

# Mechanisms of T Cell Tolerance and Suppression in Cancer Mediated by Tumor-Associated Antigens and Hormones

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**Abstract:** Despite recent advances in vaccine technology, vaccines designed to elicit T cell-based anti-tumor immunity have only achieved partial success in the clinic. The underlying reason probably stems in part from the ability of tumors to repress cognate T cell responses, which appears to operate at two separate levels. In some cases, tumors engage a variety of immunosuppressive pathways that inhibit primed effector T cells from functioning when they enter the tumor microenvironment. Some of these immunosuppressive mechanisms include the production of cytokines such as TGF- $\beta$  and the recruitment or differentiation of regulatory T cells. In contrast, other types of tumors induce a systemic impairment in the function of tumor-reactive T cells (i.e., tolerance). Tolerance to tumor antigens can be mediated through the same mechanisms that induce T cell tolerance to normal self-antigens in order to avoid autoimmunity, and can develop not only towards differentiation antigens that are expressed on both tumors as well as on the normal tissues from which they derive, but can also develop rapidly towards tumor-specific antigens. Additionally, both naive and effector T cells are susceptible to tolerization, suggesting that tolerance can potentially dampen both the priming and effector phases of anti-tumor T cell responses. Certain hormones can influence both tumorigenesis as well as T cell function and tolerance, and thus hormonal therapies could potentially impact the efficacy of T cell-based therapies. An example of this type of interaction that will be discussed in detail is the relationship between androgens and prostate cancer.

**Keywords:** T cell tolerance, T cell suppression, effector T cells, cancer, prostate cancer.

## INTRODUCTION

Recent advances in the ability to prime robust effector and memory T cell responses has fueled the development of therapeutic strategies to elicit anti-tumor immunity. Nonetheless, results from recent clinical trials testing a variety of T cell-based immunotherapeutic approaches have only demonstrated partial successes [1, 2]. This is likely to be at least partially due to the ability of tumors to dampen cognate T cell responses. Thus, the development of successful immunotherapeutic strategies to treat cancer will likely require the combination of elements that both optimize tumor-reactive effector T cell responses while simultaneously neutralizing the inherent immune-dampening activities associated with tumorigenesis.

Tumors utilize multiple mechanisms to dampen cognate T cell responses, which include the direct inactivation of tumor-reactive T cells at the systemic level (i.e., tolerance) that restricts the repertoire of tumor-reactive specificities that can be primed through vaccination, as well as creating an immunosuppressive microenvironment that limits the ability of primed tumor-reactive T cells to express their effector functions when they migrate into the tumor. The rules governing which mechanism(s) a particular tumor utilizes to dampen cognate T cell responses are not well established, but are likely influenced by a number of factors such as the tissue of origin, the pathway of oncogenesis, sites of

metastasis, expression of immunoregulatory molecules and hormonal factors.

## THE TUMOR MICROENVIRONMENT CAN SUPPRESS EFFECTOR T-CELL FUNCTION

The first evidence demonstrating that tumors can suppress cognate T cell responses ironically came from the same studies demonstrating that tumors can elicit T cell responses. Mice harboring established carcinogen-induced transplantable tumors can reject a second transplant of the same tumor, and T cells harvested from mice with established tumors can confer protection against tumor growth when transferred into naive syngeneic mice that are simultaneously challenged with the same tumor. This phenomena of concomitant immunity (reviewed in [3]) thus indicated that while tumors have the potential to prime specific T cells responses, they are also capable of suppressing the function of these effector T cells when they enter the tumor microenvironment. Although initial murine studies suggested that concomitant immunity was more likely to occur with high dose carcinogen-induced tumors compared to spontaneously arising tumors [3], the subsequent observation that T cells with tumor-specificity commonly infiltrate certain human tumors such as melanoma [4] suggested that naturally arising tumors are also capable of eliciting cognate T cell responses while simultaneously inhibiting these T cells from expressing their effector functions in the tumor microenvironment.

Some of the first mechanistic insights into how immunogenic tumors can suppress cognate T cells responses

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were made by North and colleagues who demonstrated that eliminating CD4 cells allowed CD8 cells to mediate regression of advanced tumors [5, 6]. This result suggested that tumor-induced T cell responses can be somewhat complex in as far as that there could be simultaneous development of effector CD8 cell as well as suppressive/regulatory CD4 cell activities. During the last decade numerous regulatory T cell sub-sets have been defined, although the best studied are CD4<sup>+</sup>CD25<sup>+</sup> cells [7] whose regulatory activity is programmed by the forkhead transcription factor Foxp3 [8-10]. Several murine studies have indicated that systemic neutralization of this regulatory T cell population (commonly referred to as Tregs) via anti-CD25 mAb treatment augments tumor immunity [11-14], and conversely large numbers of CD4<sup>+</sup>CD25<sup>+</sup> Tregs are present in certain human tumors [15-17]. Additionally, in human ovarian cancer increased frequency of tumor-infiltrating Tregs [18] or increased ratio of Tregs:CD8<sup>+</sup> T cells in tumors [19] correlates with poorer prognosis.

The precise mechanisms by which CD4<sup>+</sup>CD25<sup>+</sup> Tregs dampen anti-tumor T cell responses are only now beginning to be elucidated, although it appears that they might function directly in the tumor microenvironment to prevent infiltrating tumor-reactive effector T cells from expressing their effector functions. In a murine fibrosarcoma model in which Tregs infiltrate the tumor at high frequency at the later stages of tumor progression, local depletion of CD4<sup>+</sup> cells within the tumor can elicit tumor regression if the depletion is induced during the effector, but not priming, phase of the anti-tumor immune response. Furthermore, this treatment resulted in a shift from anti-inflammatory to pro-inflammatory cytokine expression within the tumors, and also enhanced the ability of tumor-infiltrating CD8<sup>+</sup> T cells to proliferate and express IFN- $\gamma$  [20]. The ability of Tregs to directly inhibit T cell responses during the effector (rather than the priming) phase has also been observed in the NOD type-1 diabetes model, where an absence of Tregs resulting from a null mutation in the *Foxp3* gene leads to more rapid tissue destruction by diabetogenic effector T cells once they have infiltrated the pancreatic islets [21].

An important question in understanding the relationship between tumor-infiltrating Tregs and the suppression of tumor immunity is how Tregs localize to the tumor microenvironment. In some cases tumors appear to secrete factors that recruit Tregs, as has been shown in human ovarian cancer where tumor cells as well as infiltrating macrophages recruit CCR4<sup>+</sup> Tregs into the local microenvironment via the chemokine CCL22 [18]. Tumors might also induce the differentiation of Tregs. Although the major pathway of Treg differentiation appears to occur in the thymus, which is programmed in thymocytes expressing T cell receptors (TCRs) with affinity/avidity for MHC-peptide complexes that are intermediate between those that induce positive and negative selection (i.e., natural Tregs) [22], CD4<sup>+</sup>CD25<sup>-</sup> cells can also be induced to differentiate into CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the periphery via a TCR- and TGF- $\beta$ -dependent pathway [23]. Given the propensity of tumors to express TGF- $\beta$  [24-26], this might represent an efficient mechanism by which tumors induce the differentiation of Tregs directly in the microenvironment. In

a murine B cell lymphoma model CD4<sup>+</sup>CD25<sup>-</sup> cells that recognize tumor-associated antigens are induced to differentiate into Tregs [27], although the potential involvement of TGF- $\beta$  in this process has not been determined. It has also been shown that a variety of tumors exhibit constitutive activation of Stat3 which induces the expression of various immunoregulatory factors that suppress tumor immunity through a variety of mechanisms, including increasing the number of Tregs in the tumor microenvironment [28].

Related to the question of how Tregs localize to the tumor-microenvironment is the role of tumor-associated antigen in this process. Given that TGF- $\beta$ -mediated differentiation of Tregs in the periphery requires TCR stimulation [23], it would seem likely that if CD4<sup>+</sup>CD25<sup>-</sup> cells differentiate into CD4<sup>+</sup>CD25<sup>+</sup> Tregs within the tumor-microenvironment that they would recognize tumor-associated antigens. Alternatively, it might also be possible that natural Tregs with specificity for tumor-associated antigens undergo expansion [29-31]. Consistent with either possibility, Tregs specific for tumor-associated antigens have been isolated from tumors [32]. This result does not necessarily imply that all tumor-infiltrating Tregs recognize tumor-associated antigens, although these tumor-specific Tregs might be the most potent in dampening tumor immunity given that Tregs appear to exert the greatest influence on T cells with similar antigen specificity [33, 34].

In addition to potentially facilitating the accumulation of Tregs, TGF- $\beta$  expressed by tumors (and possibly by infiltrating Tregs as well) might directly inhibit the function of tumor-reactive effector T cells. Exposure of CTL to TGF- $\beta$  inhibits the expression of several effector molecules such as perforin, granzyme A & B, Fas ligand and IFN- $\gamma$ , and neutralization of TGF- $\beta$  *in vivo* results in both re-expression of these effector molecules as well as enhanced tumor immunity [35, 36]. In some cases tumor-infiltrating CTL are unable to lyse tumor cells despite the expression of effector molecules. In response to TCR stimulation these CTL express IL-2 and IFN- $\gamma$ , but are unable to relocalize their microtubule-organizing center to the immunological synapse and thus release their lytic granules due to a defect in p56<sup>lck</sup> function [37]. Interestingly, these defective CTL fully recover their lytic function following a brief period of culturing in the absence of tumor [38], perhaps illustrating one way that tumors can suppress the function of tumor-reactive effector T cells locally in their microenvironment without dampening the function of these T cells systemically.

There are a number of other mechanisms employed by cells within the tumor microenvironment that appear to be immunosuppressive such as expression of factors that impair antigen presenting cell (APC) function [39], as well as enzymes that impair T cell function by altering amino acid metabolism [40, 41]. These mechanisms will not be detailed here since they will be covered more extensively in some of the accompanying reviews, but it nevertheless appears that there are likely to be a multitude of mechanisms employed by tumors to suppress the function of tumor-reactive effector T cells.

## TUMORS CAN INDUCE SYSTEMIC TOLERIZATION OF COGNATE T CELLS

The studies discussed above clearly indicate that certain tumors can elicit effector T cell responses, and must therefore simultaneously express immunosuppressive activities to block the function of these tumor-reactive effector T cells when they migrate into the tumor microenvironment. The immunogenic properties of these tumors may be related to their potential to generate inflammation when they invade surrounding tissue or metastasize [42]. Conversely, other tumors might not elicit inflammation either because they are able to grow and spread without causing tissue damage (e.g., hematopoietic tumors), or because they express activities that minimize inflammation when they do cause tissue destruction [43]. Overall, the potential of tumors to grow while eliciting minimal inflammation would be consistent with the potential to induce immunological tolerance [42]. For the purpose of this review, tolerance will be defined as an impaired ability of specific T cells to respond to antigenic challenge at the systemic level, as opposed to the previously described immunosuppressive mechanisms that impair T cell function locally in the tumor microenvironment. Evidence from numerous models indicates that T cell tolerance to tumor-associated antigens can occur, and that this tolerance can negatively impact tumor immunity.

Since many human and mouse tumor antigens fall into the category of differentiation antigens that are expressed on both tumors as well as the normal tissues from which they derive (e.g., tyrosinase [44, 45], TRP2 [46, 47] and Pmel-17/gp100 [48, 49]), it is likely that the pathways which tolerize the T cell repertoire to tissue-specific self-antigens in order to avoid autoimmunity will also negatively impact the ability of these same T cell specificities to mediate tumor immunity. To model the impact of pre-existing T cell tolerance to differentiation antigens on tumor vaccine efficacy, Hu et al. developed a transgenic mouse model in which the Friend murine leukemia virus envelope protein (env) was expressed under the control of a lymphoid-specific promoter. Env-specific T cells were tolerant in these animals as demonstrated by their failure to expand following vaccination with an env-expressing recombinant vaccinia virus, and this tolerance was associated with a failure of this vaccine to protect against subsequent challenge with an env-expressing transplantable erythroleukemia [50]. While this result illustrates that pre-existing tolerance to tumor-associated differentiation antigens can severely dampen tumor vaccine efficacy, tolerance is probably not always absolute given that T cell responses to certain tumor differentiation antigens can be elicited. Using a somewhat analogous transgenic mouse and transplantable tumor system, Sherman and colleagues demonstrated that when a tumor differentiation antigen is expressed on normal tissues in a more restricted fashion (limited mainly to pancreatic  $\beta$  islet cells) T cell tolerance is only partial - vaccination primes CTL expressing TCRs with reduced avidity for the tumor differentiation epitope relative to CTL primed in control non-transgenic mice. Furthermore, these low-avidity CTL could mediate protection to subsequent tumor challenge [51]. The possibility that tumor differentiation antigen-specific T cells that can be primed may tend to express low-avidity TCRs may help to explain why tumor

vaccines are sometimes only able to elicit partially effective tumor immunity.

The finding that T cell tolerance to tumor-associated differentiation antigens exists and can negatively impact the efficacy of tumor vaccines targeting these antigens is not particularly surprising given that tolerance induction through both central and peripheral mechanisms will have presumably been operative long before the initiation of tumorigenesis. It might therefore seem reasonable that tolerance would be less apparent for tumor-specific antigens such as those deriving from oncogenic viruses or mutated self-antigens given that they would in all probability not be present in the thymus to facilitate negative selection of cognate developing T cells, and would not be accessible to peripheral tolerance mechanisms prior to tumorigenesis. Nevertheless, numerous studies have indicated that T cell tolerance can develop rapidly towards tumor-specific antigens. When Bogen and colleagues transplanted a plasmacytoma into transgenic mice expressing a TCR specific for a class II-restricted peptide that derives from the hypervariable region of the idiotypic immunoglobulin expressed by that plasmacytoma, the anti-idiotypic CD4 cells underwent deletion [52]. Given that bolus injection of soluble foreign antigens induces immunological tolerance (in contrast to particulate antigen or antigen admixed with adjuvant that induces immunity) [53, 54], the potent tolerogenic nature of the tumor-specific antigen (i.e., idiotypic immunoglobulin) may have been related to its secretion into the blood stream at very high levels, a situation that would probably not be the case for most other tumor-specific antigens that are either expressed at lower levels or that remain cell-associated. To assess whether T cell tolerance can develop towards less abundant non-secreted tumor-specific antigens, Levitsky and colleagues developed a model in which naive TCR-transgenic CD4 cells specific for the model antigen influenza hemagglutinin (HA) are adoptively transferred into mice bearing a transplantable B cell lymphoma that expresses a low level of HA. Over several weeks, these naive HA-specific CD4 cells progressively lost the ability to both proliferate and secrete cytokines in response to *in vitro* or *in vivo* antigenic challenge [55].

Subsequent studies from various groups have confirmed that both CD4 and CD8 cell tolerance can develop towards antigens expressed on both transplantable as well as spontaneously arising tumors [56-60]. Tolerance does not develop in all tumor systems [61-64], underscoring the notion that different types of tumors vary in their capacity to induce tolerance. As discussed in the previous section, those tumors that elicit cognate effector (rather than tolerogenic) T cell responses must elaborate immunosuppressive mechanisms to inhibit the activity of tumor-reactive effector T cells that have migrated into the tumor microenvironment. Given the dynamic nature of tumorigenesis [65], it might be possible that the capacity of a given tumor to either prime or tolerize cognate T cells might change during disease progression. Indirect support for this possibility stems from the observation that melanoma patients can exhibit clonally expanded populations of non-functional tumor-associated antigen-specific CD8 cells [66], consistent with a scenario in which these tumor-reactive T cells are initially primed to undergo expansion but subsequently inactivated.

## MECHANISMS OF PERIPHERAL SELF-ANTIGEN AND TUMOR-ANTIGEN-INDUCED T CELL TOLERANCE

Since tolerization of tumor-associated antigen-specific T cells can restrict the repertoire of T cell specificities that can be primed through vaccination to mediate anti-tumor immunity, manipulations that can either block the development and/or restore the function of tolerant tumor-reactive T cells could enhance tumor vaccine efficacy. In this regard, understanding the cellular and molecular pathways that mediate tolerance will be critical.

For tumor-associated differentiation antigens that are also expressed on normal tissues, T cell tolerance should be mediated through the central and peripheral pathways that normally operate to prevent autoimmunity. Thus, the majority of self-reactive T cells undergo negative selection during development in the thymus, where immature T cells expressing TCRs that recognize MHC molecules presenting self-epitopes at high affinity/avidity undergo deletion [67-70]. Subsequently, mature T cells that recognize parenchymal self-antigens that are not presented in the thymus can be subjected to a variety of peripheral tolerance mechanisms such as deletion [71-73], functional inactivation (also referred to as anergy [74]) or suppression [75, 76].

It was initially thought that central tolerance functioned specifically to delete developing T cells with reactivity to self-antigens that were either ubiquitously expressed or that could gain access to the thymus via the circulation, while peripheral mechanisms performed the task of inactivating mature T cells specific for tissue-restricted self-antigens. More recent evidence, however, suggests a degree of overlap between central and peripheral tolerance. Expression of the transcription factor AIRE in thymic medullary epithelial cells (mTECs) induces low-level expression of a variety of tissue-restricted self-antigens that can mediate the deletion of developing cognate T cells [77-80]. Although AIRE extends the range of thymic tolerance, several lines of evidence strongly implicate that peripheral mechanisms are still essential for preventing autoimmunity. First, not all tissue-restricted self-antigens appear to be expressed in mTECs, and those that are expressed are generally present at low levels [81], suggesting that there is likely to be a high level of leakiness in this process. In fact, a substantial fraction of self-reactive T cells do escape thymic deletion [82], and it is well established in a variety of inbred mouse strains and other species that self-reactive T cells in the periphery of normal individuals can be induced to mediate autoimmunity following vaccination with cognate auto-antigen plus adjuvant [83]. The spontaneous development of autoimmunity in mice that either exhibit defective DC apoptosis [84] or that lack negative regulators of peripheral T cell responsiveness such as Foxp3 [9, 10], TGF- $\beta$  [85] and CTLA-4 [86] provides additional evidence that peripheral tolerance is critical for preventing autoimmunity.

Tissue-restricted self-antigens expressed in mTECs include certain tumor-associated antigens [87], suggesting that central tolerance does impact tumor immunity. Nevertheless, the understanding and ability to manipulate peripheral tolerance will likely have a greater potential to increase the efficacy of T cell-based therapies to treat cancer. Thus, thymic deletion will have occurred for the most part

prior to clinical diagnosis and administration of therapy, and T cell deletion cannot be reversed. In contrast, peripheral tolerance can involve mechanisms such as anergy/hypo-responsiveness that could potentially be reversed in the context of vaccination, and strategies that prevent the tolerization of adoptively transferred tumor-reactive effector T cells in the context of adoptive immunotherapy might also enhance anti-tumor immunity (as will be discussed shortly).

Since tumor-associated differentiation antigens exist as normal self-antigens prior to tumorigenesis, cognate T cells should be subject to normal tolerance mechanisms. Interestingly, mounting evidence suggests that these same mechanisms might also induce T cell tolerance to tumor-specific antigens. The studies by Bogen and colleagues demonstrated that plasmacytomas can secrete sufficiently large enough levels of idiotypic antibody into the circulation to reach the thymus and induce the deletion of developing anti-idiotypic T cells [52, 88]. Since most other tumor-specific antigens derive from mutated self-proteins, these unique epitopes cannot be encoded in the genome of thymic APCs, and assuming that they are not released into the circulation at high levels, it is unlikely that cognate T cells will undergo thymic deletion. It does appear, however, that tumor-specific antigens can be processed by similar peripheral tolerization machinery as normal parenchymal self-antigens. As a corollary to the system described previously in which naive TCR transgenic HA-specific CD4 cells become tolerant following adoptive transfer into mice harboring a transplantable tumor expressing HA (i.e., tumor-HA) [55], an analogous system was developed in which the same HA-specific CD4 cells are adoptively transferred into transgenic mice that express HA under the control of the rat C3(1) promoter (C3-HA mice) where HA is expressed in a wide variety of normal parenchymal tissues (i.e., self-HA) [89, 90]. In both the tumor-HA and self-HA models, the clonotypic CD4 cells initially display a surface marker phenotype indicative of activation, but ultimately develop a non-responsive phenotype similar to anergy [74] where they lose the ability to proliferate and secrete IL-2 following secondary exposure to antigen.

In addition to the similarity in the non-responsive phenotype of CD4 cells exposed to tumor-HA vs self-HA, tolerance in both cases was mediated through a similar antigen-processing pathway. Prior to the development of transgenic model systems to study peripheral T cell tolerance (e.g., [91, 92]), *in vitro* tolerance studies using Th1 clones indicated that anergy is induced when TCR-ligation occurs in the absence of costimulation (reviewed in [74]). This observation led to the notion that TCR-engagement without costimulation leading to non-responsiveness/anergy could potentially occur *in vivo* when T cells encounter their cognate antigens presented on either normal parenchyma or tumors (neither of which normally express costimulatory ligands). Additionally, even though B cell lymphomas do express costimulatory ligands such as B7 [55], the overall levels of costimulatory ligand expression is substantially less compared to dendritic cells (DCs) which represents the most potent APC subset [93], and normal B cells which also express low levels of costimulatory ligands have been reported to possess the capacity to induce T cell tolerance [94-97]. Thus, it was somewhat surprising when bone marrow chimera studies revealed that CD4 cell tolerance to

self-HA was not mediated through direct interaction between the HA-specific CD4 cells and HA-expressing parenchyma, but rather tolerogenic antigen presentation was mediated indirectly via bone marrow-derived APCs that had acquired parenchymal-HA [89]. This indirect or cross-presentation pathway can also facilitate the peripheral tolerization of self-reactive CD8 cells [98], and subsequent work has suggested that the cross-tolerizing APC is most likely a DC [99-101]. The ability of DCs to prime both effector and tolerogenic T cell responses appears to be regulated by the environment in which antigen is acquired. Thus, when DCs acquire pathogen-derived antigens the presence of invariant pathogen-derived inflammatory mediators (i.e., pathogen-associated molecular patterns or PAMPs) induce high expression levels of costimulatory molecules and cytokines that endow DCs with the ability to prime cognate naive T cells to develop effector and memory functions. In contrast, when DCs acquire self-antigens under steady-state conditions the absence of PAMPs results in a default expression level of sub-optimal costimulation that programs a tolerogenic T cell differentiation program that can involve the induction of anergy generally followed by deletion [102-107].

Returning to the HA-expressing B cell lymphoma model [55], given that the tumor appears to exhibit a tolerogenic sub-optimal costimulatory ligand expression profile, and also that it metastasizes to lymphoid organs, it seemed reasonable to presume that tumor cells would directly present HA to naive HA-specific CD4 cells to induce tolerance. Thus, it was notable that cross-presentation proved to be the predominant pathway of tolerance induction [108]. The finding that peripherally-tolerized self-reactive and tumor-reactive T cells can exhibit similar functionally-impaired phenotypes that can be induced by the same indirect antigen presentation pathway suggests that the peripheral tolerance machinery that normally operates to prevent autoimmunity might also help tumors to evade immune-neutralization by inducing the tolerization of tumor-specific T cells. The similarities between the tumor-HA and self-HA models do not necessarily exclude the possibility that there might be aspects of peripheral tolerance that are unique to tumors, but these similarities do suggest that a more detailed mechanistic understanding of peripheral tolerance to normal self-antigens will be relevant to understanding tolerance to tumor-specific antigens.

With regard to studying peripheral tolerance mechanisms that are common to both tumor and normal self-antigens, transgenic systems designed to examine the latter have certain advantages. For example, different founder lines generated using the same model antigen expression vector can express different levels of the model antigen due to differences in either the genomic location of transgene integration or the number of integrated transgene copies. This allows examination of the effect of antigen dose on T cell tolerization without introducing other variables such as differences in tumor burden. Additionally, tumor antigen presentation (and hence cognate T cell recognition and response) in systems where tumors develop in discrete anatomical locations generally occurs only in tumor-draining lymph nodes [59-61, 109]. While this restricted pattern of tumor-antigen presentation is important to examine with regard to understanding T cell tolerization induced by specific types of tumors, the disadvantage is that relatively

few tolerized T cells can be recovered for functional and biochemical analyses. In contrast, transgenic model self-antigen expression systems can be engineered so that the model self-antigen is expressed in multiple tissues, resulting in tolerance induction occurring in multiple lymphoid organs, and hence the potential to recover larger numbers of tolerized T cells for analysis.

Some of the initial model self-antigen TCR transgenic adoptive transfer studies indicated that *in vivo* tolerance is more complex than had been predicted from *in vitro* models. Thus, *in vitro* TCR ligation of CD4 Th1 clones in the absence of costimulation results in a lack of proliferation as well as a rapid (less than 24 hours) induction of anergy that is defined by the inability to produce IL-2 and proliferate in response to subsequent stimulation with antigen plus costimulation [74]. In contrast, when naive TCR transgenic clonotypic CD4 or CD8 cells are adoptively transferred into recipients expressing the cognate self-antigen they generally proliferate (as measured either by BrdU incorporation or CFSE dilution) for several days prior to becoming anergic and/or undergoing deletion [91, 97, 98, 110]. It was subsequently observed that clonotypic T cells encountering cognate tumor-derived antigen can also proliferate prior to becoming tolerant [56, 60, 111]. Interestingly, the kinetics of this initial proliferative response elicited by self-antigen that ultimately leads to tolerance can be comparable to that elicited by the same antigen when expressed within a recombinant viral vector that programs Th1 effector differentiation [90, 112], indicating that the strength of initial proliferation per se does not dictate functional outcome. Because the theoretical expansion in clonotypic T cell frequencies estimated by the average number of cell divisions far exceeded the actual T cell expansions, these data also suggested that *in vivo* anergy may simply represent an intermediate step in the pathway that ultimately leads to deletion [90]. Further supporting this notion, several studies that have defined deletion as the operative tolerance mechanism have also observed a residual population of T cells that exhibit an anergic phenotype [91, 113, 114].

That naive T cells encountering cognate self-antigen proliferate vigorously prior to becoming tolerant, and that tolerant cells can maintain an anergic phenotype prior to deletion seems somewhat counterintuitive in as far as proliferation expends a significant amount of metabolic energy and anergic cells take up space within lymphoid organs. In other words, why don't self-reactive T cells in the periphery simply apoptose without initially proliferating, as they do in the thymus? One possibility is that anergic cells might express an important regulatory function, and that proliferation is required for the development of this function. Consistent with this possibility, it has been observed in several peripheral tolerance systems (including when the tolerizing antigen is tumor-derived) that anergic CD4 cells do exhibit regulatory function [27, 115-117], although regulatory activity is not always defined by the expression of standard Treg markers such as CD25 and Foxp3 [118].

Peripheral T cell tolerance was initially thought to act mainly on naive rather than effector T cells. Thus, although it had been shown in various autoimmunity models that effector T cells can be tolerized following exposure to large boluses of cognate exogenous soluble antigen (reviewed in

[119]), it had generally been thought that effector T cells would not become tolerant under physiological conditions such as when cognate self-antigen might be expressed at relatively low levels. This notion derived largely from the ability of effector T cells to become activated without optimal costimulation [120-125], which might have made them resistant to the effects of steady state APCs (which induce naive T cells to become tolerant because they express sub-optimal costimulation [104-107]). It was therefore surprising when it was found that virally-primed effector and memory T cells are equally susceptible to peripheral tolerance induction compared to naive counterparts following adoptive transfer into recipients that express cognate self-antigen [126, 127]. This effector/memory T cell tolerization pathway might exist to limit the extent autoimmune damage that ensues during molecular mimicry scenarios (reviewed in [128]) where naive self-reactive T cells that have not yet been tolerized are primed by pathogens that express cross-reactive antigens [129]. However, this pathway could also have the potential to inactivate tumor-reactive effector T cells that are either primed through vaccination or injected following *ex vivo* expansion (i.e., adoptive immunotherapy [130]), and might therefore represent yet another level at which tolerance can negatively impact tumor immunity.

The cellular and molecular mechanisms that regulate peripheral T cell tolerance *in vivo* have been studied mostly in systems where naive T cells encounter tolerizing forms of antigen. However, given that peripheral tolerization of effector and memory T cells is likely to be highly relevant to tumor immunity, elucidation of the unique aspects associated with these tolerance pathways will also be important. Thus far, it appears that there are both similarities as well as interesting differences in the mechanisms by which effector and memory T cells undergo tolerization compared to naive T cells. Similar to naive T cells, both memory CD8 cells [127] and Th1 effector CD4 cells [126] undergo an initial proliferative response prior to becoming tolerant. Additionally, steady state bone marrow-derived APCs that indirectly present parenchymally-derived self-antigen are required for Th1 effector CD4 cell tolerization [126]. Effector T cells are distinguished from their naive progenitors by the expression of effector molecules such as IFN- $\gamma$  (Th1 effector CD4 cells and effector CD8 cells), IL-4 (Th2 effector CD4 cells) as well as perforins and granzymes (effector CD8 cells) [131, 132]. It was therefore of interest to assess whether the regulation of these effector molecules is altered during tolerization. In the case of Th1 effector CD4 cells exposed to self-antigen, their potential to express the effector cytokines IFN- $\gamma$  and TNF- $\alpha$  becomes impaired as early as 24 hours, while non-effector functions such as the ability to express IL-2 and to proliferate are lost only after several days [133]. In addition to indicating that the Th1 effector CD4 cell tolerization process is complex, this observation likely has physiological relevance since IFN- $\gamma$  and TNF- $\alpha$  can both play critical roles in mediating tumor-immunity [134-138]. Thus, effectors that can produce IL-2 and proliferate, but not express IFN- $\gamma$  or TNF- $\alpha$  would probably not be very effective in destroying tumors.

The TCR transgenic adoptive transfer experiments demonstrating that effector T cells are highly susceptible to peripheral tolerization were somewhat analogous to adoptive immunotherapy approaches for treating cancer where *ex vivo*

expanded tumor-reactive effector T cells are adoptively transferred into cancer patients [130]. The relevance of effector T cell tolerization to adoptive immunotherapy, however, was a bit unclear given that adoptive immunotherapy has demonstrated a degree of clinical efficacy [139, 140] despite the possibility that in these patients the targeted tumor-associated antigens might be presented by tolerogenic steady state APCs. In this regard it is worth noting that these and other adoptive immunotherapy protocols use cytotoxic drugs such as cyclophosphamide (Cytoxan) to condition patients prior to receiving tumor-reactive effector T cells and/or exogenous IL-2 administered thereafter. Cytoxan and IL-2 can also enhance the efficacy of anti-tumor adoptive immunotherapy in mouse models [50, 141, 142]. The mechanism(s) by which Cytoxan and IL-2 enhance anti-tumor adoptive immunotherapy has not been precisely established, although some studies have suggested that Cytoxan can eliminate tumor-specific regulatory T cells [141, 143], or elicit the expression of T cell growth factors [144] or type I interferons [145]. Given the cytotoxic activity of Cytoxan, it might also enhance the engraftment of adoptively transferred tumor-reactive effector T cells [142] by creating space [146-148]. IL-2 has been reported in some systems to enhance the proliferation and survival of effector T cells [149, 150]. Rather than being mutually exclusive, these different potential mechanisms might be synergistic. Along similar lines, Cytoxan plus IL-2 impeded the tolerization of TCR transgenic clonotypic Th1 effector CD4 cells that were adoptively transferred into cognate self-antigen expressing recipients [151], suggesting that the empirically-developed adoptive immunotherapy protocols might be effective in part because they minimize tolerization of the adoptively transferred tumor-reactive effector T cells. It should be noted, however, that in the transgenic mouse model Cytoxan plus IL-2 delayed rather than prevented tolerization; for example, the capacity to express IFN- $\gamma$  was extended by approximately 4 days [151]. This result may in part explain why multiple T cell infusions enhance adoptive immunotherapy protocols, and underscores that the efficacy of adoptive immunotherapy might be further improved by strategies that more effectively preserve T cell function in the face of tolerizing antigen.

Mitigating T cell tolerance in the context of T cell-based immunotherapeutic approaches to treating cancer will require a detailed understanding of the intrinsic molecular defects that are associated with T cell non-responsiveness. Using both *in vitro* anergy models as well as TCR transgenic adoptive transfer systems in which naive T cells are exposed to tolerizing antigen, a variety of cytoplasmic signaling defects that are positioned down-stream of the TCR signaling apparatus have been characterized that contribute to impaired IL-2 expression and proliferation (reviewed in [74, 152]). Some of these lesions might also play a role in the tolerization of effector T cells, since they also lose the ability to proliferate and express IL-2. Since there are functional defects that are unique to Th1 effector CD4 cell tolerization such as the rapid loss in effector cytokine expression potentials [133], there are also likely to be unique intrinsic defects that are associated with this tolerance pathway. Recent work has revealed the existence of a yet to be identified TCR-proximal signaling defect(s) that

contributes to impaired expression of IL-2, IFN- $\gamma$  and TNF- $\alpha$ , as well as at least two additional defects that selectively impair IFN- $\gamma$  and TNF- $\alpha$  expression. One of these defects has been identified as the down-modulated expression of the Th1 master regulatory factor T-bet, which contributes to impaired IFN- $\gamma$ , but not TNF- $\alpha$ , expression [153]. Given the tumoricidal activities of IFN- $\gamma$  and TNF- $\alpha$ , further identification and characterization of these defects that selectively impair their expression should aid the development of strategies to enhance tumor immunity.

### THE RELATIONSHIP BETWEEN HORMONES, T CELL TOLERANCE AND TUMOR IMMUNITY

Certain hormones can influence both tumorigenesis as well as T cell function, therefore understanding how these effects interact will be critical in tailoring appropriate T cell-based therapies. An example of this interplay is the relationship between androgens and prostate cancer (the most common malignancy in American men [154]). Androgens are required for the normal growth and differentiation of prostate epithelial cells (the cells that give rise to prostate cancer), and castration (i.e., androgen ablation) induces the apoptotic degeneration of the prostate epithelium [155, 156]. Since most prostate tumor cells also require androgens for their growth and survival, androgen ablation has become a standard therapy for advanced prostate cancer [157]. Unfortunately, disease relapse usually occurs following androgen ablation because a sub-set of tumor cells have developed alterations in either the expression or activity of the androgen receptor that allows activation in the absence of normal androgen levels [158-162].

From an immunological perspective, androgen levels are inversely related to disease severity in certain autoimmunity models [163, 164], and androgen ablation can both reverse the decline in thymic output associated with aging [165] as well as enhance peripheral T cell responsiveness [166, 167]. Since androgen ablation is a standard therapy for advanced prostate cancer, many clinical trials utilizing T cell-based therapies will likely involve patients who have either already undergone or who will be scheduled to undergo androgen ablation. Thus, understanding the effects of androgen ablation on the function of prostate-specific T cells will be critical for considering how T cell-based therapies should be administered relative to hormonal therapy.

To study the effects of prostate tumorigenesis and androgen ablation on the function of prostate-specific T cells, Drake *et al.* [60] generated Pro-HA transgenic mice in which the prostate epithelial-specific probasin promoter was used to drive the expression of the model self-antigen influenza HA that had been modified to be secreted rather than expressed on the cell surface to model secreted prostatic antigens such as PSA. In contrast to the aforementioned C3-HA transgenic mice in which HA is expressed as a self-antigen in multiple parenchymal tissues and thus causes adoptively transferred naive clonotypic HA-specific CD4 cells to become tolerant in multiple secondary lymphoid organs [89, 112], in Pro-HA mice the same adoptively transferred naive HA-specific CD4 cells retain their naive phenotype (i.e., they remain "ignorant") despite the expression of HA in the prostate epithelium [60]. This lack of antigen recognition in the Pro-HA mice did not appear to

be caused solely by a low level of expression (as has been observed in other systems [168]), but rather more likely because HA was being secreted into the prostatic lumen and not the draining lymphatics [169] such that it could not be acquired by tolerance-inducing steady state APCs [89]. Thus, disruption of the normal prostatic architecture induced either by androgen ablation (which induces apoptosis of the prostate epithelium) or tumorigenesis (induced by crossing the Pro-HA mice to TRAMP transgenic mice that develop spontaneous prostate tumors resulting from SV40 T antigen expression also under the control of the probasin promoter [170]) caused adoptively transferred naive HA-specific CD4 cells in the prostate-draining lymph nodes to undergo an abortive proliferative response that suggested that these T cells were becoming tolerant [60]. The duration of HA presentation in the draining lymph nodes of healthy androgen ablated mice was relatively short (~3 days) [60], perhaps because epithelial degeneration occurs in a synchronous wave and the phagocytic DCs that likely acquire HA from apoptotic epithelia [171, 172] have a lifespan in the lymph nodes of only a couple of days [173]. The sustained HA presentation associated with prostate cancer, but not the transient presentation caused by androgen ablation in healthy mice, was sufficient to render these prostate-specific T cells systemically tolerant as defined by an impaired ability to respond to subsequent viral immunization [60]. Notably, androgen ablation of mice with prostate cancer elicited a transient increase in HA presentation in the draining lymph nodes, followed by a diminution (but not complete elimination) of HA presentation. This pattern appeared to correlate with the apoptosis and subsequent clearance of the androgen ablation-sensitive sub-population of HA-expressing tumor cells. Most importantly, this diminution in tolerogenic antigen presentation allowed the HA-specific CD4 cells to retain their capacity to respond to vaccination, indicating that while prostate tumorigenesis promotes the tolerization of prostate-specific T cells, androgen ablation mitigates this effect.

From a clinical standpoint, the observation in the Pro-HA system that androgen ablation reduces the tolerance inducing capacity of prostate tumors suggests that T cell-based therapies to treat prostate cancer might be the most effective when administered following rather than preceding androgen ablation. Mechanistically, this enhancement could potentially operate at multiple levels. It has been reported in some systems that T cell anergy can be reversed following removal of the tolerizing antigen [110, 174]. Thus, androgen ablation might allow anergic prostate-specific T cells to regain the ability to respond to vaccination. Since effector T cells are susceptible to tolerization [129], adoptive immunotherapy targeting prostatic antigens might also have a better opportunity to eliminate the residual androgen ablation-resistant tumor cells after the level of tolerizing antigen has been reduced. Additionally, one of the inherent challenges in developing prostate cancer vaccines is that disease incidence increases with age, and aging is associated with a reduction in thymic output that contributes to a constriction in the repertoire of naive T cells. Since androgen ablation reverses the age-associated reduction in thymic output [165], in the context of prostate cancer androgen ablation might thus enhance vaccine efficacy by increasing the repertoire of naive prostate-specific T cells.

Hormones may influence immunity to other types of cancer as well. For example, breast cancer is similar to prostate cancer in many respects that might influence tumor immunity; breast tumors arise from glandular epithelial cells that require estrogens for their growth and differentiation, and tumor cells can often be eliminated through treatment with estrogen receptor antagonists such as tamoxifen, but hormonal therapy-resistant tumor cells often cause disease relapse [175-177]. Thus, similar to prostate cancer, the possibility exists that hormonal blockade in the context of breast cancer might enhance the efficacy of T cell-based therapies by reducing the levels of tolerizing antigen.

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