Tumor-Induced Immune Suppression

Dmitry I. Gabrilovich · Arthur A. Hurwitz
Editors

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Mechanisms and Therapeutic Reversal

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Immune-Suppressive Mechanisms and Cancer: Understanding the Implications, Paradoxes, and Burning Questions

Arthur A. Hurwitz and Dmitry I. Gabrilovich

Since Paul Ehrlich's 1909 prediction that the immune system is capable of suppressing the growth of tumors, a large volume of evidence produced by the work of many investigators has demonstrated the existence of a natural immune protection against cancer. As a tumor develops, it acquires novel epitopes as a result of mutations in self-proteins, frame shifts, or protein splicing identified in some tumor cells. Many tumors acquire an anaplastic or de-differentiated histologic phenotype, losing tissue differentiation antigens and acquiring expression of embryonic or "cancer-testis" antigens. In addition, changes in glycosylation or levels of expression may also change the antigenic repertoire of tumor cells. Finally, virally transformed cells may harbor strongly immunogenic viral antigens.

As a whole, these changes in antigenicity of tumor cells may permit the adaptive immune system to recognize a tumor as "foreign", despite the fact that tumors arise from "normal" self-tissues, against which tolerance is maintained. All these data justify the concept of immunosurveillance of tumors, which proposes that as mutations that lead to transformation occur, the immune system can detect these changes as "foreign" and eliminate the "invader". Recently, this concept has evolved into the concept of "immunoediting", which postulates that as a tumor develops, the immune system can shape the repertoire of a tumor's inherent immunogenicity.

It is now clear that tumors can be recognized and eliminated by the host immune system. However, this idea raises two main questions that have confronted researchers and physicians for many years: why the immune system does not always prevent tumor progression, and how to manipulate the immune system to achieve tumor eradication. The last 20 years have brought a clear realization that one of the major mechanisms of tumor escape that limits the clinical success of cancer immunotherapy is the inadequate function of the host immune system in the context of a developing tumor. During recent years, there has been an explosion of information about the potential immunosuppressive strategies employed by tumor cells.

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Intensive studies from many different groups have resulted in the discovery of numerous cellular mechanisms of immune suppression in cancer. With the identification of T-cell priming pathways, it became clear that aberrant T-cell activation can lead to non-responsiveness or anergy. T-cell receptor ligation in the absence of costimulatory signals is generally recognized as a potent way to anergize T cells. Thus, tumor cells that express MHC but lack costimulatory ligands, or immature APCs that cross-present tumor antigens, may be capable of tolerizing tumor-reactive T cells. Developing tumors can also induce production of a variety of suppressive cells. They include regulatory T cells, B cells, myeloid-derived suppressive cells, and different types of macrophages and dendritic cells. These cells suppress T-cell responses via suppressive surface molecules like CTLA-4, PDL-1, PDL-2, galectins, etc., production of inhibitory cytokines like IL-10, TGF-β, VEGF, etc., depletion of T cells of tryptophan and arginine, release of reactive oxygen species and nitric oxide and many others (many of which are discussed in this monograph).

Like suppressor cells, tumors can also express factors that create a suppressive environment. Tumors can express catabolic enzymes like indoleamine dioxygenase or arginase. Tumors have also been demonstrated to express ligands to inhibitory receptors on T cells and expression of these ligands has an inverse correlation to survival, suggesting that tumors use these receptors to evade immune recognition.

Discovery of this multitude of different immune-suppressive factors helped to develop new experimental and clinical methods to improve the immune response in cancer and the effect of cancer vaccines. However, these discoveries also raise several fundamental questions that need to be addressed in order to understand fully the biology of antitumor immunity and effective approaches to its use in therapeutic settings.

1. *Specific vs. non-specific suppression in cancer.* Most of the suppressive mechanisms that have been demonstrated in cancer and described in this monograph do not require the presence of tumor-specific antigens for their negative effect on T cells. The paradox is that despite the apparent presence of a large number of potent immune-suppressive factors, neither tumor-bearing mice nor cancer patients are profoundly immune compromised. Even at a relatively advanced stage of cancer, the host immune system retains the ability to respond to stimulation with viral and bacterial antigens or lectins. At the same time, tumor-specific immune response is repressed. The question arises that if those multiple suppressive mechanisms are truly operational, why is more profound immune deficiency not observed in tumor-bearing hosts? This paradox is currently not resolved. Currently, it appears that the understanding of the mechanisms of tumor escape requires identification of the precise role of tumor-specific immune tolerance vis-à-vis non-specific immune suppression. It is possible that the role of multiple immunosuppressive mechanisms in cancer is exaggerated due to the nature of experimental models employed. However, another explanation is much more likely. It relates to the phenomenon of compartmentalization of immune suppression in cancer. There is certainly a need for development of more experimental models that more closely reflect the "real" situation present in cancer patients. Such models might allow more accurate

characterization of multiple immunosuppressive mechanisms active at the same time.

2. *Spatial characteristics of immune suppression in cancer.* Immune suppression in cancer is not a universal process. It has become increasingly clear that the nature of immune suppression in peripheral lymphoid organs and inside the tumor site is different. Available data may suggest that in peripheral lymphoid organs, tumor-specific T-cell tolerance is more likely to be responsible for tumor escape than non-specific immune suppression. T cells retain their ability to respond to other stimuli. In contrast, tumor microenvironment creates a milieu that inhibits any type of immune reactivity and this immune suppression is not antigen-specific. Multiple studies demonstrated that tumor-infiltrating T lymphocytes are profoundly suppressed. Their function could be recovered only if they are cultured ex vivo in the presence of appropriate cytokines and effective stimulation. However, it is still unclear whether T cells are rendered non-responsive inside a tumor or if they migrate to the tumor site, having already been tolerized in peripheral lymphoid organs. This question is especially important for the attempts to use adoptive transfer of previously activated, antigen-specific T cells. Although adoptive immunotherapy holds promise, the local immunosuppressive environment of the tumor may hamper those attempts. It is very important to establish whether immune suppression at the tumor site is indeed able to block the antitumor effect of adoptively transferred T cells and to determine therapeutic approaches to tilt the balance toward effector T cells. There are no clear answers to these questions. However, the overview of current data presented in this monograph may help to develop them in the future.

3. *Strategies to target negative regulatory pathways.* The fact that tumors develop and progress is a good indication that immune surveillance of cancer is not completely efficient. Successes of cancer vaccines at this time are not impressive. The failure of antitumor immune responses is presumably the consequence of the environment of a large network of tumor-associated immune-suppressive factors. This makes targeting of this network very attractive for the goal of improvement of overall antitumor reactivity.

How best to target immune-suppressive regulatory pathways remains unclear. Negative regulatory mechanisms discussed in detail in this monograph are also essential in preventing excessive immune responses to foreign antigens and autoimmune abnormalities. It is logical that the elimination of these factors will result in the activation of the immune system. The question is whether this activation alone will be sufficient. The potential problem is that the removal of negative “brakes” would result in an accumulation of T cells reactive to any available antigens. Most of the viral and bacterial antigens are much stronger immunogens than the self-antigen present in tumors. The proportion of tumor-specific T cells among this pool of reactive T cells could be quite small. They can still be easily detected since investigators are specifically looking for these cells. However, whether they are sufficient to prevent tumor progression is not apparent.

In addition, antitumor effects will most likely be associated with autoimmune abnormalities. The more effective the antitumor response generated by a potent therapy, the more severe the potential side effects that could be developed. Often,

successful anti-melanoma responses are associated with autoimmune vitiligo, where the immune system destroys melanocytes as well as melanoma cells. However, some therapies give rise to more system autoimmune sequellae. It was reported that some of those side effects could be alleviated by corticosteroids (Ribas et al., 2005). However, it is not clear how this may affect the clinical efficacy of the treatment. Current clinical studies will undoubtedly help to address these questions. However, accumulated data presented in this monograph strongly argue in favor of a direct combination of immunostimulatory therapy with targeting immune-suppressive pathways. Partial removal of suppressive mechanisms in the presence of tumor-specific T cells may dramatically enhance their antitumor effect. A number of clinical trials testing this hypothesis have been initiated in recent years. The results of these trials will undoubtedly help to shape future therapeutic strategies.

4. *Combination of immunotherapy and other therapeutic modalities in cancer as a future of cancer treatment.* Another approach to cancer therapy has emerged in recent years. It employs conventional chemotherapy in direct combination with immunotherapy. This approach seems to be counterintuitive since it is well established that potent cancer chemotherapy blunts the immune responses. However, this perception was recently challenged by unexpected results from several clinical trials demonstrating substantial clinical benefits when immunotherapy was immediately followed by chemotherapy (Antonia et al., 2006; Arlen et al., 2006; Gribben et al., 2005; Wheeler et al., 2004). These data, in combination with the results of pre-clinical studies (Emens and Jaffee, 2005), suggest a synergistic effect of immunotherapy and chemotherapy. One of the potential mechanisms of this synergistic effect could be the elimination of immune-suppressive factors by chemotherapy. Chemotherapy is known to be able to deplete regulatory T cells, myeloid-derived suppressor cells, as well as tumor-associated macrophages. Eventually, CTL responses are also ablated by chemotherapy. However, apparently the effect of chemotherapy on tumor microenvironment precedes the effect on CTL, which may explain the clinical benefits of this approach. In addition, chemotherapy may disrupt tumor stroma, which would improve CTL penetration into tumor parenchyma. As discussed in this monograph, it is also possible that chemotherapy can help load stromal cells with tumor-associated antigens and thus help to facilitate antitumor immune responses. This field is at an early phase of development now and more studies are needed to clarify the mechanisms of this phenomenon.

The data accumulated in recent years provide strong indication that targeting immune-suppressive mechanisms in combination with induction of antitumor immune responses may profoundly enhance the effect of cancer immunotherapy. We have become more sophisticated in our understanding of the mechanisms of immune suppression in cancer and in developing new approaches to targeting those mechanisms. This monograph presents the “state of the art” in our understanding of the mechanisms of suppression of tumor immunity. By presenting a comprehensive understanding of how these suppressive mechanisms reduce the ability to elicit potent tumor immunity, we hope to stimulate the study of more powerful and presumably synergistic approaches to treating cancer.

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Mechanisms of Tumor-Associated T-Cell Tolerance

Adam J. Adler

1 Introduction

The great challenge in the treatment of cancer has been to develop modalities that destroy tumor cells without damaging healthy tissues. In fact, modalities such as chemotherapeutics that are standardly used to treat a wide variety of cancers work on the principle that tumor cells are slightly more sensitive to their cytotoxic effects than are healthy cells, and thus treatment regimens are administered that may or may not fully eradicate the cancer (depending upon the outgrowth of drug-resistant tumor cells) but generally inflict significant side effects on the patient. In this regard, there has been a long-standing interest in programming the adaptive immune system to mediate anti-tumor immunity through the targeting of antigens expressed specifically by tumors. This effort has been accelerated during recent years by advances in the ability to prime robust cytotoxic T-lymphocyte responses. Nevertheless, results from recent clinical trials testing a variety of T cell-based immunotherapeutic approaches have only demonstrated partial successes (Rosenberg et al., 2004; Srivastava, 2006). This is likely to be at least partially due to the ability of tumors to dampen cognate T-cell responses.

Ironically, the first evidence demonstrating that tumors can suppress cognate T-cell responses came from the same studies establishing that tumors can elicit T-cell responses. Thus, 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 phenomenon of concomitant immunity (reviewed in Gorelik, 1983) thus indicated that while tumors can possess immunogenic properties that allow them to prime cognate T-cell responses, they can simultaneously suppress the function of these effector T cells when they enter the tumor

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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 (Gorelik, 1983), the subsequent observation that T cells with tumor specificity commonly infiltrate certain human tumors such as melanoma (Topalian et al., 1989) suggested that naturally arising tumors can also elicit cognate T-cell responses while simultaneously inhibiting T-cell effector functions in the tumor microenvironment. Understanding how the tumor microenvironment is able to locally suppress the function of tumor-infiltrating tumor-reactive effector T cells has been the subject of intense study and will be reviewed in detail in several of the accompanying chapters.

The immunogenic properties of certain tumors may be related to their potential to generate inflammation when they invade surrounding tissue or metastasize (Pardoll, 2003). 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 (Wang et al., 2004). Overall, the potential of tumors to grow while eliciting minimal inflammation would be consistent with the potential to induce immunological tolerance (Pardoll, 2003). For the purpose of this discussion, *tolerance will be defined as an impaired ability of antigen-specific T cells to respond to antigenic challenge at the systemic level*, as opposed to the above-mentioned immunosuppressive effects that impair T-cell effector 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.

Ultimately, the development of effective T cell-based strategies to treat cancers that have a propensity to induce T-cell tolerance will likely require a component to prevent or reverse tolerance to tumor-associated antigens, which will be facilitated through a detailed understanding of the cellular and molecular mechanisms that regulate tolerance.

2 Tumors Can Tolerize Cognate T cells

Since many human and mouse tumor antigens are expressed on both tumors and the normal tissues from which they derive (i.e., differentiation antigens, e.g., tyrosinase (Wolfel et al., 1994), TRP2 (Wang et al., 1996) and Pmel-17/gp100 (Cox et al., 1994)), it is likely that the pathways which tolerize the T-cell repertoire to tissue-specific self-antigens in order to avoid autoimmunity 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 the vaccine to protect against subsequent challenge with an env-expressing transplantable erythroleukemia (Hu et al., 1993). 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. For instance, when a transgenic tumor differentiation antigen is expressed on normal tissues in a more restricted fashion, naive CD8 cells expressing T-cell receptors (TCRs) with low avidity for the tumor differentiation epitope escape tolerization and can be primed through vaccination to mediate tumor immunity (Morgan et al., 1998). The possibility that tumor differentiation antigen-specific T cells that can be primed may tend to express low-avidity TCRs might represent one facet explaining 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 the peripheral tolerance-inducing machinery prior to tumorigenesis. Nevertheless, numerous studies have indicated that T-cell tolerance can develop rapidly toward 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 idiotype-specific CD4 cells underwent deletion (Bogen, 1996). Given that bolus injection of soluble foreign antigens induces immunological tolerance (in contrast to particulate antigen or antigen admixed with adjuvant that induces immunity) (Chiller et al., 1971; Dresser, 1962), 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 toward 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 subsequent in vitro or in vivo antigenic challenge (Stavely-O'Carroll et al., 1998).

Subsequent studies from various groups have confirmed that both CD4 and CD8 cell tolerance can develop toward antigens expressed on transplantable as well as spontaneously arising tumors (Doan et al., 2000; Drake et al., 2005; Lyman et al.,

2004; Schell et al., 2000; Shrikant et al., 1999). Tolerance does not develop in all tumor systems (Hanson et al., 2000; Nguyen et al., 2002; Ochsenbein et al., 2001; Spiotto et al., 2002), underscoring the notion that different types of tumors vary in their capacity to induce tolerance. As discussed in the introduction, those tumors that elicit cognate effector (rather than tolerogenic) T-cell responses must elaborate immunosuppressive mechanisms to inhibit the tumoricidal activity of the tumor-reactive effector T cells that have infiltrated into the tumor microenvironment. Given the dynamic nature of tumorigenesis (Lengauer et al., 1998), 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 (Lee et al., 1999), consistent with a scenario in which these tumor-reactive T cells are initially primed to undergo expansion but subsequently inactivated.

3 Mechanisms of Peripheral Self-Antigen- and Tumor-Associated Antigen-Induced T-Cell Tolerance

Since tolerization of tumor antigen-specific T cells can restrict the repertoire of T-cell specificities that can be primed through vaccination, manipulations that can either block the development of 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 high-avidity TCRs that recognize MHC-self-peptide complexes presented by thymic antigen-presenting cells (APCs) undergo apoptosis (Kappler et al., 1987; Kisielow et al., 1988; Sebzda et al., 1994; Surh and Sprent, 1994). Subsequently, mature T cells specific for parenchymal self-antigens that are not presented in the thymus can be subjected to a variety of peripheral tolerance mechanisms such as deletion (Jones et al., 1990), functional inactivation (also referred to as anergy; Schwartz, 2003) or suppression by regulatory T cells (Sakaguchi, 2000; Shevach, 2001).

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 (Anderson et al., 2002). 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 (Derbinski et al., 2005), 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 (Bouneaud et al., 2000), 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 (von Budingen et al., 2001). The spontaneous development of autoimmunity in mice that either exhibit defective DC apoptosis (Chen et al., 2006) or lack negative regulators of peripheral T-cell responsiveness such as Foxp3, Cbl-b (Bachmaier et al., 2000), TGF-β (Gorelik and Flavell, 2000) and CTLA-4 (Tivol et al., 1995) provides additional evidence that peripheral tolerance is critical for preventing autoimmunity.

Tissue-restricted self-antigens expressed in mTECs include certain tumor-associated antigens (Bos et al., 2005), 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 mostly occurred 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 tumor-specific T-cell tolerance. The studies by Bogen and colleagues demonstrated that plasmacytomas can secrete sufficient levels of idiotypic antibody into the circulation to reach the thymus and induce the deletion of developing anti-idiotypic T cells (Bogen, 1996; Bogen et al., 1993). Since many 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 pathways 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) (Stavely-O'Carroll et al., 1998), an analogous system was developed in which the same HA-specific CD4 cells are adoptively transferred into C3-HA transgenic mice that express HA in a wide variety of normal parenchymal tissues (i.e., self-HA) (Adler et al., 1998,

2000). 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 (Schwartz, 2003) 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., Kearney et al., 1994; Rocha and von Boehmer, 1991), in vitro tolerance studies using Th1 clones indicated that anergy is induced when TCR ligation occurs in the absence of costimulation (reviewed in Schwartz, 2003). This observation led to the notion that TCR engagement without costimulation leading to non-responsiveness/anergy might 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 (Stavely-O'Carroll et al., 1998), the overall level of costimulatory ligand expression is substantially less compared to dendritic cells (DC) which represent the most potent APC subset (Banchereau and Steinman, 1998), and normal B cells which also express low levels of costimulatory ligands can induce T-cell tolerance in vivo (Eynon and Parker, 1992; Fuchs and Matzinger, 1992). 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 (Adler et al., 1998). This indirect or cross-presentation pathway can also facilitate the peripheral tolerization of self-reactive CD8 cells (Kurts et al., 1997). Subsequent work has suggested that steady-state DC likely represent the predominant cross-tolerizing APC (Belz et al., 2002; Hagymasi et al., 2007; Kurts et al., 2001), although other APC populations also appear to cross-tolerize (Hagymasi et al., 2007). The ability of DC to prime both effector and tolerogenic T-cell responses appears to be regulated by the environment in which the antigen is acquired. Thus, when DC 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 DC with the ability to prime cognate naive T cells to develop effector and memory functions. In contrast, when DC 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 (Finkelman et al., 1996; Hawiger et al., 2001; Janeway and medzhitov, 2002; Jenkins et al., 2001; Matzinger, 1994; Medzhitov, 2001).

Returning to the HA-expressing B-cell lymphoma model (Stavely-O'Carroll et al., 1998), 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 (Sotomayor et al., 2001). That peripherally tolerized self-reactive and tumor-reactive T cells can exhibit similar 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. The similarities between the tumor-HA and self-HA models do not necessarily exclude the possibility that there may 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 are localized to discrete anatomical locations tends to be concentrated in tumor-draining lymph nodes (Drake et al., 2005; Marzo et al., 1999). 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 (Long et al., 2006).

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 h) induction of anergy that is defined by the inability to produce IL-2 and proliferate in response to subsequent stimulation with antigen plus costimulation (Schwartz, 2003). 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 (Kurts et al., 1997; Pape et al., 1998; Rocha and von Boehmer, 1991). It was subsequently observed that clonotypic T cells encountering cognate tumor-derived antigen can also proliferate prior to becoming tolerant (Anderson et al., 2007; Drake et al., 2005; Shrikant et al., 1999; Zhou et al., 2004). 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 (Adler et al., 2000; Higgins et al., 2002b), indicating that the kinetics of initial proliferation per se does not dictate functional outcome, but rather the context in which the antigen is presented to the T cell may have a more critical role in determining T-cell fate. 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 (Adler et al., 2000). 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 (Rocha and von Boehmer, 1991; Webb et al., 1990).

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 seem somewhat counterintuitive insofar as proliferation expends a significant amount of metabolic energy and anergic cells take up space within lymphoid organs. Thus, it is not clear why self-reactive T cells in the periphery do not 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 (Apostolou and von Boehmer, 2004; Jooss et al., 2001; Zhou et al., 2006).

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 auto-antigen (reviewed in Liblau et al., 1997), 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 in vitro without optimal costimulation (Croft et al., 1994; Horgan et al., 1990; Sagerstrom et al., 1993), 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; Hawiger et al., 2001; Janeway et al., 2002; Jenkins et al., 2001; Matzinger, 1994). 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 (Higgins et al., 2002a; Kreuwel et al., 2002). This effector/memory T-cell tolerization pathway might exist to limit the extent of autoimmune damage that ensues during molecular mimicry scenarios (reviewed in Oldstone, 1998) where naive self-reactive T cells that have not yet been tolerized are primed by pathogens that express cross-reactive antigens (Adler, 2005) (Fig. 1). However, this pathway might also have the undesirable effect of inactivating tumor-reactive effector T cells that are either primed through vaccination (Fig. 2) or injected following ex vivo expansion (i.e., adoptive immunotherapy; Yee

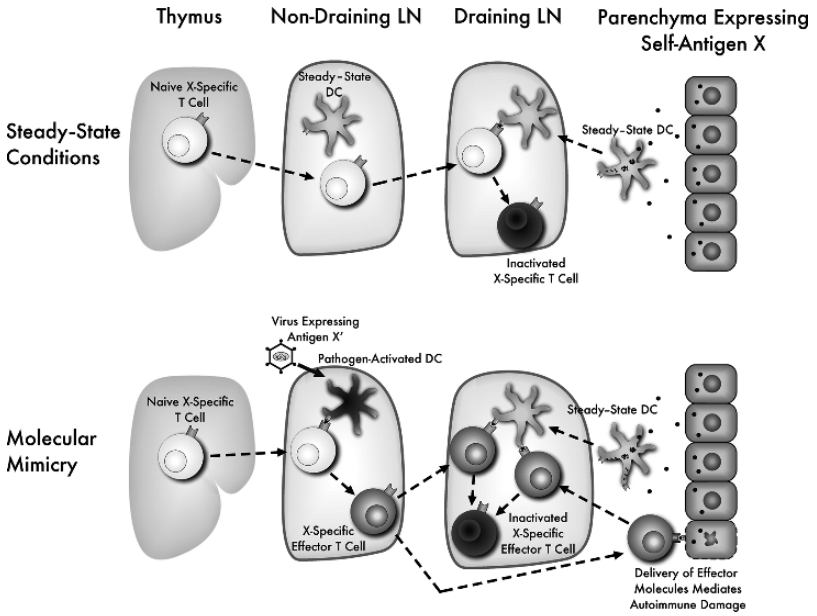

Fig. 1 The normal physiological role of the effector T-cell tolerization pathway might be to limit the extent of autoimmune pathology that ensues during molecular mimicry scenarios. During steady-state conditions naive T cells specific for the self-antigen X leave the thymus and migrate between peripheral lymph nodes (*LN*) until they enter LN draining tissues expressing X, where they are inactivated (i.e., tolerized) following encounter with steady-state tolerogenic DC presenting X. During molecular mimicry, infection with a pathogen expressing antigen X′ that is structurally similar to X leads to activation of DC presenting X′ and subsequent priming of naive X-specific T cells to differentiate into effectors that migrate into X-expressing parenchymal tissues and inflict autoimmune damage. Presentation of X by steady-state DC in the draining LN inactivates the X-specific effectors and thus shortens the duration of the autoimmune effector T-cell response

et al., 1997) and might therefore represent yet another level at which tolerance can negatively impact tumor immunity. Consistent with this possibility, naive prostate tumor-reactive T cells can be primed through vaccination to develop effector functions and partially control tumor growth, but over time effector functions and control of tumor growth diminish (Anderson et al., 2007).

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 the relevance of peripheral tolerization of effector and memory T cells to tumor immunity, elucidating the unique aspects associated with these tolerance pathways will also be important. Thus far, it appears that there are 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 (Kreuwel et al., 2002) and Th1 effector CD4 cells (Higgins et al., 2002a) undergo an initial proliferative response prior to becoming tolerant. Additionally, steady-state bone marrow-derived APCs that

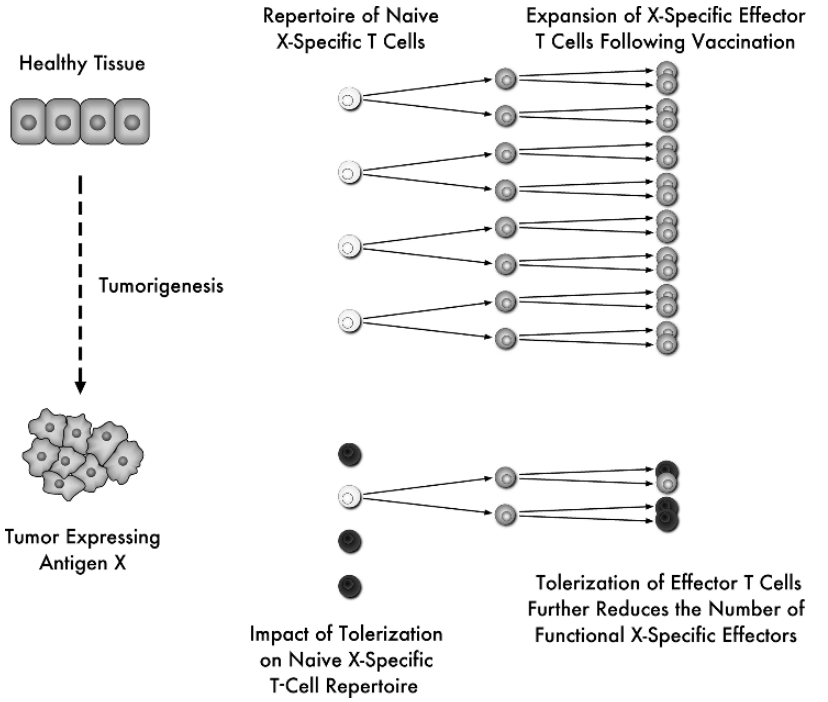

Fig. 2 An undesirable facet of the effector T-cell tolerization pathway is that it might represent an additional level at which tolerance can negatively impact tumor immunity. Tumorigenesis can result in the tolerization of a significant fraction of naive T cells specific for cognate tumor-associated antigens, thus restricting the repertoire of specificities that can respond to vaccination. Tolerization of the expanded tumor-reactive effector T-cell population (that is already reduced in number) could potentially impair tumor immunity even further. Although not shown in this figure, the effector T-cell tolerization pathway might also impede tumor immunity in the context of adoptive immunotherapy, where tumor-reactive effector T cells expanded ex vivo might be inactivated following injection into patients

indirectly present parenchymally derived self-antigen are required for Th1 effector CD4 cell tolerization (Higgins et al., 2002a). Effector T cells are distinguished from their naive progenitors by the expression of effector molecules such as IFN-γ (Th1 effector CD4 cells and effector CD8 cells), IL-4 (Th2 effector CD4 cells) as well as perforins and granzymes (effector CD8 cells) (Glimcher et al., 2004; Murphy and Reiner, 2002). 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-γ and TNF-α becomes impaired as early as 24 h, while the abilities to express IL-2 and to proliferate are lost only after several days (Long et al., 2003). In addition to indicating that the Th1 effector CD4 cell tolerization process is complex, this observation likely has physiological relevance since IFN-γ and TNF-α can both play critical roles in mediating tumor immunity (Hung et al., 1998; Ikeda et al.,

2002; Poehlein et al., 2003; Qin and Blankenstein, 2000). Thus, since neither T-cell proliferation nor IL-2 production is directly tumoricidal, effectors that can produce IL-2 and proliferate but have lost the ability to express IFN-γ and TNF-α would probably not be very effective at 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 (Yee et al., 1997). 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 (Dudley et al., 2002; Yee et al., 2002) 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 (Greenberg and Cheever, 1984; Hu et al., 1993; North, 1982). 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 (North, 1982) or elicit the expression of T-cell growth factors (Proietti et al., 1998) or type I interferons (Schiavoni et al., 2000). Given the cytotoxic activity of Cytoxan, it might also enhance the engraftment of adoptively transferred tumor-reactive effector T cells (Greenberg and Cheever, 1984) by creating space (Dummer et al., 2002; Hu et al., 2002). IL-2 has been reported in some systems to enhance the proliferation and survival of effector T cells (Blattman et al., 2003; D'Souza, 2003). 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 (Mihalyo et al., 2004), 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-γ was extended by approximately 4 days (Mihalyo et al., 2004). 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 treat 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 and 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 and that contribute to impaired IL-2 expression and proliferation have been characterized (reviewed in Mueller, 2004; Schwartz, 2003). 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 unique functional defects associated with Th1 effector CD4 cell tolerization such as the rapid loss in effector cytokine expression potentials (Long et al., 2003), 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-γ and TNF-α, as well as at least two additional defects that selectively impair IFN-γ and TNF-α 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-γ, but not TNF-α, expression (Long et al., 2006). Given the tumoricidal activities of IFN-γ and TNF-α, further identification and characterization of these defects that selectively impair their expression should aid the development of strategies to enhance tumor immunity.

4 The Relationship Between Hormones, T-Cell Tolerance and Tumor Immunity

Certain hormones can influence both tumorigenesis and T-cell function, and 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; Jemal et al., 2005). 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 (Furuya et al., 1995; Sugimura et al., 1986). Since most prostate tumor cells also require androgens for their growth and survival, androgen ablation has become a standard therapy for advanced prostate cancer (Denmeade and Isaacs, 2002). Unfortunately, disease relapse usually occurs following androgen ablation because a subset of tumor cells develop alterations in either the expression or activity of the androgen receptor that allows activation in the absence of normal androgen levels (Chen et al., 2004; Hakimi et al., 1996; Han et al., 2005; Zhao et al., 2000).

From an immunological perspective, androgen levels are inversely related to disease severity in certain autoimmunity models (Fox, 1992; Roubinian et al., 1978), and androgen ablation can reverse the decline in thymic output associated with aging (Sutherland et al., 2005) as well as enhance peripheral T-cell responsiveness (Roden et al., 2004; Viselli et al., 1995). 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 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. [2005] generated Pro-HA transgenic mice in which the prostate epithelial-specific probasin promoter drives the expression of HA antigen that has 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 self-HA expressed in multiple parenchymal tissues programs adoptively transferred naive HA-specific CD4 cells to undergo tolerization (Adler et al., 1998; Higgins et al., 2002b), the same HA-specific CD4 cells retain their naive phenotype following adoptive transfer into Pro-HA mice (i.e., they remain "ignorant") (Drake et al., 2005). 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; Kurts et al., 1998), but rather more likely because HA was being secreted into the prostatic lumen rather than the draining lymphatics (Whitmore and Gittes, 1977) where it could potentially be acquired by tolerance-inducing steady-state DC (Adler et al., 1998; Mihalyo et al., 2007). Thus, disruption of the normal prostatic architecture induced by androgen ablation-mediated apoptosis of the prostate epithelium caused adoptively transferred naive HA-specific CD4 cells in the prostate-draining lymph nodes to undergo an abortive proliferative response suggestive of tolerization. Additionally, the development of prostate cancer (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; Greenberg et al., 1995) resulted in a similar abortive proliferative response (Drake et al., 2005) regardless of the stage or rate of disease progression (Mihalyo et al., 2007). Notably, the duration of HA presentation in the draining lymph nodes of healthy androgen ablated mice was relatively short ($\sim$3 days) (Drake et al., 2005), perhaps because epithelial degeneration occurs in a synchronous wave and the phagocytic DCs that likely acquire HA from apoptotic epithelia (Liu et al., 2002; Steinman et al., 2000) have a lifespan in the lymph nodes of only a few days (Kamath et al., 2002). 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 (Drake et al., 2005). 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 parallel 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 (Fig. 3).

From a clinical standpoint, the observation in the Pro-HA system that androgen ablation reduces the tolerance-inducing capacity of prostate tumors suggests that

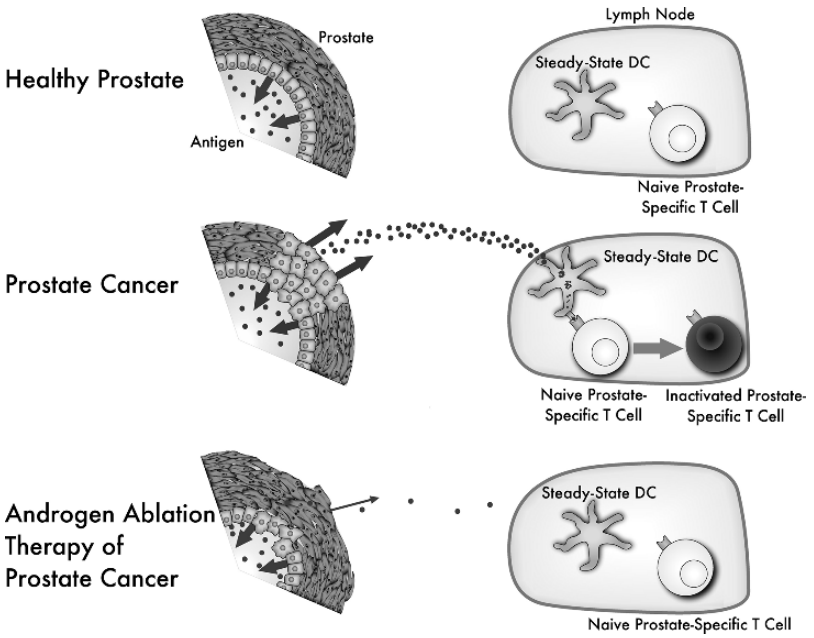

Fig. 3 The influence of prostate tumorigenesis and androgen ablation on the tolerization of prostate-specific T cells. In healthy prostates, prostate epithelial antigens are preferentially secreted in the prostatic lumen, rather than the draining lymphatics, and thus cognate T cells remain in a naive state due to a lack of presentation by steady-state tolerogenic DC. Alterations in the prostatic architecture caused by prostate tumorigenesis allow prostate epithelial/tumor antigen to reach the draining LN and to be presented by tolerogenic DC to inactivate cognate T cells. Androgen ablation induces the apoptosis of a large fraction of prostate epithelia and tumor cells, causing the level of prostate epithelial/tumor antigen to drop below the threshold required for tolerogenic antigen presentation

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 (Pape et al., 1998; Ramsdell and Fowlkes, 1992). 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 (Adler, 2005), 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 (Sutherland et al., 2005) as well as transiently augments antigen responsiveness in mature T cells (Roden et al., 2004), in

the context of prostate cancer androgen ablation might thus enhance vaccine efficacy by both expanding the repertoire of naive prostate-specific T cells and augmenting the ability of these T cells to respond to vaccination.

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 (Coffey, 2001; Cosman and Lindsay, 1999; Lopez-Otin and Diamandis, 1998). 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.

5 Conclusion

As detailed above, tumors often exploit T-cell peripheral tolerization pathways that normally operate to prevent autoimmunity, to delete or inactivate tumor-reactive T cells. Understanding how these tolerance pathways operate under normal conditions will undoubtedly provide key insights into how tolerance might be mitigated in order to allow tumor vaccines to more effectively prime tumor-reactive effector T-cell responses. It is also becoming apparent that standard treatments for certain cancers can not only impact disease progression, but also influence the functional capacity of tumor-reactive T cells. For instance, chemotherapeutic drugs such as Cytoxan can deplete T cells; however, when administered in the proper sequence they can actually augment certain T cell-based anti-tumor modalities. Additionally, androgen ablation therapy for prostate cancer can induce a state of minimal residual disease that leads to a reduction in the level of tolerizing prostate tumor antigen and hence might restore the ability of cognate T cells to respond to vaccination. In the future it will be important to study in more depth how these other complex processes interact.

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