

OX40 engagement and chemotherapy combination provides potent antitumor immunity with concomitant regulatory T cell apoptosis

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Expansion and recruitment of CD4⁺ Foxp3⁺ regulatory T (T reg) cells are mechanisms used by growing tumors to evade immune elimination. In addition to expansion of effector T cells, successful therapeutic interventions may require reduction of T reg cells within the tumor microenvironment. We report that the combined use of the alkylating agent cyclophosphamide (CTX) and an agonist antibody targeting the co-stimulatory receptor OX40 (OX86) provides potent antitumor immunity capable of regressing established, poorly immunogenic B16 melanoma tumors. CTX administration resulted in tumor antigen release, which after OX86 treatment significantly enhanced the antitumor T cell response. We demonstrated that T reg cells are an important cellular target of the combination therapy. Paradoxically, the combination therapy led to an expansion of T reg cells in the periphery. In the tumor, however, the combination therapy induced a profound T reg cell depletion that was accompanied by an influx of effector CD8⁺ T cells leading to a favorable T effector/T reg cell ratio. Closer examination revealed that diminished intratumoral T reg cell levels resulted from hyperactivation and T reg cell-specific apoptosis. Thus, we propose that CTX and OX40 engagement represents a novel and rational chemoimmunotherapy.

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Abbreviations used: CTX, cyclophosphamide; DLN, draining LN; MFI, mean fluorescence intensity; strep-PE, streptavidin-PE; TDLN, tumor DLN.

Clinically relevant cancers must establish efficient mechanisms of escaping destruction by the immune system. One of the most successful strategies of tumor-induced immune evasion is the recruitment, expansion, and activation of CD4⁺ Foxp3⁺ T reg cells (1, 2). T reg cells can potentially inhibit immune responses and constitute a major barrier for eliciting effective antitumor immunity (3). Cancer patients suffering from a variety of malignancies have elevated numbers of tumor-associated T reg cells, and increased T reg cell levels correlate with poor prognosis (4–10).

Not surprisingly, the inactivation or depletion of T reg cells has been actively pursued as means to develop more potent immune therapies. Depletion of T reg cells has been accomplished with alkylating agents such as cyclophosphamide

(CTX) (11–17). As a chemotherapeutic drug, CTX is administered clinically at doses that directly kill tumor cells. However, immunologists have known for decades that CTX has significant effects on the immune system that can promote a favorable antitumor immune response (14, 18).

In addition to specific effects of CTX on T reg cells, lymphocytes recovering from CTX-induced lymphopenia undergo homeostatic proliferation that can activate tumor-specific T cells (19). Reconstitution from CTX-induced lymphopenia is associated with elevated levels of numerous proinflammatory cytokines, which favors an antitumor immune response (20, 21). Moreover, direct tumor cell death can be associated with tissue necrosis and the release of

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danger signals that enhance tumor antigen cross-presentation and cross-priming (22, 23). To increase antitumor immune responses, CTX has been successfully combined with vaccination and adoptive immunotherapy strategies (chemoimmunotherapy) in multiple modalities and tumor models (24, 25).

OX40 is a co-stimulatory molecule and member of the TNFR family constitutively expressed on T reg cells and inducibly expressed by effector CD4⁺ T cells upon activation (26). Signaling through OX40 up-regulates the expression of anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, leading to increased clonal expansion and memory responses (17). OX40 signaling can also provide co-stimulation to activated CD8⁺ T, NK, and NKT cells (27–30).

Although the function of OX40 has been established for effector CD4⁺ and CD8⁺ T cells, the biological role that OX40 plays on T reg cells is still controversial. Early reports have shown that OX40 ligation is crucial for T reg cell homeostasis (31). Young mice deficient in OX40 have reduced levels of CD4⁺ CD25⁺ T reg cells, and mice overexpressing OX40L possess elevated levels in the spleen and thymus. However, engaging OX40 can abrogate the T reg cell suppressive function and down-regulate Foxp3 expression (31–34). Additionally, it has been reported that OX40 ligation prevents the conversion of naive CD4⁺ T cells into T reg cells induced by TGF- β and antigen (33, 35).

Given that OX40 engagement can potently stimulate T cells and potentially inhibit T reg cells, it has been successfully used in the treatment of a variety of transplantable tumors in mice (34, 36). Overexpression of OX40L in tumor cell lines and dendritic cells induced substantial antitumor immunity (37–39). The triggering of OX40 with recombinant soluble OX40L or the anti-OX40 agonist monoclonal antibody OX86 are additional strategies proven to be effective in treating immunogenic tumors (40–45). However, targeting OX40 alone or in combination with other therapeutic approaches has only marginal effects on less immunogenic, more clinically relevant mouse tumors.

We hypothesized that OX40 ligation after administration of CTX would provide strong antitumor immunity. Indeed, we found that OX86 in combination with CTX synergized to mediate the regression of established, B16 mouse melanoma tumors. We demonstrate that CTX treatment resulted in the release of tumor antigens capable of priming effector CD4⁺ and CD8⁺ T cells, and that OX40 engagement amplified these responses. Furthermore, we show that the combination therapy targets T reg cells, inducing previously uncharacterized changes in this population. Unexpectedly, OX86 administration increased the number of T reg cells in the periphery, an effect that was exacerbated when OX86 was administered with CTX. In contrast, the combination therapy significantly reduced the number of T reg cells infiltrating the tumor, with a substantial, increased infiltration of effector CD8⁺ T cells. Closer mechanistic examination revealed that the combination therapy resulted in T reg cell-specific apoptosis, which accounted for the reduced intratumoral infiltration. Thus, we report a potent, clinically translatable chemoimmunotherapy

and provide insight into a novel mechanism that involves elimination of T reg cells in the tumor microenvironment.

RESULTS

CTX synergizes with OX40 engagement to promote potent antitumor immunity

Administration of OX86 yields weak, ineffective responses to poorly immunogenic tumors (43, 44). We sought to increase the potency of OX86 treatment by combining it with CTX, a drug with both immune-enhancing properties and direct antitumor effects. Mice with 6-d established B16 tumors were treated with a single 250 mg/kg dose of CTX followed by 0.5 mg OX86 the next day. Treatment resulted in the survival of 75% of animals at day 50 (Fig. 1 A) and regressed established tumors (Fig. 1 B). Neither therapy alone provided significant tumor protection.

To evaluate the clinical potential of OX86 and CTX therapy, we compared it with the combination of CTX and monoclonal antibodies with proven efficacy in preclinical models, such as anti-CTLA4 (9D9) or anti-CD40 (FGK45) (46, 47). We found that OX86 in combination with CTX

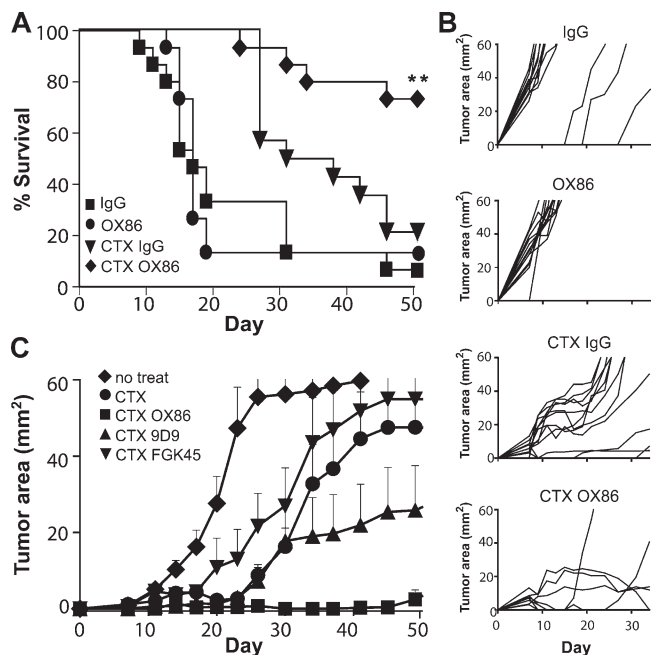


Figure 1. OX40 engagement synergizes with CTX to provide potent antitumor immunity.

(A) C57BL/6 mice ($n = 15$ per group) were injected intradermally in the flank with a lethal dose of B16 melanoma. On day 6, once tumors were palpable, CTX or PBS was injected. On day 7, mice were injected with OX86 or rat IgG. The Kaplan-Meier plot depicts overall survival. Similar results were obtained from four independent experiments.

** $P = 0.0061$. (B) C57BL/6 mice ($n = 10$ – 15 per group) were treated as in A and tumor area was measured periodically. Plots represent the tumor diameter of individual mice for each treatment. Similar results were obtained from four independent experiments. (C) C57BL/6 mice ($n = 10$ per group) were treated as in A. In some groups, OX86 injection was replaced with 9D9 (anti-CTLA4) or FGK45 (anti-CD40). Tumor area was measured periodically. Results represent mean tumor areas \pm SEM for each treatment.

showed a much higher potency than any of the other treatments. Combination therapy with FGK45 had a comparable effect to CTX alone, whereas the 9D9 combination provided some benefit (Fig. 1 C). Furthermore, the combination therapy promoted the regression of established tumors and/or significantly prolonged survival in other poorly immunogenic tumor models (4T1 mammary carcinoma, Lewis lung carcinoma, and Tramp C2 prostate adenocarcinoma; unpublished data). These experiments demonstrate that administration of a single dose of CTX synergizes with anti-OX40 antibody to provide antitumor immunity that can eradicate poorly immunogenic tumors.

CTX-induced tumor destruction releases B16 tumor antigen that is capable of priming an antitumor immune response in the presence of OX86

We hypothesized that partial destruction of the tumor by CTX releases antigen capable of priming a tumor-specific response, and that OX86 enhances the function of tumor-specific T cells. To test this hypothesis, we transferred CFSE-labeled TCR transgenic CD8⁺ T cells specific for the melanoma antigen gp100 (pmel-1 cells) (48) into tumor-bearing mice pretreated with CTX or PBS. Mice were injected the next

day with OX86 or IgG, and 5 d later proliferation by CFSE dilution was measured in the tumor draining LN (TDLN) or at a distant LN (non-DLN) by flow cytometry. In the TDLN, pmel-1 cells from the CTX plus OX86-treated group showed higher proliferation than any of the therapies alone (Fig. 2 A). Although some proliferation was found at the non-DLN in both CTX-treated groups (presumably as a result of homeostatic proliferation caused by CTX administration), a preferential proliferation was found in mice that received CTX plus OX86 compared with animals that received CTX plus IgG. Thus, proliferation of tumor-specific T cells was observed primarily in TDLN compared with non-DLN.

To further substantiate our hypothesis, we purified CD8⁺ and CD4⁺ T cells from spleens of tumor-bearing or naive mice subjected to the combination therapy and tested their ability to react against B16 and melanoma-specific peptides in IFN- γ ELISPOT assays. Only the CD4⁺ or CD8⁺ T cells from tumor-bearing mice treated with the combination therapy responded to B16 or APCs pulsed with B16-specific peptides (Fig. 2 B). These results strongly suggest that, upon CTX administration, antigen is made available for priming by drug-induced tumor destruction, and that OX86 aids in the expansion of antitumor T cells.

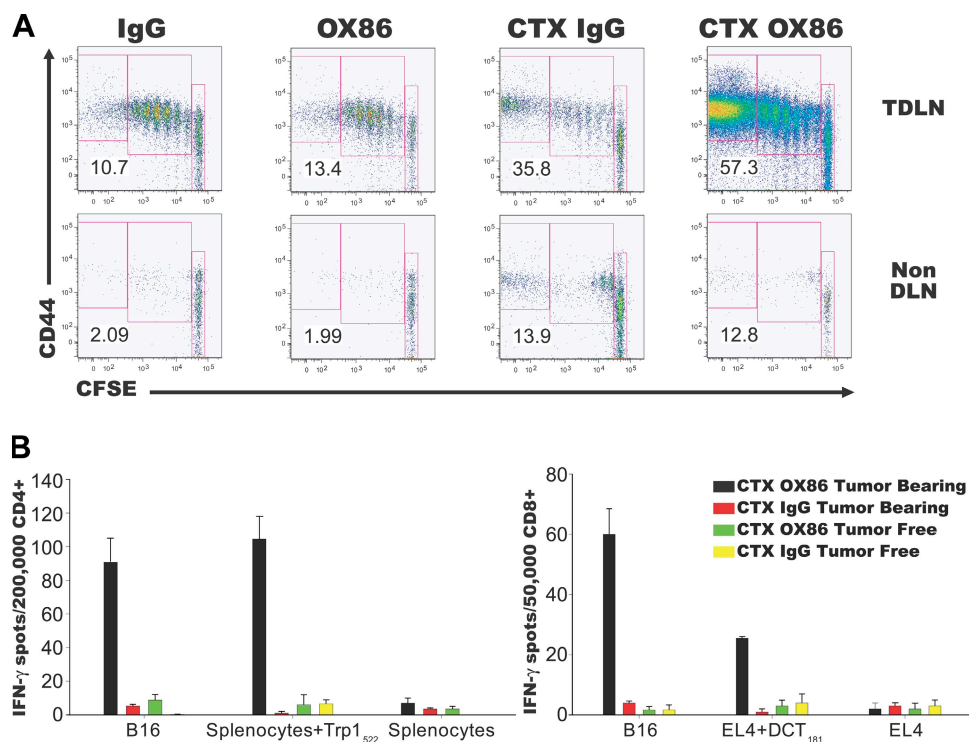


Figure 2. Tumor destruction by CTX provides antigen that can prime an antitumor immune response in the presence of OX86. (A) C57BL/6 Thy1.2 mice ($n = 4$ per group) were injected with five times the lethal dose of B16 tumor cells in Matrigel. On day 6, groups of mice were treated with CTX or PBS. On day 7, all mice received 2×10^6 purified CFSE-labeled CD8⁺ pmel-1 cells (Thy1.1) and OX86 or rat IgG. On day 13, mice were sacrificed, and a single-cell suspension was prepared from inguinal (TDLN) or contralateral cervical LN (non-DLN) and analyzed by flow cytometry. Cells were gated on DAPI⁻, CD8⁺, and Thy1.1⁺. One representative plot from three independent experiments is shown (percentages are shown). (B) C57BL/6 mice ($n = 5$ per group) were either injected with B16-Matrigel or left as non-tumor-bearing controls. On day 6, mice were injected with CTX, followed by administration of OX86 or rat IgG on day 7. 21 d after tumor inoculation, purified CD8⁺ and CD4⁺ T cells from pooled spleens were tested by IFN- γ ELISPOT. Values represent mean numbers of spots per well \pm SEM ($n = 3-4$ wells). Data shown are representative of four independent experiments.

CTX administration modulates the expression of OX40 on T reg cells

Only cells expressing OX40 can be directly receptive to the effects of an agonist anti-OX40 antibody. Hence, we investigated how CTX affects the expression of OX40 in different cell subsets. Mice were injected with CTX and spleens were harvested on days 0, 1, 2, 4, 7, 11, 17, and 24 after injection. Single-cell suspensions were prepared from spleens and analyzed by flow cytometry for OX40 expression in different cell populations. We found that CTX modulated the expression of OX40 primarily in CD4⁺ T cells, but little or no change was observed in CD8⁺ T cells (Fig. 3 A). OX40 expression was down-regulated at the nadir of CTX-induced cytopenia (day 4) and was up-regulated as cells were recovering from lymphopenia (day 11) compared with baseline expression. On closer examination, we observed that OX40 expression was modulated primarily in the CD4⁺ Foxp3⁺ T reg cell population, with minimal changes in OX40 expression in CD4⁺ Foxp3⁻ T cells (Fig. 3 B). These results suggest that T reg cells are likely to be a direct target of the combination therapy, because CTX administration modulated the expression of OX40 most dramatically in this subset.

Preferential binding of OX86 to T reg cells after CTX administration

To determine the *in vivo* cellular targets responsible for the effects of the combination therapy, we injected biotinylated OX86 into tumor-bearing mice treated with CTX. On day 21 after tumor challenge, single-cell suspensions prepared from tumor, TDLN, and spleen were stained *ex vivo* with streptavidin-PE (strep-PE) and analyzed by flow cytometry for the

presence of OX86-biotin in different cell populations. Fig. 4 A shows that OX86-biotin was bound preferentially to CD4⁺ T cells. Approximately 50% of CD4⁺ T cells in the tumor, ~20% in the TDLN, and ~30% in the spleen were OX86-biotin positive, whereas only a small percentage of CD8⁺ cells bound OX86-biotin in the tumor (~3%). When we examined OX86-biotin binding to different CD4⁺ subsets, we found that OX86 was primarily bound to T reg cells in each of the organs tested (Fig. 4 B). These results confirm the previous observation that T reg cells are a direct cellular target of the CTX plus OX86 combination therapy. These results also validate early reports showing that OX86 does not deplete OX40-expressing cells and remains bound to its cellular target for prolonged periods (49, 50). We propose, then, that CTX may increase the availability and potency of OX86 by depleting cells important for antibody clearance, additionally explaining the basis for CTX and OX86 synergy.

Combination therapy expands T reg cells in the periphery

Previous results show T reg cells to be a primary target of the CTX plus OX86 treatment. Therefore, we investigated the biological effects of the combination therapy on T reg cells *in vivo*. We injected groups of mice with CTX or PBS, followed by OX86 or rat IgG 1 d later, and analyzed populations of splenocytes at day 15. Interestingly, we found an increase in the percentage of T reg cells when OX86 or CTX was administered alone (Fig. 5, A and B). Moreover, T reg cell expansion was further amplified with the combination therapy, as we observed an ~2.5-fold increase in the percentage of T reg cells in mice treated with CTX plus OX86 compared with control treatments (Fig. 5, A and B).

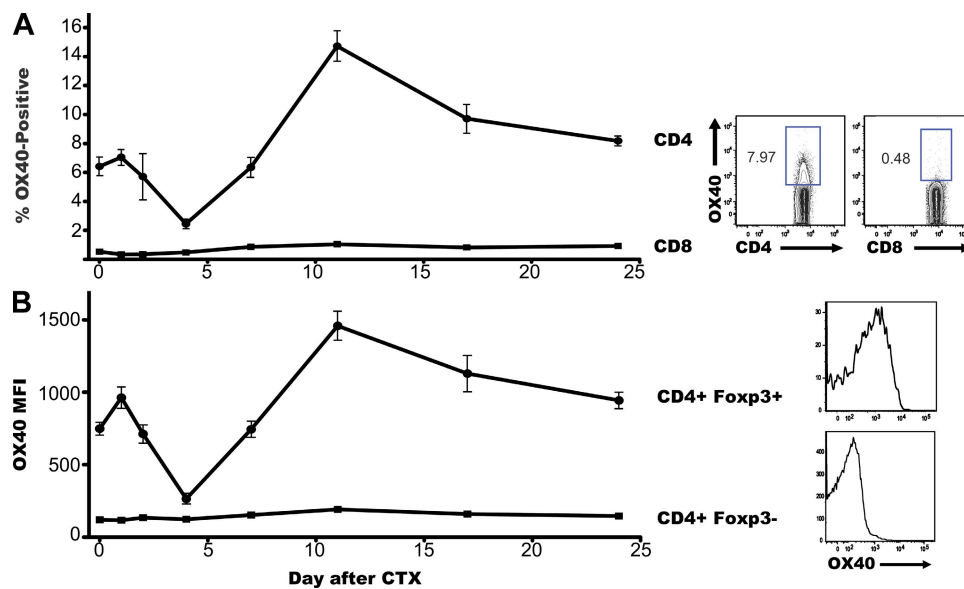


Figure 3. CTX administration modulates OX40 expression on T reg cells. C57BL/6 mice were injected with CTX. Groups of four mice were sacrificed on days 0, 1, 2, 4, 7, 11, 17, and 24 after CTX injection, and splenocytes were analyzed by flow cytometry for OX40 expression. (A) The percentage of OX40⁺ events gated on CD4⁺ and CD8⁺ T cells. A representative histogram depicting day 0 is shown (right). (B) OX40 mean fluorescence intensity (MFI) of CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cells. A representative histogram depicting day 0 is shown. Values represent means \pm SEM. The experiment was repeated three times with similar results.

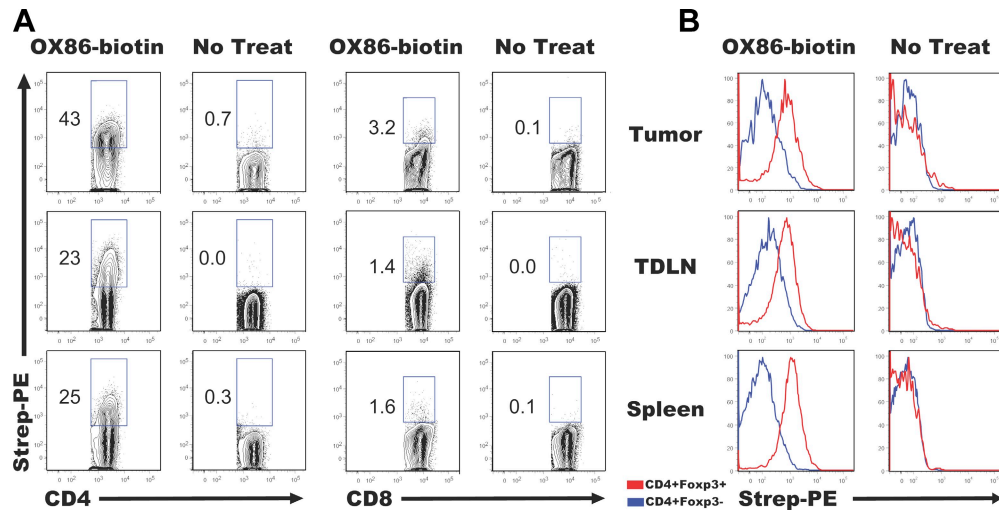


Figure 4. Preferential binding of OX86 in vivo to T reg cells after CTX administration. C57BL/6 mice ($n = 3-4$ per group) were inoculated with B16-Matrigel. On day 6, mice received CTX and were then injected with biotinylated OX86 or PBS on day 7. Single-cell suspensions were prepared on day 21 from tumor, TDLN, and spleen, and were stained with strep-PE in addition to phenotypic markers. The samples were analyzed by flow cytometry. (A) Representative histograms of CD4⁺ and CD8⁺ T cells (percentages are shown). (B) Representative histograms of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells. The experiment was repeated two times with similar results.

T reg cell expansion was confirmed by the proliferation marker Ki67, where greater numbers of T reg cells stained positive with the combination therapy compared with controls (Fig. 5 A). Only a small increase in Ki67⁺ CD4⁺ Foxp3⁻ cells was observed in the CTX-treated groups, presumably as a result of homeostatic proliferation of effector CD4⁺ T cells. Thus, the combination therapy leads to a T reg cell-specific expansion.

Recent reports have shown that OX40 engagement can block the suppressive function of Foxp3 T reg cells (32–34).

We purified CD4⁺ CD25⁺ cells from treated mice and performed ex vivo suppression assays to test if this observation holds true for T reg cells expanded in vivo. We found no difference in the suppressive capabilities of T reg cells purified from mice treated with combination and control therapies (Fig. S1). Similar results were obtained when sorted CD4⁺ Foxp3_{eGFP}⁺ cells were used in the assay (unpublished data).

Overall, these results indicate that OX40 engagement promotes the expansion of bona fide T reg cells in vivo, an effect that is greatly amplified by the combination with CTX. This

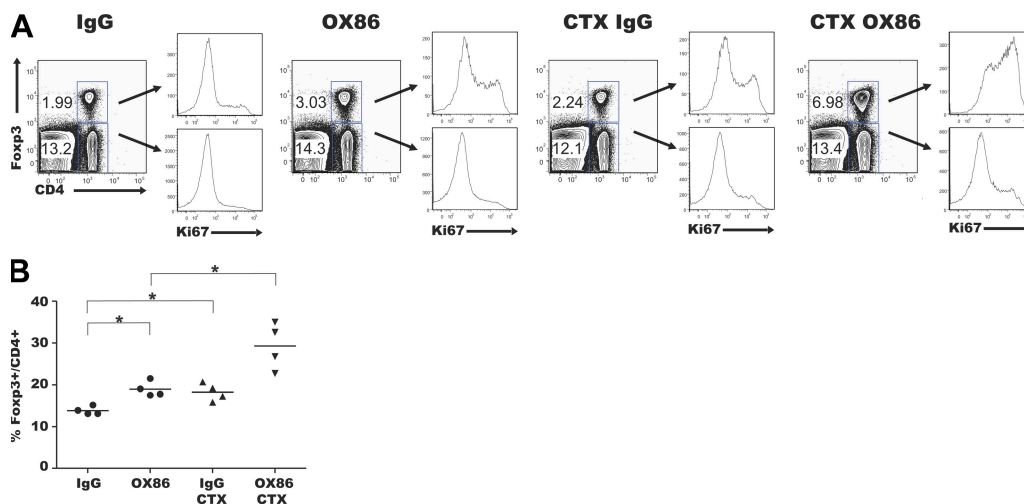


Figure 5. CTX OX86 combination therapy induces T reg cell expansion. C57BL/6 mice ($n = 4$ per group) were injected with CTX or PBS on day 1 and received OX86 or rat IgG on day 2. On day 15, splenocytes were prepared and analyzed by flow cytometry. (A) Representative histogram showing the Ki67 profiles of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells from different treatment groups (percentages are shown). (B) Percentage of Foxp3⁺ cells in the CD4⁺ gate with different treatments. The experiment was repeated five times with similar results. Horizontal bars represent means. *, $P < 0.05$.

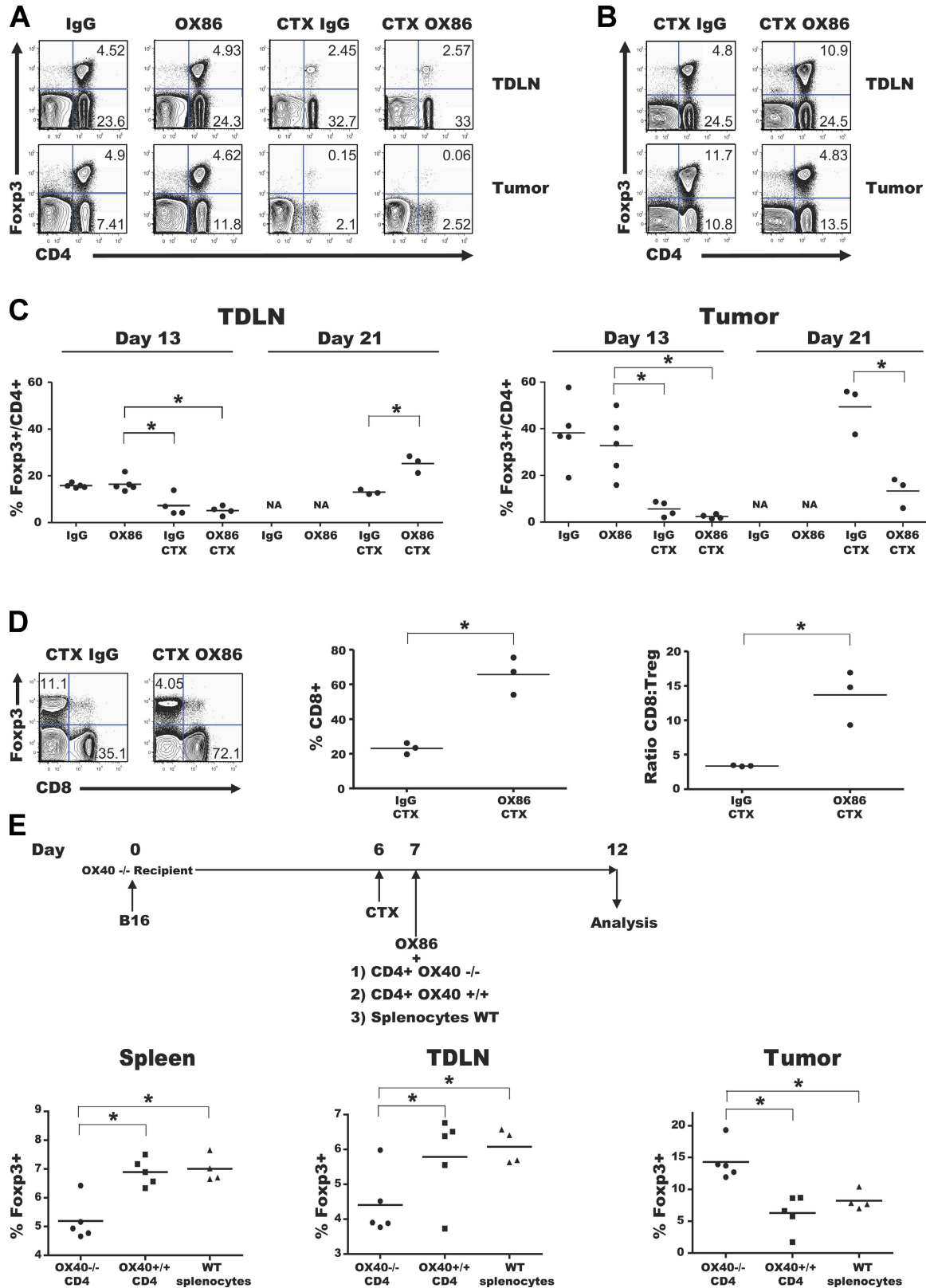


Figure 6. CTX OX86 combination therapy decreases the levels of tumor-infiltrating T reg cells concomitantly with CD8⁺ T cell tumor infiltration. (A–D) C57BL/6 mice ($n = 3-5$ per group) were inoculated with B16–Matrigel. On day 6, mice were injected with CTX or PBS followed by OX86 or rat IgG on day 7. Single-cell suspensions were prepared on day 13 or 21 from tumor and TDLN, and were analyzed by flow cytometry. Non-CTX-treated

observation is quite puzzling given the potent antitumor effects of the combination therapy.

Combination therapy decreases T reg cells in the tumor concomitantly with an increased infiltration of CD8⁺ T cells that results in a favorable T effector/T reg cell ratio

Although elevated T reg cell levels have been associated with a clinically unfavorable prognosis (51), our data show an increase in the proliferation of splenic T reg cells after treatment. Therefore, we investigated the effect of the combination therapy on cells present within the tumor microenvironment. Mice bearing B16–Matrigel tumors were treated with CTX on day 6 and OX86 or rat IgG on day 7, and tumors and TDLNs were analyzed by flow cytometry 1 wk later (day 13). As shown in Fig. 6 (A and C), we observed a profound depletion of T reg cells in the tumor and TDLN in groups treated with CTX regardless of antibody treatment. This resulted in decreased absolute numbers of T reg cells at these sites (unpublished data).

To understand the antitumor synergy of CTX plus OX86, we evaluated the effect of the combination therapy on cells present within the tumor and TDLN 2 wk after treatment (day 21). We observed that ~50% of CD4⁺ T cells infiltrating the tumor were T reg cells in the CTX plus IgG control group, whereas CTX plus OX86–treated mice showed a substantial reduction in T reg cell infiltration (~13%; Fig. 6, B and C). In contrast, the frequency of T reg cells present in the TDLN increased with the combination therapy compared with controls. We did find a modest increase of ~30% in the number of CD4⁺ Foxp3⁻ cells infiltrating the tumor; however, the combination therapy consistently decreased the absolute number of T reg cells in the tumor and increased T reg cell levels in the TDLN on day 21. (Fig. 6 B and not depicted).

On day 21 we also observed a substantial increase in CD8⁺ T cells within tumors of mice that received CTX plus OX86 combination therapy (Fig. 6 D). Thus, the combination therapy decreased the numbers of tumor-infiltrating T reg cells while simultaneously increasing the levels of effector T cells. A decreased intratumoral T reg/T effector cell ratio is a hallmark of many successful immunotherapies, as demonstrated for a variety of human cancers and mouse tumor models (2, 46, 52).

To test whether the lower numbers of infiltrating T reg cells in the tumor and the higher numbers in the periphery were caused by a direct effect of the combination therapy on T reg cells, we adoptively transferred OX40^{+/+} or OX40^{-/-} purified CD4⁺ cells into tumor-bearing OX40^{-/-} recipients

that were treated with CTX. As a control, we included a group that received wild-type splenocytes. 4 h after adoptive transfer, OX86 was administered to all groups. 5 d later, spleens, TDLN, and tumors were analyzed by flow cytometry. As shown in Fig. 6 E, lower levels of T reg cells infiltrated the tumor in mice that received OX40^{+/+} CD4⁺ cells or wild-type splenocytes compared with the OX40^{-/-} group. Higher levels of T reg cells were found in the spleen and TDLN of mice receiving wild-type CD4⁺ cells or splenocytes compared with mice that received OX40^{-/-} CD4⁺ T cells. These results confirm that the therapy-induced changes in the T reg cell population are caused at least in part by the direct targeting of T reg cells.

CTX plus OX86 increases T reg cell activation and apoptosis

We next examined the mechanism leading to lower intratumoral T reg cell infiltration with CTX plus OX86 treatment. Several reports have demonstrated that the tumor microenvironment is conducive to the peripheral conversion of CD4⁺ Foxp3⁻ to T reg cells (53). In addition, it was recently reported that OX40 engagement can block peripheral conversion of induced T reg cells (33, 35). To evaluate the possibility that OX86 might block peripheral conversion in our model, we transferred FACS-sorted CD4⁺ Foxp3_{eGFP}⁺ or CD4⁺ Foxp3_{eGFP}⁻ cells into tumor-bearing mice that were pretreated with CTX. The next day, mice were injected with OX86 or rat IgG. 5 d after cell transfer, we found no tumor-induced peripheral conversion in mice that received CD4⁺ Foxp3_{eGFP}⁻ cells (Fig. S2). However, in recipients of CD4⁺ Foxp3_{eGFP}⁺ cells, we noted increased accumulation of the transferred cells in the TDLN and decreased levels in the tumor. This is consistent with our previous observation and suggests that decreased T reg cell infiltration after combination therapy does not result from blocked peripheral conversion.

Lower T reg cell levels in the tumor could be explained, then, by a preferential lack of proliferation and/or survival. We found an increase in T reg cell proliferation in mice treated with the combination therapy, as shown by Ki67 staining (Fig. 7 A). Furthermore, in the TDLN of mice treated with the combination therapy, T reg cells down-regulated CD62L, indicating a higher activation phenotype (Fig. 7 B). CD62L down-regulation is typically accompanied by the migration of T cells to effectors sites. T reg cells present at the tumor site were all CD62L low regardless of treatment. We observed that T reg cells from mice treated with the combination therapy showed a greater down-regulation of CD25 in the TDLN, whereas in the tumor, T reg cells from both treatment groups

mice succumbed to disease before day 21. Representative histograms of CD4 versus Foxp3 cells for day 13 (A) and day 21 (B) are shown (percentages are shown). (C) Percentage of CD4⁺ T cells that are Foxp3⁺. (D, left) Representative histogram of CD8⁺ versus Foxp3⁺ cells in the tumor on day 21 (percentages are shown). (middle) Percentage of CD8⁺ T cells. (right) Ratio of CD8⁺ T cells/T reg cells. Experiments were repeated five times with similar results. (E) C57BL/6 OX40^{-/-} mice (*n* = 4–5 mice per group) were injected with B16–Matrigel. On day 6, CTX was administered, and on day 7, 2 × 10⁷ purified CD4⁺ T cells from OX40^{-/-} or wild-type mice were i.v. injected. A control group received 3 × 10⁷ splenocytes. On the same day, mice were injected with OX86. On day 12, tumors, TDLN, and spleens were analyzed by flow cytometry. The experiment was performed two times with similar results. Horizontal bars represent means. *, *P* < 0.05.

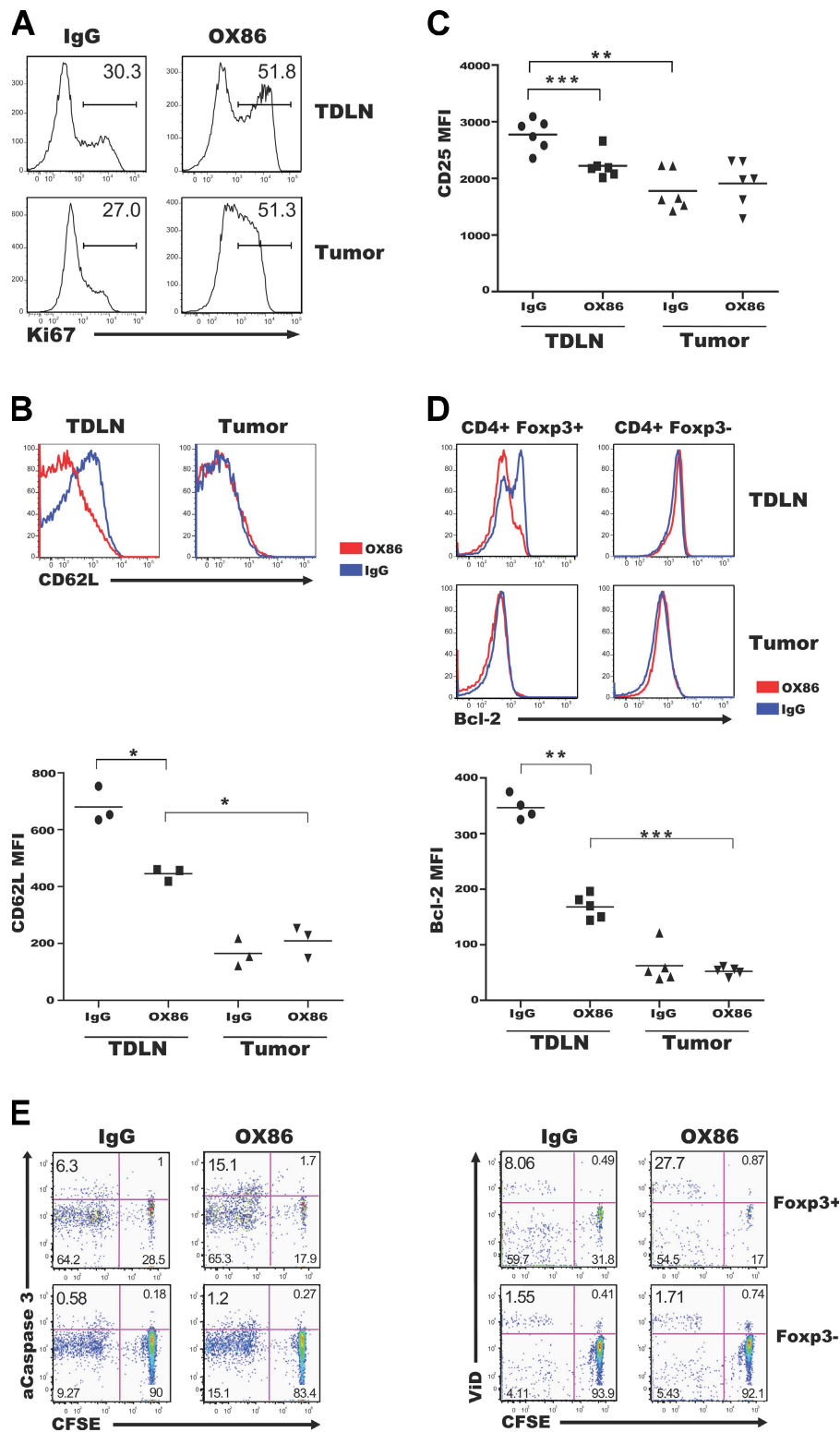


Figure 7. The combination of CTX and OX86 increases T reg cell activation and apoptosis. C57BL/6 mice ($n = 3-5$ per group) were inoculated with B16-Matrigel. On day 6, all mice received CTX. On day 7, the mice received a dose of OX86 or rat IgG. Single-cell suspensions were prepared on day 21 from tumors and TDLNs and were analyzed by flow cytometry. (A) Representative histogram of Ki67 intracellular staining gated on CD4⁺ Foxp3⁺ T cells (percentages are shown). (B, top) Representative plot of CD62L staining gated on CD4⁺ Foxp3⁺ T cells; (bottom) CD62L MFI of T reg cells in tumor or TDLN. (C) CD25 MFI of T reg cell in tumors and TDLNs. (D, top) Representative plot of Bcl-2 intracellular staining of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells; (bottom) Bcl-2 MFI of T reg cells in tumors and TDLNs. Experiments were repeated four times with similar results. (E) C57BL/6 CD45.1

were CD25 low (Fig. 7 C). Given that IL-2 signaling is required for T reg cell survival (54), we hypothesized that T reg cell-specific apoptosis caused by hyperactivation by the combination therapy accounts for lower intratumoral T reg cell infiltration. Bcl-2 down-regulation occurs at the early stages of programmed cell death and has been shown to be a marker of T reg cell apoptosis *in vivo* (55). Bcl-2 was significantly down-regulated on T reg cells in the TDLN of mice treated with the combination therapy (Fig. 7 D). Bcl-2 was unchanged on CD4⁺ and CD8⁺ T cell effector populations, indicating T reg cell-specific cell death (Fig. 7 D and not depicted). The expression of Bcl-2 in all T cell populations tested was lower in the tumor than in the TDLN. We observed only marginal increases in the frequency of Annexin V⁺ and active caspase 3⁺ T reg cells after combination therapy. As apoptotic cells should be rapidly removed by the scavenger system *in vivo*, detection of low levels might be expected (56). To increase detection sensitivity, we transferred congenic, CFSE-labeled, purified CD4⁺ T cells into B16-bearing mice that were pretreated with CTX and later injected with OX86 or control IgG. The combination therapy induced a significantly higher percentage of active caspase 3⁺ and viability dye-positive (57) cells (Fig. 7 E). These populations were primarily CFSE low, consistent with the previous finding of increased T reg cell proliferation after combination therapy (not depicted). Collectively, these results imply that lower levels of intratumoral T reg cells caused by CTX plus OX86 therapy occur as a result of specific hyperactivation and T reg cell-specific cell death.

DISCUSSION

In this paper, we provide evidence that a chemotherapeutic agent routinely used in the clinic (CTX) can greatly enhance the antitumor potency of a novel form of immunotherapy (OX40 engagement) in an aggressive tumor model in which neither therapy alone shows a significant antitumor effect. The combination of CTX and OX86 resulted in regression and long-term protection of established, poorly immunogenic tumors. Mechanistically, we found that killing of the tumor by CTX provides the antigen necessary to prime an antitumor immune response, and in this setting, OX86 greatly enhances effector T cell function. On closer examination, we found T reg cells to be an important target of the combination therapy. Surprisingly, OX86 administration alone expanded T reg cells in the periphery, an effect that was exacerbated in combination with CTX. In the tumor microenvironment, however, the combination therapy promoted a favorable T effector/T reg cell ratio by increasing the number of CD8 T⁺ cells and decreasing the number of T reg cells. We observed elevated levels of T cell activation

together with elevated apoptotic markers on T reg cells from mice treated with the combination therapy, indicating that hyperactivation leads to T reg cell-specific apoptosis within the tumor microenvironment.

Numerous studies have shown that alkylating drugs such as CTX can potentiate an antitumor immune response (58). Our data suggest that CTX-induced tumor destruction leading to antigen cross-presentation is a mechanism that contributes to the synergy of the combination therapy. This is because (a) tumor-bearing but not naive mice subjected to the combination therapy were able to mount detectable antigen-specific T cell responses to proteins expressed on B16, and (b) tumor-specific T cells (pmel-1) proliferated more vigorously with the combination therapy at the TDLN but not at a distant site. These results suggest that cross-presentation of antigen provided by damaged tumor is an effective way to generate an antitumor T cell response and does not require the laborious identification of antigenic tumor epitopes.

Several observations indicate that T reg cells are a primary target of OX86 when CTX plus OX86 combination therapy is administered. First, after CTX administration, T reg cells modulated the expression of OX40 (the sole receptor of OX86), whereas the effect on other T cell subsets was minimal. Second, we found OX86-biotin to be bound primarily to T reg cells in the tumor, TDLN, and spleen after CTX administration, whereas only a small proportion was bound to CD8⁺ and CD4⁺ T cells. Third, we observed increased T reg cell expansion in the spleen and TDLN and decreased levels of T reg cell infiltration in the tumor, an effect that was lost in an OX40^{-/-} background.

When we examined the role of OX40 engagement on T reg cells, we found that OX86 injection preferentially expanded T reg cells in the periphery. Because OX40 engagement promotes the clonal expansion of T cells (26), it is conceivable that OX86 promotes T reg cell expansion because T reg cells are in a perpetually proliferative state. (59, 60). Additionally, T reg cells are the only OX40-expressing population in naive mice (31, 33, 34). Our observations are consistent with early reports showing that chronic OX40 engagement in mice overexpressing OX40L promotes higher T reg cell levels (31). It has also been reported that OX40 engagement on T reg cells can reduce Foxp3 levels *in vitro* (33). We observed no difference in Foxp3 expression levels after OX86 administration in any of our treatments. The reason for this discrepancy is yet to be determined, but the degree of TCR stimulation or cytokine milieu that occurs *in vivo* versus *in vitro* could account for this inconsistency. In addition, we found no difference in the suppressive function of OX86-expanded T reg cells in *ex vivo* assays.

mice ($n = 4$ per group) were inoculated with B16-Matrigel. On day 6, mice received CTX. On day 7, mice were injected with purified CD4⁺, CD45.2⁺ CFSE-labeled cells, and OX86 or rat IgG. On day 12, single-cell suspensions were prepared from TDLN and analyzed by flow cytometry. Events were gated on CD45.2⁺ CD4⁺ cells. (right) Activated caspase 3 versus CFSE is shown; (left) ViD versus CFSE is shown (percentages are shown). One representative plot ($P < 0.05$ for the Foxp3⁺ CFSE low active Caspase 3⁺ or ViD⁺ population) of two independent experiments is shown. Horizontal bars represent means. *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$.

At certain doses or administration regimens, CTX preferentially depletes T reg cells (12, 13, 16, 61). At the higher doses such as those reported in this paper, CTX induced profound lymphopenia. As recovery takes place and T cells undergo homeostatic proliferation, T reg cells are proliferating more vigorously than other populations analyzed (Fig. 5). This may be because homeostatic proliferation is driven by self-MHC-peptide complexes (62), and because the T reg cell repertoire is skewed toward self (63), there are greater MHC-TCR interactions for T reg cells than for effector T cells. OX86 can provide co-stimulation to an already cycling population of cells. Therefore, the addition of OX86 will fuel T reg cell expansion even further.

Increased levels of T reg cells are typically associated with a negative prognosis in preclinical tumor models and cancer patients (51). It is therefore counterintuitive that OX86 and CTX results in the peripheral expansion of this population because the combination therapy provides such strong anti-tumor effects. To understand this apparent paradox, we measured T reg cell levels within the tumor microenvironment and found a striking decline in intratumoral T reg cell levels in mice subjected to the combination therapy. Our data indicate that this decline occurs in two phases: first, an antibody-independent phase occurs where CTX induces a profound depletion of T reg cells in the tumor microenvironment. It is plausible that actively proliferating T reg cells within the tumor microenvironment are particularly susceptible to the effects of CTX and T reg cell repopulation is locally delayed. Alternatively, CTX may eliminate cells present within the tumor stroma that are responsible for the recruitment, maintenance, and induction of T reg cells within the tumor. Second, at a later time point, tumors repopulate with T reg cells in the CTX plus IgG control, whereas with CTX plus OX86 there is still a deficiency of T reg cells in the tumor. This is concurrent with significant tumor infiltration of CD8⁺ effector T cells that causes a favorable shift in the T effector to T reg cell balance. A positive T effector/T reg cell ratio has been shown to be a hallmark of favorable disease prognosis and positively correlates with successful therapies (46, 52). Similar observations were recently reported by Gough et al., where OX86 administration in mice with immunogenic tumors promoted a favorable T effector/T reg cell ratio (64).

Closer examination of the tumor microenvironment revealed higher proliferation and activation of T reg cells resulting from combination therapy. T reg cells in the TDLN expressed higher Ki67 and lower CD62L levels in mice treated with the combination therapy, indicating higher proliferation and activation. Furthermore, T reg cells in the TDLN expressed lower levels of CD25. Given that IL-2 signaling is essential for T reg cell homeostasis, a loss of IL-2 sensitivity will limit T reg cell survival (65). Furthermore, T reg cells from mice lacking CD25 or IL-2 exhibit an activated phenotype, suggesting that CD25 down-regulation is characteristic of activated T reg cells under certain circumstances (65).

Therefore, we hypothesized that hyperactivation of T reg cells within the tumor microenvironment induces T reg cell-

specific cell death. Indeed, we detected apoptosis specifically within the T reg cell population, but not in CD4⁺ and CD8⁺ T effectors in the TDLN when mice were treated with the combination of CTX and OX86. At the tumor site in our model, however, T reg cells were apoptotic regardless of treatment, consistent with the notion that T reg cells at effector sites down-regulate Bcl-2 and then die (63).

Based on these results, we propose that the clinical measurement of peripheral T reg cells might not be of diagnostic value and that intratumoral levels are of greater importance when novel immunotherapies are evaluated. Behaviorally, T reg cells might expand or contract depending on the local milieu provided by differences in co-stimulation or cytokine stimuli. Recently, it was reported that OX40 engagement in combination with TNF- α induces CD4⁺ T cell apoptosis *in vitro* (66). Given that the tumor microenvironment is abundant in TNF- α and OX86 is bound primarily to T reg cells, it is conceivable that this is a potential mechanism for T reg cell-specific cell death.

Notably, an imbalance in the ratio of T reg/T effector cells as a result of T reg cell-specific apoptosis has been reported in a variety of autoimmune diseases. Higher apoptosis was found in CD4⁺ CD25⁺ T reg cells from patients with type 1 diabetes (67), whereas elevated numbers of CD4⁺ CD25⁺ cells undergoing apoptosis in the thyroid are found in patients with autoimmune thyroid disease (68). In the nonobese diabetic mouse strain, T reg cell apoptosis correlates with diabetes onset as a result of IL-2 deficiency (55), whereas immune steatohepatitis caused by oxidative stress has been associated with a down-regulation of Bcl-2 and an increase in T reg cell apoptosis (69). Expression of the proapoptotic molecule Bax under the CD25 promoter leads to T reg cell apoptosis and increased susceptibility to vasculitis in mice (70). To our knowledge, this is the first study to report that potent antitumor immunity can be achieved by inducing T reg cell-specific apoptosis.

The suppressive function of T reg cells constitutes a major barrier to effective cancer immunotherapy. Most cancers "hijack" T reg cells to evade the immune response by active recruitment (2), local expansion (4, 71, 72), and conversion of naive CD4⁺ T cells (73, 74). Therefore, strategies that can remove or abrogate their function are highly desirable. Clinically, the elimination of T reg cells has been achieved by targeting CD25 using a fusion protein consisting of IL-2 linked to diphtheria toxin (enileukin diftotox) (51). This strategy has the unfortunate caveat of potentially eliminating activated CD25⁺ effector T cells as well. In this paper, we present evidence that induction of T reg cell-specific cell death is an attractive alternative to T reg cell elimination because, in addition to decreasing T reg cell levels, the CTX plus OX86 combination therapy can enhance the activation of T cell effectors, as shown in Fig. 2. Furthermore, intratumoral administration of OX86 has been shown to deactivate the suppressive function of T reg cells locally (34).

Given the multifactorial nature of cancer as a disease, it is unlikely that a single therapy will be an efficacious treatment. For this reason, combining conventional cytotoxic therapy

with immunotherapy is an attractive approach. A major advantage of the reported strategy is that it does not require *ex vivo* manipulation or a priori knowledge of tumor antigens to provide potent antitumor immunity. Hence, we expect it could be easily applicable in patients suffering from a variety of malignancies regardless of MHC restriction.

Overall, we present a novel mechanism by which potent tumor immunity is attained with the combination of CTX and OX40 engagement and show that this treatment induces a favorable T effector/T reg cell ratio as a result of T reg cell-specific apoptosis within the tumor environment.

MATERIALS AND METHODS

Mice and tumor cell lines. All mouse procedures were performed in accordance with institutional protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC) under an approved protocol. C57BL/6j (8–10-wk-old females) and C57BL/6j Thy1.1 animals, and C57BL/6j CD45.1 animals were obtained from the Jackson Laboratory. pmel-1 TCR transgenic mice (Thy1.1) were a gift from N. Restifo (National Institutes of Health, Bethesda, MD) (48); Foxp3_{eGFP} mice were provided by A. Rudensky (MSKCC, New York, NY) (65). OX40^{-/-} mice were provided by N. Killeen (University of California, San Francisco, San Francisco, CA) (75). All mice were bred at MSKCC. The B16-F10 mouse melanoma line was originally obtained from I. Fidler (M.D. Anderson Cancer Center, Houston, TX) and passaged intradermally in mice several times to ensure tumor growth. Immunization with 5×10^4 B16-F10 cells was determined to be a lethal dose. For experiments where isolation of tumor lymphocytes was performed, B16-Matrigel was subcutaneously injected (2.5×10^5 B16-F10 cells in 0.2 ml of Matrigel Matrix Growth Factor Reduced; BD). Without treatment, mice bearing B16-Matrigel tumors survived up to days 14–16 after tumor challenge.

Monoclonal antibodies and drug treatment. OX86 hybridoma was obtained from the European Collection of Cell Cultures. FGK45 hybridoma was obtained from the American Type Culture Collection. 9D9 hybridoma was a gift of J.P. Allison (MSKCC, New York, NY). The antibodies were produced, purified, and biotinylated where indicated in the figures by the Monoclonal Antibody Core Facility at MSKCC. Anti-Rat IgG was purchased from Sigma-Aldrich and reconstituted in sterile PBS before use. For all treatments, 0.5 mg OX86 or rat IgG was administered. 0.25 mg FGK45 and 0.1 mg 9D9 were administered as a single dose, as indicated in the figures. Cyclophosphamide monohydrate (Sigma-Aldrich) mixed in sterile PBS was administered at 250 mg/kg. Both agents were administered as a single dose intraperitoneally.

Antibodies, FACS analysis, and cell sorting. The following antibodies were used for flow cytometry analysis: CD8-PE-Texas red, CD4-PerCP, Ki67-FITC, CD44-Alexa Fluor 700, CD62L-Alexa Fluor 750, CD25-PE-Cy7, active caspase 3-biotin, strep-PE-Texas red, and CD3 functional grade (BD); and OX40-PE, Foxp3-allophycocyanin, Bcl-2-PE, and CD45.2-Alexa Fluor 750 (eBioscience). All antibodies were used according to the manufacturer's instructions with the recommended buffers. The Vybrant CFDA SE Cell Tracer kit (CFSE), DAPI, and the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (ViD) were obtained from Invitrogen and used according to the manufacturer's instructions.

Samples from TDLNs, spleens, and tumors were mechanically dissociated to obtain a single-cell suspension. Splenocytes were depleted of erythrocytes with ACK lysing buffer (Invitrogen). Lymphocytes were isolated from tumors using Percoll (GE Healthcare) gradient centrifugation. Before staining, cells were treated with saturating anti-CD16/CD32 (BD) in staining buffer (2% bovine serum albumin and 10 mM EDTA in PBS) on ice for 15 min. Staining of surface antigens was performed in staining buffer on ice for 40 min. All intracellular staining was conducted using the Foxp3 fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed on a flow cytometer

(LSRII; BD). FlowJo software (version 8.6.2; Tree Star, Inc.) was used for all flow cytometry analysis. FACS sorting was conducted on a cell sorter (FACSDiva; BD).

ELISPOT assay and peptides. Peptides were synthesized by Genemed Synthesis, Inc. and used at >80% purity, as confirmed by HPLC. Peptide from dopachrome tautomerase/tyrosinase-related DCT₁₈₁ (sequence VYDFFVWL) is restricted by K^b (76). Tyrosinase-related protein 1, Tyrp1₅₂₂, is an I-A^b-restricted peptide (sequence RYAYDYEELPNPNHS; unpublished data).

Multiscreen-IP plates (Invitrogen) were coated with 100 μ l anti-mouse IFN- γ antibody (10 mg/ml; clone AN18; Mabtech) in PBS, incubated overnight at 4°C, washed with PBS to remove unbound antibody, and blocked with RPMI 1640 plus 7.5% FBS for 2 h at 37°C. CD8⁺ or CD4⁺ T cells were harvested from pooled spleens from sacrificed mice (3–4 mice per group), purified using anti-CD8 or anti-CD4 MACS magnetic beads (Miltenyi Biotec), and plated at a concentration of 5×10^4 CD8⁺ T cells or 2×10^5 CD4⁺ T cells per well. For antigen presentation, 5×10^4 irradiated B16 cells, EL-4 leukemia cells (American Type Culture Collection), or 10^5 T cell-depleted irradiated splenocytes that had been pulsed with 10 μ g/ml of peptide for 1 h were added to a final volume of 100 μ l/well. After incubation for 20 h for CD8⁺ T cells and 48 h for CD4⁺ T cells at 37°C, plates were extensively washed with PBS plus 0.05% Tween and incubated for 2 h at 37°C with 100 μ l/well of biotinylated antibody against mouse IFN- γ (2 mg/ml; clone R4-6A2; Mabtech). Spot development was performed as previously described (77). Spots were counted with an automated ELISPOT reader system with KS 4.3 software (Carl Zeiss, Inc).

Adoptive transfer experiments. In some experiments, animals received 2×10^6 CFSE-labeled CD8⁺ pmel-1 cells intravenously via the lateral tail vein. CD8⁺ T cells were purified (~98% pure) from pooled spleens and LNs from pmel-1 mice by positive selection using CD8a (Ly-2) microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Purified cells were stained in PBS with 10 μ M CFSE at 37°C for 10 min and quenched in complete media. The cells were washed twice in PBS before transfer.

In other experiments, OX40^{-/-} animals received 2×10^7 purified CD4⁺ T cells from OX40^{-/-} or wild-type mice or 3×10^7 splenocytes from wild-type mice. CD4⁺ T cells were purified by MACS using CD4 (L3T4) microbeads (Miltenyi Biotec). Purity was confirmed by flow cytometry to be ~98%.

For the *in vivo* conversion assay, FACS-sorted CD4⁺ Foxp3_{eGFP}⁺ and CD4⁺ Foxp3_{eGFP}⁻ cells were transferred into B16-Matrigel tumor-bearing mice pretreated with CTX and then treated with either OX86 or IgG.

In vivo apoptosis experiments were performed by injecting CD45.1 mice with B16-Matrigel. 6 d later, the mice were injected with CTX. On day 7, the mice were injected with 10^7 purified CD4⁺ cells from CD45.2 mice labeled with 10 μ M CFSE. 4 h later, the mice were injected with OX86 or rat IgG. On day 12, single-cell suspensions were prepared from DLNs and analyzed by flow cytometry.

T reg cell suppression assays. T reg cell suppression assays were performed with a modified method from Thornton et al. (78). In brief, CD4⁺ CD25⁺ T reg cells were purified by MACS using a regulatory T cell isolation kit (Miltenyi Biotec) from spleens of treated animals. 5×10^4 T reg cells were co-cultured at a 1:1 ratio with CFSE-labeled (1 μ g/ml) Thy1.1 congenic CD8⁺ T cells purified by MACS positive selection (Miltenyi Biotec) in the presence of 1 μ g/ml of plate-bound anti-CD3 antibody. After 96 h, the CD8⁺ T cell proliferation was analyzed by flow cytometry.

Statistical calculations. Statistical differences between experimental groups were determined by the two-tailed Student's *t* test using Prism software (GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows that the CTX plus OX86 combination therapy does not alter the suppressive capability of T reg cells *in vivo*. Fig. S2 shows that the blocking of peripheral T reg cell conversion does not cause reduced levels of intratumoral T reg cells after treatment with

the combination therapy. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082205/DC1>.

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