Vaccination against the Forkhead Family Transcription Factor Foxp3 Enhances Tumor Immunity

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Abstract

Depletion of CD4+CD25+ regulatory T cells (Treg) by treatment with αCD25 antibody synergizes with vaccination protocols to engender protective immunity in mice. The effectiveness of targeting CD25 to eliminate Treg is limited by the fact that CD25, the low-affinity interleukin-2 receptor, is up-regulated on conventional T cells. At present, foxp3 is the only product known to be exclusively expressed in Treg of mice. However, foxp3 is not expressed on the cell surface and hence cannot be targeted with antibodies. In this study, we tested the hypothesis that vaccination of mice against foxp3, a self-antigen expressed also in the thymus, is capable of stimulating foxp3-specific CTL that will cause the depletion of Treg and enhanced antitumor immunity. Vaccination of mice with foxp3 mRNA-transfected dendritic cells elicited a robust foxp3-specific CTL response and potentiated vaccine-induced protective immunity compared with that of αCD25 antibody administration. In contrast to αCD25 antibody treatment, repeated foxp3 vaccination did not interfere with vaccine-induced protective immunity. Importantly, foxp3 vaccination led to the preferential depletion of foxp3-expressing Treg in the tumor but not in the periphery, whereas αCD25 antibody treatment led to depletion of Treg in both the tumor and the periphery. Targeting foxp3 by vaccination offers a specific and simpler protocol for the prolonged control of Treg that may be associated with reduced risk of autoimmunity, introducing an approach whereby specific depletion of cells is not limited to targeting products expressed on the cell surface. [Cancer Res 2006;67(1):371–80]

Introduction

The immune system has established an elaborate network of central and peripheral tolerance mechanisms to discriminate between self and nonself. An important component of this network are the CD4+CD255 regulatory T cells (Treg), which mediate self-tolerance and immune homeostasis by acting in a dominant cell intrinsic manner to regulate immune functions (1–3). Two sets of observations also implicate Treg in suppression of tumor immunity. First, Treg accumulation at the tumor sites of cancer patients correlated with disease progression (4, 5). Second, elimination of Treg in mice by treatment with an αCD25 antibody enhances the immune-mediated rejection of tumors (6, 7) and synergizes with vaccination protocols (8, 9). In a recent clinical trial, depletion of Treg in renal cancer patients using an interleukin-2 (IL-2)/diphtheria toxin fusion product (ONTAK) led to enhanced vaccine-induced antitumor immune responses (10). Thus, elimination of Treg could represent an important adjunct to cancer immunotherapy.

The low-affinity α-chain IL-2 receptor CD25 is constitutively expressed on Treg and is up-regulated on conventional antigen-activated T cells, including the vaccine-induced antitumor effector T cells. This poses certain limitations on targeting CD25 to deplete Treg. (a) Treg rebound following depletion of the CD25-expressing cells within a 3- to 5-week period in mice (11, 12). Indeed, administration of αCD25 antibody subsequent to tumor vaccination was detrimental to vaccine-induced protective immunity (8, 13). Thus, depletion protocols must be confined to a period before vaccination, thus precluding the ability of controlling Tregs over time in the vaccinated patients. (b) Although the thymus is a major source of Treg, Treg can be also generated and/or expanded in the periphery as a result of suboptimal antigenic stimulation (14). Two recent studies have shown that vaccination of tumor-bearing mice led to the de novo conversion (15) or the amplification of preexisting (16) Treg populations, potentially exacerbating tumor-specific immune suppression. Thus, depletion of vaccine-induced Treg would be also beneficial but cannot be accomplished by targeting CD25. (c) Depletion of CD25-expressing cells could potentially interfere with an ongoing T cell–mediated control of subclinical pathogenic infections, with dire consequences that cannot be predicted in advance. (d) A significant fraction of Treg that, can reach up to 50%, express low to undetectable levels of CD25 (12, 17, 18) and hence will not be subject to elimination by αCD25 antibody therapy. (e) In addition, because αCD25 antibody–mediated Treg depletion is nonspecific, it can induce or exacerbate autoimmune pathology (11, 19–21).

Other products that are up-regulated on the surface of Treg are GITR, CTLA-4, CD103 or LAG-3, and OX-40, but like CD25, they are not expressed exclusively on Treg (12, 17, 18) as well as in CD4+CD25low/foxp3– Treg (12, 17, 18) as well as in subsets of CD8+ T cells expressing immune-suppressive properties (27). Thus, targeting foxp3 offers distinct advantages over targeting CD25 to eliminate immune-suppressive cells in vivo.

Unlike CD25, foxp3 is a nuclear product and is not expressed on the cell surface. Hence, antibodies or ligand-based reagents, such as ONTAK, cannot be used to eliminate foxp3-expressing cells in vivo. Because CD8+ CTLs can recognize antigenic determinants
derived from any cellular compartment expressed on the cell surface in association with MHC class I molecules, we tested the hypothesis that vaccination of mice against foxp3 is capable of stimulating a foxp3-specific CTL response leading to the elimination of foxp3-expressing cells and enhanced antitumor immunity. In this study, we show that despite the fact that foxp3 is a self-antigen expressed in the thymus (25, 26, 28), immunization of mice against foxp3 elicits a robust foxp3-specific CTL response, enhances vaccine-induced antitumor immunity, and, in contrast to αCD25 antibody treatment, does not interfere with vaccine-induced antitumor immunity. Notably, foxp3 vaccination leads to the preferential depletion of intratumoral, but not peripheral, Treg that could translate to reduced risk of autoimmunity.

**Materials and Methods**

**Mice.** Four- to 6-week-old C57BL/6 mice (H-2b) were obtained from the Jackson Laboratory (Bar Harbor, ME). In conducting the research described in this article, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals” as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**Cell lines.** The F10.9 clone of the B16 melanoma of C57BL/6 origin is a highly metastatic, poorly immunogenic, and a low class I expressing cell line (29). Other cell lines used were EL4 cells (C57BL/6, H-2b, and thymoma). Cells were maintained in DMEM supplemented with 10% FCS, 25 mmol/L HEPES, 2 mmol/L l-glutamine, and 1 mmol/L sodium pyruvate.

**Cloning of murine foxp3, tyrosinase-related protein-2, and actin mRNA and electroporation of dendritic cells.** Creation of pSP73-Sph/A64 and cloning of pSP73-Sph/TRP-2/A64 and pGEM4Z/murine actin/A64 were previously described (30). Truncated foxp3 was amplified by reverse transcription-PCR (RT-PCR) from F10.9 tumor grown in naïve C57/B6 mice using the following primers: 5'-TATATAAAGCTTGCCACCATGGCTCC-TTCCTTGGCCCTTGGCCCATCC-3' and 5'-ATATATTCTAGACTAGGCGAA-CATGCGAGTAAAC-3'. This amplifies a fragment from nucleotide 28 of the coding region, a naturally occurring ATG, to position 1120, mutated from TAC → TAG, tyrosine → stop, thereby disrupting the forkhead that is encoded by nucleotides 1010 to 1260 and deleting one of two putative

**Figure 1.** Induction of foxp3-specific CTL in mice vaccinated with foxp3 mRNA–transfected dendritic cells. Dendritic cells (DC) were transfected with mRNA and injected into mice. Ten days later, splenocytes were isolated and used in a CTL assay as described in Materials and Methods. A, expression of foxp3. Dendritic cells were transfected with mRNA encoding foxp3 or actin, stained with phycoerythrin-labeled anti-foxp3 antibody, and analyzed by flow cytometry as described in Materials and Methods. B, CTL recognition of foxp3-expressing dendritic cell targets. Splenocytes from mice immunized with TRP-2 mRNA–transfected dendritic cells, foxp3 mRNA–transfected dendritic cells, or treated with PBS were used in a CTL assay against TRP-2, foxp3, or actin mRNA–transfected dendritic cell targets. C, CTL recognition of foxp3-expressing CD25+ splenocytes. Splenocytes were fractionated into CD25+ and CD25− populations and used as targets in CTL assays with splenocytes. Splenocytes were first depleted of RBC followed by adherence for 1 h at 37°C. T cells were isolated using the EasySep Mouse T-cell enrichment kit from StemCell Technologies (Vancouver, Canada) as per manufacturer’s protocol. T cells were labeled with europium and incubated overnight as described in Materials and Methods. CD25+ T cells were isolated from the total T cells by positive selection. The cells were first labeled with the αCD25 antibody (PC61) followed by EasySep PE selection kit from StemCell Technologies. The cells that were not CD25+ T cells were used as control. DC, susceptibility of CD25+ cells to CTL lysis. Mice were immunized with chicken OVA peptide–pulsed dendritic cells and splenocytes used in a CTL assay against CD25+ cells, CD25− cells, or dendritic cells pulsed with either a class I restricted OVA peptide (SIINFEKL) or a control peptide.
nuclear localization signals encoded in nucleotides 1241 to 1270. The PCR fragment was cloned into psP73-Sph/64 to create psP73-Sph/foxp3/A64. The Spl-e linearized template was used in T7 mMessage mMachine (Ambion, Austin, TX) reactions to produce foxp3 in vitro transcribed RNA. Dendritic cell electroporation was carried out as previously described (30).

**CTL induction in vivo.** Bone marrow precursor–derived dendritic cells were generated and electroporated with RNA as previously described (30). Naive syngeneic mice were immunized s.c. at the base of the ear pinna with 1 to 1.25 × 10^5 RNA-transfected dendritic cells per ear pinna in 50 μL PBS for a total of 100 μL per mouse. For experiments testing the combination of tyrosinase-related protein-2 (TRP-2) and Foxp3 or TRP-2 and actin, mice were immunized with 2 to 2.5 × 10^5 dendritic cells for each antigen for a combined 4 to 5 × 10^5 dendritic cells in 100 μL per mouse. Splenocytes were harvested 10 days after immunization and depleted of RBC with ammonium chloride Tris buffer followed by adherence for 1 h. Non-adherent splenocytes (10^6) were cultured with 2 × 10^5 stimulator cells (dendritic cells electroporated with RNA) in 5 mL RPMI with 10% FCS, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5 × 10^-5 mol/L 3-mercaptopethanol per well in a six-well tissue culture plate. The responders were stimulated with the same antigen as used for the immunization. Cells were cultured for 5 days at 37°C and 5% CO₂. Effector were harvested on day 5 for CTL assay. In vitro cytotoxicity assays were done as previously described (30).

**Tumor immunotherapy.** For the F10.9-B16 melanoma model, mice were implanted s.c. with 2.5 × 10^5 B16/F10.9 tumor cells and, 2 d later, immunized with mRNA-transfected dendritic cells as shown (10 mice per group). All mice received a constant amount of mRNA-transfected dendritic cells. Mice were monitored for the appearance of palpable tumors. Statistical analysis: actin versus TRP-2 or foxp3, P = 0.002; foxp3 or TRP-2 versus TRP-2 + foxp3, P = 0.01.

**Figure 2.** Inhibition of B16/F10.9 melanoma growth in mice immunized against TRP-2 and foxp3. Mice were implanted with 2.5 × 10^5 B16/F10.9 tumor cells and, 2 d later, immunized with mRNA-transfected dendritic cells as shown (10 mice per group). All mice received a constant amount of mRNA-transfected dendritic cells. Mice were monitored for the appearance of palpable tumors. Statistical analysis: actin versus TRP-2 or foxp3, P = 0.002; foxp3 or TRP-2 versus TRP-2 + foxp3, P = 0.01.

**Results**

Foxp3 is a self-antigen expressed not only in circulating Treg but also in the thymus (25, 26, 28). It was, therefore, expected that foxp3 will have triggered significant tolerance, and that induction of foxp3-specific immune responses would be difficult. To test this, we immunized mice against foxp3 using foxp3 mRNA-transfected dendritic cells, an efficient method of stimulating CD8+ CTL responses in mice (31). For comparison, mice were immunized against TRP-2, a melanocyte-specific product against which CTL responses and protective immunity can be routinely stimulated in mice (32). To reduce the possibility that ectopic foxp3 expression could adversely affect dendritic cell function, a truncated function-inactivated foxp3 product was used by disrupting the forkhead structure and deleting one of the two putative nuclear localization signals (see Materials and Methods). Figure 1A shows that dendritic cells transfected with foxp3 mRNA but not actin mRNA express foxp3 protein, as measured by flow cytometry. Because the truncated foxp3 product may bind weakly the antibody and/or exhibit reduced stability, the steady-state foxp3 levels measured by flow cytometry shown in Fig. 1A are likely to represent an underestimate of the transfection efficiency and the generation of T-cell determinants. Figure 1B shows that immunization of mice against the truncated foxp3 elicited a CTL response, which lysed foxp3 mRNA-transfected dendritic cells, but not TRP-2 or actin mRNA-transfected dendritic cells. Importantly, the magnitude of foxp3 CTL was comparable with that of TRP-2 CTL stimulated in a similar manner. Thus, immunization against foxp3 was capable of stimulating a robust foxp3-specific CTL response despite the fact that foxp3 is a self-antigen expressed in the thymus.

Because Treg are capable of directly suppressing the cytolytic functions of antigen-activated CD8+ T cells (33, 34), the use of mRNA-transfected dendritic cells as targets left unanswered the question of whether the foxp3-specific CTL shown in Fig. 1B can also kill their physiologic targets, the Treg. To address this issue, we sorted splenocytes for CD25+ and CD25- cells enriched for Treg and conventional naive T cells, respectively. Using real-time RT-PCR, the CD25+ population that constituted 2% to 3% of total splenocytes expressed 45-fold more foxp3 than the CD25- population (data not shown). Figure 1C shows that the CD25+foxp3+, but not the CD25+foxp3-, cells were killed by the splenocytes from the foxp3-immunized mice. This experiment shows that Treg are susceptible to lysis by the vaccine-induced foxp3 CTL. In vitro killing of the Treg targets (Fig. 1C) was clearly inferior to that of foxp3 mRNA-transfected dendritic cells (Fig. 1B). This could reflect either inherent differences in the susceptibility of the bone marrow–derived dendritic cells and the splenic Treg to lysis by CTL, or direct inhibition of the foxp3 CTL effector functions by the cognate Treg targets. To distinguish between these two possibilities, CD25+ and CD25- T cells, as well as dendritic cells, were pulsed with the class I restricted ovalbumin (OVA) peptide and used as targets in a CTL assays. Splenocytes isolated from mice immunized with OVA peptide–pulsed dendritic cells were used as effectors. As shown in Fig. 1D, both foxp3-expressing CD25+ T cells and “conventional” foxp3-negative CD25- T cells pulsed with OVA peptide were killed at comparable efficiency, which was significantly lower than that of OVA peptide–pulsed dendritic cells. This result shows that the low efficiency of foxp3 CTL-mediated lysis of the spleen-derived Treg compared with the lysis of foxp3 mRNA-transfected dendritic cells is due to reduced susceptibility of splenic T cells to CTL lysis compared with bone marrow–derived dendritic cells, and not to Treg-mediated inhibition.

Having shown that vaccination against foxp3 induces CTL responses in vivo, we tested whether co-vaccination of mice against...
a tumor antigen and foxp3 can potentiate vaccine-induced antitumor immunity. As a prototype tumor antigen, we targeted TRP-2, which constitutes the dominant tumor antigen in the B16 melanoma tumor (32). To test whether foxp3 vaccination enhances protective antitumor immunity, mice were implanted s.c. with B16/F10.9 tumor cells, a poorly immunogenic variant of the original B16 tumor cell line (29), and immunized 2 days later by a single injection of either TRP-2 mRNA–transfected dendritic cells, foxp3 mRNA–transfected dendritic cells, or both TRP-2 and foxp3 mRNA–transfected dendritic cells. A control group of mice was immunized with actin mRNA–transfected dendritic cells. Tumor growth was monitored by determining the time to appearance of palpable tumors. As shown in Fig. 2, a single immunization with TRP-2 mRNA–transfected dendritic cells resulted in a significant delay in tumor growth, but eventually all mice succumbed to tumor. Likewise, vaccination against foxp3 also resulted in a modest delay in tumor growth similar to that of vaccination against TRP-2. However, when TRP-2 vaccination was combined with foxp3 vaccination a significant inhibition of tumor growth was seen, a proportion of mice remaining tumor-free long term (3 of 10 mice in Fig. 2). These observations support the view that removal of Treg in the tumor-bearing mice by vaccination against foxp3 enhances both a naturally occurring, albeit weak, antitumor response and synergizes with antitumor vaccination. The magnitude of the antitumor response resulting in the apparent cure of a proportion of the tumor-bearing animals following a single vaccination cycle is indicative of the benefits of combining a suboptimal antitumor vaccination protocol with vaccination against foxp3.

Current methods used to deplete Treg in vivo target CD25 by systemic administration of αCD25 antibody. The experiment shown in Fig. 3A compares the antitumor effects of foxp3 vaccination (left) and αCD25 antibody treatment (right), showing that both treatments elicit an essentially identical response as also seen in Fig. 2 (i.e., a significant retardation, but no long-term survival when foxp3 vaccination or antibody is given to
actin-vaccinated mice, and potentiation of TRP-2 vaccination-induced tumor inhibition with a fraction of mice remaining tumor-free). Although conditions for either antibody depletion or foxp3 vaccination have not been optimized, these results suggest that the antitumor effect of foxp3 vaccination is comparable with that of αCD25 antibody treatment. To determine whether enhanced protective immunity correlates with CTL responses, induction of TRP-2–specific CTL was determined in ex vivo stimulated splenocytes using a standard cytotoxicity assay. As shown in Fig. 3B, despite the fact that TRP-2 is a self-antigen, even a single vaccination elicited, albeit a modest, CTL response, which killed TRP-2–expressing B16/F10.9 tumor cells, but not TRP-2–negative EL4 tumor cells (left). As expected, CD25 antibody–mediated depletion of Treg led to a significant enhancement of the vaccine-induced TRP-2–specific CTL response (middle). Consistent with our hypothesis and the data shown in Figs. 2 and 3A, co-immunization against foxp3 also resulted in an enhanced CTL response, which was comparable with that seen in the antibody-treated mice (right). To determine the role of CD4+ and CD8+ T-cell subsets in protective immunity when vaccinated mice are co-vaccinated against foxp3 or given αCD25 antibody, CD4+ or CD8+ T cells were depleted with corresponding antibodies 1 week after immunization. Figure 4 shows that both CD4+ and CD8+ T cells were necessary to confer protective immunity in the TRP-2–vaccinated mice depleted of Treg by either co-vaccination against foxp3 or αCD25 antibody administration.

Following depletion, Tregs eventually rebound and reach normal levels within 3 to 5 weeks (11, 12). Moreover, the tumor (15) or the vaccination protocol itself (16) could contribute to the de novo generation and/or further expansion of the Treg pool. Thus, from a therapeutic standpoint, regulating Treg levels over time by periodically repeating the depletion protocol will be highly desirable. We hypothesized that because foxp3 expression, unlike CD25 expression, is restricted to Treg, repeated foxp3 vaccination should not interfere with a vaccine-induced antitumor T-cell response. To test this hypothesis, B16/F10.9 tumor-bearing mice were vaccinated against TRP-2 and foxp3 2 days after tumor implantation and monitored for tumor growth. Another group of mice received a second foxp3 vaccination 1 week after the first vaccination (9 days after tumor implantation). For comparison, instead of foxp3 vaccination, mice were treated with αCD25 antibodies once or twice using the same schedule. Consistent with the results shown in Fig. 3A, a single treatment with αCD25 antibody (Fig. 5A) or vaccination against foxp3 (Fig. 5B) combined with TRP-2 vaccination was superior to either antibody treatment or foxp3 vaccination given in the absence of TRP-2 vaccination. (As shown in Fig. 3A, αCD25 antibody administration or foxp3 vaccination alone had a modest antitumor effect, which was comparable with that of TRP-2 vaccination). As was previously
shown (8, 13), a second administration of αCD25 antibody 7 days after TRP-2 vaccination led to accelerated tumor growth (Fig. 5A), most likely due to depletion of the vaccine-activated TRP-2 T cells. In contrast to αCD25 treatment, a second foxp3 vaccination had no adverse effect on the vaccine-induced protective antitumor response, although it did not result in better protection (Fig. 5B). In a second experiment, a slight enhancement of protective immunity was seen following a second foxp3 vaccination, but it did not reach statistical significance (data not shown). A likely reason why a second foxp3 vaccination did not result in further enhancement of antitumor immunity is that it takes 3 to 5 weeks for Treg to rebound (11, 12), coupled with the aggressive growth of the B16/F10.9 tumors.

The fate of foxp3-expressing Treg in the periphery and the tumors of mice vaccinated against either TRP-2, TRP-2 + αCD25 antibody treatment, or TRP-2 + foxp3 was determined by multi-parameter flow cytometry. As shown in Fig. 6A, administration of αCD25 antibody resulted in a 2-fold reduction of foxp3-expressing cells in the periphery, measured in the spleen or the vaccine-draining lymph node. As expected, the majority of foxp3-expressing cells in the lymph node or spleen were CD4+ T cells. Notably, and consistent with recent findings (12, 17, 18), not all CD4+foxp3+ T cells expressed CD25. In fact, the 50% reduction in foxp3-expressing cells following αCD25 antibody administration was due to the fact that a significant fraction of the CD4+foxp3+ cells expressed low to undetectable levels of CD25 and not because αCD25 antibody depletion was inefficient, underscoring one of the limitations of using αCD25 antibody to control Treg activity in vivo. Surprisingly, as shown in Fig. 6B, foxp3 vaccination did not affect the percentage of foxp3-expressing cells in the draining lymph node or the spleen. We have consistently seen that in the spleen, but not the lymph nodes, of the foxp3-vaccinated mice, a numerical redistribution of CD25+foxp3+ to CD25- foxp3+ has taken place. The mechanism and significance of this phenomenon is currently being investigated.

The effect of foxp3 vaccination or αCD25 antibody treatment on tumor-infiltrating foxp3-expressing cells is shown in Fig. 6C. Both foxp3 vaccination or αCD25 antibody treatment led to a significant reduction in the numbers of intratumoral foxp3-expressing cells (85% and 80%, respectively). Both treatments also resulted in a modest 2-fold decrease in tumor infiltrating CD4+ T cells, mostly reflecting the depletion of the foxp3+CD4+ T cells. Interestingly, foxp3 vaccination, and to a lesser extent αCD25 antibody treatment, was also accompanied by a decrease in (foxp3 negative) CD8+ T cells in the tumor. Importantly, however, both methods of Treg depletion resulted in ~2-fold increase in the ratio of conventional T cells/Treg in the tumors of the treated mice (Fig. 6D). In summary, flow cytometric analysis has shown that αCD25 antibody treatment and foxp3 vaccination result in the intratumoral depletion of Treg, but whereas αCD25 antibody treatment also affected the peripheral pool of Treg, foxp3 vaccination did not. Thus, foxp3 vaccination, in stark contrast to αCD25 antibody treatment, seems to cause the preferential depletion of Treg in the tumor, but not in the periphery.

Foxp3 vaccination or CD25 depletion protocols used in this study were not associated with morbidity, mortality, or signs of vitiligo, and no anti-DNA antibody were detected in the foxp3-immunized mice (data not shown). It remains to be seen if the differential effect of foxp3 vaccination on tumor-infiltrating Treg will translate to reduced autoimmune manifestations.

Figure 6. Flow cytometry analysis of foxp3-expressing cells in mice vaccinated against foxp3 or treated with αCD25 antibody. A and B, mice were vaccinated against TRP2 and either depleted of CD25+ cells with antibodies or co-vaccinated against foxp3 as described in Materials and Methods and Fig. 4. Seven to 10 d later, spleens and auricular lymph nodes were harvested, and cells were stained with antibodies against foxp3, CD25, and CD4 and analyzed by flow cytometry. Cells were labeled for surface markers for 20 min on ice with αCD25 antibody/APC and CD4 antibody/FITC before intracellular staining for Foxp3. Staining for Foxp3 was done with the phycoerythrin anti-mouse Foxp3 antibody staining kit and according to the manufacturer’s protocol. All antibodies and isotype controls were obtained from eBioscience (San Diego, CA). Foxp3high cells (left) as gated populations were analyzed for CD4 and CD25 expression (right). C, mice were implanted s.c. with B16 melanoma cells immediately before foxp3 vaccination or αCD25 antibody treatment, as described in Fig. 2 legend. Tumors were harvested as soon as they were palpable and pooled (Trp-2 group, 4 of 5 tumors; Trp-2 + foxp3 group, 4 of 5 tumors; Trp-2 + αCD25 antibody group, 3 of 5 tumors). Single tumor cell suspensions were generated, and cells were stained with APC-conjugated αCD25 antibody and FITC-conjugated αCD4 antibody and phycoerythrin/Cy5-conjugated αCD8, fixed, permeabilized, and stained with phycoerythrin-conjugated anti-foxp3 antibody. D, ratio of conventional T cells (Foxp3+CD4+ and CD8+ cells) to Treg cells (Foxp3–) was calculated from the flow cytometry data shown in (C).
Foxp3 Vaccination–Mediated Treg Depletion

A

TRP-2

Lymph node

Spleen

TRP-2 + αCD25 Ab

B

TRP-2

TRP-2 + Foxp3

C

TRP-2

D

TRP-2 + Foxp3

TRP-2 + αCD25 Ab
Discussion

The underlying hypothesis of this study was that immunization against foxp3 would potentiate vaccine-induced antitumor immunity. We have shown that (a) vaccination of mice with foxp3 mRNA-transfected dendritic cells elicits a robust foxp3-specific CTL response, which is capable of killing foxp3-expressing Tregs in vitro (Fig. 1); (b) co-vaccination of mice against a tumor antigen (TRP-2) and foxp3 potentiates the vaccine-induced protective immunity (Figs. 2 and 3A), which correlates with enhanced induction of TRP-2-specific CTL responses (Fig. 3B); and (c) the effect of foxp3 vaccination on tumor immunity is comparable with that of αCD25 antibody administration (Fig. 3), the current gold standard in Treg depletion-mediated enhancement of tumor immunity (6–9). Nevertheless, despite having established the concept that induction of foxp3-specific CTL responses can enhance tumor immunity, neither the foxp3 vaccination protocol nor the αCD25 antibody treatment have been optimized to permit a conclusion about their relative potency. Considering the aggressive nature and low immunogenicity of the B16/F10.9 melanoma tumor cell line, the antitumor effects seen in the tumor-bearing mice immunized once without boosting against an endogenous "self" tumor antigen and foxp3 (Figs. 2 and 3A) is not unremarkable. In vitro elimination of T cells by vaccinating against T cell–specific products is not without precedence. Cohen et al. have shown that vaccination with irradiated T cells corresponding to a T-cell clone specific to a myelin basic protein (MBP) epitope can protect mice from MBP-induced experimental autoimmune encephalomyelitis. Notably, the T-cell response was not directed against idiotypic determinants of the MBP T-cell clone but rather to determinants expressed on activated T cells; yet, no adverse effects were seen in the immunized mice (35, 36).

Foxp3 is a self-antigen that is also expressed in the thymus, both in thymocytes destined to become Treg (25, 26) and in thymic stromal cells (28). It was, therefore, surprising that we did not detect significant tolerance against this thymic antigen. As shown in Fig. 1B, immunizing mice against foxp3 elicits a CTL response of a magnitude comparable with that of a CTL response induced against TRP-2. In preliminary experiments, foxp3-specific CTL responses could be also stimulated in vitro from the PBMC of human volunteers, suggesting that the absence of tolerance to foxp3 may also extend to human settings (data not shown). Although providing evidence for lack of operational tolerance against foxp3 in the CD8+ T-cell compartment, which can be exploited for vaccination purposes, these experiments do not exclude the existence of effective tolerance in vivo especially in the CD4+ T-cell and humoral compartments.

Because CD25 is also up-regulated on conventional T cells upon activation, targeting CD25 to deplete Treg must be restricted to a period before vaccination, precluding the long-term control of Treg, which in mice rebound within 3 to 5 weeks of depletion (11, 12). Indeed, administration of αCD25 antibody following vaccination abrogated protective immunity conceivably by depleting the vaccine-induced tumor-specific effector T cells (refs. 8, 13 and this study; Fig. 5A). A main advantage of targeting foxp3 is that foxp3 expression is highly restricted to Treg and, at least in mice, is not expressed in antigen-activated T cells (1). Consistent with the Treg-restricted expression of foxp3 and in stark contrast to αCD25 antibody administration, a second foxp3 vaccination subsequent to tumor vaccination did not adversely affect the vaccine-induced protective immune response (Fig. 5B), suggesting that targeting foxp3 will offer a strategy to control Treg over time. The recent demonstration that the vaccination protocol itself can generate de novo and/or amplify a preexisting Treg pool (15, 16) underscores the potential benefits of regulating Treg function after vaccination when the tumor-specific conventional T cells express CD25.

Whether foxp3 expression is restricted to Treg in humans is less clear. Several studies have shown that foxp3 expression is Up-regulated in polyclonally activated “conventional” CD4+CD25− T cells (37–39), whereas one study that used a purified naive CD4+CD45RA+CD25− T cell subset failed to observe up-regulation of foxp3 expression upon antigenic stimulation (40). Yet, another study has reported a 10-fold difference in foxp3 expression between the polyclonally activated conventional T cells and Treg (41). A recent study has shown that a proportion (<25%) of polyclonally activated conventional T cells up-regulated foxp3 transiently and at significantly lower levels compared with Treg (42). Thus, at present, the weight of evidence favors the view that the detection of foxp3 in ex vivo cultured human T cells represents either the up-regulation of foxp3 in precommitted Treg, the expansion of a small number of preexisting foxp3+ Treg, or the transient and low level up-regulation of foxp3 in a fraction of T cell receptor–stimulated conventional T cells (which could possibly reflect suboptimal in vitro stimulation conditions favoring the de novo generation of Treg, as shown in murine systems; ref. 14). However, the possibility that in humans, in stark contrast to rodents, foxp3 represents an activation marker necessary but not sufficient to confer a suppressive phenotype, cannot be excluded.

A second potential advantage of targeting foxp3 for Treg depletion is that foxp3 expression defines a broader set of immune suppressive cells, including a subset of CD8+ T cells exhibiting suppressive potential (27). In particular, a significant fraction of the foxp3-expressing CD4+ Treg-expressing low to undetectable levels of CD25 yet retains suppressive functions (12, 17, 18, 26). In humans, CD25 expression on CD4+ T cells as determined by flow cytometry does not provide a clear demarcation between conventional T cells and Treg (43). This will require the calibration of the depletion intensity, a procedure that may be difficult to achieve in clinical settings and could vary from patient to patient (10). The existence of CD4+CD25− Foxp3+ Treg explains why effective depletion of >90% of CD4+CD25+ Treg using αCD25 antibody correlated with only a modest 2- to 3-fold depletion of foxp3− cells (Fig. 6A).

Flow cytometry analysis has shown that foxp3 vaccination, in contrast to αCD25 antibody depletion, leads to the preferential depletion of Treg from the tumor (Fig. 6). Both methods targeting Treg were accompanied by a significant reduction in the number of tumor infiltrating foxp3-expressing cells (Fig. 6C). However, whereas αCD25 antibody treatment led to a 2- to 3-fold depletion of Treg in the periphery as measured in the lymph nodes and spleen (Fig. 6A and the bone marrow (data not shown), vaccination against foxp3 had no effect on the number of Treg in the periphery (Fig. 6B). αCD25 antibody treatment can induce, and more often exacerbate, autoimmunity (11, 19–21) conceivably because the across-the-board depletion of Treg consistent with the 2- to 3-fold depletion of peripheral Treg shown in Fig. 6A. The differential depletion of Treg limited to the tumor by foxp3 vaccination raises the possibility that foxp3 vaccination will be associated with reduced risk of autoimmunity. Future studies will test this hypothesis. What could be the reasons for this differential
effect? One possibility is that the activated foxp3-specific T cells will preferentially home to the tumor sites (enriched for foxp3 expressing Treg) according to the same principles that the activated T cells home to the inflamed sites and tumors. This is reminiscent of studies showing that activated CTL targeted to antigens expressed in the tumor and peripheral tissue can inhibit tumor growth with minimal or no adverse effect on the normal tissues (44–46). A second reason could be that Treg in the tumor tissue are in an activated state having recently encountered tumor antigens, and, as reported, activated Treg up-regulate Foxp3 expression up to 5- to 10-fold (25, 47–50), thus becoming more susceptible for CTL recognition and elimination than their quiescent peripheral counterparts. Future studies will need to explore the mechanism underlying these observations.

Because mutations in foxp3 are the primary defect in multi-organ autoimmune syndromes in mice (scurfy) and humans (IPEX syndrome; ref. 24), effective, even if transient, depletion of foxp3-expressing cells, which will encompass a broader spectrum of immune suppressive cells than CD25-targeted protocols, could have significant adverse effects. The foxp3 vaccination or CD25 depletion protocols used in this study were not associated with signs of morbidity or mortality. No signs of vitiligo were seen in the treated C57BL/6 mice, nor were anti-DNA antibody generated in the Treg-depleted mice. Nevertheless, a concern associated with active immunotherapy is that it could lead to uncontrolled persistence of (anti-foxp3) immunity and long-term depressed levels of Treg. This, however, is not likely because a sustained immune response is dependent on continued access to antigen in a proinflammatory context, which, in this instance, is provided, and hence can be controlled, by vaccination. A specific concern of vaccinating against foxp3 is that it is expressed in the thymus, and that vaccination against foxp3 could adversely affect thymic functions. Thus, elimination or reduction, even if transient, of foxp3-expressing cells by active immunotherapy could have undesirable adverse effects and will require careful examination using optimized and extended Treg depletion protocols.

As an adjunct to immunotherapy, targeting foxp3 by vaccination is a highly suitable protocol for depleting Treg in clinical settings. Targeting cell surface products using antibodies or ligand-based reagents requires the development, optimization, and validation of a separate procedure and often relies on reagents that are not easily accessible for clinical use. On the other hand, foxp3 vaccination does not involve a separate procedure because patients would be co-vaccinated against foxp3 and tumor antigen(s) using the same protocol. In this study, we used mRNA-transfected dendritic cells to stimulate immunity against tumor and foxp3 (31). There is no reason to think that the foxp3 vaccination approach will not apply to other vaccination strategies as well. Thus, pending further optimizations and careful testing for adverse effect, targeting foxp3 by vaccination could rapidly progress to clinical testing.

In summary, in this study, we described a strategy to deplete foxp3-expressing cells in vivo by stimulating a foxp3-specific CTL response. Unlike antibody- or ligand-based methods, stimulating CTL responses against products exhibiting differential expression pattern represents a broadly applicable method to selectively deplete deleterious in vivo, which is not limited to targeting products expressed on the cell surface.

Acknowledgments

Received 8/4/2006; revised 10/31/2006; accepted 11/3/2006.

Grant support: NIH/National Cancer Institute grants R01 CA102500 and R01 CA098637.

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