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OX40 Agonists can drive Treg expansion if the cytokine milieu is right

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Abstract

We report that OX40 stimulation drives all lineages of CD4 T cell development including Treg and the plasticity of the response is dependant on local cytokines. In TGF-β1-treated cultures, OX40 agonist increased IFN-γ and IL-4 production and diverted T cells from the Treg lineage. However, cytokine blockade in the context of OX40 stimulation promoted also enhanced Treg *accumulation*. This observation was evident in naive mice, as OX40 engagement enhanced Treg proliferation and accumulation in vivo. Lastly, OX40 agonist administration influenced EAE disease severity in opposing directions depending on the timing of administration. Given during Ag priming, OX40 agonist drove Treg expansion and inhibited disease, whereas, given later it enhanced T cell effector cytokine production in the CNS and exacerbated disease. Hence, OX40 signaling can augment the accumulation of all CD4 T cell lineages; however its accentuation of immune responses may have vastly different biologic outcomes depending upon the local cytokine milieu.

Introduction

Ligation of OX40, a costimulatory molecule transiently expressed on recently activated T cells, is known to augment effector T cell expansion, function, and survival (1–3); however, the biological role of OX40 signaling on regulatory T cells (Tregs) is not fully clear. Although, Tregs constitutively express OX40 on their surface (4, 5), the generation of thymic Tregs was not significantly influenced by OX40 signals (5). In contrast, OX40

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stimulation was shown to prevent inducible Treg conversion of effector T cells in the presence of TGF-β1 (4, 5). The OX40-imparted resistance to TGF-β1-mediated Treg conversion appears to be the result of IL-4, IL-6, and IFNγ produced by OX40-stimulated T cells (6, 7).

We sought to further understand the mechanisms underlying the ability of OX40 signaling to prevent Treg conversion, as well as explore the relationship between OX40 and Treg proliferation. In vitro experiments demonstrated the ability of OX40 to block TGF-β1-mediated Treg conversion of activated T cells through enhanced production of Th1/2 cytokines. In the absence of IFNγ or IL-4 agonist-OX40 stimulation enhanced the accumulation of Tregs. These data suggested OX40 induced Treg expansion and this was confirmed in vivo, where administration of OX40 agonists to naïve mice elicited Treg proliferation. Finally, OX40 agonist administration expanded Tregs if given during priming in an experimental autoimmune encephalomyelitis (EAE) model, which led to a decrease in disease severity. In contrast, if OX40 agonist administration was delivered at the onset of disease when effector T cells had already infiltrated the central nervous system (CNS) we observed increased effector cytokine production in the CNS, and an increase in disease severity. Thus, it appears that the local cytokine milieu generated during an immune response ultimately determines the fate of CD4 T cell linage after OX40 stimulation, which subsequently enhances the expansion of all T cell lineages, including Tregs.

Materials and Methods

Mice

Female C57BL/6 mice, SJL mice IFNγ$^{-/-}$, and FoxP3eGFP knock-in mice 6 to 8 weeks of age were used in accordance with the appropriate institutional guidelines and committees.

Treg conversion cultures

CD4^{+}CD25^{-} T cells were isolated from LNs by MACS depletion of CD25^{+} and CD4^{-} cells (97% pure) (Miltenyi Biotec). Enriched T cells were cultured with 20 U/mL IL-2 and TGF-β1 (2 ng/mL: 24 well plates or 1 ng/mL; 96 well plates) and activated with plate bound αCD28 (2 μg/mL) and αCD3 (2.0 μg/mL: 24 well plates or 0.5 μg/mL: 96 well plates). αOX40 (IgY), previously shown to activate OX40 and induce IL-12R (Ruby-IL-12 ref) (10 μg/mL: 24 well plates or 5 μg/mL: 96 well plates) and blocking IL-4, IL-6, and IFNγ Abs at 0.5 μg/mL/Ab (eBioscience) were added. Cultures were harvested after 72 hrs and cells stained for CD4, CD25 and FoxP3 (eBioscience).

Adoptive transfer

Cells from spleens of FoxP3eGFP CD45.2 mice were stained with APC-CD4 and DAPI (Invitrogen) and sorted by DAPI^{-}CD4^{+}GFP^{+} or GFP^{-}. Purified populations (>97% purity) of Tregs (GFP^{+}) or CD4^{+} T cells (GFP^{-}) were adoptively transferred into CD45.1 mice at 10^{6} or 5 X 10^{6} respectively. Mice received 500 μg of αOX40 (OX86) or rat IgG, and splenocytes were analyzed by FACS (d6).

Treg suppression assay

The assays were performed as previously described (8). Mice were treated with (500 μg) of αOX40 or rat IgG i.p. Splenic $CD4^+CD25^+$ Tregs were purified by MACS (d6) and were mixed with CFSE-labeled (1 μg/ml) MACS-purified Thy1.1 $CD8^+$ T cells at various ratios in the presence of 1 μg/mL plate-bound αCD3. After 96 hrs T cell proliferation was analyzed.

EAE

SJL mice were injected in the flanks with a 200 μL emulsion containing 150 μg $PLP_{139–151}$ (NeoMPS) with an equal volume of CFA (200 μg *M. tuberculosis)* (d0). Mice were divided into 3 treatment groups: 1) 200 μg rat IgG (d0 and d4), 2) 200 μg αOX40 (d0 and d4) and 3) 200 μg αOX40 at disease onset and 2 and 4 days later (i.p.) and monitored daily for disease according to a 6 point scale: 0=healthy, 1=limp tail or mild hindlimb weakness, 2=moderate hindlimb weakness, 3=moderately severe hindlimb weakness, 4=severe hindlimb weakness, 5=paraplegia with no more than moderate forelimb weakness, 6=moribund condition.

Analysis of cells obtained from EAE mice

Spleen and LNs were prepared by homogenizing the tissue through fine mesh screens. Cells from the CNS were obtained as previously described (9). Cells were analyzed by FACs for CD4, CD25 and FoxP3. Additionally, 4 X 10^6 spleen and LN cells/well (24-well) or 10^5 brain cells/well (96-well) were re-stimulated with 10 μg $PLP_{139–151}$ for 48 hrs. Supernatants were collected and analyzed for cytokines using a Luminex Bio-Plex kit (Bio-Rad).

Results and Discussion

TGFβ-1 conversion of T cells into Tregs is influenced by OX40-mediated IFNγ and IL-4 production

The effect of an agonist OX40 antibody (αOX40) on TGF-β1-mediated Treg generation was studied by stimulating naïve enriched $FoxP3^-CD4^+$ T cells with αCD3 and αCD28 in the presence of IL-2 and TGF-β1 (4, 10). Anti-OX40 or rat IgG was added to cultures and Tregs assessed after 72 hrs. As previously seen (4, 10), OX40 stimulation decreased the percentage of Tregs ($FoxP3^+$) (Fig. 1A). To determine the role that differentiating cytokines, played in the OX40-mediated decrease in Treg conversion, blocking antibodies to these cytokines were added. IFNγ and IL-4 have shown to impart resistance to TGFβ-1-mediated Treg conversion (11), and the inflammatory cytokine, IL-6, in conjunction with TGFβ-1 directs Th17 differentiation (12). The addition of blocking IL-4, IL-6, and IFNγ antibodies to cultures stimulated with αOX40 increased the frequency of $FoxP3^+$ T cells compared to TGFβ-1 treatment alone (38.5% to 55.1%), and cell numbers were not different (0.60 X 10^5 ± 0.08 vs. 0.70 X 10^5 ± 0.03) (Fig. 1A, 1B, and data not shown). Furthermore, the presence of either IFNγ or IL-4 in culture prevented the OX40-enhanced TGF-β1-Treg accumulation, but not IL-6 (Fig. 1C). Analysis of the culture supernatants, revealed αOX40 stimulation significantly increased the production of both IFNγ and IL-4 (Fig. 1D). Both OX40 stimulated and control cultures displayed similar levels of IFNγ producing T cells (data not shown), suggesting OX40-stimulation enhanced effector T cell production of IFNγ and not

the differentiation of IFNγ-producing T cells. In addition, to further understand the role of IFNγ in OX40-stimulated Treg cultures, IFNγ-deficient T cells were cultured with TGFβ-1 (2 ng/ml), αOX40, and αIL-4. Various doses of exogenous IFNγ were then added and Treg conversion measured. A concentration of IFNγ needed to reduce Treg conversion was four-fold less than TGFβ-1 (0.5 ng/ml vs 2 ng/ml) in these cultures, suggesting this ratio (IFNγ:TGFβ-1=0.25) may delineate the effect of OX40 stimulation expanding Tregs or reducing conversion. These results demonstrate that OX40-imparted resistance to TGF-β1-Treg conversion is mediated in part by increasing Th1/2 differentiation cytokine production, but more importantly OX40 stimulation appears to drive Treg accumulation in the absence of these cytokines.

OX40 stimulation increased the accumulation of cycling Tregs in naïve mice

The findings that OX40 stimulation appeared to drive Treg accumulation in the absence of T helper differentiating cytokines (Fig. 1B), prompted investigations into the relationship between OX40 stimulation and Treg proliferation in naive mice. Because of the transient nature of OX40 expression on activated T cells, administration of αOX40 to naïve mice likely engages constitutively expressed OX40 on Tregs. A single injection of αOX40 increased the numbers of $FoxP3^+$ Tregs four fold in the spleens in a dose-dependant manner when compared to controls six days after injection, the peak of the response (Fig. 2A and 2B). To determine if αOX40 enhances proliferation of both natural Tregs (nTregs), the predominate Treg population in naive mice, and inducible TGFβ-1 Tregs (iTregs), we assessed Ki-67 expression, a marker of dividing cells. TGFβ-1-iTregs, generated in as previously described, were transferred into congenic recipients (Thy1.1) and treated with αOX40 or rat IgG. Six days later both OX40-stimulated transferred iTregs) and endogenous nTregs expressed increased Ki-67 compared to controls (Fig. 2C). To determine if αOX40 stimulation expanded existing Tregs or induced new Tregs in naive mice, $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ T cells were sorted from the spleens of naive FoxP3eGFP transgenic mice, transferred to naive congenic recipients and injected with αOX40 or rat IgG. OX40-stimulation increased the frequency of transferred $FoxP3^+$ T cells compared to controls, while Treg conversion of the transferred $FoxP3^-$ T cells following αOX40 treatment was minimal (Fig. 2D). Collectively these data show systemic agonist OX40 administration can expand $CD4^+FoxP3^+$ Treg populations in naïve mice.

It has been shown that OX40-engagement can abrogate the suppressive effects of Tregs. To determine whether systemic administration of αOX40 could abrogate the function of the expanded Tregs in naïve mice, Tregs were isolated from αOX40- and IgG-treated mice and cultured with naïve CFSE-labeled CD8 T cells at various ratios. After TCR activation we observed no significant differences in the suppression of T cell proliferation by Tregs from αOX40-treated mice compared to IgG-treated mice (Fig. 2E). This result is in contrast to other studies that showed OX40-stimulated Tregs abrogated their suppressive function (5, 13, 14). An explanation for the divergent effects of OX40-signals seen on Treg-suppression remains to be determined.

Timing of OX40 agonist administration in mice with EAE is critical in determining disease severity

EAE can be induced in mice by the administration of an associate myelin peptide, ($PLP_{139–151}$). In this model of autoimmunity, self-reactive CD4 T cells are primed in the draining LNs, and then infiltrate the CNS, resulting in progressive paralysis 10–12 days after immunization. OX40 is selectively expressed on the pathogenic T cells isolated from sites of inflammation in the diseased CNS tissue (15) and increased OX40 signaling exacerbates disease severity (16). In contrast, blockade of OX40 signaling at disease onset ameliorates disease severity (17), suggesting that the key window of OX40 signaling in the pathogenesis of EAE appears to be when autoreactive T cells enter the CNS and re-express OX40 (18).

The role of OX40 signaling during PLP priming, compared to disease onset, is less well known. Thus, experiments were performed in $PLP_{139–151}$/CFA immunized SJL mice that received αOX40 at either priming (days 0 and 4), or disease onset (day of onset, +2 and +4 days). Mice receiving αOX40 at priming experienced amelioration of the disease, which was in contrast with mice receiving αOX40 at disease onset where the clinical signs of disease were worse than controls (Fig. 3A). Analysis of $PLP_{139–151}$-stimulated cytokine profile of lymphocytes isolated from CNS tissue day 13 after priming revealed the contrasting effects αOX40. T cells isolated from the CNS of mice administered αOX40 at disease onset produced greater levels of the pathogenic cytokines, IL-2, IL-6, IL-17, and IFNγ, compared to T cells isolated from the CNS in mice administered αOX40 during Ag priming (Fig. 3B).

To understand a potential mechanism of disease amelioration mediated by the OX40 agonist at priming, Treg levels were assessed after immunization, but prior disease onset. It has been shown Tregs play a critical role in EAE disease amelioration and protection (19). To this end, LNs and spleens were harvested from αOX40- or IgG-treated mice seven days after immunization and Tregs were analyzed. OX40-engagement at Ag priming increased the frequency of both $FoxP3^+CD25^+$ and $FoxP3^+CD25^-$ Tregs in both the spleen and LNs (Fig. 3C), which was reflected in the total number of $FoxP3^+$ Tregs found in the lymph nodes (IgG: 0.95×10^6 vs. αOX40: 2.22×10^6; p=0.016). Thus, the increased ratio of Tregs in the αOX40-treated mice may have dampened immunity during priming leading to a decrease in development of self-reactive T cells that could initially enter the CNS and lessen disease severity.

These findings resolve some of the inconsistencies regarding the effect of OX40 on Treg proliferation and peripheral Treg generation. Our data suggests the make-up of the local cytokine milieu at the time of OX40 engagement can direct either resistance to Treg conversion or facilitate Treg proliferation. It is well established that differentiation cytokines antagonize the TGF-β1-mediated development of Tregs by imprinting Th phenotypes that supplant the formation of a Treg phenotype (11, 12). Enhanced production of differentiation cytokines from activated T cells by OX40 signaling appears to underlie the imparted resistance to Treg conversion. Yet in a non-inflammatory environment OX40 signals effectively drive proliferation and expansion of functional Tregs (5, 13). Simply put, OX40 signals tend to stimulate all CD4 T cell populations and its enhancement of lineage development appears to be dependent on the local cytokine milieu.

The implications for the perceived dichotomy in OX40 effects on Tregs are apparent in the initiation and progression of autoimmunity in the EAE model. Induction of the disease by priming with associate myelin-specific Ags and CFA not only drives auto-reactive T cells, but also produces conditions for increased Treg generation (20, 21). Thus, the administration of the OX40 agonist during priming resulted in the increased accumulation of Tregs and a coincident decrease in disease progression and severity. Conversely, enhanced OX40 engagement at the time of disease onset, where alterations in the CNS cytokine environment have already begun, and the auto-reactive T cells have broken the blood-brain barrier and re-expressed OX40, resulted in an increase in effector cytokines within the CNS and increased disease severity. In apparent contrast to the EAE results, OX40 stimulation at priming has also been shown to boost cancer vaccine responses (ref). The difference in OX40 action at priming in these two disease models may be explained by the use of CFA in EAE that drives an inflammatory microenvironment in the LN more conducive to Tregs compared to tumor draining LNs. Other potential reasons are differences in Ag-affinity or amount of Ag; however a definitive explanation remains to be determined.

In conclusion, the differential effects of OX40 signaling on Treg generation and proliferation appear to be established by the cytokines present at the site of T cell activation. Moreover, the data generated in the EAE model showed two different biologic outcomes when the same OX40 agonist was delivered just 10 days apart. We attribute these opposing effects to different immune environments (draining LNs and inflamed CNS) where the conditions drove two different biologic outcomes using the same immune stimulus. Thus, it appears that engagement of OX40 can augment all lineages of CD4 T cells, generating different biologic outcomes depending upon the influence from the local cytokine milieu.

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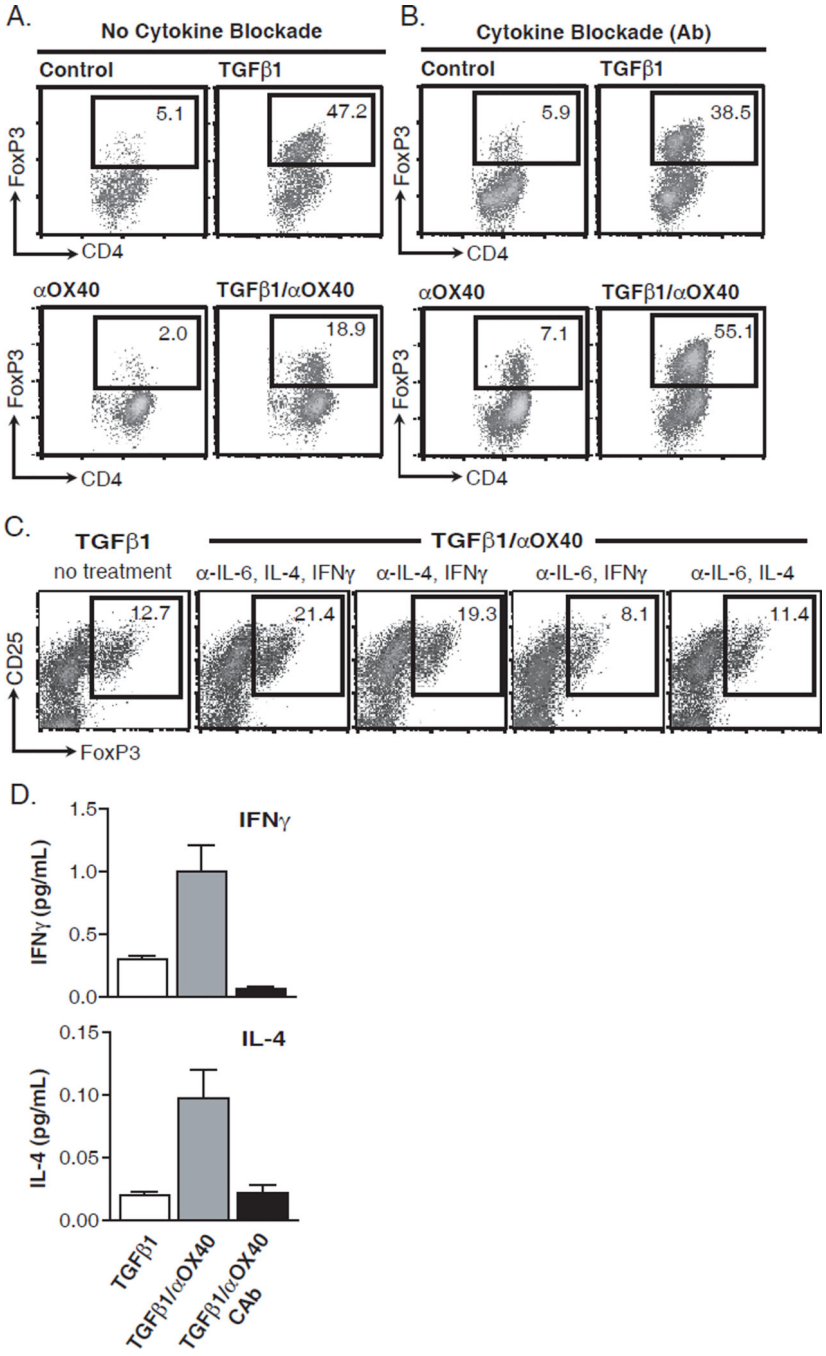

FIGURE 1.

The cytokines IFNγ and IL-4 determine the effect of OX40 stimulation on activated T cells in the presence of TGFβ-1. Isolated $CD25^{-}FoxP3^{-}$ T cells were stimulated by αCD3 and αCD28 in the presence of IL-2. (A) Cultures were then treated with TGFβ-1 and/or agonist OX40 antibody (αOX40) and incubated for 72 hrs. (B) Blocking Abs specific for IL-4, IL-6, and IFNγ or (C) combinations of IL-4, IL-6, and IFNγ Abs were added to cultures. (D) Levels of IFNγ and IL-4 from cultures (72 hrs). (E) Dose

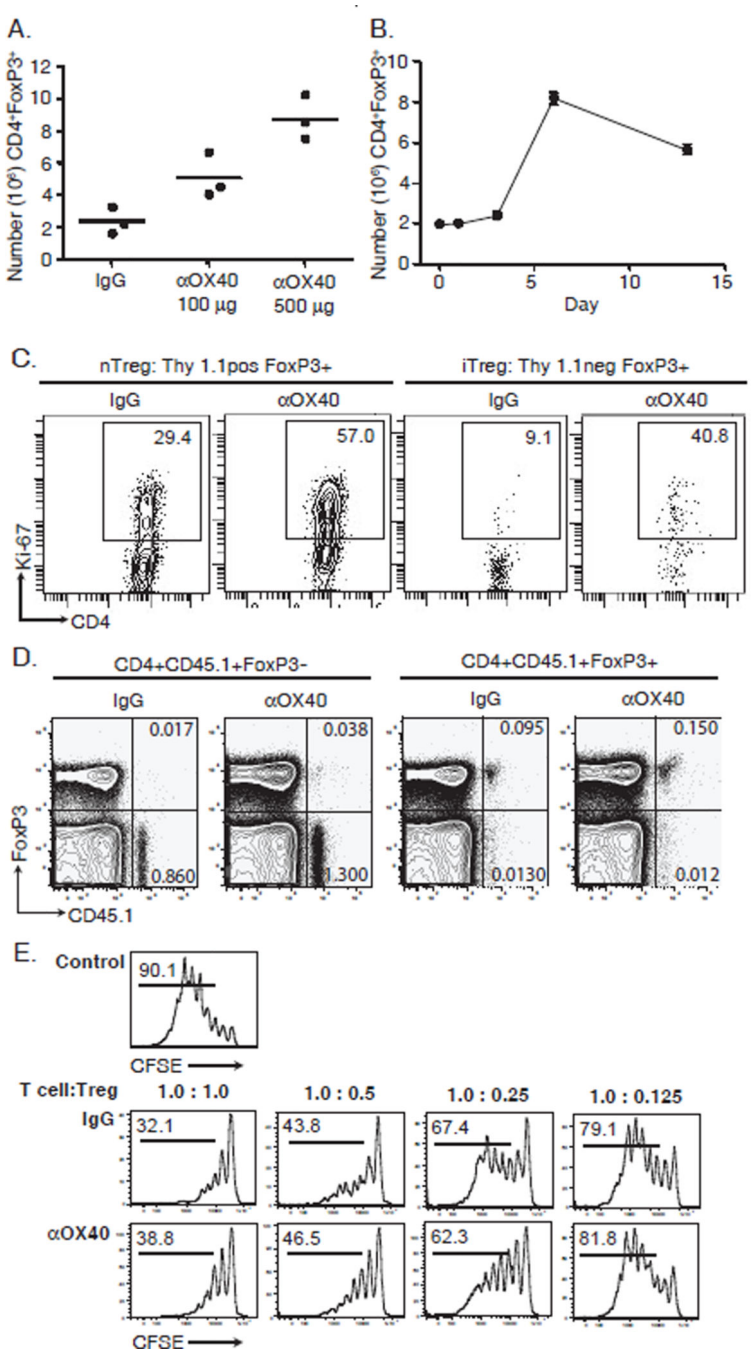

FIGURE 2.

Agonist OX40 augments the accumulation and cycling of functionally suppressive $CD4^+FoxP3^+$ T cells in vivo. (A) Naïve wt mice were injected with rat IgG or αOX40 (100 or 500 μg) i.p. and splenic $CD4^+FoxP3^+$ T cells were enumerated six days later. (B) Splenocytes from mice injected with 500 μg αOX40 were analyzed for $CD4^+FoxP3^+$ T cells at various times. (C) TGFβ-1-generated Tregs (10^6) (Thy1.2) were injected into Thy1.1 congenic hosts and treated with rat IgG or αOX40 (500 μg) one day later. Ki-67 expression was analyzed six days after treatment in $Thy1.1^+CD4^+FoxP3^+$ and $Thy1.1^-CD4^+FoxP3^+$ T

cells. (D) FoxP3^{+} and FoxP3^{-} CD4 T cells were isolated by FACs sorting from FoxP3eGFP spleens by gating for CD4^{+}GFP^{+} and CD4^{+}GFP^{-} respectively. FoxP3^{+} T cells (10^{6}) or FoxP3^{-} T cells (5 X 10^{6}) were transferred to CD45.1 congenic mice and treated with rat IgG or αOX40 (500 μg). Spleens were analyzed for CD45.2^{+}FoxP3^{+} T cell expansion (d6). (E) Isolated CD4^{+}FoxP3^{+} T cells (50,000) from the spleens of mice six days after rat IgG or αOX40 treatment were mixed with naïve CFSE-labeled CD8 T cells and stimulated with αCD3. Two-three days later CFSE dilution was measured.

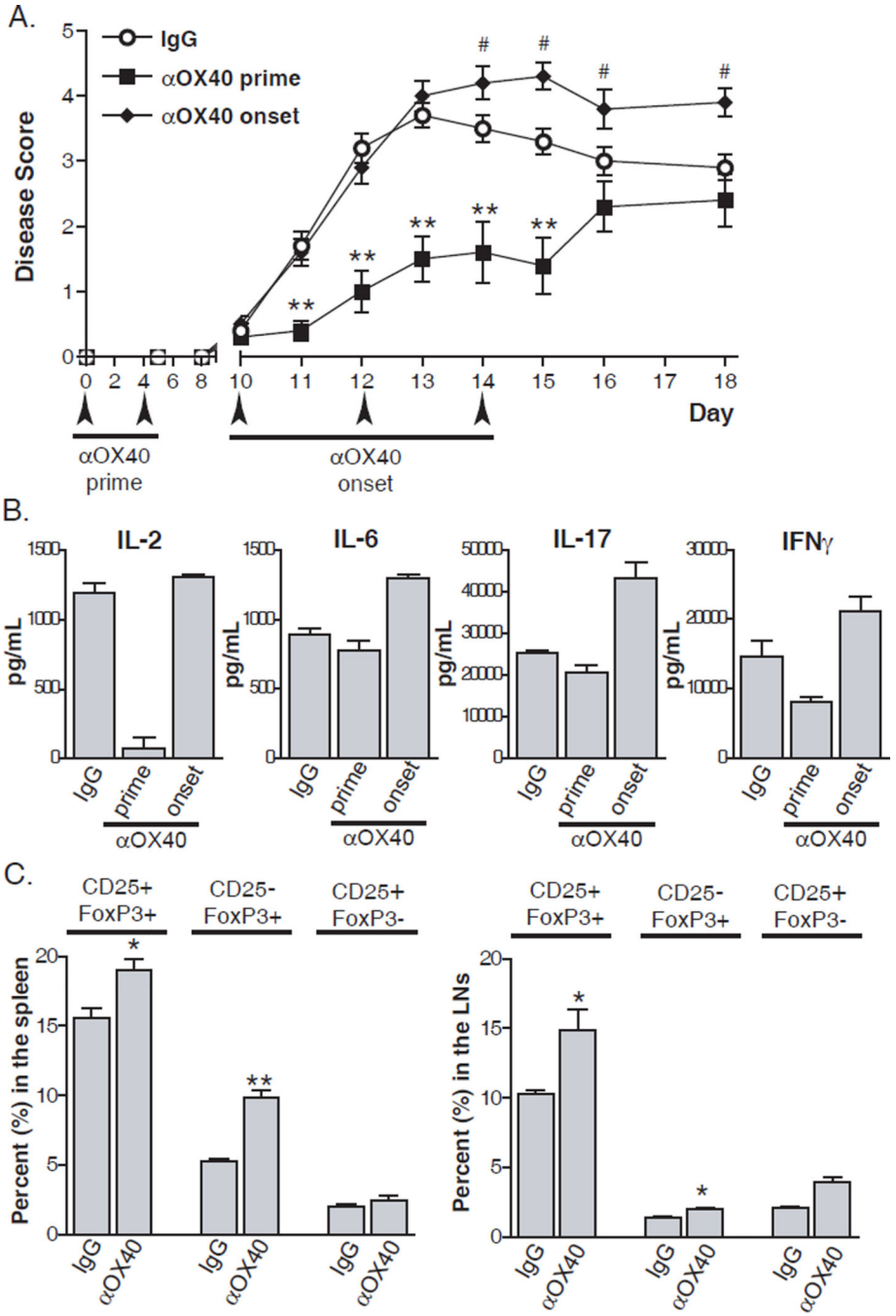

FIGURE 3.

The timing of OX40-engagement in a model of EAE influences disease severity by alterations in Tregs. (A) EAE disease progression and severity is decreased following αOX40 administration (250 μg) compared to rat IgG at Ag-priming (days 0 and 4), but mediated an increase in severity when administered at disease onset (day of onset, day +2 and +4). (B) αOX40 treatment at disease onset increases IL-2, IL-6, IL-17, and IFNγ produced by isolated CNS cells. (C) αOX40 treatment at PLP/CFA-priming alters the

frequency of FoxP3$^+$ CD4 cells in the spleen and LNs of EAE mice after seven days. Values of $p \leq 0.05$ were considered significant and expressed as */# $p \leq 0.05$ and **/## $p \leq 0.001$.