

## Tumor Immunology: Multidisciplinary Science Driving Basic and Clinical Advances

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### Abstract

The fourth AACR Special Conference "Tumor Immunology: Basic and Clinical Advances" was held in Miami, FL in December 2012. The overall objective of this meeting was to discuss emerging concepts in cancer immunology and immunotherapy. The key findings that emerged from this meeting included: (i) multiple immune checkpoints should be inhibited to increase effective T-cell therapy, (ii) successful adoptive T-cell therapy will rely on obtaining the proper T-cell phenotype, (iii) chimeric antigen receptors have shown promise in treating some B-cell malignancies, and (iv) multiple pathways of inflammation within the tumor microenvironment are immunotherapy targets. *Cancer Immunol Res*; 1(1); 16–23. ©2013 AACR.

### Introduction

The overall objective of the fourth Special AACR Conference on Tumor Immunology organized by the Cancer Immunology Working Group was to discuss multidisciplinary approaches that overcome the obstacles to T-cell immunotherapy established by the tumor microenvironment (TME). With the advent of U.S. Food and Drug Administration (FDA)-approved immunotherapies for melanoma and prostate cancer and many more agents in clinical trials, the possibilities have expanded for mainstream use of immunotherapy alongside traditional cancer treatments. However, many of the data presented at the conference and reviewed in this article reiterate that until the barriers created by tumor-associated inflammation and immunosuppression, including cellular, structural, and molecular components, have been elucidated and targeted, we will not reach the true potential of immunotherapeutic cancer treatment. Combinatorial and multimodality therapy will be required to overcome the multiple mechanisms of tumor-induced immunosuppression and escape. Thus, the specific themes that emerged from the meeting were: (i) the contribution of the TME to T-cell tolerance, (ii) the role of checkpoint inhibitor blockade in tumor immunotherapy, and (iii) strategies for improving adoptive T-cell transfer therapies using chimeric/engineered T-cell receptors (TCR) and better defined T-cell phenotypes.

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### Interplay between the tumor microenvironment and T cells

Tumor immunotherapy has seen progress in recent years and celebrated successes with the FDA approval of Provenge and ipilimumab as immunotherapies for prostate cancer and melanoma, respectively. Although these therapies have focused on activating tumor-specific T cells, it has become increasingly clear in recent years that activating T cells in the periphery does not translate into clinical success, due, in large part, to the interactions of the immune system with the TME.

The interplay between the immune system and tumor is clearly shown by the concept of immunoediting, where the immune system, by targeting developing tumors, selects for those variants that can escape and become malignant (1). Yet the question remains, are edited tumors really non-immunogenic? The answer would seem to be "No" as simple therapies such as anti-CTLA-4 or anti-PD1 have shown dramatic reductions in tumor size in some clinical settings (2). One possible explanation for the somewhat surprising result is that in mouse models, tumors develop quickly, whereas in reality, tumors develop over the course of years, giving them the opportunity to develop both driver and passenger mutations that the immune system can recognize. Still not all tumors treated with single immunotherapeutic agents regress, showing that though important, activating T cells is not the only challenge to successful immunotherapy (3).

### Tregs

One of the major tolerogenic influences in the TME are regulatory T cells (Treg). Although thought of as directly tolerizing tumor-specific T cells, Tregs may also play a role in altering the TME, specifically in prostate cancer (4). Tregs are represented at a higher proportion in the bone marrow than in blood (17% vs. 6%; ref. 5). In patients with prostate cancer, the proportion of Tregs in the bone marrow may be 40% (5). Tregs are targeted to the bone marrow through CXCR4, the ligand for CXCL12 (SDF-1), which is highly expressed in bone marrow (5). In addition, Treg interactions with dendritic cells

through receptor activator of NF- $\kappa$ B/receptor activator of NF- $\kappa$ B ligand (RANK/RANKL) expand Tregs in the bone marrow, overall suppressing osteoclast formation and leading to bone deposition (5). These data delineate the mechanism of action of RANKL inhibitors, which have shown promise in prostate cancer trials (6).

### **T<sub>H</sub>2 cells and macrophages**

Even potentially tumor-specific T cells may promote a tumor permissive microenvironment. T-helper 2 (T<sub>H</sub>2) cells, while promoting effective antibody responses, can directly or indirectly lead to a tolerogenic TME (7). Immunoglobulin and lymphotoxin from T<sub>H</sub>2-activated B cells, through Fc $\gamma$ R and receptor binding, can promote the production of myeloid cells, whereas interleukin (IL)-4 secreted by T<sub>H</sub>2 cells can directly promote myeloid/macrophage development through IL-4R $\alpha$  (7, 8). In breast cancer models, IL-4R $\alpha$ <sup>+</sup> macrophages have been shown to promote tumor progression, which in turn leads to tumor secretion of colony-stimulating factor (CSF)-1 and further macrophage expansion (9). CSF-1 makes an attractive target for immunotherapy as mammary tumors secrete higher levels of CSF-1 after chemotherapy, and in a breast cancer model, the combination of paclitaxel and CSF-1R blockade led to reduced tumor growth and metastasis, which was dependent on CD8<sup>+</sup> T cells (9). However, CSF-1R blockade and chemotherapy also leads to increased Treg infiltration through either an IL-10 or CD103<sup>+</sup> dendritic cell-mediated mechanism. Nonetheless, CSF-1R blockade may hold promise in combination with other therapies.

### **Immune checkpoint ligands**

Another example of the interaction between the immune system and the tumor leading to the downmodulation of the antitumor response occurs in the case of PD-1 exhaustion of T cells. A productive antitumor response usually involves the cytokine IFN- $\gamma$ , but in some cases IFN- $\gamma$  secretion by T cells leads to the upregulation of PD-L1 on tumor cells and the surrounding stroma and, ultimately, T-cell exhaustion (10). Standard treatment of non-small cell lung carcinoma (NSCLC) with BRAF inhibitors has the unexpected result of upregulating PD-L1 through an as yet uncharacterized epigenetic mechanism. Because of these and other findings on epigenetic changes in tumor gene expression, trials have been initiated using histone deacetylase (HDAC) inhibitors and DNA methyltransferase inhibitors such as 5-azacytidine in combination with PD-1 as a way to maximize these preliminary results.

Other immunosuppressive signaling pathways activated in the TME include galectin-1 (Gal-1), a carbohydrate-binding lectin, which is overexpressed in Hodgkin lymphoma and Epstein-Barr virus positive (EBV<sup>+</sup>) posttransplant lymphoproliferative disorders (PTLD) in an AP-1-dependent manner (11, 12). Binding of Gal-1 leads to the apoptosis of effector T cells, but suppressive T-cell phenotypes such as T<sub>H</sub>2 and Treg cells lack Gal-1 ligands (12). Gal-1 expression in Reed-Sternberg cells in Hodgkin lymphoma and EBV<sup>+</sup> cells in PTLD contributes to an immunosuppressive tumor environment with few effector cells, making it an interesting target for therapy.

### **Cancer-associated fibroblasts**

The concept of standard treatments promoting a tumor permissive environment can also be seen in prostate cancer. Following androgen ablation in prostate cancer, the hypoxic environment allows the conversion of fibroblasts to myofibroblasts that secrete CXCL13. The increase in myofibroblasts may be the result of increased TGF- $\beta$  found in the microenvironment. Inhibition of TGF- $\beta$  results in inhibition of myofibroblasts and reduced CXCL13 production and lower numbers of infiltrating T cells, dendritic cells, and B cells, the latter of which has been shown to be tumor promoting. These findings show the complexity and opportunities available for immunotherapy.

### **TME endothelium**

Endothelial cells in the TME also act as a barrier to tumor-specific T cells especially in ovarian cancer. FasL expressed in endothelial cells leads to the apoptosis of effector T cells that attempt to enter the tumor. In contrast, Tregs are not affected by this control mechanism. FasL was found to be upregulated by VEGF, prostaglandin E2 (PGE2), and IL-10; however, concurrent neutralization of VEGF and PGE2 led to lower epithelial FasL expression and CD8<sup>+</sup> T-cell infiltration. In addition, local treatment targeting VEGF and PGE2 allowed CD8<sup>+</sup> T-cell infiltration of distant, untreated metastases, even those in the brain, showing the potential of targeted combination therapy for these immunosuppressive molecules.

### **Myeloid-derived suppressor cells**

The interaction of the innate immune system and tumor also plays an important role in tumor development as well as the suppression of antitumor responses. Using 2 models of liver cancer, hepatocellular carcinoma (HCC) driven by the *Akt* and *Ras* oncogenes and a Myc-driven hepatoblastoma, it was shown that the Akt/Ras-driven HCC had more infiltrating CD11b<sup>+</sup>Ly6C<sup>med</sup>Ly6G<sup>high</sup> cells and that both oncogenes were needed for this infiltration. Consequently, depletion of neutrophils with an anti-Gr1 antibody after induction of tumorigenesis lead to improved survival in the Myc-driven model compared with the Akt/Ras-driven model, further showing that different driver mutations affect the surrounding tissue and types of cellular infiltrate, and therefore must be differentially targeted.

A paradox of myeloid-derived suppressor cells (MDSC) was also described. MDSCs have at least 2 phenotypes, granulocytic (Ly6G<sup>+</sup>Ly6C<sup>low</sup>) and monocytic (Ly6G<sup>-</sup>Ly6C<sup>high</sup>; ref. 13). In tumor-bearing hosts, granulocytic MDSCs (g-MDSC) are more prevalent in tumors, yet they are less proliferative and less suppressive than monocytic MDSCs (m-MDSC), which are found in greater numbers in the spleens of tumor-bearing animals. This paradox was explained by the observation that m-MDSC can differentiate into g-MDSC, as well as mature macrophages and dendritic cells. Retinoblastoma 1 (Rb1) was shown to be a marker of MDSC differentiation, being higher in m-MDSC and losing expression as they develop into g-MDSC.

### **IDO**

MDSCs are also recruited by the expression of indoleamine 2,3-dioxygenase (IDO) in the TME, supporting procarcinogenic

inflammation and increasing vascular genesis (14). In addition to attracting MDSCs, IDO is an enzyme that depletes tryptophan, and thus suppresses T-cell activity (14). Recent findings have shown that IDO is a unique target for therapy. In addition to working through the stress response kinase GCN2, IDO-mediated tryptophan depletion inhibits the kinases mTOR and PKC- $\theta$  (15). These pathways, though complimentary, are separate. A novel experimental inhibitor of IDO, 1-methyl-D-tryptophan (D-1MT), relieves the inhibition of mTOR and PKC- $\theta$  by acting as tryptophan mimetic (15). Thus, it is an attractive potential therapy due to its reduced safety concerns compared with nonspecific inhibition of IDO (16).

### GM-CSF and tumor stroma

Pancreatic cancer is notorious for its large stromal component compared with tumor volume and uniquely illustrates the interaction between the microenvironment and the immune system. Using a Kras- and p53-mutated mouse model (KPC) of pancreas cancer, the interaction of cancer and the microenvironment in inhibiting the immune system is evident (17). Pancreatic ductal adenocarcinoma (PDA), like many tumors, secretes granulocyte macrophage colony-stimulating factor (GM-CSF), which is known to induce MDSC (18). In addition, macrophages are a significant component of the PDA stroma (17). In the KPC model, macrophages and CD11b<sup>+</sup>Gr1<sup>+</sup> cells are found throughout the tumor and surrounding metastases, indicating a role in PDA spread; however, treatment with anti-CD40 alone is enough to slow or stop tumor growth in a T-cell-independent fashion (19). GM-CSF and T cells, however, can also be important mediators of PDA regression. In human trials, GM-CSF-secreting tumor vaccines have been shown to be partially effective at treating some patients with PDA as long as Treg-depleting therapy is given with vaccine (20). Long-term survival following a GM-CSF-secreting pancreatic cancer vaccine is associated with the induction of a vigorous T-cell response (21). In the KPC model, T-cell-dependent control of PDA can also be seen when Tregs are depleted and a Listeria-based Kras-specific vaccine is given. Interestingly, both CD8<sup>+</sup> and T<sub>H</sub>17 cells are important effectors in this model. T<sub>H</sub>17 cells have become of particular interest given their stem-like abilities such as secreting both IFN- $\gamma$  and IL-17, their ability to change T<sub>H</sub> phenotype, and their long life *in vivo* (22, 23).

The dual role of the innate immune system is also seen in lung cancer through study of the molecule progranulin. Sera from GVAX and anti-CTLA-4-treated mice showed anti-progranulin antibodies and antitumor activity. Progranulin, made by the tumor and myeloid cells, is a survival signal that acts through caspase-3 and an inhibitor of macrophage activation following phagocytosis of necrotic tumor cells (24). Neutrophils can degrade and counteract the effects of progranulin using elastase; however, elastase can in turn be deactivated by secretory leukocyte protease inhibitor (SLPI; ref. 25). GVAX platforms have been shown to induce antibodies against both progranulin and SLPI molecules. Furthermore, anti-progranulin has been shown to induce tumor-specific necrosis, making these molecules attractive targets for immunotherapy that targets the innate immune system.

The interaction between the immune system and the TME has led to the discovery of numerous ways that the TME suppresses the immune system. These mechanisms, though seemingly daunting, have opened up numerous opportunities to develop new tumor-therapeutic immunotherapeutic strategies.

### The Role of Checkpoint Inhibitor Blockade in Immunotherapy

Immune checkpoints have recently come into the cancer drug spotlight with the approval of Yervoy (ipilimumab), an anti-CTLA-4 monoclonal antibody, for metastatic melanoma, and numerous clinical trials investigating the use of immune checkpoint inhibitors targeting LAG-3, PD-1, Tim3, B7-H3, and B7-H4 (26). Although the concept behind the inhibition of immune checkpoints is to counteract inhibitory T-cell signaling encountered in the TME, the end results may be widely different as each molecule is involved in various and different components of T-cell signaling, as well as having effects in other cell types. Furthermore, one or another immune checkpoint may be more or less critical to T-cell function depending on tumor type and the phenotype of T cells recruited to the TME.

### Mechanisms of ligand upregulation

The rationale for targeting a particular immune checkpoint may rely on the immune checkpoint ligands that are upregulated in that cancer type. Several immune checkpoints are upregulated after T-cell encounter with antigen, as part of the normal immune response for protection against autoimmunity, but are chronically stimulated via ligands present on tumor cells and inhibitory immune cells (27). In the case of PD-1 ligands, PD-L1 and PD-L2, there were 2 main mechanisms of tumor upregulation, termed either innate or adaptive resistance (26). The innate resistance of tumors includes genetic or epigenetic alterations, which allow for the upregulation of PD-L1 and PD-L2. In classical Hodgkin lymphoma (cHL), gene amplification of PD-L1 and PD-L2 occurs as part of the 9p24.1 chromosomal amplification and also as a result of JAK2 overexpression, which is also part of the amplification region and induces PD-1 ligand expression (28). In cHL with normal copy number, constitutive activation of AP-1 regulates transcription of PD-L1 through an AP-1-dependent enhancer (29). In addition, EBV antigens can contribute to the process of PD-L1 overexpression in PTLN (29). Some tumors require the induction of IFN- $\gamma$ -expressing T cells and exposure to IFN- $\gamma$  in the tumor site to upregulate PD-1 ligands, which subsequently signal through PD-1 to suppress infiltrating cytotoxic T cells (adaptive resistance; ref. 10). Regardless of mechanism, the upregulation of checkpoint ligands by tumors can serve as a biomarker for patients who may respond to immune checkpoint inhibitors.

### Combinatorial checkpoint blockade

On the basis of these successes of anti-CTLA-4 in clinical trials for melanoma and other cancer types, immune checkpoint blockade is now being tested in combination with other immunotherapies. In mouse models, anti-PD1 or PD-L1 and

anti-CTLA-4 given together with a vaccine are twice as effective as either on their own (30). With the combination blockade of both the CTLA-4 and PD-1 pathways, there is increased IFN- $\gamma$  production and increased potency of CD8<sup>+</sup> T cells (30). It has been previously shown that anti-CTLA-4 efficacy relies on both activation of effector T cells and the inhibition of Tregs and in this latest set of data, combination blockade also decreases the fraction of MDSCs infiltrating tumors (30, 31).

Anti-CTLA-4 has emerged as a very potent immunotherapy for patients with cancer with dramatic tumor shrinkage and achieving complete responses for some patients with metastatic disease; however, autoimmune side effects have been observed with the use of this monoclonal antibody, some life-threatening (32). Therefore, other immune checkpoints conferring enhanced antitumor immunity coupled with less autoimmunity are desirable for use in the clinic. LAG-3 is an immune checkpoint, which does not cause autoimmune tissue damage when inhibited or genetically deleted (33). In an OT-1 mouse model, adoptive transfer of OT-1-specific T cells given with blockade of PD-1 and LAG-3 increased the trafficking of T cells to tissues. The dual blockade of PD-1 and LAG-3 also increases the memory T-cell pool (CD127<sup>high</sup> and KLRG1<sup>low</sup>), resulting in later functional memory cells upon rechallenge to a *Listeria* vaccine. In the PRO-TRAMP prostate cancer model, there was a slight increase in CTLs early on following adoptive transfer of T cells, checkpoint blockade, and *Listeria* vaccine, but the most pronounced difference was enhanced memory T cells.

#### Checkpoint blockade with vaccines or adoptive T-cell therapy

In addition to combination with other immune checkpoints, the solution to combining potency with less nonspecific autoimmunity may be achieved by combining vaccines targeting tumor antigens with checkpoint inhibitors. Pancreatic cancer-infiltrating T cells induced with pancreatic tumor vaccine given with low dose cyclophosphamide in clinical trials show upregulation of PD-1, providing a rationale for testing immune checkpoint blockade in combination with vaccine and Treg-depleting therapies such as low dose cyclophosphamide in a mouse model. In this mouse study combining GM-CSF-secreting pancreatic tumor vaccine with blockade of PD-1 and Treg-depletion, T-cell responses to mesothelin, a tumor antigen involved in pancreatic cancer metastasis, were enhanced, numbers of effector memory CD8<sup>+</sup> T cells increased, and mice treated with anti-PD-1 in addition to the other therapies had statistically improved survival compared with vaccine and cyclophosphamide alone (34). In an adoptive transfer mouse model, multiple checkpoint inhibitors targeting CTLA-4, LAG-3, and PD-1 were all required to achieve complete clearance of leukemic cells by the adoptively transferred cells that become tolerized and deleted because of tolerance to self-antigen shared by the leukemic cells and normal cells (35). Thus, immune checkpoint blockade can enhance the efficacy of vaccine-induced or adoptively transferred T cells by allowing them to overcome suppressive signaling induced by ligands expressed by the tumor.

#### Costimulatory molecules with immune checkpoint blockade

Along with the class of T-cell molecules that serve to dampen the immune response to antigen, there are also costimulatory molecules, which help activate immune responses to foreign antigens. As many tumor antigens are also self-antigens, the activation of these costimulatory signals may be suboptimal in the context of tumor immunity. Vaccines and agonist antibodies present 2 ways to target these costimulatory molecules while also blocking signaling through checkpoint molecules. Targeting one of these costimulatory molecules, OX40, with an agonist antibody has been found to stimulate T cells *in vivo* in mouse models and augment antitumor immunity by providing a costimulatory signal (36). The tumor clearance seen in mouse models with the anti-OX40 agonist antibody is long lasting, with immunity to rechallenge, and relies on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (36). A mouse anti-human OX40 antibody was recently tested in a clinical trial for advanced cancer with several tumor regressions, but no partial or complete responses. Antitumor responses increased and T cells showed signs of activation following administration; however, the antibody could only be given in a single dose due to anti-mouse antibodies that formed in patients. Further trials will use human anti-OX40 in multiple doses and in combination with other therapies to enhance efficacy, such as a TGF- $\beta$  inhibitor and anti-CTLA-4.

PSA-TRICOM combines 3 immunostimulatory molecules, ICAM-1, LFA-3, and B7.1, with prostate-specific antigen (PSA) in a poxvirus vector. It has shown to be safe, effective in increasing overall survival, and to induce immune responses to PSA in metastatic castration-resistant prostate cancer (37). PSA-TRICOM was combined with ipilimumab in a phase I dose-escalation trial for prostate cancer. Adverse events were similar to that seen with ipilimumab in terms of autoimmune side effects but were not exacerbated compared with ipilimumab alone. The combination showed increased survival with anti-CTLA-4 compared with a previous trial of the vaccine alone (38). A combination of immunostimulatory treatments also showed promise in a murine lymphoma model where CpG deoxyoligonucleotides injected into a primary tumor, in combination with anti-CTLA-4 blocking and agonistic anti-OX40 treatment, led to the destruction of the primary tumor, depletion of Tregs, and the regression of metastases, thus showing the promise that combination immunotherapies may have in the future (39).

Inducible T-cell costimulator (ICOS) was identified as a marker on activated, IFN- $\gamma$ -producing, tumor-infiltrating T cells following therapy with anti-CTLA-4 (40). While ICOS has been shown to have various roles in immunity, the use of ICOS-deficient mice showed that ICOS is necessary for T-cell function in anti-CTLA-4-treated mice (41). This provided a rationale for adding ICOS ligand (ICOSL) to the surface of irradiated B16 cells and treating mice with combined anti-CTLA-4. This combination was superior to a control vaccine with anti-CTLA-4 or anti-CTLA-4 alone, showing that the activity of anti-CTLA-4 can be significantly augmented with a costimulatory signal.

The success of checkpoint inhibitors in the clinical and preclinical mouse models has led to novel uses and new

discoveries, including those that target additional immune checkpoint or costimulatory molecules. On the basis of knowledge about gene families such as the B7/CD28 family, a computational and genetic screen for novel B7-related proteins was carried out by Levy and colleagues. Characterization of these potentially B7-related family members fused to an IgFc domain in T-cell stimulation assays led to the discovery of several previously uncharacterized proteins with T-cell suppressive activity. Use of these immunoglobulin (Ig) fusions led to suppression of autoreactive T cells in a mouse model of multiple sclerosis. These proteins now are interesting targets to release the inhibition of tumor-specific T cells. By targeting multiple molecules at once, T-cell suppression can be overcome and T-cell signaling can be restored and enhanced.

### Strategies for Improving Adoptive T-cell Transfer Therapies

Even when developing tumors induce a T-cell response that results in tumor infiltration, the resulting T-cell response is hyporesponsive and not protective (42). Adoptive T-cell therapy can be effective by administering large doses of T cells, which are engineered to recognize tumor antigens with high affinity, and thus are not yet tolerized. Another approach used in adoptive cell therapy has been to engineer chimeric antigen receptors (CAR); the fusion of a tumor antigen-specific B-cell receptor to the signaling apparatus of a T cell and administering the modified lymphocytes. As there have been many documented clinical responses using these 2 related cell therapies, recent efforts are focusing on increasing the efficiency of these therapies with a focus on optimizing T-cell phenotype for long-lived, potent responses to tumor antigens.

#### TCR specificity and avidity

Adoptive T-cell therapy requires a large infusion of high-avidity T cells targeting appropriate tumor antigens with the ability to infiltrate and function within the TME. Ideally, target antigens will be expressed by the tumor, but not by normal, healthy tissue, thus limiting autoimmunity. However, many tumor antigens are overexpressed self-proteins or mutated forms of self-proteins, resulting in central deletion of T cells with high-avidity TCRs for these antigens. Therefore, the number of naturally occurring high-avidity TCRs from patients with cancer for expansion and adoptive therapy is limited. One solution to this problem is to clone a high-avidity TCR and transduce this TCR into existing T cells. This approach has been refined by 2 new methods for bypassing the improper pairing of the transferred TCR sequence with endogenous  $\alpha$  and  $\beta$  chains: the use of zinc-finger nucleases that disrupt the endogenous TCR genes, and a single-chain TCR composed of variable  $\alpha$  and  $\beta$  chain sequences linked to intracellular signaling domains (43, 44). Both of these approaches have proven safe in mouse models and efficacious at targeting antigens without recognition of nonspecific targets. Affinity of TCRs for self-antigens can be further improved with CDR3 region mutations or by identifying TCR sequences found in thymic double-negative T-cell populations that were destined to become  $\gamma\delta$  T cells based on their strong recognition of self-antigen by the pre-TCR. This technique identifies high-avidity

TCRs, which would not normally be seen in the  $\alpha\beta$  T-cell repertoire after development in the thymus and a source of potentially tumor-reactive TCRs.

#### Viral antigens as targets

Virally associated cancers make attractive targets for adoptive cell therapy, as they are foreign proteins not expressed by normal cells, eliciting polyfunctional T-cell responses. However, viral cancers develop mechanisms of immune evasion, including expression of molecules, which can exclude CD8<sup>+</sup> T cells from the epithelium in cervical cancer, providing a barrier for vaccine-induced or adoptively transferred T cells. In cervical intraepithelial dysplasia, lesions that excluded CD8<sup>+</sup> T cells downregulated the adhesion molecule MAdCAM-1, which is responsible for tissue infiltration of  $\alpha4\beta7$ -expressing T cells, thus avoiding immunosurveillance (45). Another known mechanism is the downregulation of MHC I, and therefore, limited presentation of antigens, which has been described in Merkel cell carcinoma with the Merkel cell polyomavirus T antigen (46). However, intratumoral IFN- $\beta$  and radiation administered before adoptive cell therapy can augment HLA-I expression on tumor cells, making this viral antigen once again a viable target for T-cell therapy. Polyclonal virus-specific T cells were expanded in culture with the cytokines IL-2, -7, and -21 and then administered into a patient with metastatic Merkel cell carcinoma. This patient had 60% shrinkage of existing lesions and no new metastasis, associated with the persistence of transferred T cells and infiltration into the tumor.

#### Bispecific T cells

Attempts to target viral antigens in the treatment of virally associated cancers such as EBV antigens in EBV-associated malignancies have had some success in the clinic. EBV-specific T cells administered to patients following stem cell transplant and in the setting of nontransplant-associated EBV<sup>+</sup> cancers have been effective in eradicating disease (47, 48). The potency of these virus-targeted T cells can be harnessed with bispecific T cells that are derived from antiviral T cells transduced with a CAR targeting a tumor antigen such as CD33 in acute myelogenous leukemia (AML). These CD33-specific T cells were shown to traffic to tumor sites and control tumor progression in a murine xenograft model (49). This technique is currently being expanded to use Varicella zoster virus (VZV)-specific T cells to take advantage of the preexisting immunity many patients have to VZV.

#### Irradiation pretreatment for adoptive T-cell therapy

Among the main goals of adoptive T-cell therapy is to create a pool of cells, which will be resistant to tumor-derived tolerance mechanisms and be long-lived *in vivo*, with the ability to expand and destroy cancer should it recur. One strategy shown to be efficacious is to treat patients with high doses of chemotherapy or radiation to rid the body of existing immune populations before administration of expanded antigen-specific T cells. This treatment regimen allows for the depletion of tolerizing immune cells as well as for the homeostatic proliferation and preferential expansion of transferred T cells (50). After this strategy was validated in mouse models, a clinical

trial was designed to incorporate high-dose cyclophosphamide and fludarabine with or without total body irradiation along with adoptively transferred T cells expanded from TIL. Objective response rates for patients with metastatic melanoma were 49%, 52%, and 72%, with cyclophosphamide and fludarabine alone, chemotherapy with 2 Gy irradiation or chemotherapy with 12 Gy irradiation, respectively, showing that high doses of irradiation combined with high-dose chemotherapy to precondition patients for adoptive T-cell therapy increases the response to immunotherapy (51).

### Checkpoint blockade and adoptive T-cell therapy

Finally, modifying the phenotype of adoptively transferred T cells or CARs can be crucial in the success and long-term benefits of therapy. Adoptively administered T cells or CARs, despite expansion *ex vivo* and genetic manipulation to bind tumor cells with higher avidity, are subject to the same mechanisms of tumor-specific tolerance as endogenous T cells including upregulation of immune checkpoint molecules and the resulting T-cell exhaustion. Investigators hypothesized that anti-CTLA-4 monoclonal antibody therapy would be effective in the case of adoptive T-cell therapy as it is when given to augment preexisting or vaccine-induced T-cell responses. Anti-CTLA-4 was given 1 day following infusion of MART1-specific T cells to patients with metastatic melanoma that underwent cyclophosphamide pretreatment and low-dose IL-2 administration with the T-cell infusions throughout the trial. Several patients, including those who previously failed adoptive T-cell therapy or anti-CTLA-4 monotherapy, had partial responses or stable disease, which was associated with the persistence of anti-MART1 CD8<sup>+</sup> T cells, which had higher effector cytokine expression than baseline. Notably, the induction of additional tumor antigen-specific T cells was observed following therapy, providing evidence that transfer of T cells with antigen-restricted specificity does not limit the development of new T-cell responses, but rather augments it.

### T-cell phenotype

It has been previously shown that less differentiated central memory T cells, T<sub>cm</sub>, persist longer after infusion and are associated with tumor regression (52, 53). T<sub>cm</sub> represent a greater ability to transition into different T-cell subsets including effector memory T cells (T<sub>em</sub>) and effector T cells (T<sub>eff</sub>), in a process that is controlled by epigenetic regulation (54, 55). However, these T-cell subsets differentially regulate glucose metabolism. T cells with a T<sub>cm</sub> transcriptional profile exhibit lower glucose uptake than T<sub>em</sub>/T<sub>eff</sub> cells. In mouse models, administration of a glycolytic inhibitor to T cells reduces their glucose intake and improves their longevity *in vivo* and *in vitro*. Another strategy to enrich for T<sub>cm</sub> is isolation of virus-specific CD8<sup>+</sup> T cells, followed by transduction with tumor antigen-specific CARs, creating bispecific CD19 and viral T cells or using a magnetic bead-based system to isolate T<sub>cm</sub> and transduce them with CD19 CARs (56, 57).

### Chimeric antigen receptors

CARs represent another adoptive T-cell therapy approach in tumor immunotherapy. CARs combine a specific antigen

receptor with an intracellular signaling domain for the targeting of tumor antigens by adoptively transferred T cells. CARs with a receptor targeting CD19 and with a CD3 $\zeta$  and 4-1BB signaling domains have been administered to patients with chronic lymphocytic leukemia (CLL) and pre-B cell acute lymphocytic leukemia (ALL). Several complete responses have been observed with the main adverse event associated with treatment being tumor lysis syndrome (58, 59). In patients showing a complete response, the CAR-modified T cells can be identified for up to 2 years after infusion. In a clinical trial using CD19-targeted CARs to treat B-cell malignancy, the main toxicity observed in patients following CAR infusion was associated with high levels of IFN- $\gamma$  and TNF- $\alpha$  produced by anti-CD19 T cells, as well as chronic B-cell depletion (60). The repertoire of antigens that CARs can target is unlimited; however, as with adoptive T-cell therapy using specific TCRs, this method relies on the definition of antigens expressed by an individual's tumor and developing receptors directed against this antigen. CAR T cells are also susceptible to the same upregulation of inhibitory molecules such as PD-1 and 2B4 and decreased T-cell function in the TME as traditional adoptively transferred T cells.

### T-cell targets in stroma

Adoptive cell therapy to attack tumors is not limited to the tumor cells; another target can also be the surrounding stromal cells, which promote tumor growth and metastasis. Fibroblast activation protein (FAP) is expressed by fibroblasts in the TME (cancer-associated fibroblasts) that contribute to tumor progression, treatment resistance, and angiogenesis (61). The administration of FAP-specific CAR T cells resulted in decreased tumor growth compared with control T cells, with increased apoptotic tumor cells, decreased collagen content, inhibition of angiogenesis, and decreased M2-polarized macrophages. Importantly, no toxicity was seen in mice, showing the specificity of FAP for cancer-associated fibroblasts. In another mouse model, it was observed that FAP-specific CARs were able to reduce tumor growth of tumors that did not express FAP, but had FAP<sup>+</sup> stroma cells, both by administering CAR-expressing T cells locally and systemically.

### Safety of CARs

While the development of transgenic TCRs and CARs represents an effective new strategy for targeting tumor antigens and controlling tumors, associated toxicities such as tumor lysis syndrome and continued B-cell aplasia (with anti-CD19 CARs) highlight both the potency and the hazard of using these therapies. It has been shown that these cells can persist for long periods of time and may account for their ability to keep patients in remission (62). To improve these new therapies and to guarantee their safety, there needs to be a mechanism for turning off the activity of T cells once tumors are eradicated. Ideally, small molecules can be given at prescribed time points to deactivate or turn down the activity of adoptively transferred T cells. One strategy is the cotransduction using a self-inactivating lentivirus vector with CARs into T cells of a truncated EGF receptor (EGFR) with the binding epitope of cetuximab retained, for the deletion of CAR T cells (63). This

can be further combined with a methotrexate-resistant dihydrofolate reductase (DHFR) variant to confer methotrexate-resistance to clones that retain the CD19-CAR and truncated EGFR (63). With new methods to insure the safety of these therapies, adoptive cell therapy promises to be an effective tool for eradicating cancer.

## Summary

The role and status of immunotherapy as treatment of cancer has increased as the effectiveness of standard therapy has plateaued. Our knowledge of T-cell activation and tolerance has led to promising first-line cancer treatments; however, the TME remains a formidable barrier to tumor destruction. This AACR conference highlighted discoveries that will enable us to improve tumor immunotherapy from

both a T-cell activation and TME perspective. New discoveries about T-cell physiology combined with new knowledge of the TME should lead to combinatorial therapies that amplify the potency of tumor immunotherapy. Combining treatments that target checkpoint controls (CTLA-4, LAG-3, PD-1, and OX40), T-cell phenotype (T<sub>cm</sub> vs. T<sub>em</sub> and T<sub>H</sub>1/T<sub>H</sub>17 vs. T<sub>H</sub>2), the innate components (macrophages, neutrophils, and MDSC), plus advances made in TCR engineering has the potential to significantly advance the early promise of tumor immunotherapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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