TUMOR-SPECIFIC T CELL MEMORY: CLEARING THE T_{reg} HURDLE

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Abstract

Anti-tumor immune responses can be stimulated by interfering with regulatory T cell (T_{reg}) function. However this effect is short-lived unless T cell memory to tumor antigens can be generated. Our recent studies show that T_{reg} cells not only limit primary responses to tumor/self antigens in tumor-bearing hosts, but also prevent the natural generation of T cell memory to such antigens. Here we discuss the role of regulatory T cells in suppressing T cell memory after surgical excision of tumors, and the potential clinical benefits of overcoming this suppression.

Keywords

T cell memory; tumors; regulatory T cells; CD8 T cells; surgery; melanoma

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 demonstrated that memory T cells are required for pathogen clearance and protection against re-infection. More recently, these lessons are being applied to cancer models. While 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 a majority of 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, Engelhard and co-workers 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 (5). 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 *IL-18* (6). Co-stimulatory 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 demonstrated 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 post-vaccination (8). Such studies illustrate that some aspect of active tumor rejection may lead to the development of immunological memory.

Interestingly, historical data show that progressive tumors themselves can induce functional T cell memory. In the 1980's North found that protective T cell memory resulted after surgical excision of a highly-immunogenic methylcholanthrene-induced tumor (9). 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, post-surgical 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 North may likely have been tumor-induced regulatory T cells (T_{reg}). T_{reg} 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). T_{reg} 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 T_{reg} cells prevent primary T cell responses against poorly-immunogenic cancers (11,12). We previously demonstrated that $CD4^+CD25^+$ T_{reg} cells suppress the *de novo* priming of CD8 T cells in response to growth of the poorly-immunogenic B16 melanoma (14). If T_{reg} 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 T_{reg} cells also developed concomitant immunity, evidenced by the rejection of a secondary melanoma inoculated at a different site. Thus, T_{reg} 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 T_{reg} cells during tumor growth drives the natural development of T cell memory

We recently asked whether tumor growth and T_{reg} 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_{reg} cells were eliminated with a CD4 depleting antibody. This strategy eliminates $CD4^+CD25^+$ T cells, as well as any $CD4^+$ precursors of induced T_{reg} cells. Following T_{reg} depletion, intradermal primary tumors were surgically excised to attenuate T cell priming and to extend the lifespan of the mice. Defining T cell memory was challenging because classical 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 employ 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, 40–60% 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 intravenously. 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_{reg}-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 immunological 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 post-surgical immunity. TRP-2/DCT-specific T cells were present at least 30 days post-surgery, and IFN-γ 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_{reg} cells induced durable T cell memory against self antigens expressed by the tumor.

Because little was known about T cell memory against tumor/self antigens, it was also important to characterize these T cells with regards 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_{CM}) and effector (T_{EM}) memory (16). *In vitro* generated T_{CM} have been shown to be more potent than T_{EM} for mediating tumor rejection (2). However, it was unknown whether tumor antigen-specific T_{CM} could be generated in hosts with persistent peripheral self antigen. Interestingly, we found that mice with post-surgical immunity developed a mixed population of antigen-specific T_{EM} and T_{CM}. T_{EM} dominated the population and were found in lung as well as lymphoid tissues, whereas T_{CM} 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_{EM} and T_{CM}, even in the face of persistent antigen.

Finally, we observed that a high proportion of T_{reg}-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 progressing to other locations with time. This demonstrated that melanoma growth can induce an immune response against normal host melanocytes, and that such autoimmunity is normally prevented by T_{reg} cells. Melanocyte-specific autoimmunity also provided further confirmation of a potent post-surgical immune response against shared tumor/self antigens.

Implications

The study of post-surgical immunity has demonstrated that T_{reg} cells are a fundamental obstacle to the development of T cell memory in hosts bearing poorly-immunogenic tumors (Figure 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^{+} T_{reg} 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 contribution of naturally-occurring and tumor-induced T_{reg} cells to the suppression of priming remains unknown, and future work will be required to characterize the kinetics of T_{reg} responses in tumor-bearing mice (Fig. 1). However, based on the robust and

undiminished tumor protection that we observe following surgery, one could speculate that the T_{reg} cells that re-emerge in tumor-excised hosts are less suppressive than those that arise in tumor-bearing hosts.

T_{reg} depletion several days prior to surgery could potentially be modeled in the clinic for the treatment of minimal residual disease. The best strategy for T_{reg} depletion *in vivo* remains to be determined, because cell-surface markers specific for T_{reg} 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-diptheria toxin fusion protein is another option that has already proven effective for the depletion of T_{reg} 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_{reg} function (11). Importantly, in this model of post-surgical immunity, the tumor itself serves as the source of antigen, presumably priming T cell responses against a multitude of tumor antigens. While 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_{reg} depletion and surgery, we observe long-lived tumor protection and persistent populations of functional T_{CM} and T_{EM}. 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 to 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 post-surgical 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 which employ active immunization, our study shows that poorly-immunogenic tumors themselves can induce tumor-specific T cell memory after the hurdle of T_{reg} suppression is overcome. This work stresses the importance of exploring immunotherapies in conjunction with T_{reg} depletion and the surgical treatment of cancer to provide long-lived and meaningful control of recurrent and metastatic disease.

Acknowledgements

REFERENCES

1. Klebanoff CA, Gattinoni L, Restifo NP. CD8+ T-cell memory in tumor immunology and immunotherapy. Immunol Rev 2006;211:214–224. [PubMed: 16824130]
2. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 2005;102:9571–9576. [PubMed: 15980149]

3. van der Most RG, Murali-Krishna K, Lanier JG, Wherry EJ, Puglielli MT, Blattman JN, Sette A, Ahmed R. Changing immunodominance patterns in antiviral CD8 T-cell responses after loss of epitope presentation or chronic antigenic stimulation. Virology 2003;315:93–102. [PubMed: 14592762]

4. Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. J Virol 2004;78:5535–5545. [PubMed: 15140950]

5. Mullins DW, Sheasley SL, Ream RM, Bullock TN, Fu YX, Engelhard VH. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. J Exp Med 2003;198:1023–1034. [PubMed: 14530375]

6. Luo Y, Zhou H, Mizutani M, Mizutani N, Liu C, Xiang R, Reisfeld RA. A DNA vaccine targeting Fos-related antigen 1 enhanced by IL-18 induces long-lived T-cell memory against tumor recurrence. Cancer Res 2005;65:3419–3427. [PubMed: 15833877]

7. Cohen AD, Diab A, Perales MA, Wolchok JD, Rizzuto G, Merghoub T, Huggins D, Liu C, Turk MJ, Restifo NP, Sakaguchi S, Houghton AN. Agonist anti-GITR antibody enhances vaccine-induced CD8 (+) T-cell responses and tumor immunity. Cancer Res 2006;66:4904–4912. [PubMed: 16651447]

8. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med 1999;190:355–366. [PubMed: 10430624]

9. Bursuker I, North RJ. Immunological consequences of tumor excision: from active immunity to immunological memory. Int J Cancer 1986;37:275–281. [PubMed: 3943923]

10. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. J Natl Cancer Inst 1957;18:769–778. [PubMed: 13502695]

11. Yamaguchi T, Sakaguchi S. Regulatory T cells in immune surveillance and treatment of cancer. Semin Cancer Biol 2006;16:115–123. [PubMed: 16376102]

12. Wang HY, Wang RF. Regulatory T cells and cancer. Curr Opin Immunol 2007;19:217–223. [PubMed: 17306521]

13. Murakami M, Sakamoto A, Bender J, Kappler J, Marrack P. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. Proc Natl Acad Sci U S A 2002;99:8832–8837. [PubMed: 12084927]

14. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med 2004;200:771–782. [PubMed: 15381730]

15. Zhang P, Cote AL, de Vries VC, Usherwood EJ, Turk MJ. Induction of postsurgical tumor immunity and T-cell memory by a poorly immunogenic tumor. Cancer Res 2007;67:6468–6476. [PubMed: 17616708]

16. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–763. [PubMed: 15032595]

17. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science 2003;300:339–342. [PubMed: 12690202]

18. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E, Vieweg J. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005;115:3623–3633. [PubMed: 16308572]

19. Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol 2004;172:4204–4214. [PubMed: 15034033]

20. Obar JJ, Fuse S, Leung EK, Bellfy SC, Usherwood EJ. Gammaherpesvirus persistence alters key CD8 T-cell memory characteristics and enhances antiviral protection. J Virol 2006;80:8303–8315. [PubMed: 16912282]
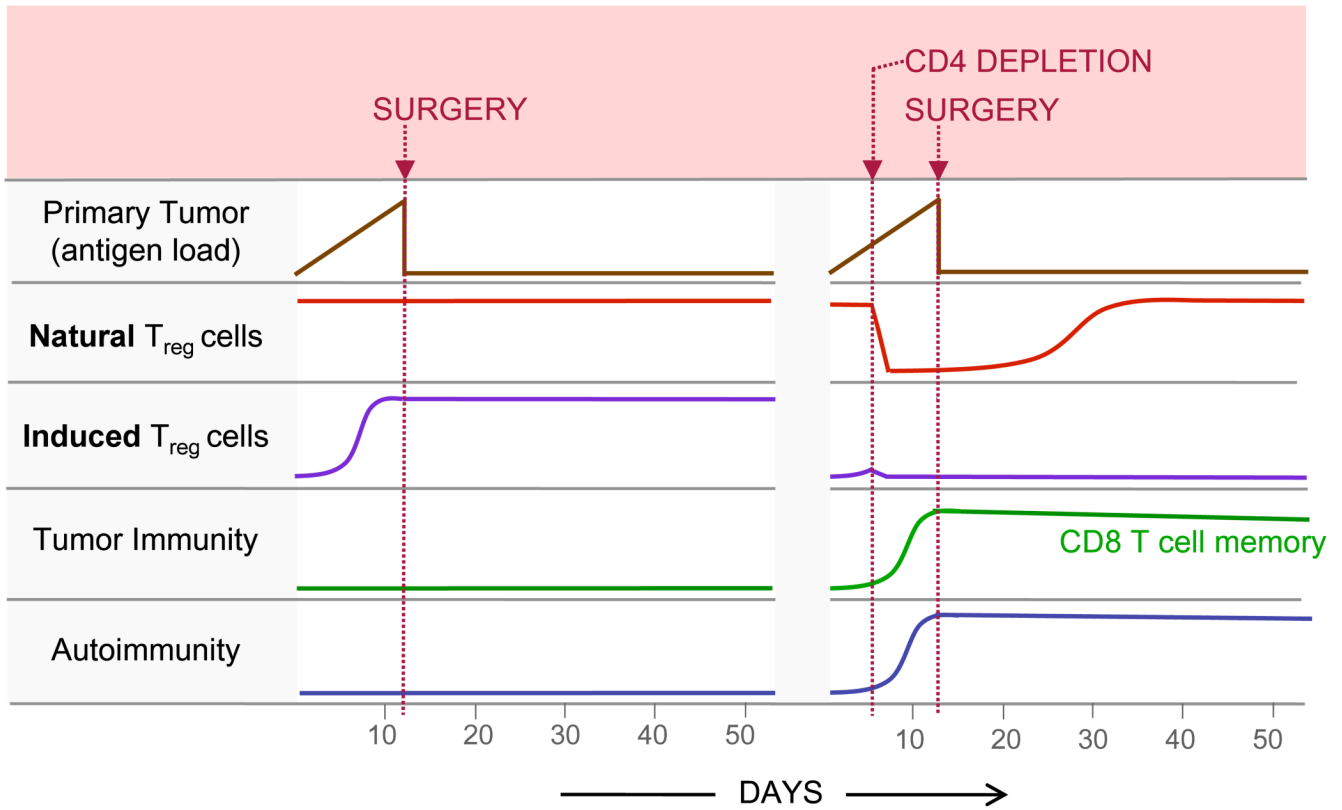
Figure 1. Model for the generation of post-surgical tumor protection and T cell memory in hosts bearing poorly-immunogenic tumors

Surgery alone is insufficient for providing immunity against poorly-immunogenic tumors which do not naturally prime functional CD8 T cell responses, and may instead induce T_{reg} development (left panel). However, the depletion of CD4 T cells eliminates natural and induced populations of T_{reg} cells, thereby enabling the priming of protective anti-tumor immunity during growth of a poorly-immunogenic tumor (right panel). This tumor-primed immune response develops into functional CD8 T cell memory against tumor/self antigens following surgical excision of the primary tumor (right panel). CD4 depletion in tumor bearing mice also leads to concurrent autoimmunity against the normal tissue counterpart of the tumor (right panel). This model demonstrates that T_{reg} depletion in hosts bearing poorly-immunogenic tumors is sufficient for the generation of CD8 T cell memory following surgical tumor excision