Tumor-Specific T-Cell Memory: Clearing the Regulatory T-Cell Hurdle

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Abstract
Antitumor immune responses can be stimulated by interfering with regulatory T-cell (Treg) function. However, this effect is short lived unless T-cell memory to tumor antigens can be generated. Our recent studies show that Treg cells not only limit primary responses to tumor/self-antigens in tumor-bearing hosts but also prevent the natural generation of T-cell memory after surgical excision of tumors. Here, we discuss the role of Treg cells in suppressing T-cell memory after surgical excision of tumors and the potential clinical benefits of overcoming this suppression. [Cancer Res 2008;68(6):1614–7]

Background
The generation of CD8 T-cell memory is one of the major goals of tumor immunotherapy. For years, studies in infectious disease models have shown that memory T cells are required for pathogen clearance and protection against reinfection. More recently, these lessons are being applied to cancer models. Although surgery currently remains the leading cure for solid tumors, memory T-cell responses may be required for the durable prevention of tumor recurrence and metastasis following surgery. In vitro–generated memory CD8 T cells have also been shown to be highly effective at treating large established melanomas (1, 2). However, in stark contrast with infectious disease models, most human tumors are poorly immunogenic, and most tumor antigens are unaltered self-proteins. This presents a significant challenge, as mechanisms of central and peripheral tolerance prevent the priming of T-cell responses against self-antigens. Even if tolerance is broken, T cells remain exposed to self-antigens in the periphery, which may lead to the development of functionally impaired memory, as observed with chronic viral infections (3, 4).

Despite these challenges, some vaccination strategies have been capable of inducing long-lived protective T-cell responses against poorly immunogenic tumors. In one of the earliest examples, Mullins et al. (5) showed that a CD40L-matured dendritic cell vaccine could generate CD8 T-cell recall responses against the melanocyte differentiation antigen tyrosinase, as well as long-term protection against melanoma. Cytokines can also drive the development of memory in vivo, as shown by the development of a durable and protective central and effector memory CD8 T-cell response following administration of a DNA vaccine encoding the tumor antigen Fra-1 and the cytokine interleukin-18 (IL-18; ref. 6). Costimulatory molecules may also play an important role. A xenogeneic DNA vaccine encoding gp100, which typically induces only short-term immunity, induced long-lived T-cell responses and tumor protection when coupled with a stimulatory antibody to GITR (7). However, aside from these studies, there are few examples of active immunotherapy inducing durable T-cell memory to tumor/self-antigens. More frequently, memory is shown following the immune-mediated rejection of primary tumors. For example, regression of B16 melanoma, induced by a GM-CSF–producing tumor cell vaccine and CTLA-4 blockade, protected mice from tumor challenge as long as 100 days after vaccination (8). Such studies illustrate that some aspect of active tumor rejection may lead to the development of immunologic memory.

Interestingly, historical data show that progressive tumors themselves can induce functional T-cell memory. In the 1980s, Bursuker and North (9) found that protective T-cell memory resulted after surgical excision of a highly immunogenic methylcholanthrene-induced tumor. This memory arose naturally in response to tumor growth and without a need for vaccination. However, it was crucial to excise primary tumors when they were small to prevent the generation of “suppressor” cells that would attenuate the response. Notably, postsurgical immunity was only a phenomenon of highly immunogenic tumors and was not observed in hosts bearing poorly immunogenic cancers (10).

In hindsight, the suppressor cells identified by Bursuker and North may likely have been tumor-induced regulatory T cells (Treg). Treg cells are crucial mediators of peripheral tolerance (11). They possess a CD4+CD25+Foxp3+ phenotype and arise both in the thymus and through the conversion of Foxp3−CD4+ T cells in the periphery (11, 12). Treg cells suppress the development of CD8 T-cell memory in infectious disease models (13), but until recently, their role in preventing memory against poorly immunogenic tumors had not been shown.

Many studies have illustrated that Treg cells prevent primary T-cell responses against poorly immunogenic cancers (11, 12). We previously showed that CD4+CD25+ Treg cells suppress the de novo priming of CD8 T cells in response to the poorly immunogenic B16 melanoma (14). If Treg cells were depleted during growth of the melanoma, mice primed CD8 T cells against differentiation antigens expressed by both the tumor cells and normal melanocytes. Melanoma tumor-bearing mice that lacked Treg cells also developed concomitant immunity, evidenced by the rejection of a secondary melanoma inoculated at a different site. Thus, Treg cells functioned early to suppress the de novo priming of immunity against this poorly immunogenic tumor. However, whether such tumor/self-antigen–specific T cells could develop into functional T-cell memory remained unknown.

Removing Treg Cells during Tumor Growth Drives the Natural Development of T-Cell Memory
We recently asked whether tumor growth and Treg depletion could induce functional T-cell memory by studying immunity following curative surgery in mice bearing B16 melanoma (15).
As in our previous work (14), mice were inoculated with B16 melanoma, and then T<sub>reg</sub> cells were eliminated with a CD4-depleting antibody. This strategy eliminates CD4<sup>+</sup>CD25<sup>+</sup> T cells as well as any CD4<sup>+</sup> precursors of induced T<sub>reg</sub> cells. Following T<sub>reg</sub> depletion, i.d. primary tumors were surgically excised to attenuate T-cell priming and to extend the life span of the mice. Defining T-cell memory was challenging because classic memory T cells are defined based on their ability to persist following the clearance of antigen (4). Because tumor/self-antigens are never cleared, we chose to use an operational definition of memory as a functional T-cell response present at least 1 month following surgery.

To assess the development of T-cell memory, mice were challenged with B16 tumor cells in the flank 1 month after surgery. Not surprisingly, mice that had received surgical treatment alone were overtaken by secondary tumors. Mice that had received CD4 depletion alone, but no primary tumor, also succumbed to the second tumors. However, 40% to 60% of mice that had been depleted of CD4 T cells during growth of their primary tumors were protected against secondary tumors given as long as 2 months after surgery. Moreover, these mice developed systemic immunity, evidenced by their rejection of lung tumors inoculated i.v. Lung tumors that were already established at the time of surgery were also rejected, indicating a potential control of metastatic disease. Importantly, the depletion of CD8 T cells abrogated this long-lived tumor protection, providing evidence of CD8 T-cell memory.

These data established that growth of a poorly immunogenic tumor could induce functional T-cell memory, although the specificity of these memory T cells was not known. Our previous work had shown that short-term CD8 T-cell responses in T<sub>reg</sub>-depleted, B16 melanoma-bearing mice were specific for tumor/self-antigens (14). Among these antigens were the melanosomal membrane proteins TRP-2/DCT and gp100 (14). However, due to immunologic tolerance and antigen persistence, it seemed unlikely that T cells against melanosomal proteins would be sustained following surgery. Because of this, we were surprised to find memory CD8 T cells specific for both TRP-2/DCT and gp100 in mice with postsurgical immunity. TRP-2/DCT–specific T cells were present at least 30 days after surgery, and IFN-γ–producing and IL-2–producing transgenic T cells specific for gp100 were found as long as 150 days following surgery. Thus, tumor growth in the absence of T<sub>reg</sub> cells induced durable T-cell memory against self-antigens expressed by the tumor.

**Figure 1.** Model for the generation of postsurgical tumor protection and T-cell memory in hosts bearing poorly immunogenic tumors. Left, surgery alone is insufficient for providing immunity against poorly immunogenic tumors that do not naturally prime functional CD8 T-cell responses and may instead induce T<sub>reg</sub> development. Right, however, the depletion of CD4 T cells eliminates natural and induced populations of T<sub>reg</sub> cells, thereby enabling the priming of protective antitumor immunity during growth of a poorly immunogenic tumor. This tumor-primed immune response develops into functional CD8 T-cell memory against tumor/self-antigens following surgical excision of the primary tumor. CD4 depletion in tumor-bearing mice also leads to concurrent autoimmunity against the normal tissue counterpart of the tumor. This model shows that T<sub>reg</sub> depletion in hosts bearing poorly immunogenic tumors is sufficient for the generation of CD8 T-cell memory following surgical tumor excision.
Because little was known about T-cell memory against tumor/self-antigens, it was also important to characterize these T cells with regard to their phenotype and localization. Based on infectious disease models, the memory CD8 T-cell compartment can be divided into two phenotypically and functionally distinct subpopulations: central (T<sub>CM</sub>) and effector (T<sub>EM</sub>) memory (16). In vitro-generated T<sub>CM</sub> are more potent than T<sub>EM</sub> for mediating tumor rejection (2). However, it was unknown whether tumor antigen–specific T<sub>CM</sub> could be generated in hosts with persistent peripheral self-antigen. Interestingly, we found that mice with postsurgical immunity developed a mixed population of antigen-specific T<sub>CM</sub> and T<sub>EM</sub>. T<sub>EM</sub> dominated the population and were found in lung as well as lymphoid tissues, whereas T<sub>CM</sub> represented a smaller population that was only found in lymphoid tissues. These data illustrated that T cells recognizing tumor/self-antigens can develop into long-lived populations of T<sub>EM</sub> and T<sub>CM</sub> even in the face of persistent antigen.

Finally, we observed that a high proportion of T<sub>reg</sub>-depleted tumor-excised mice also developed an autoimmune response against normal melanocytes. This was evidenced by the outgrowth of white hair (on black mice) beginning at the surgery site and against normal melanocytes. This was evidenced by the outgrowth of white hair (on black mice) beginning at the surgery site and progressing to other locations with time. This showed that melanoma growth can induce an immune response against normal host melanocytes and that such autoimmunity is normally prevented by T<sub>reg</sub> cells. Melanocyte-specific autoimmunity also provided further confirmation of the potent postsurgical immune response against shared tumor/self-antigens.

**Implications**

The study of postsurgical immunity has shown that T<sub>reg</sub> cells are a fundamental obstacle to the development of T-cell memory in hosts bearing poorly immunogenic tumors (Fig. 1). As seen in most patients with cancer, our model shows that surgery alone does not induce protection against poorly immunogenic tumors (Fig. 1, left). However, in our model, surgery in conjunction with the depletion of CD4<sup>+</sup> T<sub>reg</sub> cells enables the development of long-lived tumor protection and CD8 T-cell memory as well as melanocyte-specific autoimmunity (Fig. 1, right). The relative proportion of naturally occurring and tumor-induced T<sub>reg</sub> cells to the suppression of priming remains unknown, and future work will be required to characterize the kinetics of T<sub>reg</sub> responses in tumor-bearing mice (Fig. 1). However, based on the robust and diminished tumor protection that we observe following surgery, one could speculate that the T<sub>reg</sub> cells that reemerge in tumor-excised hosts are less suppressive than those that arise in tumor-bearing hosts.

T<sub>reg</sub> depletion several days before surgery could potentially be modeled in the clinic for the treatment of minimal residual disease. The best strategy for T<sub>reg</sub> depletion in vivo remains to be determined because cell surface markers specific for T<sub>reg</sub> cells have remained elusive. Total CD4 depletion is one possibility, as it clearly induces durable protective memory despite the temporary loss of CD4 helper T cells, which have been thought to be crucial for the development of memory (17). Targeting CD25 with an IL-2-diphtheria toxin fusion protein is another option that has already proven effective for the depletion of T<sub>reg</sub> cells in the peripheral blood of cancer patients (18). Alternatively, GITR stimulation and CTLA-4 blockade, which have been used in conjunction with vaccines to induce T-cell memory (7, 8), may act directly or indirectly to attenuate T<sub>reg</sub> function (11). Importantly, in this model of postsurgical immunity, the tumor itself serves as the source of antigen, presumably priming T-cell responses against a multitude of tumor antigens. Although our studies analyzed memory responses against tumor/self-antigens, it is likely that T cells specific for many antigens collectively provide tumor protection. Such a diverse T-cell repertoire may help to prevent the emergence of antigen loss variants, in contrast to immunotherapies that target only single antigens.

Following T<sub>reg</sub> depletion and surgery, we observe long-lived tumor protection and persistent populations of functional T<sub>CM</sub> and T<sub>EM</sub>. This was somewhat unexpected in light of studies with chronic viral infections, where a prolonged exposure to high loads of antigen leads to dramatic functional impairments within the memory T-cell compartment. Such responding CD8 T cells progressively lose functions and are eventually deleted (3, 19). However, it is important to note that this is not always the case. For example, we have previously observed in murine gammaherpesvirus (MHV-68) infection that most antiviral effector functions are intact (20). In fact, CD8 T cells from MHV-68 persistently infected mice mediate more efficient control of a challenge infection compared with cells from mice that have cleared the virus (20). Therefore, some memory cells generated in the face of persistent antigen may actually be better adapted for long-term immune surveillance. Memory T cells in mice with postsurgical immunity might also possess this capability.

In summary, there is now convincing evidence that functional CD8 T-cell memory can be generated against tumor/self-antigens. In contrast to studies that use active immunization, our study shows that poorly immunogenic tumors themselves can induce tumor-specific T-cell memory after the hurdle of T<sub>reg</sub> suppression is overcome. This work stresses the importance of exploring immunotherapies in conjunction with T<sub>reg</sub> depletion and the surgical treatment of cancer to provide long-lived and meaningful control of recurrent and metastatic disease.

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