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Strategies and challenges in eliciting immunity to melanoma

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Summary

The ability of CD8 T cells to recognize melanoma tumors has led to the development of immunotherapeutic approaches that use the antigens CD8 T cells recognize. However, clinical responses rates have been disappointing. Here we summarize our work to understand the mechanisms of self-tolerance that limit responses to currently utilized antigens, and our approach to identify new antigens directly tied to malignancy. We also explore several aspects of the anti-tumor immune response induced by peptide-pulsed dendritic cells. Dendritic cells differentially augment the low avidity of recall T cells specific for self-antigens, and overcome a process of aberrant CD8 T cell differentiation that occurs in tumor-draining lymph nodes. Dendritic cell migration is constrained by injection route, resulting in immune responses in localized lymphoid tissue, and differential control of tumors depending on their location in the body. We demonstrate that CD8 T cell differentiation in different lymphoid compartments alters the expression of homing receptor molecules and the presence of systemic central memory cells. Our studies highlight several issues that must be addressed to improve the efficacy of tumor immunotherapy.

Keywords

CD8 T cells; melanoma; tumor antigens; phosphopeptides; memory; regional immunity

Introduction

The role of the immune system in controlling tumor outgrowth has been explored for several decades. The presence of T cells specific for melanoma antigens (Ags) in patients and mice demonstrated the ability of the immune system to recognize these tumors (1-3). Despite the presence of T cells that recognize tumors, tumors frequently grow out and kill their host. The identification of tumor-derived peptides recognized by these T cells (reviewed in (4)) provided the possibility of inducing or enhancing a tumor-specific immune response.

Immunotherapeutic approaches have been shown to significantly enhance these immune responses, and have resulted in significantly improved control of melanoma (4;5). However, the overall clinical response rates remain disappointingly low (6). These results highlight the need to understand the immune responses that current vaccination approaches induce, and the intrinsic mechanisms by which tumors limit these responses. This review summarizes the work of our laboratory to address several elements related to these issues. The first half will focus on self-tolerance as a limiting factor in immunogenicity of most currently identified Ags, and the means by which this may be circumvented. We also describe a new category of Ag that we believe has great potential because of its relationship to cellular processes associated with

malignancy. The second half will focus on the development of effective vaccination strategies to elicit anti-tumor immunity. Critical components of an effective vaccine include overcoming self-tolerance, efficient generation of effector cells, the proper migration of effector cells to tumors, and the induction of memory.

Self-tolerance to tyrosinase is induced by a novel mechanism involving direct presentation from a radioresistant cell in lymph nodes

The largest category of peptide Ags that are both widely displayed on melanomas and recognized by CD8 T cells from multiple patients originate from proteins associated with pigment production in normal melanocytes. These proteins, classified as melanocyte differentiation proteins (MDPs), are presented by multiple class I or class II MHC restriction elements and MDP-specific T cells have been generated *in vitro* from both melanoma patients and healthy individuals (7-9). However, there is concern that immune-based treatment of melanoma based on these Ags may be impeded by self-tolerance (10;11). Interestingly, melanoma regression occurring spontaneously or in association with several forms of immunotherapy has been associated with autoimmune skin depigmentation and melanocyte destruction (vitiligo) in patients (12-15) and murine models (16-18) suggesting that breaking tolerance enables control of tumor growth. There is little understanding of the mechanisms that control self-tolerance and autoimmunity to Ags expressed in melanocytes. The development of CD8 T cell tolerance has been thoroughly studied for Ags expressed on pancreatic islets (19;20). These studies showed that cross-presentation of Ag by CD11c⁺CD8 α ⁺ DC localized to pancreatic lymph nodes (LN) caused CD8 T cells activation and proliferation, but deletion instead of accumulation. Recently, we investigated the mechanism of self-tolerance to the MDP tyrosinase to provide insight into opportunities to subvert self-tolerance in the presence of melanoma.

We have developed a murine model based on a peptide derived from tyrosinase (Tyr₃₆₉), which is highly homologous to a human peptide (Tyr₃₆₉Y) that is known to be presented on melanoma cells by HLA-A*0201 (10;21;22). The tyrosinase model system utilizes TCR transgenic (tgTCR) mice (designated FH mice) that express a TCR specific for the Tyr₃₆₉ peptide from murine tyrosinase. Transgenic mice were generated to express a recombinant human class I MHC molecule termed AAD, which enables an analysis of endogenous tyrosinase presentation and T cell activation in a model that mimics the human system (23). An additional component of this model is the use the c^{38R145L} albino mutation, which represents a complete deletion of the tyrosinase structural gene (24) backcrossed onto C57BL/6 (22). This provides the opportunity to examine FH responses in the presence and absence of self-Ag. These mice were crossed to the FH tgTCR to examine the mechanism of tolerance to endogenous tyrosinase. Although tyrosinase expression is nominally restricted to melanocytes and retinal pigment epithelium (25;26), low level transcripts are also detected in the thymus (27), and it was suggested that these might confer central tolerance. However, when FH⁺ AAD⁺ mice that were either tyrosinase⁺ or albino were compared, we found no difference in thymic cellularity, percentage of CD8 thymocytes that expressed FH, level of expression of either FH or CD8, or the percentage of the CD8 FH thymocytes that were HSA^{hi} (28). We also established that, in a non-tgTCR context, responses to Tyr₃₆₉ in neonatally thymectomized AAD⁺ albino hosts implanted with AAD⁺ tyrosinase⁺ thymi remained robust, while those in thymectomized AAD⁺ tyrosinase⁺ hosts implanted with AAD⁺ albino thymi remained undetectable. Thus, tolerance to Tyr₃₆₉ is not due to tyrosinase expression in the thymus.

We next tested the alternative hypothesis that tolerance to Tyr₃₆₉ is due to mature T cell encounter with peripheral Ag. After 3d, FH cells in peripheral LN of albino mice remain undivided, while FH cells in tyrosinase⁺ mice have undergone several divisions. However, cellular accumulation was substantially smaller than observed when cells in albino recipients

were activated with a low dose of recombinant vaccinia virus expressing murine tyrosinase. Activation by endogenous tyrosinase also led to a significantly higher percentage of apoptotic cells in each cell division. FH cells were undetectable 14d after transfer into tyrosinase⁺ hosts, while cells in albino hosts persisted at initial levels. Thus, tolerance to Tyr₃₆₉ is based in part on a peripheral deletion mechanism similar to that described for model Ags expressed in pancreatic β cells and keratinocytes (19;20;29). However, in contrast to those earlier studies, activation of FH cells in tyrosinase⁺ mice was not limited to skin draining LN, but included all other LN evaluated, but not spleen. We showed this was due to activation in these compartments by examining CD69 up-regulation 12-24h after transfer, and by evaluating activation in mice treated with FTY720, a drug that prevents egress of lymphocytes from LN (30;31). Treatment of recipients with anti-CD62L prior to adoptive transfer excluded FH cells from LN, but not spleen, and blocked T cell activation. This demonstrates that Tyr₃₆₉ presentation is occurring in LN, but not in spleen or non-lymphoid tissues. Thus, Tyr₃₆₉ presentation is not limited to LN that drain tissues known to express tyrosinase.

To gain insight into the Tyr₃₆₉-presenting cells mediating deletion, we created chimeras by transfer of bone marrow from AAD^{neg} donors into AAD⁺ recipients and vice versa. Surprisingly, adoptively transferred FH cells still underwent activation in AAD^{neg} albino→AAD⁺ tyrosinase⁺ chimeras although the number of CD11b⁺ and CD11c⁺ cells that were AAD⁺ was reduced by at least 90% (32). Thus, tolerogenic presentation of Tyr₃₆₉ from endogenous tyrosinase is mediated by radioresistant, not radiosensitive, cells. More surprisingly, FH cells were not activated to any extent in AAD⁺ albino→AAD^{neg} tyrosinase⁺ chimeras, although more than 90% of the peripheral leukocytes, lymphocytes, CD11b⁺ and CD11c⁺ cells in these animals were AAD⁺. Thus, Tyr₃₆₉ derived from “endogenous self” tyrosinase is not cross-presented by radiosensitive antigen presenting cell (APC) under steady state conditions.

The activation of T cells in AAD^{neg} albino→AAD⁺ tyrosinase⁺ chimeras might have been due to skin derived DCs, such as epidermal Langerhans cells (LC). LC develop from skin-resident precursors that are resistant to γ -irradiation, and host LC persist in bone marrow chimeras even after total body irradiation (33-35). They are also localized near melanocytes in epidermis and hair follicles (36). To test the hypothesis that LC were responsible for the presentation of Tyr₃₆₉ derived from endogenous tyrosinase, we generated AAD⁺ tyrosinase⁺ Langerin:DTR⁺ mice. These mice express the diphtheria toxin receptor exclusively on LC and enable selective depletion of these cells upon treatment of mice with diphtheria toxin (37). However, the activation and deletion of adoptively transferred FH cells in the LN of LC-depleted mice was unaltered. Thus, LC are not responsible for presentation of Tyr₃₆₉ from endogenous tyrosinase. Similar results were obtained using Mafia and CD11c:DTR mice to deplete cells expressing c-fms or CD11c (38;39). Collectively, these results suggest that bone-marrow derived cells do not mediate tolerogenic tyrosinase presentation.

We next evaluated the hypothesis that the Tyr₃₆₉ presentation in LN was due to a LN resident cell that directly expressed the protein was evaluated. Surprisingly, we detected modest levels of tyrosinase mRNA in LN, but not spleen, of tyrosinase⁺ mice. This mRNA expression is radioresistant in bone marrow chimeras, and its level is 100-fold higher in LN than thymus. This pattern of tyrosinase mRNA expression is entirely consistent with the pattern of Tyr₃₆₉ presentation that leads to peripheral, but not central deletion. A similar observation of pervasive expression and Ag presentation in LN has been observed by others (40;41). Although these investigators used a transgenic Ag model, which introduced the possibility of aberrant expression, the concordance of our results and theirs suggests that this is a previously unidentified mechanism leading to peripheral tolerance. Our results are consistent with the possibility that the LN resident cell is a melanocyte, although they are considered sessile. Alternatively, the responsible cells may be LN stromal cells (40). Whether this mechanism of

peripheral tolerance is applicable to other MDPs or other non-tissue specific proteins is of great interest. An important issue with respect to this unappreciated mechanism of peripheral tolerance is whether it is as efficient as that mediated by conventional APC. In this regard, it is also of interest to know whether this might also be related to the observation that tumor specific T cell responses in melanoma patients are more evident than those in patients with other solid tumors.

Melanoma-derived tyrosinase is cross-presented by radiosensitive APC and directly presented by tumor cells

Given the lack of cross-presentation of Tyr₃₆₉ from endogenous tyrosinase by hematopoietic cells, we were interested in how Tyr₃₆₉ was presented when the source was a melanoma tumor. We evaluated direct and cross-presentation by using B16-F1, which can provide tyrosinase for cross-presentation, but cannot directly present Tyr₃₆₉; B16-F1 transfected with AAD to enable direct presentation of Tyr₃₆₉; and chimeric mice reconstituted with AAD^{neg} bone marrow in which cross-presentation of Tyr₃₆₉ cannot occur. These experiments demonstrated that activation of T cells in mice receiving tumor 1 day (d) earlier was due entirely to cross presentation (42). Collectively, these results demonstrate for the first time that the Tyr₃₆₉ epitope is cross-presented and that this mechanism is necessary for activation of Tyr₃₆₉-specific T cells at early stages of tumor outgrowth.

Tumor cells injected 1d previously are not organized as a biologically relevant tumor mass, and many are likely to have undergone necrosis (43). Thus, while these results are relevant to the potential immunogenicity of necrotic tumor cells, they are not directly relevant to understanding the immunogenicity of Ag derived from organized tumors. We evaluated this by adoptively transferring naïve T cells into mice that had harbored B16 tumors for different lengths of time, and then assaying the extent of T cell activation at a fixed time after T cell transfer. We found that that cross-presentation of both tumor-derived Ags persists for at least 17d. After about 14d, however, we also found that B16 cells had metastasized to the draining mediastinal LN, and that T cells were activated by both direct and cross-presentation pathways at this time. These results establish the importance of both sustained cross-presentation, and direct presentation in conjunction with LN metastasis, as mechanisms for activation of Tyr₃₆₉ specific CD8 T cells.

The persistent cross-presentation of tyrosinase derived from tumor, which is localized to tumor-draining LN, is mechanistically distinct from the tolerogenic direct presentation of endogenous tyrosinase, which is pervasive among all LN (Fig. 1). The different mechanisms of presentation for endogenous and tumor-derived tyrosinase pose interesting questions as to why endogenous tyrosinase is not cross-presented. One possibility involves the limited availability of tyrosinase as an Ag in the endogenous setting. Upon tumor outgrowth a more substantial population of melanocytes is available to provide a source of tyrosinase. Furthermore, the inflammation and/or melanocyte destruction caused by tumor outgrowth may result in cross presentation. Nonetheless, the distinct modes of presentation reveal an opportunity to target self-tolerance mechanisms without inhibiting the response to tumor. An interesting possibility is to determine whether intentionally delete endogenous Tyr₃₆₉ presenting cells prior to adoptive transfer enables more effective control of subsequently introduced tumors.

Phosphopeptides as novel tumor antigens derived from proteins essential for tumor survival

Virtually all of currently identified melanoma-associated antigens (Ags) are derived from one of two sources: proteins that are expressed in normal melanocytes and concerned with pigmentation (MDPs); and proteins that are expressed only in testes and a fraction of tumors

(cancer-testes Ags) see <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>). In tumors other than melanoma, a small number of Ags have been identified that are derived from proteins that are objectively associated with cellular transformation and/or metastasis. These include epitopes from survivin, Her2/neu, p53, WT-1, and telomerase (44-48). These are attractive targets for immunotherapeutic development based on the hypothesis that immune escape through mutation of such Ags may be more difficult without compromising the malignant phenotype.

A systematic search for endogenously expressed peptide Ags derived from proteins associated with transformation or metastasis requires an approach that can identify potentially important peptides based on the function of the underlying source protein. It is well-established that many signal transduction pathways that rely on phosphorylation and dephosphorylation are altered or dysregulated in cancer cells, and some of these have been directly associated with alterations in cellular growth control, apoptosis, and metastasis (49-52). Indeed, a number of prominent activating mutations in kinases and inactivation of phosphatases involved in the MAPK and Akt signaling pathways have recently been reported and evaluated in melanoma (53-56). We hypothesized that peptides that are derived from proteins differentially phosphorylated on serine, threonine, and tyrosine as a result of these dysregulated signaling pathways would be attractive candidates for MHC associated Ags that would be associated with cancer-relevant cellular processes in melanoma. Therefore, we have analyzed mixtures of more than 10,000 peptides presented by HLA-A*0201 on the surface of two melanomas, and compared them with the peptides displayed on either an ovarian carcinoma, or an Epstein Barr virus-transformed B lymphoblastoid cell line (BLCL). Of the 36 phosphopeptides detected in association with HLA-A*0201, eleven are presented on one or more cancer cell lines, but not on the BLCL, with ten of the eleven presented on at least one of the melanoma cell lines (57). Nine of these melanoma-presented phosphopeptides were derived from known phosphorylated source proteins and two contain known phosphorylation sites (57) (Table 1). These results strongly suggest that the observed HLA-A*0201-associated phosphopeptides are not derived from aberrant phosphorylation of defective translation products (58), but instead, from degradation of properly folded, biologically active proteins that are post-translationally modified by their respective kinases.

The targeting of tumor Ags derived from proteins that are vital for the melanoma's survival and metastatic potential is attractive since down-regulation and/or mutations of genes that encode these proteins would compromise their ability to maintain malignant characteristics (59). We have identified a number of phosphopeptides that are differentially displayed on melanoma cell lines, and seven of these peptides are derived from source proteins that are either overexpressed or dysregulated in cancer cells (Table 1). Knockdown of several of these source proteins (IRS2, β -catenin, c-Jun, and BCAR3) by siRNA leads to loss in proliferative ability or migratory properties of cancer cells (60-66), suggesting they are critical to the malignant phenotype. In addition, seven of these proteins, are known to either be up-regulated or more active in several different types of cancer cells (62;67-78), again lending support for their importance as targets for cancer immunotherapy.

To support the potential use of these class I MHC-restricted phosphopeptides as immunotherapeutics against melanoma, we have demonstrated these HLA-A*0201-restricted phosphopeptides are immunogenic *in vivo* in HLA-A*0201 recombinant transgenic mice and *in vitro* after incubation with human PBMC (57;79) (unpublished results). These CD8⁺ T lymphocytes secrete IFN γ when they specifically recognize target cells that have been pulsed with synthetic peptides corresponding to the phosphorylated forms of these epitopes but not the non-phosphorylated form of the epitope. In addition, the CD8⁺ T cells also discriminate more than just the phosphate group on the peptides, as they recognize the phosphopeptide they were generated against and not another phosphopeptide with a phosphorylated residue at the

same position in the epitope sequence. These phosphopeptide-specific CD8⁺ T cells also recognize endogenously processed and presented phosphopeptide on the surface of human melanoma cells (57). Because the phosphopeptides we have identified are derived from overexpressed signaling proteins in tumor cells, T lymphocytes may be able to selectively target tumor cells while limiting self-reactivity and damage on peripheral host tissues. We are currently evaluating the functional utility of phosphopeptides in cancer immunotherapy through phosphopeptide-specific T cell adoptive therapy and active immunization protocols. This will set the stage for future *efficacy* clinical trials that will determine the value of phosphopeptides as immunotherapeutic agents for melanoma, and ultimately, for other types of cancer. Overall, these results indicate that phosphopeptides represent a new category of shared cancer antigens related to cellular growth control processes.

The roles of vaccination in promoting more effective anti-tumor immunity

The identification of class I MHC associated peptide Ags expressed on tumors is only the first step in the development of effective immunotherapies as the modalities to deliver them in an immunogenic form are also necessary. Immunization with dendritic cells (DC) pulsed with synthetic peptide Ags is attractive because these cells superior ability to activate naïve T cells (80-83). Exogenous DC are unique antigen delivery vehicles because they distribute selectively into different lymphoid compartments when injected into the body by different routes (84-88). The use of peptide-pulsed bone marrow derived DC (BMDC) enables us to vary the number of cells injected, the number of distinct peptide Ags displayed, and the density of peptide presented on each cell. We have explored this model system to identify the underlying mechanisms responsible for generating efficient anti-tumor immunity.

Vaccination promotes high avidity recall responses against self-antigens

Our early work established that self-tolerance to Tyr₃₆₉ in AAD⁺ tyrosinase⁺ mice substantially compromised their ability to control B16-AAD melanoma (10). However, vaccination with the closely related human Tyr₃₆₉Y epitope, and less effectively, murine Tyr₃₆₉, generated memory cells that enabled AAD⁺ tyrosinase⁺ mice to control B16-AAD to a similar extent as did naïve AAD⁺ albino mice (10;89;90). Bulk primary effectors elicited by vaccination of tyrosinase⁺ mice with human Tyr₃₆₉Y recognized murine Tyr₃₆₉ about 100-fold less well than Tyr₃₆₉Y, based on peptide doses that elicited half-maximal IFN γ secretion *ex vivo* (Fig. 2). Furthermore, only 65% of the human Tyr₃₆₉Y elicited cells were cross-reactive on murine Tyr₃₆₉ at the maximum peptide dose. Thus, by analogy with humans, self-tolerance to Tyr₃₆₉ in AAD⁺ tyrosinase⁺ mice is incomplete. It is not clear if the Tyr₃₆₉-reactive cells in tyrosinase⁺ mice have yet to encounter tolerogenic Ag or are ignorant because their avidity for Tyr₃₆₉ is too low, or a mixture of both.

Given these results, an important question is how immunization leads to memory T cells that are effective against a Tyr₃₆₉⁺ tumor. We addressed this by examining the functional avidity of primary and recall effector cells *ex vivo* after immunization of either AAD⁺ albino or AAD⁺ tyrosinase⁺ mice with either human Tyr₃₆₉Y or murine Tyr₃₆₉. The functional avidity of recall effector cells elicited with Tyr₃₆₉Y increased 10-20-fold over that of primary effectors in either albino and tyrosinase⁺ mice {Bullock, 2003 11747 /id} (Fig. 2). Similarly, immunization of albino mice with murine Tyr₃₆₉ increased the avidity of recall effectors 10-20 fold over primary effectors (Fig. 2). However, immunization of tyrosinase⁺ mice with Tyr₃₆₉ increased the functional avidity for murine Tyr₃₆₉ by almost 100-fold over primary effectors (Fig. 2). These high avidity T cells also recognize Tyr₃₆₉ displayed on B16-AAD tumor cells, demonstrating their potential for tumor control (92;93). These results suggest that one mechanism by which vaccination can improve anti-tumor immunity is by disproportionately augmenting the avidity of recall T cells specific for self-Ags.

Vaccination overcomes the aberrant differentiation of CD8 T cells that occurs in late stage tumor-draining LN

The results described above indicate that B16 tumors directly activate Ag specific T cells and provide Ag for cross-presentation. However, the resulting immune response is usually inadequate to control the tumor. While many studies have evaluated the suppressive microenvironment of the tumor itself {Blohm, 2002 12280 /id}{Radoja, 2001 1615 /id}, we turned our attention to the initial differentiation of CD8 T cells in the tumor-draining LN. Responses of naïve cells were analyzed after adoptive transfer into mice bearing early stage (1d after inoculation) and late stage (14d after inoculation) tumors. In both situations, the T cells activated comparably and underwent robust proliferation. However, in mice bearing late stage tumors, the cells failed to acquire the ability to secrete IFN γ , and exhibited substantially reduced cytotoxic activity (42). These CD8 T cells did not produce IL-4 or IL-10 (42), hallmark cytokines of Tc2-like and CD8 regulatory T cell differentiation programs, nor do they express Foxp3 (unpublished results). This defect in effector function accompanied by unimpaired proliferation is distinct from observations of a fully anergic phenotype that have been more commonly reported.

The direct presentation of Ag by B16 cells that occurs in late-stage tumor bearing mice suggested that this might be responsible for the selective defect in effector function, but the defect was observed when either direct or cross presentation were evaluated separately (42). However, when late stage tumor bearing animals were provided with systemic Ag, the defect was only evident in T cells activated in the tumor-draining LN: CD8 cells in non-draining LNs responded normally (42). Collectively, these results demonstrate that aberrant differentiation of these CD8 T cells is a consequence of the unique microenvironment of the tumor-draining LN.

While not attributable to direct Ag presentation by the tumor, the possibility remains that tumor cells that have metastasized to the LN are responsible for shaping this microenvironment. Accordingly, we have attempted to understand the nature of this microenvironment by evaluating cellular changes within the tumor-draining LN. Gr-1⁺ and CD11b⁺ myeloid-derived suppressor cells and CD25⁺ and/or FoxP3⁺ regulatory T cells have both been reported to accumulate in tumors and tumor-draining LN and to suppress CD8 T cell effector activity (94-96). However, we have not observed accumulation of either cell type in late stage tumor-draining LN of B16 bearing mice (unpublished results). Indolamine oxidase secreting plasmacytoid DC have been reported in tumor-draining LN for both B16 and other melanomas (97). Additionally, a substantial increase in the number of B lymphocytes in B16 tumor-draining LN has also been reported (98), which is associated with both lymphangiogenesis (98) and suppression of tumor immunity (99-101). These cells remain to be evaluated for their presence and contribution to the aberrant differentiation of tumor-specific CD8 T cells in our model system.

We have also sought to gain insight into the role inhibitory molecules may be playing in late-stage tumor-draining LN, and their involvement in aberrant differentiation. The co-inhibitory molecule B7-H1 is expressed by B16 melanoma, is up-regulated on DC exposed to tumor-derived cytokines, and has been shown in other systems to modulate T cell activation via its ligand, PD-1 (102-104). We found that PD-1 was expressed on B16-specific CD8 T cells in the tumor-draining LN and the tumor (unpublished). In keeping with this, treatment of late stage tumor-bearing mice with anti-B7-H1 enhanced the extent of proliferation of CD8 T cells in tumor-draining LN (Fig. 3A). However, these cells still did not produce IFN γ . Thus, the PD1-B7-H1 axis does not inhibit the differentiation of CD8 effector cells in tumor-draining LN. Conversely however, immunization of late stage tumor-bearing mice with CD40L-activated exogenous DC presenting tumor Ag resulted in complete differentiation of functional CD8 effectors based on IFN γ secretion {Hargadon, 2006 12376 /id}. We achieved the same

result by using IL-12 (Fig. 3B). Antigen presentation to CD8 T cells in the absence of IL-12 has been shown to result in proliferation without acquisition of effector function (106), demonstrating that it functions as a “signal 3” cytokine. Collectively, these results suggest that the aberrant differentiation of CD8 T cells in the late-stage tumor-draining LN may be caused by the failure of endogenous APC to provide IL-12. Importantly, these results also demonstrate a selective role for vaccination or immunotherapy modalities that provide mature DC functionality (BMDC matured with CD40L, IL-12, but not systemic Ag) can induce appropriate effector T cell differentiation. These results reveal potential weaknesses in vaccination protocols that rely on uptake of Ag by endogenous antigen presenting cells.

The existence of immunosuppressive networks in tumors is well established (107). Our studies highlight the tumor-draining LN as another locus for immunosuppression, and suggest that the mechanisms operating in it may be distinct from those in the tumor. Thus, effective therapeutic vaccination/immunotherapy strategies need to operate on T cells in both of these environments to achieve maximal effectiveness.

Importance of the site of T cell activation in anti-tumor efficacy

Several years ago, we demonstrated that the route of injection of peptide-pulsed BMDC influenced the ability to control tumors growing in different locations. Thus, subcutaneous (SC) delivery of Tyr₃₆₉-pulsed CD40L-activated BMDC enabled control of B16-AAD tumors growing in subcutaneous sites, with modest control of lung lesions (90). Conversely, intravenous (IV) immunized mice controlled lung lesions very well, but failed to control subcutaneous tumors. Potential explanations for these findings were based on the additional observation that BMDCs exhibited restricted trafficking to secondary lymphoid organs, limiting the sites of T cell activation. Based on the up-regulation of CD69 and CD25 on adoptively transferred T cells 24h after activation, we established that BMDC traffic to distinct secondary lymphoid organs depending on injection route. Those injected SC traffic to axillary/brachial LN, while those injected IP traffic to mesenteric and mediastinal (paratracheal and parathymic) LN, and those injected IV traffic to the paratracheal LN and spleen (88) (manuscript submitted). Thus, the site of T cell activation might dictate the distribution of memory cells, or their ability to migrate to different peripheral compartments after activation. The route might also dictate the size of the T cell response. We have subsequently evaluated these issues in more detail.

Expression of adhesion proteins and chemokine receptors after activation in distinct lymphoid organs

The extent of infiltration of tumors by CD8 T cells has been correlated with a positive prognosis in patients (108;109), but little is understood about the factors that control the trafficking of these T cells. T cell infiltration into tumors can be ablated with pertussis toxin, which blocks chemokine receptor signaling (110). In support of a role for chemokine receptors, CXCR3 and CCR5 are expressed on CD8 T cells infiltrating tumors (111;112), and CXCR3 expression is associated with increased patient survival (113;114). Further, CCR4 on human CD8 T cells can mediate migration toward melanoma cells *in vitro*. There are conflicting reports concerning the involvement of CXCR4 in CD8 T cell infiltration of melanoma (115;116), and the roles of P-selectin and E-selectin in tumor protection (117;118).

In conjunction with injection of BMDC by different routes, we have evaluated the expression of chemokine receptors and adhesion molecules on adoptively transferred naïve T cells activated in different lymphoid compartments. Most activated CD8 T cells expressed CXCR3, CCR5, and CCR9, regardless of the secondary lymphoid organ in which they were activated (88) (unpublished). The lack of selectivity of CCR9 expression is particularly surprising, since it has been shown to be induced by DCs from mesenteric LN and Peyer's patches, but not those

from axillary LN (119;120). Whether this difference reflects a difference between *in vitro* and *in vivo* influences, or if CCR9 induction is an inherent property of BMDC irrespective of the LN microenvironment remains to be determined.

In contrast to the lack of selectivity in expression of these chemokine receptors, CD8 T cells activated in the mesenteric LN after IP immunization up-regulated $\alpha 4\beta 7$ integrin, whereas those activated in most other LN and spleen did not (88) (unpublished). Co-expression of CCR9 and $\alpha 4\beta 7$ defines two subpopulations in the mesenteric LN, both of which are $\alpha 4\beta 7^+$, but only one of which expresses CCR9 and migrates to the gut. A small subset of cells activated in the mediastinal LN express an intermediate level of $\alpha 4\beta 7$ integrin, but these cells fail to co-express CCR9 and do not migrate to the gut (88) (unpublished). In contrast, most cells activated in the mediastinal LN, as well as spleen, express the $\alpha 4\beta 1$ integrin (88) (unpublished results), which is associated with generalized homing to sites of inflammation (121-123). These activated cells consistently accumulated in the lungs of immunized animals. Finally, cells activated in the axillary/brachial LN uniquely express the 1B11 epitope of CD43 (unpublished), which has recently been described as an E-selectin ligand (124). Collectively, this work establishes that CD8 T cell activation leads to 3 major subpopulations based on expression of $\alpha 4\beta 7$, $\alpha 4\beta 1$, and 1B11 which are produced in the mesenteric LN, mediastinal LN/spleen, and axillary/brachial LN, respectively. These experiments illustrate the influence the local microenvironment can have on the induction of adhesion proteins on CD8 T cells, and provide support for the idea that differences in the expression of these molecules determine the BMDC immunization route dependent differences in tumor control we have observed.

Redistribution of activated CD8 T cells to non-priming LN and its relationship to T cell memory

While the preceding results establish relevant differences in the expression of adhesion molecules on primary CD8 T cell effectors primed in distinct secondary lymphoid organs, they do not address the issue of memory cell localization, which might also influence regional tumor immunity. In the course of evaluating the progression of the primary response into memory, we made the surprising observation that, despite highly localized BMDC priming, activated T cells were present in most LN examined within 3 days. Using the drug FTY720, which blocks egress of lymphocytes from secondary lymphoid organs by interfering with S1P1 signaling {Brinkmann, 2002 12250 /id}, we demonstrated that this reflects the redistribution of activated cells from the site of activation to Ag free LN (88) (manuscript submitted). Even when T cell adoptive transfer was delayed by 4 days after DC immunization, FTY720 revealed that all activation was confined to the LN to which the DC initially migrated (Fig. 4). Most redistribution from priming to Ag-free LN occurs via a CD62L dependent mechanism (manuscript submitted), but we have also observed that cells activated in mesenteric LN to express a high level of $\alpha 4\beta 7$ redistribute to the mediastinal LN in a manner dependent on this integrin (88). Although initially done using adoptively transferred T cells, we have also observed redistribution into Ag free LN by endogenous CD8 T cells activated by BMDC (manuscript submitted). Our results indicate that early redistribution of differentiated CD8 T cells to non-priming secondary lymphoid organs is a general feature of the immune response to peptide-pulsed CD40L-activated BMDC.

The rapid redistribution of activated CD8 cells into Ag free LN suggested that they might be precursors of central memory cells. To characterize these cells further, we trapped them by delayed administration of FTY720, and evaluated their phenotype over time (manuscript submitted). We found that a significant percentage of these cells expressed a high level of CD62L, although this fraction declined when the adoptive transfer number was reduced. However, CD62L dependent redistribution of both populations was always evident. Both of these populations also expressed CD44 and $\alpha 4$ integrins, produced IFN- γ after a short *ex*

vivo peptide restimulation, and were stable for at least 6 days during FTY720 treatment. Thus, these cells exhibited characteristics of well-differentiated effectors. However, in keeping with the possibility that they contained memory precursors, they began to express CD127, which has been associated with memory T cell potential (121;122), by day 7 after immunization. To unambiguously establish the presence of memory precursors, we electronically sorted divided cells expressing either high or low levels of CD62L from Ag free LN 3d post-immunization, injected them into naïve, recipients, and established that they both persisted for at least 3 weeks and responded to Ag challenge. These studies indicate that early redistribution of activated CD8 T cells seeds Ag free LN with cells that behave as memory T cell precursors.

Collectively, these results demonstrate that initially localized primary responses lead to a systemic distribution of memory cells among LN. Thus, the injection route dependent control of tumors growing SC and in lung does not seem to be strongly tied to memory cell distribution. However, based on the demonstration that the localization of primary responses to BMDC determines the expression of homing receptor molecules, and correlates with different peripheral tissue localization of activated T cells. These results suggest that regional tumor control based on route of immunization may be due to homing characteristics imprinted on memory cells based on the site in which their precursors were activated during the primary response. Determining the contributions of the site of priming, the site of memory residence and the site of secondary priming to the homing molecule expression of CD8 T cells and the influence of these on CD8 T cell infiltration of tumors is an ongoing focus of our work.

Localization limits the size of the primary CD8 T cell response

When BMDC are injected SC in the scapular fold, about 3000 rapidly and selectively accumulate in the axillary/brachial LN, regardless of the number injected (90;125). As already mentioned, no DC accumulate in any other LN. This limited LN capacity limits the size of primary and recall immune responses and the ability to control subcutaneous tumors (125). In contrast, the size of these responses in the spleen and control of lung tumors is correlated with the number of DC injected, because DC infiltration into this organ is not limited by injection number over a very broad range (125). A second aspect of this is that the number of CD8 T cells activated is limited by their relatively slow recirculation among different LN, leading to a large residual population of naïve cells. However, we found that distributing DC into two non-contiguous LN led to discrete priming in each LN, substantially larger recall responses in both priming LN and a 3rd non-contiguous LN, and significantly decreased outgrowth of subcutaneously growing B16-AAD tumor. In addition, we found that it was possible to condition LN through an initial injection of activated, but unpulsed DC. This resulted in the subsequent influx of larger numbers of both Ag-pulsed DC and naïve T cells, and was also accompanied by both increased primary immune response magnitude and subsequent tumor control. Collectively, these results emphasize that the limited distribution of exogenous DC upon immunization results in activation of only a fraction of the available Ag-specific T cells present in the immune system as a whole. Maneuvers to enhance the size of the response, while still retaining selectivity in induction of homing receptor molecules based on the location of the priming site, represent an important and overlooked opportunity to improve the immune response to tumors.

Concluding Remarks

In this review, we have highlighted two interrelated issues relevant to enhancing the immune response to tumors: the nature of tumor Ags, and the mechanisms by which to enhance their ability to augment tumor immunity. While many tumor Ags have been defined over the last 17 years, it is not clear that they are optimal, or that we understand how to maximize their utility. Tyrosinase is a self-Ag that is nonetheless a significant target of the immune response that develops in many melanoma patients. The demonstration that tolerance to tyrosinase is

based on a distinct mode of presentation by a LN resident cell, rather than through cross-presentation by DC, offers an important opportunity to manipulate tolerance. However, to understand whether other Ags, including other MDP, engender tolerance through this mechanism is extremely important. The identification of phosphopeptides on melanoma cell lines has revealed a new category of shared cancer antigens related to dysregulated signaling pathways and growth control processes, whose full potential still will require substantial work to uncover. Using CD40L-activated BMDC, we have also uncovered several important aspects by which vaccination can augment immunity, as well as some limitations. BMDC differentially up-regulate the avidity of self-reactive T cells, and can prevent the aberrant differentiation of T cells in late-stage tumor draining LN. However, the basic mechanisms by which they do these things still remain to be determined. Finally, we have demonstrated the critical importance of the site of activation, as determined by the route of vaccination, in determining the patterns of T cell differentiation and tumor control. It is important to remember that even in an individual with metastatic disease, solid tumors are localized, and the patterns of T cell homing seem to play an important role in determining whether local control is effective. We expect that this issue will take on additional complexity as it is investigated further, based on such factors as chemokine secretion by different tumors and the nature of the tumor vasculature. Nonetheless, we believe that these factors must be taken into account in improving our current generation of active immunotherapeutic approaches.

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Fig. 1.

Endogenous Tyr₃₆₉ is presented by a radioresistant cell, while tumor-derived Tyr₃₆₉ is both cross-presented and directly presented by the tumor. In peripheral LN draining normal, on-inflamed tissue, tyrosinase is not presented by Langerhans cells or DCs draining from the skin (top, blue box). Rather, tyrosinase is expressed within the LN, and is likely directly presented to tyrosinase-specific T cells inducing activation and subsequent deletion of self-reactive cells. In contrast, in late stage tumor-bearing mice (bottom, red box), antigen is both cross-presented by dendritic cells, as well as directly presented in the LN by metastatic tumor. In this context, T cells are activated, and though they are not deleted, these cells have impaired effector function.

Fig 2.

Comparison of functional avidities of primary and recall effector CD8⁺ T cells for Tyr369 and Tyr369(Y) in tyrosinase⁻ and tyrosinase⁺ AAD⁺ mice. CD8⁺ T cells from primary (Prim) or recall (Rec) responses following immunization of albino (Alb) or tyrosinase⁺ (Tyr⁺) mice with Tyr369(Y) were incubated with C1R-AAD stimulator cells pulsed with increasing concentrations of Tyr369 (open symbols) or Tyr369(Y) (closed symbols) and assessed for activation by costaining for CD8 and the intracellular accumulation of IFN γ . Data plotted are the mean of 2 or 3 mice per group $\pm$ standard error of mean. Data has been normalized to the maximum response obtained by each T cell population after stimulation with the peptide used to generate the T cell response. Data are from one of four similar experiments.

Fig. 3.

Administration of IL-12, but not disruption of PD-1-B7-H1 interaction, induces CD81 T-cell differentiation in tumor-draining LNs of late stage tumor-bearing mice. C57BL/6 mice were injected with 4×10^5 B16-OVA at the indicated day before adoptive transfer of 2×10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-I cells. (A) Mice were injected intraperitoneally with 100 μ g of the blocking antibody to B7-H1 (10B5, provided by Dr. Leiping Chen) or control hamster IgG every other day starting 1 day before adoptive transfer. Five days after adoptive transfer, tumor-draining LNs were harvested, and OT-I cells were assessed for IFN γ production. (B) Mice were either untreated or treated with 1 μ g IL-12 at the time of adoptive transfer. Five days later, the mediastinal LN was harvested and OT-I cells were assessed for the production of IFN γ .

Fig. 4. Restricted trafficking of BMDCs and activation of CD81 T cells in the axillary/brachial LNs. C57BL/6 mice were immunized subcutaneously with 10^5 SIINFEKL-pulsed BMDCs four days prior to adoptive transfer of 10^6 CFSE-labeled OT-I cells. Mice were treated with FTY720 where indicated. The indicated lymphoid organs were harvested, and OT-I cell proliferation was analyzed by CFSE dilution.

HLA-A*0201-restricted phosphopeptides displayed on melanoma are derived from source proteins associated with malignant transformation or metastasis

Table 1

Sequence ^a / Source protein ^b	Source protein function ^d	Source protein association with transformation/ metastasis ^c
YLDpSGIHSGA / β -Catenin	<ul style="list-style-type: none"> Target of Wnt pathway Activates transcription factors TCF & Lef 	<ul style="list-style-type: none"> Over-expressed or in constitutively activated state in many cancers Knockdown inhibits proliferation of colon cancer and pediatric hepatic tumors
RVApSPTSGV / Insulin Receptor substrate-2 (IRS2)	Docking protein involved in mitogenic signaling by insulin growth factor I & insulin	<ul style="list-style-type: none"> Over-expressed in pancreatic cancer & hepatocellular carcinomas resulting in GSK-3β inhibition and block in apoptosis Knockdown promotes apoptosis of hepatocellular carcinoma cells Over-expression in mammary gland leads to hyperplasia, tumorigenesis & metastasis Regulates motility in metastatic human breast cancer cell lines
KLApSPLELRL / C-Jun/JunD	Component of AP-1 transcription factor	<ul style="list-style-type: none"> C-Jun can induce oncogenic transformation Phosphorylation at Ser⁷³ enhances AP-1 transactivator activity and c-Jun stability Jun-C knockdown blocks androgen-dependent growth of prostate carcinomas
IMDRpTPEKL / Breast cancer anti-estrogen resistance 3 (BCAR3)	<ul style="list-style-type: none"> GDP-GTP exchange factor that binds to p130^{Cas} & Src Interacts with Rac and PAK1 to promote cyclin D1 expression Activates PI-3 kinase 	<ul style="list-style-type: none"> Confers resistance to growth inhibition by anti-estrogens in metastatic breast carcinoma Knockdown inhibits migration of breast cancer cells
KLLpSPSNEKL / Ub-carboxyl terminal hydrolase 10 (USP-10)	<ul style="list-style-type: none"> Ubiquitin-specific protease Binds to androgen receptor and mediates its nuclear activity 	<ul style="list-style-type: none"> Over-expression in glioblastoma multiforme associated with poor patient survival Over-expressed in breast cancer tissue
VMIGpSPKKV / Tensin-3	<ul style="list-style-type: none"> Component of focal adhesions Substrate for EGFR-induced Src Possible regulator of motility 	<ul style="list-style-type: none"> Over-expressed in breast cancer
KLLDFGSLpSNLQV / Ribosomal protein S17	Phosphorylated by p70 S6 kinase after Rapamycin treatment	<ul style="list-style-type: none"> Over-expressed in hepatocellular carcinoma
RTTpSPTYGL / β-Synemin	Intermediate filament protein	Unknown
RLFPpSKELRC / TDI1D transcription initiation factor subunit 13	Transcription initiation factor	Unknown
RTLpSHISEA / FAM65A	Unknown	Unknown

^a pS and pT represent phosphorylated Ser or phosphorylated Thr residues, respectively. Bolded residue indicates a known phosphosite (<http://www.phosphosite.org>).

^b Bolded text indicates known phosphoprotein.

^c from the Human Protein Reference database (<http://www.hprd.org>) and the NCBI (<http://www.ncbi.nlm.nih.gov>).