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Enhancing Cancer Immunotherapy by Intracellular Delivery of Cell-Penetrating Peptides and Stimulation of Pattern-Recognition Receptor Signaling

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Abstract

The importance of T-cell-mediated antitumor immunity has been demonstrated in both animal models and human cancer immunotherapy. In the past 30 years, T-cell-based immunotherapy has been improved with an objective clinical response rate of up to 72%. Identification of MHC class I- and II-restricted tumor antigens recognized by tumor-reactive T cells has generated a resurgence of interest in cancer vaccines. Although clinical trials with cancer peptide/protein vaccines have only met a limited success, several phase II/III clinical trials are either completed or ongoing with encouraging results. Recent advances in immunotherapy have led to the approval of two anticancer drugs (sipuleucel-T vaccine and anti-CTLA-4 antibody) by the US FDA for the treatment of meta-static castration-resistant prostate cancer and melanoma, respectively. Intracellular delivery of antigenic peptides into dendritic cells (DCs) prolongs antigen presentation of antigen-presenting cells to T cells, thus further improving clinical efficacy of peptide/protein cancer vaccines. Because innate immune responses are critically important to provide sensing and initiating of adaptive immunity, combined use of cell-penetrating peptide vaccines with stimulation of innate immune signaling may produce potent anti-tumor immune responses. We will discuss the recent progress and novel strategies in cancer immunotherapy.

1. INTRODUCTION

The host immune system consisting of innate and adaptive immunity plays an essential role in immunosurveillance, recognition, and destruction of cancer cells (Schreiber *et al.*, 2011; Vesely *et al.*, 2011). T cell-mediated antitumor immunity has been demonstrated in murine tumor models 30 years ago by adoptive transfer experiments (Greenberg, 1991; Rosenberg, 1990; Rosenberg *et al.*, 2008). Recently, adoptive T-cell therapy has been successfully used to treat many human cancers such as melanoma, renal cell carcinoma, and lymphoma with varying degrees of tumor regression (Lesterhuis *et al.*, 2011; Rosenberg, 2011; Rosenberg *et al.*, 2008; Tey *et al.*, 2006). Although CD4⁺ and CD8⁺ T cells are the major components of T-cell-mediated antitumor immunity, natural killer (NK), NK1.1 T (NKT), and $\gamma\delta$ T cells may also play a role in immunosurveillance against cancer (Diefenbach and Raulet, 2001; Vesely *et al.*, 2011; Wang, 2001). Using tumor-reactive T cells, many immunogenic tumor antigens have been identified (Boon *et al.*, 1994; Wang and Rosenberg, 1999). Clinical studies using molecularly defined MHC class I-restricted tumor antigens show that peptide vaccines can induce antigen-specific immune responses in the patients who received peptide vaccines, but overall immune responses elicited by the use of CD8⁺ T-cell peptides are weak in several early clinical studies. However, several tumor antigens, including gp100 and

MAGE-3, have been used in phase II and III clinical trials with encouraging results. Recently, antigen-presenting cells (APCs) loaded with tumor antigens have been approved by US Federal Food Administration (FDA) for the treatment of prostate cancer. This is an important milestone in the field of cancer immunology. However, blood APC-based vaccines require autologous cells from the patients and are expensive (\$93,000 for a course of three treatments) with an average extension of survival of 4.1 months. To further improve broad application and clinical efficacy of cancer vaccines, we need to develop peptide/protein-based vaccines as a more broadly applicable and less expensive anticancer drugs. Further, recent studies have identified several checkpoints or roadblocks for T-cell activation and function (Callahan *et al.*, 2010; Chambers *et al.*, 2001; Zhu *et al.*, 2011). These include coinhibitory molecules and other negative regulators in T cells and dendritic cells (DCs), as well as immune suppression mediated by regulatory T cells (Zhu *et al.*, 2011). To enhance antitumor immunity, it is necessary to remove these roadblocks so that T cells can be fully activated and to mediate the eradication of cancer cells through modulating innate immune signaling. Importantly, anti-CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) antibody therapy has a proven therapeutic effect on metastatic melanoma and has recently been approved by US FDA for the treatment of metastatic melanoma (Hodi *et al.*, 2010; Royal *et al.*, 2010). With encouraging results of clinical trials and FDA approval of two anticancer immunotherapy drugs, development of synthetic peptide/protein-based cancer vaccines will be the next frontline in the field of cancer immunotherapy and vaccines. In this chapter, we discuss a novel strategy for enhancing antitumor immune responses by intracellular delivery of peptides into DCs and stimulation of innate immune signaling to overcome immune suppression.

2. ADOPTIVE CELL THERAPY: HISTORY AND RECENT PROGRESSES

2.1. LAK and cytokine-induced killer cell therapy

In the early 1980s, lymphocyte-activated killer (LAK) cells were first used in mouse models and human cancer patients (Grimm *et al.*, 1982; Muul *et al.*, 1987). LAK cells are generated from the peripheral blood mononuclear cells (PBMCs) following *ex vivo* expansion in the presence of interleukin (IL)-2 and are capable of killing fresh tumor cells without requirement of MHC restriction. Clinical studies show modest efficacy against melanoma and renal carcinoma cancer (Rosenberg *et al.*, 1987; Takayama *et al.*, 2000). In the early 1990s, cytokine-induced killer (CIK) cells are generated from PBMCs in the presence of interferon (IFN)- γ on day 0, followed by adding anti-CD3 and IL-2 on day 1, with continued addition of IL-2 during 21–28 days of culture (Schmidt-Wolf *et al.*, 1991). These CD3⁺ CD56⁺ CIK cells are converted from CD3⁺ CD56⁻ cells and expanded up to 1000-fold after 21–28 days with mixed cell populations (2% NK cells, and >90% CD3 and CD56⁺ T cells) and show strong cytotoxicity against many tumor cell targets without MHC restriction (Linn *et al.*, 2002). Thus, these cells may function similar to NK-like T cells. It is not clear how they recognize tumor targets and what antigens they recognize on tumor cells. Although CIK cells have been tested in a clinical setting for a variety of cancers, data on the efficacy of CIK therapy are limited. The first report of the international registry on CIK treatment of 426 patients shows that a large-scale expansion of CIK cells *ex vivo* is possible and cell infusion is safe (Hontscha *et al.*, 2011). The clinical response rate of CIK is 51/384 (13%) with complete and partial responses, 40/384 (10%) with minor response, and 161/384 (42%) with a stable disease (Hontscha *et al.*, 2011). Several groups report that combination of CIK or tumor-infiltrating lymphocyte (TIL) with chemotherapy produces better clinical response rates and reduces the percentage of relapse compared with chemotherapy alone (Dudley *et al.*, 2002; Jiang *et al.*, 2005). With encouraging clinical evidence of CIK therapy, it is important to conduct randomized clinical trials to determine the clinical benefit of cancer patients who receive CIK alone, chemotherapy alone, and CIK plus chemotherapy in the near future.

2.2. Adoptive T-cell therapy with TILs

Adoptive cell therapy (ACT) with TILs has emerged as the most effective treatment for patients with metastatic melanoma. TIL-based therapy was first reported in 1988, and clinical studies with TIL plus IL-2 showed 34% objective response rate compared with 16% clinical response with IL-2 alone (Rosenberg, 2000; Rosenberg *et al.*, 1988, 1994). Importantly, ACT with TILs has been dramatically improved for clinical efficacy by introducing treatment with a nonmyeloablative preparative immunodepleting regimen consisting of cyclophosphamide and fludarabine before T-cell infusion (Dudley *et al.*, 2002). Objective clinical response rates have been observed in 49–72% of patients with metastatic melanoma refractory to all other treatments (Rosenberg, 2011; Rosenberg *et al.*, 2008). This regimen depletes circulating lymphocytes as well as those at tumor sites for a week before host hematopoietic cell recovery, thus likely removing suppressive Treg cells and allowing antitumor T cells to survive and expand in patients after adoptive transfer. The unique features of ACT are that clinical responses can be durable (>5 years) and low percentages of relapse (Rosenberg *et al.*, 2011). More recently, whole body irradiation was added to the cyclophosphamide and fludarabine lymphodepleting preparative regimen before adoptive T-cell transfer. These further modifications result in the highest objective clinical responses in all patients receiving treatment (Wrzesinski *et al.*, 2010). However, because the difficulties associated with generation and expansion of TILs derived from tumor tissues and variable availability of such tumor tissues in cancer patients, ACT with autologous TILs is not suitable for many cancer patients, in particular, those with cancer other than melanoma. The success rate for generating TILs from other types of cancer is much lower than that seen in melanoma. Thus, use of T cells transduced with retro-viruses or lentiviruses encoding T-cell receptors (TCRs) that recognized tumor antigens or with genes encoding cytokines or costimulatory molecules has made ACT available for patients with different types of cancer. Alternatively, these peripheral T cells will be stimulated with antigenic peptides to generate antigen-specific T cells. This approach has been successfully used to treat lymphoma and melanoma with antigen-specific T cells (Hunder *et al.*, 2008; Leen *et al.*, 2006; Wolf *et al.*, 2003; Yee *et al.*, 2000, 2002). Despite impressive clinical response rates of ACT in cancer therapy in phase I/II clinical studies, so far no ACT treatment has been approved as an anticancer drug. This may be due to difficulties in patentability of many immunotherapeutic strategies or technologies, individualized treatment associated with high costs, and lack of funding and interests by the pharmaceutical companies. However, with recent successes and promising clinical results, ACT or other immune cell transfer therapies are likely to move into phase III trials and obtain FDA approval as anticancer drugs.

3. APC-BASED VACCINES: THE FIRST FDA APPROVED DRUG FOR PROSTATE CANCER

Professional APCs such as DCs can potently induce antigen-specific T cells. DC loaded with cancer antigens or tumor lysates has been used to vaccinate cancer patients with limited success. However, the US FDA recently approved the first therapeutic cancer vaccine drug (Provenge, Dendreon) for the treatment of metastatic castration-resistant prostate cancer. The sipuleucel-T vaccines consist of blood APCs, including monocytes and DCs, loaded with a fusion protein (PA2024) of the prostate antigen prostatic acid phosphatase linked to an immunostimulatory granulocyte-macrophage colony-stimulating factor. Although tumor regressions, radiographic, and prostate-specific antigen responses occurred very rarely, an overall survival benefit of 4.1 months was observed for the sipuleucel-T group versus the placebo group. This constitutes an important milestone in the field of cancer immunotherapy. However, because of the individual patient-specific nature and the high cost of this treatment (\$93,000 for three infusions), it is very challenging to implement this treatment in the large eligible patient population. Direct *in vivo* targeting and activation of

DC with broadly injectable immunogens for cancer treatment are desirable. Moreover, the clinical efficacy of cancer vaccines remains to be improved. To achieve this purpose, the most appropriate tumor antigens and/or immune targets for immunotherapy of distinct cancer types need to be identified.

4. TUMOR ANTIGENS RECOGNIZED BY TUMOR-REACTIVE T CELLS

4.1. Tumor antigens recognized by CD8⁺ T cells

In 1991, the first human tumor antigen was identified by screening cDNA expression library with tumor-reactive CD8⁺ T cells (Van der Bruggen *et al.*, 1991). Since then, many tumor antigens have been identified using T cells with antitumor activity (Table 6.1). These tumor antigens can be classified into several types: (1) tissue-specific tumor antigens with higher expression in cancer cells compared with normal cells; (2) tumor-specific and shared antigens that are expressed in cancer and testis, but not in other normal tissues; (3) tumor-specific and unique antigens. Some of them, including gp100, MAGE-3, and NY-ESO-1, have been testing in phase II/III clinical trials with encouraging results. It should be noted that chronic infection or chronic inflammation has caused about 20% of human cancers (Coussens and Werb, 2002; De Marzo *et al.*, 2007). Epstein–Barr virus (EBV), a human gamma herpesvirus with tropism for B cells, has been implicated in the pathogenesis of a variety of human tumors, including immunoblastic lymphoma, Burkitt lymphoma, nasopharyngeal carcinoma, and Hodgkin disease (Leen *et al.*, 2007; Young and Rickinson, 2004). Infection with oncogenic human papilloma virus (HPV) is causally linked to the development of cervical cancer (Melief and van der Burg, 2008). Hepatitis B virus (HBV) and HCV infection of liver cells is a primary factor in the development of liver cancer (Coussens and Werb, 2002; Karin *et al.*, 2006; Peto, 2001). Viral antigens derived from EBV, HPV, and HBV are not the subject of this review but have been extensively used to develop prophylactic and therapeutic vaccines against viral infection and cancer (Huang *et al.*, 2011; Kwak *et al.*, 2011; Long *et al.*, 2011; van der Burg and Melief, 2011).

4.2. Tumor antigens recognized by CD4⁺ Th and Treg cells

Given the importance of CD4⁺ T cells in antitumor immunity, it is critical to identify MHC class II-restricted tumor antigens capable of stimulating CD4⁺ Th cells. There are several strategies to identify MHC class II antigens recognized by CD4⁺ T cells: (1) genetic targeting expression (GTE) system using tumor-reactive human T cells isolated from patients with cancer or other immune-related diseases, (2) use of HLA-DR transgenic (Tg) mice for identification of MHC class II-restricted antigens *in vivo*, (3) peptide stimulation *in vitro* using candidate antigens over-expressed in cancer cells. Many tumor antigens capable of stimulating CD4⁺ T cells have been identified (Table 6.2).

4.2.1. GTE system—We have developed GTE system for identification of antigens recognized by CD4⁺ T cells (Wang and Rosenberg, 1999; Wang *et al.*, 1999a, b). The GTE system comprises two essential components: (A) generation of a highly transfectable HEK293IMDR cell line and (B) the creation of an Ii fusion library from tumor cells such that the Ii fusion proteins are targeted to the endosomal/lysosomal compartment for efficient antigen processing and presentation for T-cell recognition (Wang and Rosenberg, 1999; Wang *et al.*, 1999b). Using this system, many tumor antigens have successfully been identified. For example, we identified a mutated fibronectin as a tumor antigen recognized by HLA-DR2-restricted CD4⁺ T cells. A mutation in this gene results in the substitution of lysine for glutamic acid and gives rise to a new T-cell epitope recognized by CD4⁺ T cells (Wang *et al.*, 2002b). Analysis of cytokine profiles and suppressive activity of these T cells reveal that they are CD4⁺ Th1 cells, secreting IFN- γ and IL-2, but no suppressive function. Because elevated percentage of CD4⁺ Treg cells are present in tumor tissues, we have

recently generated many such tumor/antigen-specific CD4⁺ Treg cell clones from TILs in surgically removed tumor samples. Using the same strategy for Th1 cells, we identified LAGE1 and ARTC1 as antigenic ligands for CD4⁺ Treg cells, providing direct evidence that antigen-specific CD4⁺ Treg cells are present at tumor sites and mediate antigen-specific and local immune suppression of antitumor immunity (Wang *et al.*, 2004, 2005).

4.2.2. Use of HLA-DR Tg mice and *in vitro* peptide stimulation—MHC class II-restricted epitopes can be identified by using HLA-DR Tg mice in combination peptide stimulation *in vitro*. HLA-DR4 Tg mice were used to identify CD4⁺ T-cell epitopes from candidate antigens (Touloukian *et al.*, 2000; Zeng *et al.*, 2000). HLA-DR Tg mice might have advantages for identifying putative peptides, as they should have a high precursor frequency of antigen-specific T cells after immunization. Once candidate peptides are known, one can generate antigen-specific CD4⁺ T cells from human PBMCs stimulated with synthetic candidate peptides. Therefore, the combined use of immunization of DR Tg mice with the intact protein antigens and stimulated with the peptides predicted by a computer-assisted algorithm may avoid the need to stimulate human PBMCs with a large number of peptides. NY-ESO-1 is a potent immunogen recognized by both antibody and T cells (Chen *et al.*, 1997; Jager *et al.*, 1998; Wang *et al.*, 1998b). Of particular interest is that 10–13% of patients with advanced cancer developed a high titer of antibody (Stockert *et al.*, 1998; Zeng *et al.*, 2000). We identified a T-cell epitope presented by HLA-DP4, a predominant allele expressed in 40–70% of the population (Zeng *et al.*, 2001). Identification of DP4-restricted T-cell peptides from MAGE-3 and NY-ESO-1 could be of great benefit for more than 50% of patients with cancer. These studies suggest that, unlike LAGE-1, NY-ESO-1 may preferentially activate CD4⁺ Th cells. However, a recent study shows that NY-ESO-1 can also induce Treg cells (Vence *et al.*, 2007). Interestingly, several MHC class II peptides recognized by CD4⁺ T cells have been identified from EBNA1 as well as other viral antigens (Bickham *et al.*, 2001; Leen *et al.*, 2001; Munz *et al.*, 2000; Paludan and Munz, 2003; Voo *et al.*, 2002). T-cell peptides derived from EBV viral antigens are capable of stimulating CD4⁺ Th1 and Treg cells (Marshall *et al.*, 2003; Voo *et al.*, 2005). It appears that the same T-cell epitope from EBNA1 can stimulate both Th1 and Treg cells (Voo *et al.*, 2005). Thus, it is likely that both tumor and viral antigens can activate effector and Treg cells, depending on particular epitope affinity and cytokine milieu. Although IL-17-producing T (Th17) cells are present in many human cancer tissues, very little is known about their antigen specificity. In particular, the role of Th17 cells in cancer immunity and tumor progression is not clear and requires further study.

5. CURRENT PROGRESSES OF SYNTHETIC PEPTIDE VACCINES

Identification of these MHC class I-restricted antigens has set the stage for developing peptide-based cancer vaccines, although some evidence for a therapeutic effect on tumor growth inhibition and regression was observed in patients who received peptide vaccines (Marchand *et al.*, 1999; Rosenberg *et al.*, 1998). However, objective complete clinical responses were sporadic, even though CTL reactivity was clearly evident after one round of stimulation *in vitro* of PBMC from the majority of vaccinated patients (Rosenberg *et al.*, 2004). Analysis of the infiltrating lymphocytes in skin and tumor biopsies using T-cell-specific peptide–major histocompatibility complex tetramers showed generation of antigen-specific CD8⁺ T cells (Yee *et al.*, 2000). Recently, a multicenter phase II clinical trial of melanoma patients with gp100 peptide with or without IL-2 shows that the gp100 peptide vaccine plus IL-2-treated group had a significant improvement in overall clinical response compared with the IL-2-only group (16% vs. 6%) as well as longer progression-free survival (Schwartzentruber *et al.*, 2011). The median overall survival was also improved in the gp100 peptide vaccine plus IL-2 group compared to the IL-2-only group (17.8 vs. 11.1 months). Similarly, MAGE-3 peptide/protein vaccines are ongoing in phase III clinical trials (Bilusic

and Madan, 2011; Cecco *et al.*, 2011). The clinical efficacy of peptide vaccines may be further improved by the use of DCs loaded with cancer peptides or by the use of synthetic long peptides harboring both CD4 and CD8 T-cell epitopes (Melief and van der Burg, 2008; Chapters 3 and 4). A major challenge in cancer vaccine development is how to generate strong and long-lasting antitumor immunity through optimal delivery of well-chosen tumor-associated antigens.

6. ENHANCING ANTITUMOR IMMUNITY BY INTRACELLULAR DELIVERY OF PEPTIDES INTO DCs

Although mature DCs are more potent than immature DCs in priming and eliciting T-cell responses (Apetoh *et al.*, 2011; Delamarre and Mellman, 2011; Tacke and Figdor, 2011), they lose the ability to efficiently take up exogenous antigens, particularly for MHC class II-restricted antigens (Banchereau and Steinman, 1998). As a result, peptide-pulsed DCs as vaccines have several limitations. For example, peptide degradation, rapid MHC class I turnover, and the disassociation of peptide from MHC class I molecules during the preparation and injection of DC/peptides may result in short half-lives of MHC class I/peptide complexes on the DC surface, leading to weak T-cell responses. We developed a novel strategy to overcome these problems by the use of cell-penetrating peptides (CPPs) for intracellular delivery of cancer peptides into DCs (Wang and Wang, 2002; Wang *et al.*, 2002a). Intracellular delivery of peptides into DCs could allow DCs to continuously process and present the internalized peptides to T cells for an extended period of time. Several potent CPPs have been identified from proteins, including the Tat protein of human immunodeficiency virus, the VP22 protein of herpes simplex virus, and fibroblast growth factor (Berry, 2008; Deshayes *et al.*, 2005; Edenhofer, 2008; Gupta *et al.*, 2005; Torchilin, 2006), although some long synthetic peptides can also penetrate selectively into DCs (Melief and van der Burg, 2008). Among them, the 11-mer TAT peptide (YGRKKRRQRRR) and other CPPs have been well studied for the transduction of biologically active proteins into cells both *in vitro* and *in vivo* (Gupta *et al.*, 2005). We found that both CPP1-TRP2 or TAT-TRP2 peptides can translocate intracellularly into mature DCs and prolong DCs to process the internalized peptides and to present MHC-peptide complexes to antigen-specific T cells (Wang and Wang, 2002; Wang *et al.*, 2002a). Immunization of mice with DCs transduced with CPP1-TRP-2 or TAT-TRP2 resulted in complete protection against B16 tumor as well as significant inhibition of the preestablished B16 tumor (Wang and Wang, 2002; Wang *et al.*, 2002a). Although both DC/TRP2 and DC/TAT-TRP2 immunization increased the number of TRP2-specific CD8⁺ T cells detected by K^b/TRP2 tetramers, T-cell activity elicited by DC/TAT-TRP2 was 3- to 10-fold higher than that induced by DC/TRP2 (Wang *et al.*, 2002a). Consistent with previous studies showing that CD4⁺ T cells are required for an antitumor effect, our studies show a similar requirement for DC/TAT-TRP-2-induced antitumor immunity, suggesting that both CPP1- and TAT-mediated antigen delivery of a self-peptide may have general applications for enhancing T-cell-based cancer therapy, and CD4⁺ T-cell response is required for generating optimal antitumor immunity. More recently, we initiated a phase I clinical trial for prostate cancer using TAT-NY-ESO-1 peptides. Clinical studies show that TAT-NY-ESO-1 peptide vaccines are safe and induced antigen-specific T-cell responses (R.-F.W. Guru Sonpavde and Teresa G. Hayes, unpublished data).

7. ENHANCING IMMUNE RESPONSES AND BLOCKING IMMUNE SUPPRESSION BY STIMULATION OF INNATE IMMUNE RECEPTORS

7.1. Innate immune receptors and signaling

Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and AIM-2-like receptors have emerged as innate pattern recognition receptors (PRRs) that can

detect a variety of invading pathogens and intracellular ligands, thus serving as a first line of defense against infectious pathogens and cancer. These germline-encoded PRRs are expressed in DCs and other immune cells, and can recognize structure-conserved molecules, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Recognition of PAMPs or DAMPs by PRRs triggers the activation of several key signaling pathways, including NF- κ B, type I IFN, and inflammasome, leading to the production of inflammatory cytokines, which, in turn, promote DC maturation programs for the induction of adaptive immune responses (Iwasaki and Medzhitov, 2004; Takeda and Akira, 2005). TLRs are expressed on the cell surface (TLR1, TLR2, TLR4, and TLR5) or in the endosome (TLR3, TLR7, TLR8, and TLR9). By contrast, several intracellular PRRs such as RIG-I and MDA5 are in the cytoplasm and responsible for the recognition of invading viruses (Akira *et al.*, 2006; Kato *et al.*, 2005; Yoneyama *et al.*, 2004). NLRs represent a large family of protein receptors/regulators harboring an initiating signal domain, such as the caspase recruitment domain (CARD), pyrin domain (PYRIN) or baculovirus inhibitor-of-apoptosis repeat (BIR) domain, a nucleotide-binding oligomerization domain (NOD), and an LRR domain. Activation of such cytoplasmic receptors by invading pathogens including bacteria and viruses activates inflammasome consisting of caspase-1 and ASC and leads to the production of proinflammatory cytokines such as IL-1b and IL-18. Thus, TLRs, NLRs, and RLRs are critical in bridging innate and adaptive immune responses by activating several key signaling pathways and in producing many important cytokines as mediators. Thus, they represent a potent means of modulating immune responses in cancer immunotherapy. Both natural and synthetic ligands for TLRs and RLRs have been identified and characterized for their recognition, but only a limited number of ligands have been identified for NLRs. These ligands of PRRs, in particular TLRs, have been used as potent vaccine adjuvants to enhance immune responses. The most significant development of cancer vaccine studies is to include various TLR agonists to vaccine formulations, including TLR-3 (poly I:C), TLR-4 (monophosphoryl lipid A; MPL), TLR-5 (flagellin), TLR-7 (imiqui-mod), and TLR-9 (CpG) (Duthie *et al.*, 2011). The types of signaling and cytokines by immune cells after TLR stimulation control CD4⁺ T-cell differentiation into Th1, Th2, Th17, and Treg cells. However, stimulation of immune cells such as DCs and T cells by most TLR-based adjuvants produces proinflammatory cytokines and promotes Th1 and CD8⁺ T responses (Manicassamy and Pulendran, 2009).

7.2. Blocking negative regulators of innate immune signaling

Although innate immune responses are critically important as sensors to induce adaptive immunity, tight regulation of innate signaling pathways is essential for both innate and adaptive immunities; otherwise, aberrant immune responses may occur, leading to severe or even fatal consequences. Similarly, DC maturation and activation are controlled by both positive and negative regulators, thus leading to immunity or tolerance induction. To generate potent antitumor immunity, we need to block negative regulators in immune cells such as DCs and T cells. For example, A20-silenced DCs produce potent antitumor immunity (Song *et al.*, 2008). We recently identified several negative regulators (NLRC5 and NLRX1) that inhibit NF- κ B and type I IFN signaling (Cui *et al.*, 2010; Xia *et al.*, 2011). Similarly, several key negative regulators such as CTLA-4 and programmed cell death 1 (PD-1) play a critical role in the inhibition of T-cell activation. Blockade of CTLA-4 and/or PD-1 results in T-cell activation and enhances nonspecific immune responses (Chambers *et al.*, 2001; Zhu *et al.*, 2011). These antibody-based immunotherapies are currently under active clinical trials (Callahan *et al.*, 2010), and anti-CTLA-4 antibody therapy has been approved by US FDA for the treatment of metastatic melanoma (Hodi *et al.*, 2010).

7.3. Overcoming Treg cell-mediated immune suppression by TLR signaling

As Treg cells have accumulated in the tumor microenvironment, it is necessary to develop novel strategies to overcome this type of immune suppression; otherwise, the immune response induced by cancer vaccines will be weak and transient. To block immune suppression mediated by Treg cells, we found that TLR8 ligands (ssRNA40 and Poly-G10 oligonucleotides) can directly reverse the suppressive function of human (but not murine) Treg cells in the absence of DCs. Using RNA interference technology, we identified the TLR8–MyD88 signaling pathway that is required for the reversal of Treg cell function by Poly-G oligonucleotides (Peng *et al.*, 2005). More importantly, we demonstrated that the suppressive function of CD8⁺ Treg cells and $\gamma\delta$ -TCR Treg cells can also be reversed after Poly-G oligonucleotide treatment (Kiniwa *et al.*, 2007; Peng *et al.*, 2007), suggesting that these cells share a common TLR8 signaling-mediated mechanism. Recent studies show that TLR2 ligands can reverse the suppressive function of human Treg cells (Nyirenda *et al.*, 2011; Oberg *et al.*, 2010). However, murine TLR2-deficient mice reduce the number of CD4⁺ CD25⁺ Treg cells (Netea *et al.*, 2004). Activation of TLR2 with its ligand (Pam3Cys) directly increases the proliferation of murine Treg cells and transiently reverses their suppressive function (Liu *et al.*, 2006; Suttmüller *et al.*, 2006). However, engagement of TLR2 with polysaccharide A of *Bacteroides fragilis* enhances the suppressive function of Treg cells (Round *et al.*, 2011). These studies demonstrated that TLR signaling is critically important in modulating immune responses.

8. CONCLUSIONS

In the past 30 years, significant progress has been made in the field of cancer immunotherapy. While clinical validation and development has been slow, recent approval of two immunotherapy drugs (sipuleucel-T and anti-CTLA-4 antibody) has boosted the development of immunotherapy as important and promising treatment of patients with cancer. As ACT is the most powerful treatment of cancer with up to 72% objective clinical response rate, it is important to move forward with phase III clinical trials with tumor-specific T cells and CIK effector cells. Like APC-based therapy, the limitation of ACT is individualized medicine, and it is very challenging to broaden its application with retroviruses or lentiviruses expressing antigen-specific TCR. For these reasons, development of peptide/protein cancer vaccines has great potential. With encouraging results from recent multicenter clinical trials with gp100 and MAGE-A3, many therapeutic companies will step in the field and develop therapeutic peptide cancer vaccines for many types of cancer. The key issues will be how to further improve immune responses and clinical efficacy of peptide cancer vaccines. To achieve this goal, (1) we need to develop *in vivo* targeted delivery of peptides into APCs such as DCs with CPPs-linked cancer peptides (long peptide)/proteins, or nanoparticles; (2) we need to overcome immune suppression mediated by Treg cells and other immune cells; (3) importantly, peptide vaccines should be combined with strategies that block negative regulators or immune suppression to achieve maximal antitumor immunity and clinical responses. However, because immune responses measured in the blood do not necessarily correlate with clinical efficacy or survival, it is important to probe the reasons for these clinical observations. Because pretreatment of patients with chemodepleting regimens improves the clinical benefit of ACT, it is reasonable to believe that combined use of immunotherapy with chemotherapy can enhance immune responses and clinical outcomes. Recent advances and rapid progress in the field of cancer immunotherapy represent an unprecedented opportunity for the development of therapeutic cancer vaccines in the next few years.

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TABLE 6.1

Tumor antigens recognized by CD8⁺ T cells

Antigens	MHC class I restrictions	Peptide epitopes	References
<i>Tissue-specific antigens</i>			
gp100	A2	KTWGQYWQV	Bakker <i>et al.</i> (1994)
	A2	AMLGHTTMEV	Tsai <i>et al.</i> (1997)
	A2	MLGHTTMEV	Tsai <i>et al.</i> (1997)
	A2	SLADTNSLAV	Tsai <i>et al.</i> (1997)
	A2	ITDQVPFSV	Kawakami <i>et al.</i> (1995)
	A2	LLDGTATLRL	Kawakami <i>et al.</i> (1994a)
	A2	YLEPGPVTA	Cox <i>et al.</i> (1994)
	A2	VLYRYGSFSV	Kawakami <i>et al.</i> (1995)
	A2	RLMKQDFS	Kawakami <i>et al.</i> (1998)
	A2	RLPRIFCSC	Kawakami <i>et al.</i> (1998)
	A3	LIYRRRLMK	Kawakami <i>et al.</i> (1998)
	A3	ALNFPQSQK	Kawashima <i>et al.</i> (1998)
	A3	SLIYRRRLMK	Kawashima <i>et al.</i> (1998)
	A3	ALLAVGATK	Skipper <i>et al.</i> (1996)
	A24	VYFFLPDHL	Robbins <i>et al.</i> (1997)
	A*6801	HTMEVTVYHR	Sensi <i>et al.</i> (2002)
	B*3501	VPLDCVLYRY	Benlalam <i>et al.</i> (2003)
	Cw8	SNDGPTLI	Castelli <i>et al.</i> (1999)
MART-1/Melan-A	A2	AAGIGILTV	Coulie <i>et al.</i> (1994), Kawakami <i>et al.</i> (1994b)
	A2	ILTVILGVL	Castelli <i>et al.</i> (1995)
	A2	EAAGIGILTV	Schneider <i>et al.</i> (1998)
	B45	AEEAAGIGIL	Schneider <i>et al.</i> (1998)
gp75/TRP-1	A31	MSLQRQFLR	Wang <i>et al.</i> (1996b)
TRP-2	A2	SVYDFVWL	Parkhurst <i>et al.</i> (1998)
	A2	TLDSQVMSL	Noppen <i>et al.</i> (2000)
	A31	LLGPGRPYR	Wang <i>et al.</i> (1996a)
	A33	LLGPGRPYR	Wang <i>et al.</i> (1998a)
	A68	EVISCKLIK	Lupetti <i>et al.</i> (1998)
Tyrosinase	Cw8	ANDPIFVVL	Castelli <i>et al.</i> (1999)
	A1	KCDICTDEY	Kittlesen <i>et al.</i> (1998)
	A1	SSDYVIPIGTY	Kawakami <i>et al.</i> (1998)
	A2	YMDGTMSQV	Wolfel <i>et al.</i> (1994)
	A2	MLLAVLYCL	Wolfel <i>et al.</i> (1994)
	A24	AFLPWHLRF	Kang <i>et al.</i> (1995)
	B44	SEIWRDIDF	Brichard <i>et al.</i> (1996)
	B*3501	TPRLPSSADVEF	Benlalam <i>et al.</i> (2003)
<i>Tumor-specific shared antigens</i>			
BAGE	Cw16	AARAVFLAL	Boel <i>et al.</i> (1995)

Antigens	MHC class I restrictions	Peptide epitopes	References
CAMEL	A2	MLMAQEALAFI	Aarnoudse <i>et al.</i> (1999)
MAGE-A1	A1	EADPTGHSY	Traversari <i>et al.</i> (1992)
	A3	SLFRAVITK	Chaux <i>et al.</i> (1999a)
	A24	NYKHCFPEI	Fujie <i>et al.</i> (1999)
	A28	EVYDGREHSA	Chaux <i>et al.</i> (1999a)
	B37	REPVTKAEMI	Tanzarella <i>et al.</i> (1999)
	B53	DPARYEFLW	Chaux <i>et al.</i> (1999a)
	Cw2	SAFPTTINF	Chaux <i>et al.</i> (1999a)
	Cw3	SAYGEPRKL	Chaux <i>et al.</i> (1999a)
	Cw16	SAYGEPRKL	Van der Bruggen <i>et al.</i> (1994b)
MAGE-A2	A2	KMVELVHFL	Visseren <i>et al.</i> (1997)
	A2	YLQLVFGIEV	Visseren <i>et al.</i> (1997)
	A24	EYLQLVFGI	Tahara <i>et al.</i> (1999)
	B37	REPVTKAEMI	Tanzarella <i>et al.</i> (1999)
MAGE-A3	A1	EADPIGHLY	Gaugler <i>et al.</i> (1994)
	A2	FLWGPRALV	Van der Bruggen <i>et al.</i> (1994a)
	A24	TFPDLESEF	Oiso <i>et al.</i> (1999)
	A24	IMPKAGLLI	Tanaka <i>et al.</i> (1997)
	B44	MEVDPIGHLY	Fleischhauer <i>et al.</i> (1995), Herman <i>et al.</i> (1996)
	B52	WQYFFPVIF	Russo <i>et al.</i> (2000)
	B37	REPVTKAEMI	Tanzarella <i>et al.</i> (1999)
	B*3501	EVDPIGHLY	Benlalam <i>et al.</i> (2003)
MAGE-A4	A2	GVYDGREHTV	Duffour <i>et al.</i> (1999)
MAGE-A6	A34	MVKISGGPR	Zorn and Hercend (1999)
	B37	REPVTKAEMI	Tanzarella <i>et al.</i> (1999)
	B*3501	EVDPIGHVY	Benlalam <i>et al.</i> (2003)
MAGE-A10	A2	GLYDGMEHL	Huang <i>et al.</i> (1999)
MAGE-A12	Cw7	VRIGHLYIL	Heidecker <i>et al.</i> (2000), Panelli <i>et al.</i> (2000)
NY-ESO-1	A2	SLLMWITQCFL	Jager <i>et al.</i> (1998)
	A2	SLLMWITQC	Jager <i>et al.</i> (1998)
	A2	QLSLLMWIT	Jager <i>et al.</i> (1998)
	A31	ASGPGGGAPR	Wang <i>et al.</i> (1998b)
	B*3501	MPFATPMEA	Benlalam <i>et al.</i> (2003)
SSX-2	A2	KASEKIFYV	Ayyoub <i>et al.</i> (2002)
<i>Tumor-specific unique antigens</i>			
β -Catenin	A24 S	YLDSGIHF	Robbins <i>et al.</i> (1996)
Caspase-8	B35	FPSDSWCYF	Mandruzzato <i>et al.</i> (1997)
CDK-4	A2	ACDPHSGHFV	Wolfel <i>et al.</i> (1995)

TABLE 6.2

MHC class II-restricted melanoma antigens recognized by CD4⁺ T cells

Tumor antigens	HLA restrictions	Peptides	References
<i>Mutated/fusion antigens</i>			
TPI	HLA-DR1	GELIGILNAAKVPAD	Wang <i>et al.</i> (1999a), Pieper <i>et al.</i> (1999)
LDFP	HLA-DR1	PVIWRRAPA	Wang <i>et al.</i> (1999b)
	HLA-DR1	WRRAPAPGA	Wang <i>et al.</i> (1999b)
CDC27	HLA-DR4	FSWAMDLDPKGA	Wang <i>et al.</i> (1999a)
	Fibronectin	HLA-DR2	
		PSVGQQMIFE K HGFRRTTPP	Wang <i>et al.</i> (2002b)
Neo-PAP	HLA-DR7	RVIKNSIRLTL	Topalian <i>et al.</i> (2002)
ARTC1	HLA-DR1	YSVYFNLPADTIYTN	Wang <i>et al.</i> (2005)
<i>Nonmutated antigens</i>			
Tyrosinase	HLA-DR4	QNILLSNAPLGPQFP	Topalian <i>et al.</i> (1996)
	HLA-DR4	SYLQDSDPDSFQD	Topalian <i>et al.</i> (1996)
	HLA-DR15	FLLHHAFVDSIFEQWLQRHRP	Kobayashi <i>et al.</i> (1998)
gp100	HLA-DR4	WNRQLYPEWTEA Q RRLD	Touloukian <i>et al.</i> (2000)
	HLA-DR7	GP T LIGANASFSIALN	Kobayashi <i>et al.</i> (2001a)
	HLA-DR7/DR53	TGRAM L G T HTMEVTYH	Lapointe <i>et al.</i> (2001), Kobayashi <i>et al.</i> (2001a)
	HLA-DR7	SLAV V STQLIMPGQE	Kobayashi <i>et al.</i> (2001a)
MART-1	HLA-DR4	RNGYRALMDKSLHVGTCALTRR	Zarour <i>et al.</i> (2000)
MAGE-A1	HLA-DR13	LLKYRAREPVT K AE	Chaux <i>et al.</i> (1999a)
MAGE-A2	HAL-DR1	LLKYRAREPVT K AE	Chaux <i>et al.</i> (1999a)
MAGE-3	HLA-DR11	TSYVKVLHHMV K ISG	Manici <i>et al.</i> (1999)
	HLA-DR13	AELVHFL L LKYRAR	Chaux <i>et al.</i> (1999b)
	HLA-DR13	FLL L KYRAREPVT K AE	Chaux <i>et al.</i> (1999b)
	HLA-DP4	TQH F VQENYLEY	Schultz <i>et al.</i> (2000)
	HLA-DR1, 4, 7, 11	FFPVIFSKASS S LQL	Kobayashi <i>et al.</i> (2001b)
	HLA-DR1, 4, 11	RKVAELVHFL L LKYR	Consogno <i>et al.</i> (2003)
MAGE-A6	HLA-DR13	LLKYRAREPVT K AE	Chaux <i>et al.</i> (1999a)
LAGE1	HLA-DR13	R L L Q LHITMP F SS	Wang <i>et al.</i> (2004)
CAMEL	HLA-DR11/12	PWKRS W SA	Slager <i>et al.</i> (2003)
NY-ESO-1	HLA-DR4	LPVPGV L L K EFTVSGN L TI	Zeng <i>et al.</i> (2000)
	HLA-DP4	W IT Q C F LPVFLAQPPSG Q RR	Zeng <i>et al.</i> (2001)
hTRT	HLA-DR7	RPGLLGASV L GLDDI	Schroers <i>et al.</i> (2002)
Eph	HLA-DR11	DVTFNIACK K CG	Chiari <i>et al.</i> (2000)

Note: Amino acid sequence in bold stands for mutated or core sequence for recognition.