

Tumor Immunology: Basic and Clinical Advances

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Abstract

The third in a series of AACR conferences, entitled "Tumor Immunology: Basic and Clinical Advances," was held in Miami Beach, Florida from November 30 to December 3, 2010. The overall objective of this meeting was to discuss rapid developments in the understanding of basic principles of antitumor immunity and strategies for increasing the success rate of cancer immunotherapy. The key findings that emerged from the meeting included (i) that integrated approaches are required for the development of effective cancer immunotherapies and (ii) attention should be on multiple cellular and molecular components and their broader networks rather than on a single pathway or cell type. *Cancer Res*; 71(13); 4338–43. ©2011 AACR.

Introduction

The overall objective of this third special AACR conference organized by the Cancer Immunology Working Group was to discuss rapid developments in the understanding of basic principles of antitumor immunity, inflammation, and cancer that are leading to the design of more scientifically based strategies for increasing the success rate of cancer immunotherapy. The specific themes of the meeting included (i) mechanisms of cancer initiation by inflammatory responses that can be targeted for developing cancer prevention approaches; (ii) novel adoptive T-cell therapy approaches that exploit state-of-the-art technologies to engineer tumor-specific T-cell receptors (TCR); (iii) new therapeutic or prophylactic vaccine approaches that either target particular dendritic cell (DC) subsets for improved antigen delivery, or utilize new adjuvants or immunomodulating agents to deplete immunosuppressive cell subsets and/or block inhibitory receptors on tumor-specific effector lymphocytes to enhance vaccine efficacy; (iv) mechanism(s) of monoclonal antibody (mAb) therapy and ways to improve its efficacy; (v) complex signaling networks and cross-talk in the tumor microenvironment for targeting to enhance immune cell infiltration and effector function; and (vi) epigenetic mechanisms, such as acetylation and methylation, that regulate tumor growth and immune recognition, and the emerging role of histone deacetylases (HDAC) in immunologic networks participating in inflammatory responses.

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Advances in the Development of Cancer Immunotherapies

Given this year's approval of Dendreon's cellular immunotherapy Provenge (sipuleucel-T) by the U.S. Food and Drug Administration (FDA) and the phase III study showing a significant survival benefit in metastatic melanoma patients receiving the anti-CTLA-4 antibody ipilimumab (Bristol-Myers Squibb; ref. 1), it is clear that significant advances in immunotherapy are happening already and will continue at a rapid pace in the near future. However, despite these examples, most tumor immunotherapies currently under clinical development have been met with limited success. Consequently, regardless of the session, a central theme throughout the meeting was to identify potential new clinical and research strategies to design and achieve enhanced antitumor efficacy.

Adoptive cell therapy (ACT) is one form of immunotherapy that has shown promising results in early studies in melanoma patients (2). ACT involves the transfer of large numbers of autologous lymphocytes enriched for activated tumor antigen-specific cytotoxic T lymphocytes (CTL). Several methods have been used to generate enriched pools of tumor-specific CTLs, including expansion of tumor-infiltrating lymphocytes (TIL) and repetitive antigen-specific stimulation of peripheral blood mononuclear cells (PBMC). However, these methods are technically challenging, difficult to standardize, and not generalizable to all patient specimens. One potential way to overcome this hurdle is to engineer the transferred cells to express TCRs known to be specific for tumor antigens (3–5). Methods for introducing tumor antigen-specific TCR genes into PBMCs have been developed, and a clinical study transferring PBMCs engineered to express a TCR specific for the melanoma antigen MART-1 combined with peptide-pulsed DCs is underway. Results from preliminary studies using engineered PBMCs for ACT in melanoma patients have highlighted at least 3 challenges facing this therapy. First, although TCR genes can be introduced with relatively high efficiency, additional modifications to current protocols are required to minimize pairing with endogenous TCRs and to ensure

long-term expression of the introduced TCR genes. Second, similar to what occurs with endogenous lymphocytes, over time the transferred cells become unresponsive. Third, the majority of transferred lymphocytes do not persist long term, and this is especially important given that prior studies using TIL for ACT have suggested that clinical responses were associated with persistence of the transferred lymphocytes.

An important question to consider for ACT is whether all peripheral blood lymphocytes (PBL) are equal (6). Analysis of human PBL has indicated that there are at least 4 major subtypes of phenotypically distinct CD8⁺ memory T cells (TCM) that can be distinguished on the basis of the expression of CD62L (+ or -) and CD161 (high or low). Preclinical studies conducted in murine and primate models have shown that these lymphocyte subpopulations are not equally suited for ACT. Specifically, these studies suggest that CD62L⁺CD161^{lo} central TCMs may provide the optimal precursor cells for ACT because this subset is uniquely capable of generating all compartments of effector and TCMs following transfer. In addition, the longevity of transferred cells may be enhanced by concomitant treatment with interleukin (IL)-15. Clinical studies are required to determine whether selecting for TCMs and/or the addition of IL-15 will result in longer-term survival of transferred cells and improve the efficacy of this therapy.

Clinical studies testing ACT have been primarily limited to melanoma patients. However, ACT is now being tested in patients with multiple myeloma. In this study, lymphocytes infiltrating the bone marrow (MIL) are expanded *ex vivo* and used for cell transfer. MILs are analogous to TILs, the best source of CTL inducing objective clinical antitumor responses in up to 50% of treated melanoma patients. However, whereas TILs can only be expanded from approximately 25% of melanoma patients, MILs can be generated from 100% of patients with multiple myeloma. Although this study has not been completed, durable remissions have been observed in some patients and it will be interesting to see whether the response rate is similar to what has been observed in melanoma patients.

Another broad category of immunotherapy includes vaccine approaches designed to activate and amplify endogenous repertoires of tumor-specific lymphocytes. Numerous vaccine strategies have been tested and shown to be capable of inducing circulating tumor-specific T cells. However, these vaccines rarely result in clinically significant responses. The reasons for the failure of these therapies are numerous and complex but are mainly due to the insufficient induction of robust effector and TCM responses that can gain access to the tumor. In addition, numerous tolerance mechanisms dampen both the systemic and local antitumor immune response. Successful antitumor immunotherapy will not only require more potent vaccines but also methods for overcoming these tolerance mechanisms.

Essentially all vaccines aim to deliver tumor antigens to DCs so that they can in turn activate tumor-specific T cells. Strategies of antigen delivery include injection of defined antigenic peptides, recombinant viruses and bacteria, recombinant proteins, DNA constructs, and *ex vivo* antigen-loaded DCs. Problems with these strategies include competition with

vector-derived antigens, insufficient antigen presentation, insufficient trafficking of injected DCs to lymph nodes, and tolerance induction caused by antigen presentation by non-professional antigen-presenting cells (APC) and/or tolerogenic DCs. Interestingly, it may be possible to overcome many of these problems by using synthetic long (28–35 amino acids) peptides (SLP). An SLP vaccine consisting of 13 long peptides covering full-length human papillomavirus (HPV) E6 and E7 proteins formulated in Montanide ISA 51 has been tested in 20 women with premalignant HPV-16–induced high-grade vulvar intraepithelial neoplasia (VIN3; ref. 7). Objective clinical responses were observed in 79% of the treated patients at 12 months. Importantly, complete and durable responses were induced in 47% of the treated patients and were associated with stronger HPV-16–specific T-cell responses. In addition, patients with larger lesions containing increased frequencies of regulatory T cells (Tregs) were less likely to respond. The promising results of this study suggest that vaccines may be most effective very early in cancer development. Many vaccines that are currently being tested in advanced disease target antigens that are also present on premalignant lesions. One example is the MUC1 antigen that is abnormally expressed in epithelial adenocarcinomas as well as on premalignant lesions that can progress to cancer, such as advanced colonic polyps or inflammatory bowel disease, that can lead to colitis-associated colon cancer (8, 9). Previous studies in animal models have shown safety and efficacy of the MUC1 vaccines to prevent cancer development. A clinical trial is currently being carried out testing the immunogenicity and safety of the MUC1 peptide vaccine with the poly-ICLC (TLR3 agonist) adjuvant in patients with a history of advanced colonic adenomas, with the ultimate goal of preventing adenoma recurrence and progression to colon cancer.

Vaccines typically target tumor antigens to endogenous DCs because they are the most potent APCs capable of inducing, regulating, and maintaining T-cell responses. However, there are multiple subsets of DCs with varying capacities for inducing favorable antitumor T-cell responses (10). Many of the DCs present in cancer patients may be dysfunctional and more likely to induce tolerance or nonproductive T-cell responses. More potent vaccines may be designed if we understand which DC subsets are superior, how to target antigen to these subsets, and/or how to reprogram undesirable DC subsets. Alternatively, it may be possible to circumvent the dysfunctional endogenous DCs by using *ex vivo* generated DCs (11). However, successful vaccination will require proper conditioning of the DCs. Preclinical studies suggest that type-I–polarized DCs may be superior activators of antitumor CTL and Th1 CD4⁺ T-cell responses and are currently being tested in patients with different forms of advanced cancer.

The limited success of prior vaccine studies may also be in part due to the use of insufficiently potent immunologic adjuvants. A 3-component vaccine (TriVax) consisting of antigenic peptides, the toll-like receptor (TLR) agonist poly-IC, and anti-CD40 antibody has been shown to induce potent antitumor immune responses in mice (12). This vaccine may be capable of selectively inducing effector T cells and

not Tregs. It will be important to determine whether this novel vaccine can be successfully translated into the clinic.

A major obstacle for developing effective therapeutic vaccines is immunologic tolerance. Although the ideal vaccine would selectively induce effector T cells and not Tregs, it is not clear that this is possible. An alternative approach is to deplete or inhibit Tregs concurrently with the administration of the vaccine. Much progress has been made in understanding the various populations of these cells in humans and the phenotypic markers that can be used to enumerate them and potentially specifically eliminate them. Although better options are required, cyclophosphamide is a drug capable of temporarily depleting Tregs when given at low doses. Pretreatment with low-dose cyclophosphamide has been shown to enhance the potency of granulocyte macrophage colony-stimulating factor (GM-CSF)-secreting whole-cell vaccines in both preclinical and clinical settings and is associated with the induction of higher avidity tumor-specific T-cell responses (13, 14). Importantly, analysis of mesothelin-specific T-cell responses in vaccinated pancreatic cancer patients suggest that higher-avidity responses are associated with both prolonged survival and the ability to traffic to tumor. In addition, these data suggest that the size and avidity of the mesothelin-specific T-cell repertoire may predict who will respond to vaccination. Additional studies are required to determine whether mesothelin-specific T-cell responses can accurately predict vaccine responders and whether this antigen can serve as a specific target for vaccination.

Inhibitory receptors expressed on effector T cells, such as CTLA-4 and PD-1, provide attractive targets for immunomodulation. Antibody blockade of CTLA-4 with ipilimumab has been extensively studied and was recently shown in a phase III study to induce durable antitumor responses in approximately 10% to 20% of advanced melanoma patients (1). Although these results are encouraging, important unanswered remain, like the following: Why do only 10% to 20% of patients respond? and How can we predict who will respond? Although the first question remains largely unanswered, a recent study suggests that the post-treatment upregulation of inducible costimulator (ICOS) on effector T cells may distinguish responders from nonresponders (15). Although additional studies are required for validation, this could serve as a convenient screening method for patients eligible for treatment with ipilimumab. In addition, these data provide the rationale for investigating the role of ICOS in antitumor responses and indicate that this may serve as an additional target for immunomodulation.

More recently, a PD-1-blocking antibody has been developed (Bristol-Myers Squibb) and is currently being tested in patients with various tumors. Although fewer patients have been treated, the response rate to anti-PD-1 may be higher than to ipilimumab. The preliminary results suggest that approximately 30% of treated patients may develop objective responses (16). Interestingly, response to anti-PD-1 may correlate with surface expression of B7-H1 (one of the ligands for PD-1) on the tumor. Additional studies are underway to determine the proper dosing and efficacy of anti-PD-1 therapy.

Importantly, clinical studies testing immune checkpoint inhibitors in combination with a vaccine are just now being initiated. It remains possible that these combinations will elicit more potent antitumor responses and improved response rates.

Antibodies for Cancer Immunotherapy

Over the past decade, mAb therapy has emerged as an important therapeutic agent for cancer treatment resulting in clinical responses and survival advantages for some patients. However, these responses are only seen in a subset of patients and relapse often occurs even after prolonged treatment. As a result, understanding the mechanism(s) of mAb therapy and devising ways to improve its efficacy was a major theme at this year's meeting.

The mechanisms proposed to explain the clinical efficacy of mAb therapy had primarily focused on their ability to turn off signaling pathways crucial for the maintenance of malignant cells. More recently, attention has begun to focus on mechanisms to promote the host immune response. Preclinical studies suggest that a robust antitumor effect is dependent on antibodies with high affinity for a tumor antigen and for cellular Fc receptors. Increased Fc-Fc receptor interaction resulted in highly efficient antibody-dependent cellular cytotoxicity (ADCC). The significance of ADCC lies in its ability to promote antigen presentation and engage the full armament of the adaptive immune response.

Trastuzumab (Herceptin; Genentech, Roche) was approved by the FDA in 1998 for the treatment of HER2/*neu* overexpressing breast cancer. As with other mAb therapies, only a subset of patients treated with trastuzumab experience clinical benefit. An IgE homolog of trastuzumab was engineered with the same light and heavy chain variable regions but with an epsilon in place of the γ -1 heavy chain constant region. *In vitro* functional assays showed that the IgE homolog could induce antigen-specific mast cell degranulation. Preclinical studies testing the monoclonal IgE antibody in animal models suggested that mediators released by degranulated mast cells may initiate and potentiate effector cell activation and recruitment to the tumor site. Further testing is required to understand how the use of monoclonal IgE compares with the standardly used IgG.

IFN- α is known to have potent antitumor effects and can directly induce apoptosis in tumor cells. However, clinical use of IFN- α is limited because of its systemic toxicity. A fusion protein was constructed to determine whether targeting IFN- α specifically to the tumor can enhance its antitumor effects while limiting its systemic toxicity. The transmembrane protein CD20 is elevated on neoplastic B cells, and the fusion protein was constructed by linking IFN- α to the C-terminus of an antibody specific for CD20. Studies using a lymphoma mouse model showed that the IFN- α fusion protein had significantly enhanced proapoptotic effects against CD20-expressing tumors that appeared to be mediated through the caspase pathway and enhanced STAT1 activation.

The Tumor Microenvironment

Solid tumors develop in the context of a complex microenvironment that includes epithelial cells, fibroblasts, endothelial cells, immune cells, cytokines, and chemokines. It has been shown that the nature of this microenvironment can strongly influence the growth, progression, and metastatic potential of the tumor cells. In addition, the microenvironment can act as a barrier, limiting the efficacy of cancer immunotherapy. A central theme throughout the meeting was to highlight some of the complex signaling networks and cross-talk occurring within the tumor microenvironment and identify strategies that could alter a tumor-promoting microenvironment to one that supports tumor elimination.

Epithelial-mesenchymal transition (EMT) is a biological program that can be activated in carcinoma cells and facilitate their ability to disseminate to distant sites in the body. In addition, an outcome of EMT by epithelial cells is the acquisition of stem cell characteristics (17). When EMT-inducing signals are removed, these cells can revert to their original epithelial state. This phenotypic plasticity suggests a dynamic equilibrium within tumors that may be shifted in one direction or another by contextual signals within the tumor microenvironment. This further complicates the influence of the microenvironment on tumor cells and their metastatic potential.

Cancer-related inflammation has emerged as the seventh hallmark of cancer. The proinflammatory cytokine TNF- α is found increased in the tumor microenvironment of most solid tumors. It can be produced by malignant cells as well as myeloid cells that infiltrate the tumor microenvironment. TNF- α has been shown to have a critical role in chronic inflammatory diseases and a tumor-promoting role in preclinical mouse tumor models. To translate anti-TNF therapy into the clinical setting, a better understanding of its mechanism of action is required. In a preclinical model of ovarian cancer, TNF- α was shown to exert its tumor-promoting activity through its control of a complex cytokine/chemokine network (18). Stable knockdown of TNF- α resulted in decreased CCL2, CXCL12, VEGF, IL-6, and macrophage migration inhibitory factor (MIF), all of which are associated with tumor cell survival, stimulation of new blood vessels, and dissemination of tumor cells. Identification of the "TNF network" suggested that TNF- α may serve as a "master" cytokine and raised the question of whether silencing other members of this autocrine network could be effective in inhibiting tumor growth and metastasis. This question is currently being addressed in a phase II trial of the anti-human IL-6 antibody CNT0328 in women with relapsed ovarian cancer.

TGF- β possesses both tumor suppressor and tumor promoter activity and has been identified as a potential therapeutic target for cancer immunotherapy. The difficulty lies in determining how to abrogate the tumor-promoting effects of TGF- β while maintaining its tumor suppressor properties. Mechanistic evidence has begun to unravel the factors that facilitate its switch from tumor suppressor to promoter. In a mouse mammary carcinoma model, deletion of the type II TGF- β receptor gene in epithelial cells resulted in increased

chemokine/chemokine receptor signaling, specifically CXCL1 and CXCL5, leading to the recruitment of myeloid-derived suppressor cells (MDSC) into the invasive front of tumors (19). These tumor-isolated MDSCs were found to produce high levels of matrix metalloproteinases (MMP) and TGF- β , and subsequent coinjection of these MDSCs with tumor cells resulted in an increase in lung metastasis. This work suggests that TGF- β -driven chemokine secretion may be an important regulator of MDSC infiltration into the tumor microenvironment.

An equally important mechanism by which TGF- β can enhance tumor growth is through its ability to attract and polarize neutrophils. TGF- β blockade in mice challenged with tumors led to an influx of neutrophils into the tumor microenvironment. These neutrophils were characterized as N1 TANs (antitumorigenic) that express immunoactivating cytokines and chemokines and low levels of arginase (20). Depletion of N1 TANs abolished the antitumor effects of TGF- β blockade. Using a microarray approach, bone marrow neutrophils (BMN) from normal mice were compared with the granulocyte fraction of MDSCs (G-MDSC) from spleens of tumor-bearing mice, N2 TANs (protumorigenic) from untreated tumor-bearing mice, and N1 TANs from tumor-bearing mice that received TGF- β blockade. Major differences in immune response genes and chemotaxis genes were found among the 4 groups. However, the difference between N1 and N2 TANs was less pronounced than the difference between them and BMN or G-MDSC, with the exception of T-regulatory chemoattractant CCL17 that was upregulated in N2 compared with N1 TANs. This work suggests that the presence of N1 or N2 TANs at the tumor is not due to differential recruitment but occurs intratumorally depending on the presence or absence of TGF- β in the microenvironment.

Epigenetic Regulation of Tumor Growth and Immune Recognition

Most of the antigens expressed by tumor cells are also expressed in normal cells resulting in immune tolerance and a failure of the immune system to recognize and eliminate tumors expressing self antigen. Supporting this phenomenon, it is known that T-cell unresponsiveness occurs during the growth and progression of hematologic and solid tumors. To circumvent tumor-induced tolerance, several studies have focused on the cellular and molecular mechanisms regulating bone marrow-derived APCs and Tregs that have been implicated in tolerance induction and maintenance. A new area of interest is to determine how epigenetic modifications, such as acetylation and methylation, are involved in regulating expression of genes involved in controlling and regulating inflammatory responses.

It is well known that HDACs are enzymes that regulate transcription through chromatin modification. Although histones are the primary substrate for HDACs, they also may deacetylate nonhistone proteins, such as p53, RelA, and BCL6, and they are involved in many other processes depending on their subcellular localization and stage of

cellular differentiation. The data presented at this conference highlighted the emerging role of HDACs in immunologic networks participating in inflammatory responses. HDACs may regulate the transcriptional regulation of pro- and anti-inflammatory cytokines. HDAC11 and HDAC6 were shown to have opposite effects on the expression of IL-10, a cytokine that plays a central role in promoting tolerance induction. Overexpression of HDAC11 in APCs strongly inhibited the transcriptional activity of IL-10, whereas overexpression of HDAC6 promoted IL-10 gene transcription. Importantly, an HDAC11 mutant with a truncated deacetylase domain failed to inhibit IL-10 gene expression, suggesting that intact enzymatic deacetylase activity is required for this process. Furthermore, knocking down HDAC11 using HDAC11-specific short hairpin RNAs resulted in enhanced IL-10 gene expression in APCs. In macrophages overexpressing HDAC11, the histones H3 and H4 were not acetylated when compared with control cells, suggesting that HDAC11 may directly interact with the IL-10 promoter and induce chromatin modifications (21). Thus, HDAC11 and HDAC6 may represent novel molecular targets for cancer immunotherapy and it may be possible to use specific HDAC inhibitors (HDACi) to promote immune activation versus immune tolerance.

Acetylation has also been shown to be an important epigenetic mechanism regulating Tregs. As mentioned above, Tregs play a crucial role in the maintenance of self-tolerance. The forkhead box protein P3 (Foxp3) gene acts as a master regulator of Treg development, and its expression is required for Treg suppressor function. The N-terminal region of Foxp3 is proline rich and recruits the histone acetyl-transferase (HAT) TIP60. Foxp3 may also interact with the histone deacetylases HDAC7 and HDAC9. Thus, Foxp3 acetylation may be reciprocally regulated by HAT p300 and the HDAC

factors. Because acetylation is associated with increased Foxp3 expression, it is plausible that treatment with HAT inhibitors may abrogate the tumor suppressor activity of Foxp3⁺ Tregs.

Expression of the *Foxp3* gene is also modulated by the other major epigenetic mechanisms such as DNA methylation. Stable Foxp3 expression has been associated with demethylation of a conserved element within the Foxp3 locus called TSDR (Treg-specific demethylated region). Interestingly, the TSDR region is not demethylated in TGF- β -induced Tregs or activated effector T cells that show transient expression of Foxp3 (22). Thus, the methylation status of the TSDR region may provide a superior method for depicting Tregs from other cell types.

Taken together, these data suggest that transient versus stable Foxp3 expression seems to be directly regulated by deacetylation and de-methylation mechanisms. Controlling these post-translational events may have important consequences for the modulation of T-cell function and signaling.

In considering the results presented at this conference, it is clear that integrated approaches are required for the development of effective cancer immunotherapies. The tumor microenvironment is composed of a complex network of numerous cell types and signaling components. As such, when devising these new strategies, attention should be on multiple cellular and molecular components and their broader networks rather than on a single pathway or cell type.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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