIF₁₉₋₂₇/HLA-A2 complex is not restricted to cancers of the breast. These findings suggest that our technology can discover prevalently expressed peptide/HLA class I complexes and that TCRms made to these targets might have broad applications for detecting and diagnosing histologically distinct cancers.

The ability to distinguish invasive breast cancer suggests that the MIF₁₉₋₂₇/HLA-A2 complex could provide an alternative to the three most relevant prognostic and treatment-guiding markers for breast cancer: estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor (HER-2). Although MIF₁₉₋₂₇/HLA-A2 has not been directly compared to other breast cancer markers, the MIF₁₉₋₂₇/HLA-A2 complex showed a degree of overexpression in invasive tissues comparable to HER-2 (~30%). Furthermore, while ER, PR, and HER-2 are often observed in ductal carcinoma in situ, RL21A did not stain this non-invasive tissue type, implying that MIF₁₉₋₂₇/HLA-A2 could offer a marker for invasive phenotypes. Future studies will elucidate the full diagnostic value of the MIF₁₉₋₂₇/HLA-A2 complex.

B) Therapeutic promise of TCRms

In addition to their value for epitope validation and as potential diagnostic tools, TCRms could provide a new class of therapeutics for the treatment of cancer. Breast cancer biomarkers such as HER-2 provide a highly successful precedent for the dual use of peptide/HLA complexes as both prognostic markers and therapeutic targets. We have now shown using 4 different TCRms profound anti-tumor activity in pre-clinical models of breast cancer. However, before these studies, *in vitro* studies performed earlier in our laboratory had shown that TCRm could induce tumor cell death via CDC and ADCC. It was a logical progression of experiments that led to investigations into the therapeutic activity of TCRms. To our surprise, we found that TCRm could directly induce tumor cytotoxicity *in vitro* and *in vivo*. Because of their versatility in targeting and their profound anti-tumor effects, TCRms could be used either as direct anti-tumor agents or as targeting agents to enhance the efficacy of other therapeutics.

Direct anti-tumor effects—TCRms exhibit profound anti-tumor activity both *in vitro* and *in vivo* [17]. For example, treatment with RL6A or RL21A (Table 1) dramatically reduced the tumor burden in two orthotopic breast cancer models (data not shown and Figure 3). In nude mice implanted with the tumorigenic MDA-MB-231 cell line, RL6A reduced tumor growth [19], and RL21A treatment inhibited tumor growth by five-fold [22]. For an orthotopic model using the more aggressive BT-20-A2 cell line, RL21A inhibited tumor growth by more than two-fold. Both of these cell lines are phenotypically triple negative (ER⁻, PR⁻, HER-2⁻), suggesting that RL6A and RL21A could be valuable therapeutic tools for breast cancer patients for whom there are currently no targeted therapies. The ability of TCRm treatment to produce significant reduction of tumors *in vivo* demonstrates that tumor-specific peptide/HLA complexes may offer a prolific source of new targets for successful immune therapies.

Our in vitro and in vivo studies indicate that TCRms directly activate caspases, apoptosis, and cell death by signaling through selective and direct binding to the peptide/HLA class I complex. Inflammatory infiltrate was not obvious in the tumors of xenograft models. Since natural killer cells, monocytes, and macrophages are essential for mAbs to kill tumor cells through ADCC, this suggests that TCRm-mediated killing is at least partly independent from ADCC. In addition, TCRms lacking the Fc fragments were capable of reducing tumor growth, although to a lesser degree than whole TCRms, indicating that TCRm-mediated killing of tumor cells is partially independent of ADCC and complement, but that ADCC and complement dependent cytotoxicity may contribute to the effects of TCRms (Figure 4). Our in vitro studies indicate that activation of the caspase-dependent intrinsic pathway, disruption of mitochondrial membrane integrity, and JNK signaling contribute to TCRm-induced tumor cell apoptosis. The ability of the TCRm to kill tumor cells independently of immune effector cells could provide a significant advantage over the majority of currently approved therapeutic mAbs.

Many current mAb therapies require a functional immune system for tumor cell killing, which is less than ideal for patients who are immunocompromised, or in the common scenario that the tumor is protected from complement dependent cytotoxicity by high expression of complement regulatory proteins. Like trastuzumab and bevacizumab, TCRms appear to lead to tumor cell apoptosis directly through induction of pro-apoptotic signaling. However, unlike trastuzumab, which shows reduced efficacy when the density of target epitopes on the cell surface is low, TCRms show high efficacy in killing tumor cells despite the relatively low density of target peptide/HLA complexes on the cell surface. This represents an additional advantage of the TCRms, since the expression of tumor-specific proteins is often low. TCRms thus present a promising direction in the development of immune-based cancer therapeutics.

Targeted drug delivery—The side effects of non-specific cancer treatments can be minimized by delivering the treatment directly to the tumor. We have found that TCRms coupled directly to poly(lactide-co-glycolide) (PLGA) nanoparticles (<100 nm) containing paclitaxol can specifically target and poison tumor cells. Furthermore, since differentiated, non-malignant cells produce distinct patterns of peptide/HLA complexes, TCRms directed against cell-type specific peptide/HLA complexes could be used to target specific cell types. For example, highly specific TCRms could potentially be used to deliver drugs to the vascular bed of a specific organ. Indeed, TCRms have been used to target non-malignant cells, and our recent results provide proof of concept for the use of TCRms for brain-specific targeting and overcoming the blood-brain barrier.

The RL6A TCRm recognizes the YLL_{128–136}/HLA-A2 complex expressed in brain endothelial cells. The YLL_{128–136} peptide is derived from the p68 RNA helicase protein, which has been shown to have high mRNA expression levels in rat brain microvessels. As an initial model for the blood brain barrier, we used hCMEC/D3 cells, an HLA-A2 positive cell line derived from human brain endothelium. Flow cytometry analysis showed that hCMEC/D3 cells express the YLL/HLA-A2 complex, and that expression is increased by pretreatment with interferon γ . Confocal microscopy experiments using both fixed and live cells showed that RL6A bound to the surface of hCMEC/D3 cells and was internalized into vesicles marked by the early endosome marker EEA1. Consistent with these data, primary human brain endothelial cells also bound and internalized the RL6A TCRm, as observed by flow cytometry and microscopy [24].

These results highlight the broad potential of TCRms like RL6A for potential diagnostic or therapeutic applications. It has previously been proposed that the blood-brain barrier could be circumvented by taking advantage of physiological transport mechanisms mediated by

endothelial receptor proteins. Although numerous preclinical studies support the promise of this approach, the receptors targeted to date have not been specific to the blood-brain barrier. We predict that specific vascular endothelial cells are marked by unique peptide/HLA complexes, and that the generation of TCRm specific for these markers will allow targeting to select organ vascular beds for diagnostic and therapeutic agents. The internalization of RL6A by brain endothelial cells thus establishes proof of concept, presenting the exciting possibility that TCRms made to peptide/HLA complexes specific to brain endothelium could be used to target diagnostic agents or therapeutics to the blood-brain barrier and brain. Furthermore, the observation that inflammation, proliferation, and other physiological changes influence peptide/HLA expression patterns raises the possibility that tumor neovasculature can be differentiated from normal, resting endothelium. The benefits of targeting tumor vasculature using bevacizumab or other small molecule drugs to block angiogenesis have already been demonstrated in preclinical and clinical settings. Targeting specific peptide/HLA complexes expressed on tumor vascular tissue with TCRms or TCRm-drug conjugates could provide novel strategies for inhibiting angiogenesis. TCRms therefore offer the potential for highly specific targeting of diagnostic and therapeutic agents to the blood-brain barrier, tumor vasculature and other organ vascular beds.

Conclusion

Immunotherapy offers the potential for highly targeted and efficacious treatments for a variety of diseases. However, the development of new immunotherapies has been hindered by the paucity of suitable targets. We have shown that peptide/HLA complexes provide a rich source of disease-specific markers that can be validated and targeted with TCRms. The combination of our innovative strategies for the discovery of peptide/HLA markers, the generation of TCRms, and the application of these antibodies presents a powerful, integrated approach to HLA-based immunotherapy. First, we use soluble HLA molecules for the efficient and reliable discovery of peptide/HLA markers that are novel, disease-specific, and immunologically relevant. Next, through an inventive and reliable approach to the production of TCR-like antibodies, we generate TCRms to validate these markers. Finally, we use TCRms in the development of new clinical tools that target validated peptide/HLA markers for the diagnosis and treatment of cancer and infectious disease.

Because of their high specificity and strong binding affinity, TCRms show great promise as immunotherapeutic agents to improve diagnosis, directly kill diseased cells, and provide targeted delivery of existing drugs. We have shown that, in addition to distinguishing cancerous or virally infected cells from normal cells, TCRms can distinguish invasive breast tumors from non-invasive and normal breast tissues and from normal tissues. Furthermore, TCRms exert potent direct anti-tumor effects in orthotopic models of breast cancer, even in the absence of an intact immune response or high target density. Finally, TCRms can be used to target specific cell types, such as tumor neovasculature, and receptor-mediated endocytosis of the TCRm by brain endothelium suggests that TCRms could be used to deliver drugs to the blood-brain barrier. In summary, the integration of significant advances in marker discovery, validation and targeting presents the opportunity for a powerful, streamlined approach to immunotherapy in cancer and infectious diseases.

Acknowledgments

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Literature Cited

1. Wittman VP, et al. Antibody targeting to a class I MHC-peptide epitope promotes tumor cell death. *Journal of Immunology*. 2006; 177(6):4187–95.
2. Weidanz JA, et al. Levels of specific peptide-HLA class I complex predicts tumor cell susceptibility to CTL killing. *Journal of Immunology*. 2006; 177(8):5088–97.
3. Weidanz JA, et al. Development and implementation of a direct detection, quantitation and validation system for class I MHC self-peptide epitopes. *Journal of Immunological Methods*. 2007; 318(1–2):47–58. [PubMed: 17134715]
4. Cohen CJ, et al. Direct detection and quantitation of a distinct T-cell epitope derived from tumor-specific epithelial cell-associated mucin using human recombinant antibodies endowed with the antigen-specific, major histocompatibility complex-restricted specificity of T cells. *Cancer Research*. 2002; 62(20):5835–44. [PubMed: 12384546]
5. Denkberg G, et al. Direct visualization of distinct T cell epitopes derived from a melanoma tumor-associated antigen by using human recombinant antibodies with MHC- restricted T cell receptor-like specificity. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99(14):9421–6. [PubMed: 12093904]
6. Andersen PS, et al. A recombinant antibody with the antigen-specific, major histocompatibility complex-restricted specificity of T cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93(5):1820–4. [PubMed: 8700842]
7. Porgador A, et al. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity*. 1997; 6(6):715–26. [PubMed: 9208844]
8. Nishida M, et al. Characterization of novel murine anti-CD20 monoclonal antibodies and their comparison to 2B8 and c2B8 (rituximab). *Int J Oncol*. 2007; 31(1):29–40. [PubMed: 17549402]
9. Vega MI, et al. Rituximab-mediated cell signaling and chemo/immuno-sensitization of drug-resistant B-NHL is independent of its Fc functions. *Clin Cancer Res*. 2009; 15(21):6582–94. [PubMed: 19861448]
10. Carballido E, Fishman M. Sipuleucel-T: Prototype for Development of Anti-tumor Vaccines. *Curr Oncol Rep*.
11. Celis E, et al. Identification of potential CTL epitopes of tumor-associated antigen MAGE-1 for five common HLA-A alleles. *Mol Immunol*. 1994; 31(18):1423–30. [PubMed: 7823968]
12. Wolfel T, et al. Analysis of antigens recognized on human melanoma cells by A2-restricted cytolytic T lymphocytes (CTL). *Int J Cancer*. 1993; 55(2):237–44. [PubMed: 7690346]
13. Kawakami Y, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med*. 1994; 180(1):347–52. [PubMed: 7516411]
14. Dudley ME, et al. A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *J Immunother*. 2002; 25(3):243–51. [PubMed: 12000866]
15. Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. *Proc Natl Acad Sci U S A*. 2004; 101(Suppl 2):14639–45. [PubMed: 15381769]
16. Hughes MS, et al. Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Human Gene Therapy*. 2005; 16(4):457–72. [PubMed: 15871677]
17. Verma B, et al. TCR Mimic Monoclonal Antibodies Induce Apoptosis of Tumor Cells via Immune Effector-Independent Mechanisms. *Journal of Immunology*. 2011
18. Hawkins OE, et al. Identification of breast cancer peptide epitopes presented by HLA-A*0201. *Journal of Proteome Research*. 2008; 7(4):1445–57. [PubMed: 18345606]
19. Verma B, et al. Direct discovery and validation of a peptide/MHC epitope expressed in primary human breast cancer cells using a TCRm monoclonal antibody with profound antitumor properties. *Cancer Immunology. Immunotherapy : CII*. 2010; 59(4):563–73.

20. Verma B, et al. TCR mimic monoclonal antibody targets a specific peptide/HLA class I complex and significantly impedes tumor growth in vivo using breast cancer models. *Journal of Immunology*. 2010; 184(4):2156–65.
21. Kim S, et al. Single-chain HLA-A2 MHC trimers that incorporate an immunodominant peptide elicit protective T cell immunity against lethal West Nile virus infection. *Journal of Immunology*. 2010; 184(8):4423–30.
22. Hawkins O, et al. An HLA-Presented Fragment of Macrophage Migration Inhibitory Factor Is a Therapeutic Target for Invasive Breast Cancer. *Journal of Immunology*. 2011; 186(11):6607–16.
23. Hunt DF, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science*. 1992; 255(5049):1261–3. [PubMed: 1546328]
24. Bhattacharya R, et al. A novel vascular targeting strategy for brain-derived endothelial cells using a TCR mimic antibody. *Journal of Cellular Physiology*. 2010; 225(3):664–72. [PubMed: 20506235]

Figure 1. View of the Overall Strategy for Identifying Peptide Epitopes Uniquely Presented by the HLA A*0201 of Breast Cancer Cells

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Figure 2. RL21A reactivity to primary invasive ductal carcinoma

Cryopreserved, HLA-A*02+ IDC, DCIS, benign fibroadenoma, and matched normal adjacent tissues were stained for (A) all HLA-A2 complexes using the BB7.2 antibody and (B) the specific HLA-A2/MIF₁₉₋₂₇ complex using RL21A. Tissues received a composite score of 0–12 based on both proportion and intensity of staining. Representative staining of (C) normal adjacent tissue and (D) IDC tissue by RL21A are shown, with higher magnified inset. Expression of the MIF1a intact protein is shown using an antigen-specific mAb in panel F and corresponding control antibody in panel E. Specificity of RL21A staining was confirmed by competition with tetrameric HLA-A2/peptide complexes. Tissue staining was abrogated in the presence of HLA-A2 tetramer containing the MIF₁₉₋₂₇ peptide (G), but not HLA-A2 tetramer containing irrelevant peptides (not shown).

Figure 3. RL21A inhibits tumor growth in orthotopic murine models. Upper Panel

Athymic nude mice were implanted with MDA-MB-231 tumor cells in the right mammary fat pad in the presence of Matrigel. Once-weekly injections of 500 ug of RL21A (n=10) or control mAb (n=10) were initiated after tumor volumes exceeded 50mm³. Tumor sizes were measured twice weekly using calipers and tumor volumes determined using the standard formula: volume=L×(b²)/2 (L=longest diameter; b=shortest diameter, where the mean tumor diameter was measured in two dimensions). Data are plotted as mean tumor volume + SEM. **Lower Panel.** Once-weekly injections with 500 ug of RL21A (n=10) or control mAb (n=10) were initiated 48 hours after BT20/A2 tumor cell injection. Tumor sizes were measured twice weekly using calipers, and tumor volumes were determined as described above. * $P<0.05$; *** $P<0.001$.

Figure 4. Putative mechanisms of TCRm mediated tumor cell death

TCRm binding to specific peptide/HLA complexes on tumor cell surface leads to destruction of tumor cell death via secondary effector mechanisms (ADCC and CDC) and also independently by activation of intrinsic caspase pathways and JNK phosphorylation.

Key Properties of RL21A TCRm

To assess staining, a total score of 0–12+ was calculated as a target expression score (0–2+ = positive staining in ~25% of cells per view field; 3–4+ = ~50% of cells; 5–6+ = >75% of cells) added to an intensity score. (scale of 0–6+, determined by staining intensity and copy number quantification using the Quantibrite bead system).

Table 1

	RL21A	RL6A
Molecular Target	HLA-A*02 - Human p68 YLLPAIVHI	HLA-A*02- Human MIF1a FLSELTQQL
Affinity(K_D)	52nM	0.42nM
Normal Tissue Staining	Male Donor 0/22 Female Donor 0/19	Male Donor 0/19 Female Donor 0/16
Adjacent Tissue Staining	5/15 <1+ out of 4+	6/15 <1+ out of 4+
Invasive Grade Breast Tumor Staining	29/29 x=3+ out of 4+	30/30 x=3.5+ out of 4+
Staining of Low HER2 tissues (Hercept test 0–2+, n=13)	13/13 x=3+ out of 4+	13/13 x=3+ out of 4+
Primary Stroma	(+) Stained	(+) Stained
Tumor Vascular	(+) Stained	(+) Stained
Metastac Ovarian Tumors	6/6 x=3+ out of 4+	6/6 x=3+ out of 4+
In vivo Therapeutic Efficacy	1 MDA-MB-231	3 MDA-MB-231
	2 BT20/A2	4 MCF-7
Secondary Effector Funcon (ADCC/CDC)	1 MDA-MB-231	3 MDA-MB-231
	2 MCF-7	4 MCF-7
Direct Inducon of Apoptosis	1 Annexin V	5 Annexin V
	2 Caspase 3	6 Caspase 3
	3 PARP	7 PARP
	4 JNK Phos.	8 JNK Phos.