Published in final edited form as:

Eur J Immunol. 2016 June ; 46(6): 1480–1489. doi:10.1002/eji.201546204.

The GARP/Latent TGF-β1 complex on Treg cells modulates the induction of peripherally derived Treg cells during oral tolerance

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Abstract

Treg cells can secrete latent TGF-β1 (LTGF-β1), but can also utilize an alternative pathway for transport and expression of LTGF-β1 on the cell surface in which LTGF-β1 is coupled to a distinct LTGF-β binding protein termed glycoprotein A repetitions predominant (GARP)/LRRC32. The function of the GARP/LTGF-β1 complex has remained elusive. Here, we examine in vivo the roles of GARP and TGF-β1 in the induction of oral tolerance. When Foxp3^{-} OT-II T cells were transferred to wild-type recipient mice followed by OVA feeding, the conversion of Foxp3^{-} to Foxp3^{+} OT-II cells was dependent on recipient Treg cells. Neutralization of IL-2 in the recipient mice also abrogated this conversion. The GARP/LTGF-β1 complex on recipient Treg cells, but not dendritic cell-derived TGF-β1, was required for efficient induction of Foxp3^{+} T cells and for the suppression of delayed hypersensitivity. Expression of the integrin αvβ8 by Treg cells (or T cells) in the recipients was dispensable for induction of Foxp3 expression. Transient depletion of the bacterial flora enhanced the development of oral tolerance by expanding Treg cells with enhanced expression of the GARP/LTGF-β1 complex.

Keywords

Foxp3; GARP; TGF-β; Tolerance; Treg cell

Introduction

TGF-β1 is synthesized as a proprotein consisting of an *N*-terminal propeptide (termed latency-associated peptide [LAP]) and bioactive TGF-β1. The proprotein is cleaved intracellularly by furin, but LAP remains noncovalently associated with TGF-β1 to form the small latent complex. In many cell types, the small latent complex is coupled to latent TGF-β binding proteins (LTBP) and secreted as the large latent complex. Mouse

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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and human Treg cells can secrete TGF-β1 by this classical pathway, but can also utilize an alternative pathway for transport and expression of latent TGF-β1 (LTGF-β1) on the cell surface in which LTGF-β1 is coupled to a distinct LTBP termed glycoprotein A repetitions predominant (GARP)/LRRC32 [1–3]. GARP is expressed at low levels on resting Treg, but its expression is enhanced by TCR stimulation or by culture in IL-2 or IL-4 in the absence of TCR stimulation [4]. The expression of GARP is independent of TGF-β1 and recombinant LTGF-β1 can bind directly to GARP. Furthermore, GARP forms a disulfide bridge with Cys33 on LAP and GARP can also outcompete LTBP for binding to LTGF-β1 [5].

The release of biologically active TGF-β1 from LAP is a critical regulatory step for TGF-β1 function. Many mechanisms have been proposed for the activation of LTGF-β1, but more recent studies have shown that active TGF-β1 can be released from the LTGF-β1/LTBP complex by the action of the integrins αvβ6 and αvβ8 that bind the arginine-glycine-aspartic acid site in LTGF-β1. Integrin αvβ8 is expressed on dendritic cells (DCs) and conditional deletion of *Igtb8* from DCs resulted in severe inflammatory bowel disease and systemic autoimmunity [6]. Integrin β8 (αvβ8) is selectively expressed on Treg and can process LTGF-β1 on activated Treg in a cell intrinsic manner, producing biologically active TGF-β1 [7, 8].

The role of cell surface TGF-β1 as a mediator of the Treg suppressor function is controversial. A modest reduction in the in vitro suppressor capacity was observed following siRNA-mediated knock down of human GARP [9]. However, GARP-deficient mouse Treg cells were as suppressive as WT Treg cells in vitro [4]. Although GARP-deficient Treg cells can still secrete LTGF-β1, it is unlikely that that secreted LTGF-β1 plays any role in Treg suppression in vitro because the suppressive capacity of TGF-β1 deficient Treg cells is also unimpaired [4, 10].

Cell surface-associated TGF-β1 on Treg cells may also mediate immunoregulatory functions. We have suggested an alternative role for Treg-expressed cell surface TGF-β1. Activation in vitro of cocultures of activated Treg cells and näive T cells resulted in induction of Foxp3 expression in a low percentage of the responder T cells in a TGF-β1 dependent fashion [11]. Other studies suggested that activated Treg cells could induce Th17 cells in the presence of IL-6 [12]. Although both the induction of $Foxp3^+$ T cells and Th17 cells could have been mediated by TGF-β1 that had been secreted by Treg cells, we have demonstrated that the major source of the TGF-β1 involved in both induction of $Foxp3^+$ T cells and IL-17 producing T cells is the GARP/LTGF-β1 complex [4].

Despite the conservation of GARP/LTGF-β1 complex between mouse and man, very little is known about its functional role in vivo. In some studies the production of TGF-β1 has been claimed to be required for the suppression of inflammatory bowel disease [13, 14], while in others TGF-β1 deficient Treg were shown to be capable of suppressing T-cell mediated colitis [15, 16], but the suppression was dependent on TGF-β1 produced by a non-Treg [15]. Worthington et al. [8] have recently reported that Treg cell-specific deletion of αvβ8 does not result in spontaneous inflammation, but that Treg lacking this integrin were unable to suppress ongoing inflammatory responses. Cuende et al. [17] have recently developed novel anti-GARP antibodies that blocked the activation of TGF-β1 and were found to reverse

the Treg-induced attenuation of xenogeneic graft versus host disease. The reversal of Treg suppression was interpreted to be exclusively due to blocking the activation of LTGF-β1 and not due to depletion of the Treg cells.

Here, we examine in vivo the cell extrinsic roles of GARP and TGF-β1 in the induction of oral tolerance. We demonstrate the importance of Treg cells for de novo establishment of oral tolerance via the GARP/LTGF-β1 pathway. In addition, we demonstrate that the intestinal microbiota played a role in the induction of oral tolerance by promoting a tolerance-inducing environment resulting in expression of the surface GARP/LTGF-β1 complex on Treg cells in the MLN.

Results

Foxp3$^+$ Treg cells and IL-2 are required for the induction of pTreg cells during oral tolerance

It has previously been established by several groups that when naïve Foxp3$^-$ OVA-specific CD4$^+$ T cells are transferred into mice followed by oral administration of OVA in the drinking water that a proportion (10–15%) of the transferred cells in the MLN and Peyer's patches will gain expression of Foxp3. As our group [11] and others [18, 19] have previously postulated that Treg cells may play a role in de novo differentiation of CD4$^+$Foxp3$^-$ T cells to Foxp3$^+$ T cells (so-called "infectious tolerance"), we first investigated if the transient depletion of recipient Treg cells could influence the conversion. We made use DEREG animals [20], which express the Diphtheria Toxin (DT) receptor (DTR) under the control of the Foxp3 promoter. Following administration of DT to these animals, Foxp3$^+$ Treg cells are rapidly depleted, but largely return to normal levels within 7–14 days. Both WT and DEREG animals received two injections of DT (1 μg on days −1 and 0), followed by transfer of OT-II cells on day 0, and administration of OVA-containing water on days 1–8. While −13% of OT-II cells became Foxp3$^+$ following transfer to DT-treated WT mice, a 50–60% reduction in OT-II Foxp3$^+$ cells was seen in the DT-treated DEREG recipients (Fig. 1A and B). At the time of the assay (day 8 following transfer), a significant depletion of the recipient Foxp3$^+$ T-cell population was still seen in the DT-treated DEREG mice (Fig. 1B).

As IL-2 plays a nonredundant role in vitro [21] for the induction of iTreg cells, we evaluated the role of IL-2 in vivo in the oral tolerance model. OT-II cells were transferred on day 0 and OVA-containing water was provided on days 1–8. Animals were injected with anti-IL-2 (S4B6, 400 μg/mouse) on days 1, 3, 5, and 7 of oral antigen feeding. Mice that were treated with anti-IL-2 had a drastic reduction in the generation of OT-II Foxp3$^+$ T cells in both the MLN and the Peyer's patches (Fig. 1C and D). These data demonstrate for the first time in this transfer model that both host Foxp3$^+$ T cells and IL-2 are critical for the induction of Foxp3$^+$ Treg cells.

Treg-cell expression of GARP is essential for the optimal induction of oral tolerance

As we have previously established that Treg-cell derived TGF-β1 and GARP can serve as the source of TGF-β1 needed for conversion of CD4$^+$ Foxp3$^-$ cells to CD4$^+$Foxp3$^+$ cells

in vitro [4], we next determined if TGF-β1 or GARP were essential for the conversion of $CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ T cells during the process of oral tolerance in vivo. OT-II cells were transferred to WT recipients or TGF-$\beta1^{fl/fl}$-Foxp3-Cre mice. Induction of Foxp3 expression by the transferred OT-II cells was significantly diminished following transfer to TGF-$\beta1^{fl/fl}$-Foxp3-Cre mice (Fig. 2A and C). The extent of reduction was similar to that seen in Treg-depleted DEREG mice. Furthermore, a similar reduction was seen when the OT-II T cells were transferred into $GARP^{fl/fl}$-CD4-CRE mice, indicating that GARP-derived TGF-β1 was required for maximum conversion in vivo (Fig. 2B and D).

Treg cell-expressed GARP/LTGF-β1 complex controls suppression of DTH induced by oral tolerance

While the OT-II transfer model is ideal for the quantitation of the induction of antigen-specific $Foxp3^+$ peripherally induced Treg cells (pTreg cells) in vivo, it was important to also determine if the results obtained using this model were of functional importance in vivo. Therefore, we fed WT or TGF-$\beta1^{fl/fl}$-Foxp3-Cre animals OVA or water for 1 week. OVA-fed and control animals were then immunized with OVA in CFA and 7 days later delayed-type hypersensitivity (DTH) was assayed by challenging the animals with OVA in one footpad and PBS in the contralateral footpad. WT animals pretreated with oral OVA had a diminished DTH response, while OVA-fed TGF-$\beta1^{fl/fl}$-Foxp3-Cre animals were not tolerized and developed DTH responses (Fig. 2E). This result is consistent with the marked reduction in the induction of $Foxp3^+$ T cells following oral OVA in the TGF-$\beta1^{fl/fl}$-Foxp3-Cre animals.

GARP-derived TGF-β1 does not modulate the frequency or function of MLN DC populations

It has previously been reported that the production of retinoic acid by migratory $CD103^+CD11b^+$ DC subpopulations in the MLN and gut are required for the induction of Foxp3 expression during oral tolerance [22, 23]; it therefore remained possible that effects of GARP-derived TGF-β1 were secondary to modulation of the frequency or function of these DC subpopulations. However, the frequency of each of the subpopulations was identical in WT to $GARP^{fl/fl}$-CD4-CRE mice (Fig. 3A and B), as was their ability to produce retinoic acid as determined ex vivo by the ALDEFLUOR assay (Fig. 3C and D).

Oral tolerance induction can occur independently of DC-derived TGF-β1 and T-cell expressed αvβ8

As the reduction in the induction of $Foxp3^+$ T cells following exposure to oral antigen was never complete, we considered the possibility that DC-derived TGF-β1 also contributed to the induction of $Foxp3^+$ T cells as previously suggested [22]. Induction of Foxp 3 expressing OT-II cells in the MLNs and Peyer's patches was similar following transfer into WT or TGF-$\beta1^{fl/fl}$-CD11c-Cre mice (Fig. 4A and D).

To determine the contribution of OT-II derived TGF-β1 to the process of conversion, we generated *OT-II* × *Foxp3-GFP* × *Tgfb1*$^{fl/fl}$ × *CD4-Cre* animals, sorted naïve $CD4^+Foxp3(GFP)^-CD45RB^{hi}CD44^{low}$ cells, and transferred them into WT $CD45.1^+$ recipients. The deletion of TGF-β1 only in the OT-II cells resulted in a modest reduction

in the induction of Foxp3 expression following oral antigen and significant differences were only seen in the MLN, but not in Peyer's patches (Fig. 4B and E).

As we have previously established that Treg cells require the expression of αvβ8 for the processing of LTGF-β1 from GARP to efficiently produce biologically active TGF-β1 in vitro [7], we determined whether αvβ8 expressed on Treg played a similar role in vivo. OT-II cells transferred into WT and ITGB8$^{fl/fl}$-CD4-Cre mice had similar percentage of Foxp3^{+} pTreg after oral antigen, thus indicating that expression of αvβ8 by Treg (or T cells) in the recipients was dispensable for induction of Foxp3 expression (Fig. 4C and F). This is most likely due to αvβ8 working in trans from DC, whose expression has previously been demonstrated to be required for Foxp3 expression during oral tolerance induction [24].

Induction of pTreg cells in vivo during antigen exposure is modulated by gut flora

The studies described above demonstrate that active TGF-β1 derived from the GARP/LTGF-β1 complex plays an important role in the induction of oral tolerance. However, the GARP/LTGF-β1 complex is expressed at very low levels on freshly explanted Treg, but markedly upregulated following Treg activation in vitro. Since gut-associated Treg have a more activated phenotype than Treg in other peripheral sites, we evaluated the contribution of normal flora to pTreg induction. OT-II cells were transferred to WT-specific pathogen-free mice that had been depleted of their flora by treatment with multiple antibiotics for 1 week. The mice were then fed OVA water for 1 week and induction of Foxp3^{+} Treg OT-II cells measured in the MLN. Surprisingly, the conversion of OT-II cells from Foxp3^{-} to Foxp3^{+} was markedly enhanced in the MLN (Fig. 5A and B) of animals whose flora was depleted compared to animals not pretreated by antibiotics. One possible explanation for this enhancement in the conversion of the OT-II cells is the increased number of recipient Foxp3^{+} T cells observed 7 days following the cessation of oral antibiotics (Fig. 5C and D). Furthermore, GARP expression was also modestly enhanced on the Foxp3^{+} T cells in these recipients resulting in an environment that could potentially promote Treg induction (Fig. 5E).

Discussion

It is widely assumed that TGF-β1 is one of the major mediators used by Foxp3^{+} Treg cells in the execution of their suppressive functions [25, 26]. While many in vitro and in vivo studies have supported this view [8, 13, 14], an equal number have failed to find a significant contribution of Treg-produced TGF-β1 to Treg cell suppressor function [15, 16, 27]. TGF-β1 is produced by multiple cell types and it has been difficult to demonstrate that the ability of Treg to produce TGF-β1 is actually greater than that of other activated T cells [27]. Notably, what distinguishes Treg cells from other T cells is the ability of Treg to secrete TGF-β1 by multiple pathways. Treg can release LTGF-β1 coupled to LTBP and Treg can also express LTGF-β1 on their cell surface coupled to GARP [1, 2]. Treg cells also express integrin αvβ8 that catalyzes the release of active TGF-β1 moiety from LTGF-β1. αvβ8 may function in cis or trans to release active TGF-β1 from the GARP/LTGF-β1 complex or in trans to release active TGF-β1 from the large latent complex [7] or potentially from a secreted form of the GARP/LTGF-β1 complex [28].

The goal of the present studies was to define the potential role of the GARP/LTGF-β1 complex in the induction of oral tolerance. While it has been well established that the $Foxp3^+$ Treg can be induced in culture in vitro in the presence of TCR stimulation, TGF-β1 and IL-2 [21], the requirements for the induction of pTreg in vivo are less clear. It is likely that TGF-β1 contributes to the induction of pTreg, as it is difficult to induce pTreg in mice that express a dominant negative TGF-βR2 [29]. As oral tolerance represents one of the best-characterized models for pTreg induction, we have analyzed the cellular source of TGF-β1 and the contribution of IL-2 to the induction of oral tolerance. It has previously been proposed that soluble oral antigens are taken up and processed by $CX3CR1^+$ macrophages in the small intestine and that antigen is then transferred to $CD103^+$ DC via gap junctions [30]. $CD103^+$ DC, through their production of retinoic acid and TGF-β1, as well as their expression of integrin αvβ8 induce the conversion of naïve $CD4^+Foxp3^-$ cells to Treg during the oral administration of antigen [22, 24] in the MLN.

We demonstrate here for the first time that IL-2 plays a nonredundant role in the induction of $Foxp3^+$ pTreg induced by the oral administration of antigen. We believe that the effects of IL-2 are directly mediated on the conversion of the OT-II cells from $Foxp3^-$ to $Foxp3^+$, but it is possible that IL-2 could also be acting on the recipient Treg cells. More importantly, we demonstrate in the adoptive transfer model that the induction of pTreg is much less efficient when DTR^+ $Foxp3^+$ Treg cells are depleted from the recipient animal following treatment with DT.

The contribution of recipient Treg to pTreg induction was mediated by TGF-β1 derived from the GARP/LTGF-β1 complex on Treg cells of the recipient, as pTreg induction was impaired when the recipient Treg cells could not produce TGF-β1 ($TGF\text{-}β1^{fl/fl}$-Foxp3-Cre) or failed to express $GARP^{fl/fl}$ (GARP-CD4-Cre). The decreased efficiency of induction of $Foxp3^+$ T cells was paralleled by a decrease in the suppression of delayed hypersensitivity in vivo. Surprisingly, the induction of oral tolerance was normal in animals whose Treg were deficient in αvβ8. It is therefore likely that TGF-β1 derived from the GARP/LTGF-β1 complex on Treg is activated by αvβ8 expressed by $CD103^+$ DC. In contrast to previous in vitro studies [22] that suggested that DC were the major cellular source of TGF-β1 mediating oral tolerance, deletion of TGF-β1 production from DC using CD11c-Cre had no effect on oral tolerance induction.

Since the GARP/LTGF-β1 complex is only expressed at high levels after T-cell receptor stimulation [4], we examined whether antigens derived from bacterial flora played a role in the process of oral tolerance induction. Prior to the transfer of naïve OT-II cells and oral ovalbumin administration, we depleted the gut bacterial flora with water containing multiple antibiotics. Paradoxically, we observed a markedly enhanced induction of Foxp3 expression in the transferred OT-II cells. In addition, the percentages of $CD4^+Foxp3^+$ T cells among $CD4^+$ T cells were consistently enhanced in the MLN, but not in the spleen, of recipient animals after 1 week of bacterial reconstitution. It remains possible that following cessation of antibiotic treatment, the microenvironment in the gut during repopulation of the flora promotes the activation and expansion of $Foxp3^+$ Treg cells, enhances their expression of the GARP/LTGF-β1 complex, and enhanced conversion of OT-II cells to Treg cells. However, we have no direct evidence that repopulation of the flora leads to expansion of $Foxp3^+$ Treg.

We could not analyze the responses of mice treated with multiple antibiotics both before and during the induction of oral tolerance as these animals had very small MLNs and very few cells could be recovered.

In summary, we demonstrate for the first time that the GARP/LTGF-β1 complex on Treg cells is a major source of TGF-β1 needed for induction of pTreg cells during the process of oral tolerance. These results raise the possibility that a dysfunction of Treg or a defect in the expression of GARP/LTGF-β1 complex may play a role in the development of food allergies.

Materials and methods

Mice

C57BL/6 were obtained from Taconic. C57BL/6-CD45.1^{+} and OVA-specific TCR transgenic OT-II (CD45.1, Rag1$^{-/-}$) mice were obtained by NIAID and were maintained by Taconic Farms (Germantown, NY, USA) under contract by NIAID. *Itgb8*$^{fl/fl}$ mice, *Lrrc32*$^{fl/fl}$ (GARP), and *Tgfb1*$^{fl/fl}$ mice have been previously described [4, 10, 31]. GARP$^{fl/fl}$ mice, which were originally obtained on a mixed background, were backcrossed for at least eight generations to C57/BL6. Floxed mice were crossed to CD4-CRE mice, CD11c-CRE, or Foxp3-YFP/CRE. GARP$^{fl/fl}$-CD4-Cre mice were used as GARP is expressed on CD4^{+}Foxp3^{+} T cells, but not conventional CD4^{+}Foxp3^{-} cells. OT-II Foxp3-GFP mice, which were generated by crossing OT-II mice to Foxp3-GFP mice, were further crossed to *Tgfb1*$^{fl/fl}$ × CD4-CRE to generate *Tgfb1*$^{fl/fl}$ × *CD4-CRE* × *OT-II Foxp3-GFP*. DEREG mice were generously provided by Tim Sparwasser [20]. Transient Treg depletion was achieved by injection of 1 μg of recombinant Diphtheria Toxin (Millipore) on two consecutive days intraperitoneally in PBS. All animal protocols used in this study were approved by the NIAID Animal Care and Use Committee.

Cell isolation and flow cytometry

CD4^{+} cells from OVA-specific TCR transgenic OT-II (CD45.1, Rag1$^{-/-}$) mice, cells were labeled with anti-CD4 beads and purified on the AutoMACS Pro Cell Separator (Miltenyi Biotec). T cells were isolated from the peripheral LNs, MLN, and spleen by mechanical disruption. For the analysis of MLN DC, the MLNs were cut into small pieces then digested in complete media containing Liberase TL Research Grade and DNase (both at 0.1 mg/mL).

All cell sorting was performed on FACSAria flow cytometers (BD Biosciences). Single-cell suspensions were stained using antibodies from eBioscience or BioLegend according to the manufacturer's protocol. For staining of Foxp3, cells were fixed and permeabilized using the Foxp3 fixation/permeabilization staining kit (eBiosciences), followed by intracellular staining. All flow cytometry was performed on an LSRII (BD Biosciences).

Induction of oral tolerance

Briefly, 10^6 CD4^{+} cells from OVA-specific TCR transgenic OT-II (CD45.1, Rag1$^{-/-}$) were injected intravenously into various WT or conditional KO (cKO) strains on Day 0 (21). On days 1–8, mice were given filter sterilized 1.5% OVA containing drinking water. On

day 8, animals were sacrificed and the MLN and Peyer's patches were collected. Animals were individually analyzed for the conversion of OT-II cells into Foxp3$^+$ cells. Transferred OT-II cells were gated based on: CD4$^+$TCRβ$^+$CD45.1$^+$CD45.2$^-$, as indicated in Supporting Information Fig. 1A. Animals were aged 8–12 weeks for these experiments.

In some experiments, animals were injected on days 1, 3, 5, and 7 with 400 μg anti-IL-2 (S4B6, BioXcell) or PBS. In experiments involving DEREG mice, both WT and DEREG mice were injected with DT on days −1 and 0. In experiments with multiple antibiotic water, animals were given drinking water containing 0.5% Vancomycin, 1.0% Neomycin, 1.0% Metronidazole, and 1.0% Ampicillin on days −7 through day 1, then switched to OVA water for days 1–8. This antibiotic treatment has been previously described [32, 33].

For DTH experiments, OT-II cells were not given. Oral tolerance was induced by supplementing the drinking water for 6 days with 1.5% OVA. One day after the end of OVA feeding, mice were immunized with 10 μg OVA in CFA (100 μL total volume: 50 μL OVA with 50 μL of CFA). Ten days after immunization, mice were challenged with 10 μg of OVA in 10 μL of PBS in the footpad. As an internal control, the opposite footpad was injected with PBS alone. Footpads were subsequently measured at 24 and 48 h.

ALDEFLUOR assay

ALDEFLUOR assays were performed according to the manufacturer's instructions (Stem Cell Technologies) with minor adjustments (1.5 μM ALDEFLUOR reagent, 30 μM DEAB inhibitor). Digests of MLN were stained for Thy1.2, CD19, CD11c, MHC-class II, and CD103 (10^6 cells), and ALDEFLUOR as per manufacturer's instructions.

mAb used in FACS analysis

Anti-mouse CD45.1 (A20), CD45.2 (104, CD4 (RM4–5), Foxp3 (FLK-16s), TCRβ (H57–597), CD103 (2E7), CD45RB (C363.16A), CD44 (IM7), CD8α (53–6.7, CD11c (N418), Thy1.2 (53–2.1, B220 (RA3–6B2), CD11b (M1/70), and I-A^b (AF6–120.1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by funds from the National Institutes of Health. The authors would like to thank Julie Edwards, Carol Henry, and Ke Weng from the NIAID Flow Cytometry Section for sorting their cells. J.P.E. designed and performed most experiments and prepared the manuscript; T.W.H, D.M.F., and Y.B. designed and performed the DTH experiments; D.D.G. assisted in performing several experiments; and E.M.S. supervised the studies and prepared the manuscript.

Abbreviations:

cKO	conditional KO
DT	diphtheria toxin
DTH	delayed-type hypersensitivity

GARP	glycoprotein A repetitions predominant
Itgb8	integrin β8
LAP	latency-associated peptide
LTBP	latent TGF-β binding proteins
LTGF-β1	latent TGF-β1
pTreg cell	peripherally induced Treg cell

References

1. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D and Shevach EM, GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. Proc. Natl. Acad. Sci. USA 2009. 106: 13445–13450. [PubMed: 19651619]
2. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H and Unutmaz D, Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. Proc. Natl. Acad. Sci. USA 2009. 106: 13439–13444. [PubMed: 19666573]
3. Stockis J, Colau D, Coulie PG and Lucas S, Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. Eur. J. Immunol. 2009. 39: 3315–3322. [PubMed: 19750484]
4. Edwards JP, Fujii H, Zhou AX, Creemers J, Unutmaz D and Shevach EM, Regulation of the expression of GARP/latent TGF-beta1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation. J. Immunol. 2013. 190: 5506–5515. [PubMed: 23645881]
5. Wang R, Zhu J, Dong X, Shi M, Lu C and Springer TA, GARP regulates the bioavailability and activation of TGFbeta. Mol. Biol. Cell 2012. 23: 1129–1139. [PubMed: 22278742]
6. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, Wang Y et al. , Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. Nature 2007. 449: 361–365.
7. Edwards JP, Thornton AM and Shevach EM, Release of active TGF-beta1 from the latent TGF-beta1/GARP complex on T regulatory cells is mediated by integrin beta8. J. Immunol. 2014. 193: 2843–2849. [PubMed: 25127859]
8. Worthington JJ, Kelly A, Smedley C, Bauche D, Campbell S, Marie JC and Travis MA, Integrin alphavbeta8-mediated TGF-beta activation by effector regulatory T cells is essential for suppression of T-cell-mediated inflammation. Immunity 2015. 42: 903–915. [PubMed: 25979421]
9. Wang R, Wan Q, Kozhaya L, Fujii H and Unutmaz D, Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. PLoS One 2008. 3: e2705. [PubMed: 18628982]
10. Li MO, Wan YY and Flavell RA, T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 2007. 26: 579–591. [PubMed: 17481928]
11. Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ and Shevach EM, CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J. Exp. Med. 2008. 205: 1975–1981. [PubMed: 18710931]
12. Xu L, Kitani A, Fuss I and Strober W, Cutting edge: regulatory T cells induceCD4+CD25-Foxp3-TcellsorareselF-inducedtobecomeTh17cells in the absence of exogenous TGF-beta. J. Immunol. 2007. 178: 6725–6729.
13. Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H and Strober W, TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J. Immunol. 2004. 172: 834–842. [PubMed: 14707053]

14. Pesu M, Watford WT, Wei L, Xu L, Fuss I, Strober W, Andersson J et al. , T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. Nature 2008. 455: 246–250.
15. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA and Powrie F, T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J. Exp. Med. 2005. 201: 737–746. [PubMed: 15753207]
16. Kullberg MC, Hay V, Cheever AW, Mamura M, Sher A, Letterio JJ, Shevach EM et al. , TGF-beta1 production by CD4+ CD25+ regulatory T cells is not essential for suppression of intestinal inflammation. Eur. J. Immunol. 2005. 35: 2886–2895. [PubMed: 16180248]
17. Cuende J, Lienart S, Dedobbeleer O, van der Woning B, De Boeck G, Stockis J, Huygens C et al. , Monoclonal antibodies against GARP/TGF-beta1 complexes inhibit the immunosuppressive activity of human regulatory T cells in vivo. Sci. Transl. Med. 2015. 7: 284ra256.
18. Qin S, Cobbold SP, Pope H, Elliott J, Kioussis D, Davies J and Waldmann H, “Infectious” transplantation tolerance. Science 1993. 259:974–977.
19. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, Hori S et al. , Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. J. Exp. Med. 2011. 208: 2043–2053. [PubMed: 21875958]
20. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A et al. , Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J. Exp. Med. 2007. 204: 57–63. [PubMed: 17200412]
21. Davidson TS, DiPaolo RJ, Andersson J and Shevach EM, Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J. Immunol. 2007. 178: 4022–4026. [PubMed: 17371955]
22. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y and Powrie F, A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J. Exp. Med. 2007. 204: 1757–1764. [PubMed: 17620361]
23. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, Wohlfert EA et al. , Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity 2011. 34: 435–447. [PubMed: 21419664]
24. Worthington JJ, Czajkowska BI, Melton AC and Travis M., Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphav-beta8. Gastroenterology 2011. 141: 1802–1812. [PubMed: 21723222]
25. Shevach EM, Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 2009. 30: 636–645. [PubMed: 19464986]
26. Vignali DA, Collison LW and Workman CJ, How regulatory T cells work. Nat. Rev. Immunol. 2008. 8: 523–532. [PubMed: 18566595]
27. Gutcher I, Donkor MK, Ma Q, Rudensky AY, Flavell RA and Li MO, Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation. Immunity 2011. 34: 396–408. [PubMed: 21435587]
28. Gauthy E, Cuende J, Stockis J, Huygens C, Lethe B, Collet JF, Bommer G et al. , GARP is regulated by miRNAs and controls latent TGF-beta1 production by human regulatory T cells. PLoS One 2013. 8: e76186. [PubMed: 24098777]
29. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC and von Boehmer H, Inducing and expanding regulatory T cell populations by foreign antigen. Nat. Immunol. 2005. 6: 1219–1227. [PubMed: 16244650]
30. Mazzini E, Massimiliano L, Penna G and Rescigno M, Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) macrophages to CD103(+) dendritic cells. Immunity 2014. 40:248–261. [PubMed: 24462723]
31. Proctor JM, Zang K, Wang D, Wang R and Reichardt LF, Vascular development of the brain requires beta8 integrin expression in the neuroepithelium. J. Neurosci. 2005. 25: 9940–9948. [PubMed: 16251442]

32. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB et al. , Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 2008. 4: 337–349. [PubMed: 18854238]

33. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S and Medzhitov R, Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004. 118: 229–241. [PubMed: 15260992]

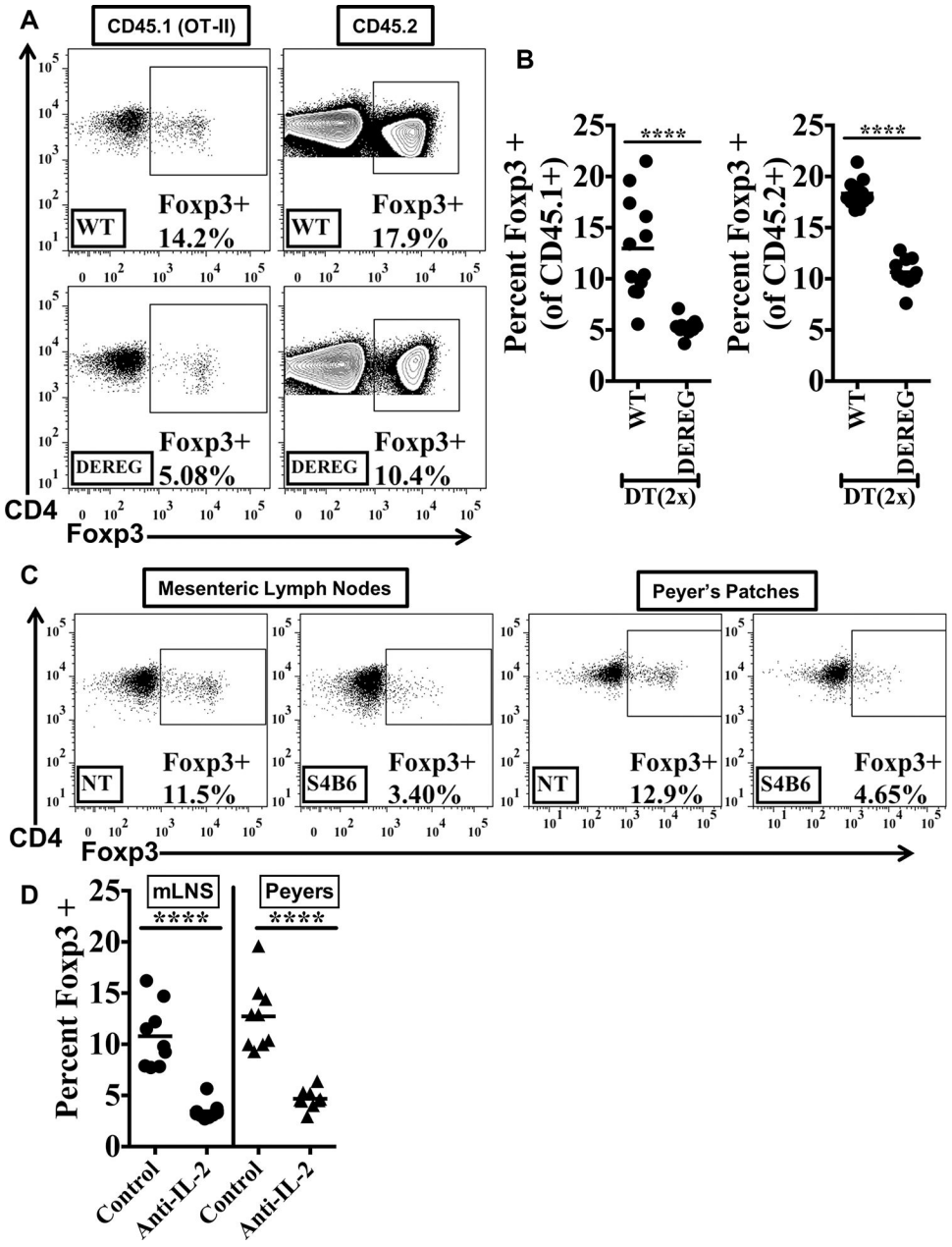

Figure 1.

The presence of Treg cells and IL-2 is required for the induction of pTreg cells during oral tolerance. (A and B) WT and DEREG animals were injected with DT on two consecutive days intraperitoneally in PBS on days −1 and 0. OT-II ($CD4^+CD45.1^+$ $Rag1^{-/-}$) cells were transferred on day 0, followed by OVA containing drinking water for days 1–8, and then sacrificed on day 8. (A) Representative dot plot from WT (top panels) or DEREG (bottom panels) animals gated on transferred OT-II cells ($CD4^+TCR\beta^+CD45.1^+CD45.2^-$, left panels) or representative contour plot of recipient $CD4^+$ T cells ($CD4^+TCR\beta^+CD45.1^-CD45.2^+$), as indicated in Supporting Information Figure 1A. Samples were from the MLN. (B) Pooled results from (A), representing four independent experiments plotting the results from individual animals. (C and D) WT mice were injected on days 1, 3, 5, and 7 with 400 μg

anti-IL-2 (S4B6) or PBS (NT). Animals received OT-II cells and OVA containing water as in (A). (C) Representative dot plot from PBS- or S4B6-treated animals gated on transferred OT-II cells. Samples were obtained from the MLN (left panels) or the Peyer's patches (right panels). (D) Pooled results from (C), representing three experiments plotting the results from individual animals. Bars represent means. $^{****}p < 0.0001$, as determined by *t*-test.

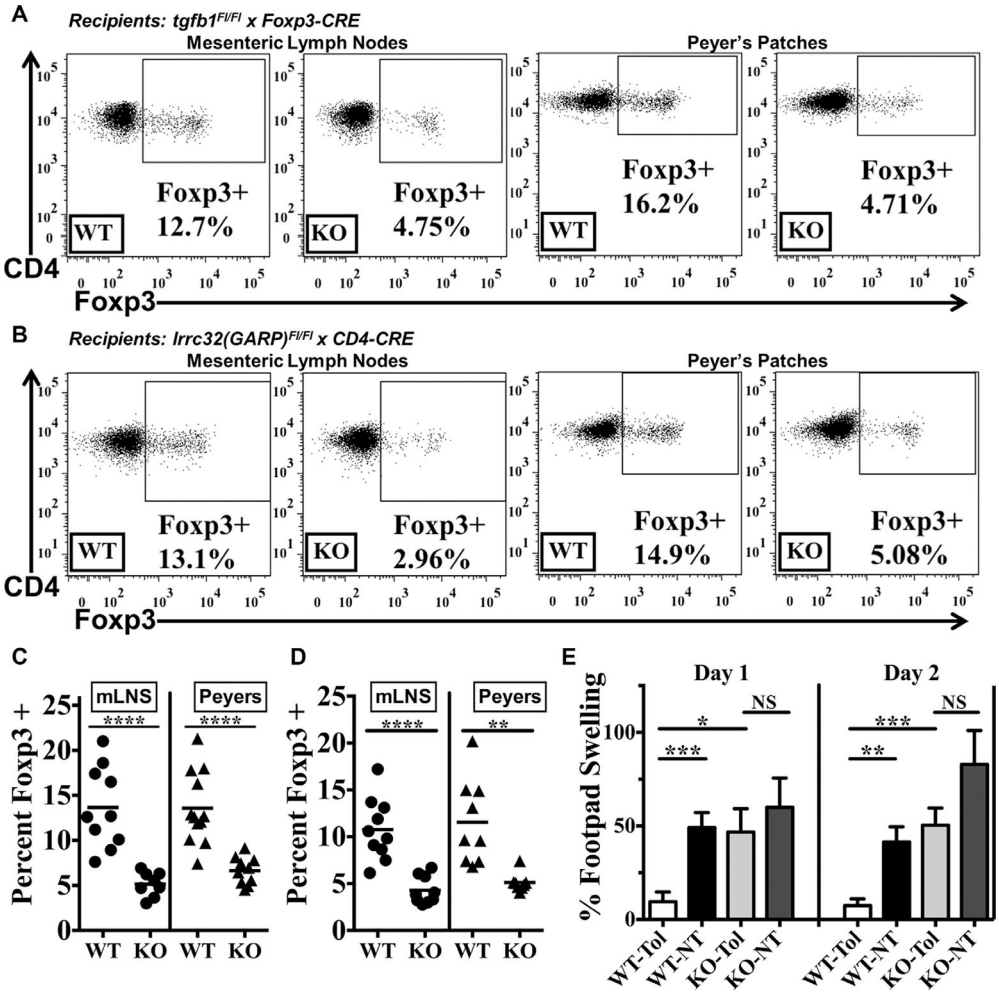

Figure 2.

Treg-cell derived TGF-β1 through the GARP molecule is essential for the maximum establishment of de novo Treg-cell induction during oral tolerance. (A–D) OT-II ($CD4^+CD45.1^+$ $Rag1^{-/-}$) cells were transferred on day 0, followed by OVA containing drinking water for days 1–8. Animals were sacrificed on day 8. Recipients were TGF-β1 WT or cKO (A and C, *tgfb1*$^{fl/fl}$ ± Foxp3-CRE) GARP WT or cKO (B and D, *lrrc32*$^{fl/fl}$ ± CD4-CRE). (A and B) Representative dot plot from TGF-β1 WT/cKO (A) or GARP WT/cKO (B) treated animals gated on transferred OT-II cells ($CD4^+TCR\beta^+CD45.1^+CD45.2^-$), as indicated in Supporting Information Figure 1A. Samples were obtained from the MLN (left panels) or the Peyer's patches (right panels) (C and D) Pooled results from (A and B), representing five (C) or four (D) experiments plotting the results from individual animals. Bars represent means. (E) Oral tolerance experiment. Oral tolerance was induced by supplementing the drinking water for 6 days with OVA water (Tol) or not (NT, plain water). One day after the end of OVA feeding, mice were immunized with 10 μg OVA in CFA. Ten days after immunization, mice were challenged with OVA in one footpad. As an internal control, the contralateral footpad was injected with PBS alone. Footpads were subsequently measured at 24 and 48 h. Data are shown as means ± SEM of

31 total (eight WT-Tol, seven WT-NT, nine KO-Tol, seven KO-NT) mice pooled from three experiments.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as determined by *t*-test.

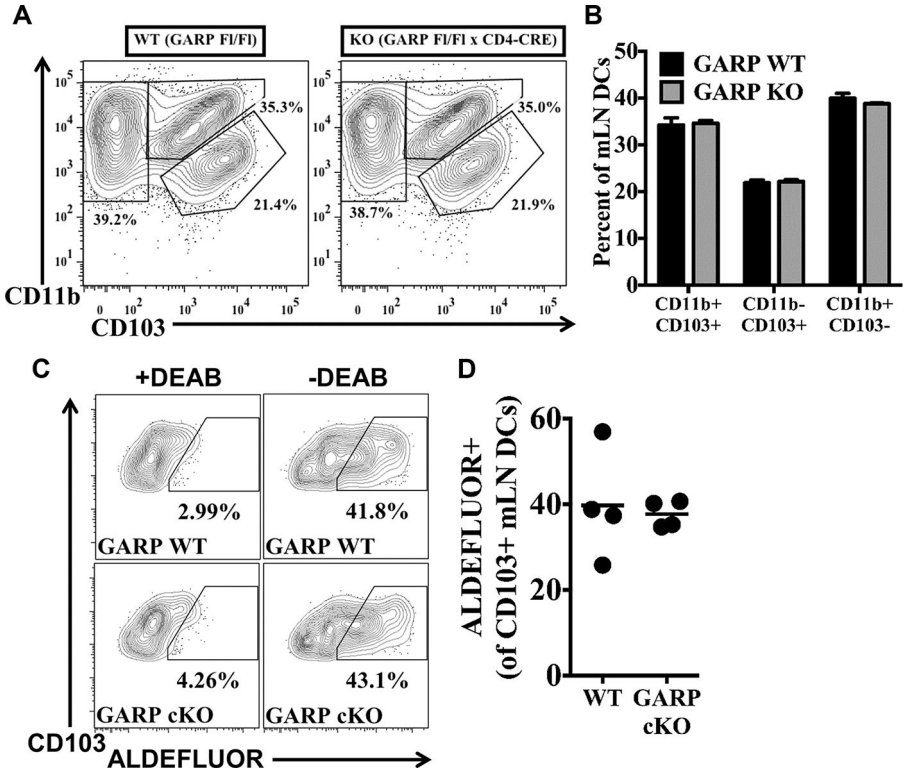

Figure 3.

Deletion of GARP does not alter DC phenotypes or production of retinoic acid. (A and B) Total MLN DC populations (Thy1.2^{-}B220-, CD11c^{hi}MHC-II^{hi}, as indicated in Supporting Information Fig. 1B) from WT and GARP cKO animals (*Irrc32*^{flfFl} ± CD4-CRE). (A) Representative contour plots demonstratingtheproportionofCD11b-and CD103-expressing DCs and (B) a graphical representation of two animals shown as the means ± SD. These results are representative of two independent experiments. (C and D) ALDEFLUOR (aldehyde dehydrogenase) assay. Gated on CD103^{+} DC (Thy1.2^{-}CD19^{-}MHC-II^{+}CD11c^{+}CD103^{+}) from GARP WT or cKO animals. Samples were derived from the MLN. Some samples were incubated with the aldehyde dehydrogenase inhibitor DEAB (serves as negative control). Values represent the proportion ALDEFLUOR^{+} CD103^{+} DC after 30 min incubation at 37° with the reagent. (D) Graphical representation of the proportion of ALDEFLUOR^{+} CD103^{+} in (C) from several different animals from a single experiment. Bars represent the means. Results are representative of two independent experiments.

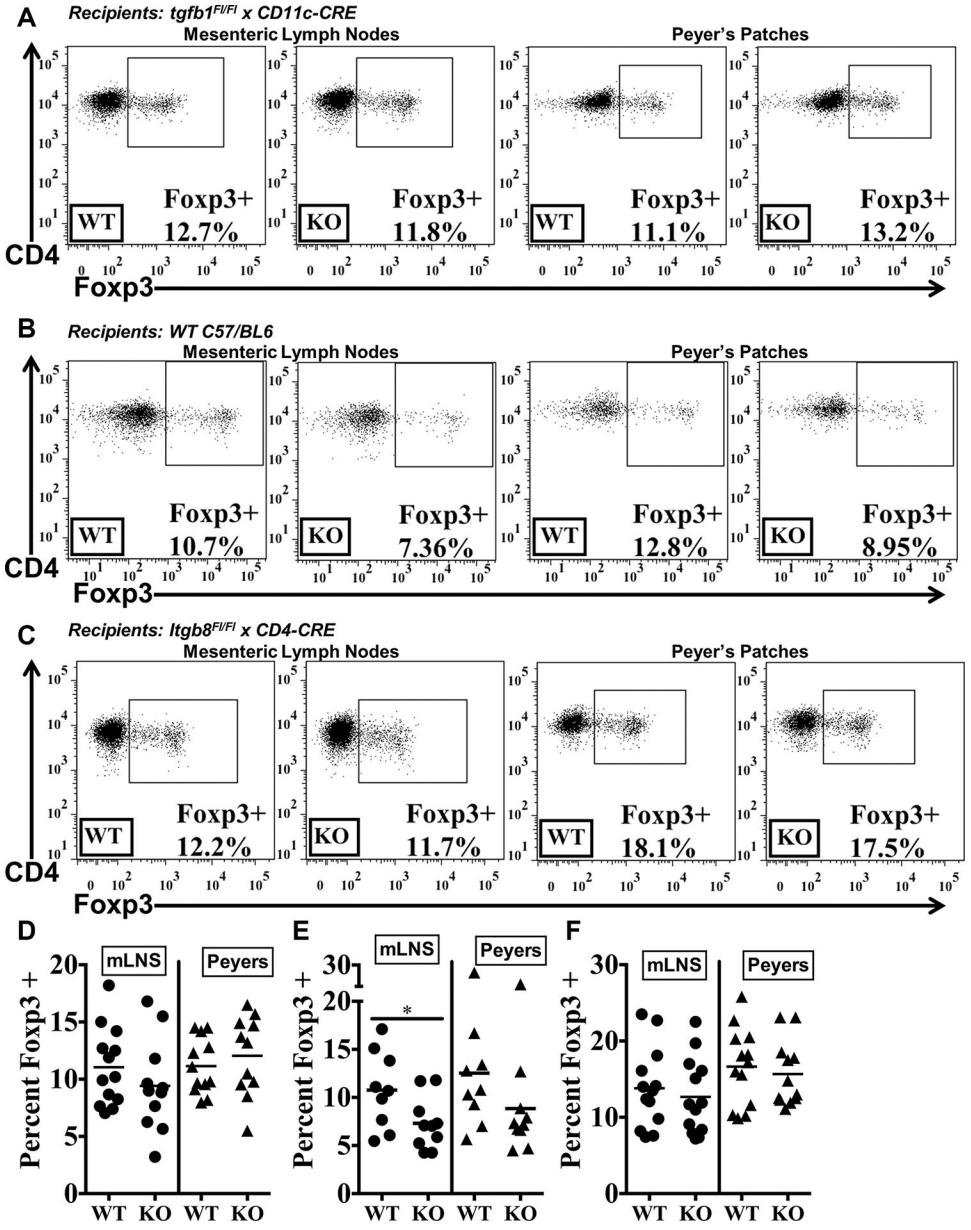

Figure 4.

Establishment of de novo Treg-cell induction during oral tolerance is independent of DC-derived TGF-β1 and T-cell expressed ITGB8, while only modestly affected by OT-II derived TGF-β. (A and C) OT-II ($CD4^+CD45.1^+$ $Rag1^{-/-}$) cells or TGF-β1 WT/cKO OT-II cells ($CD4^+CD45RB^+CD44^{low}Foxp3(GFP)^-$) were transferred on day 0, followed by OVA in the drinking water for days 1–8. Animals were sacrificed on day 8. Recipients were (A) TGF-β1 WT or DC cKO (CD11c-CRE), (B) WT, or (C) ITGB8 WT or cKO (CD4-CRE). Samples were obtained from the MLN (left panels) or the Peyer's patches (right panels). (D and E) Pooled results from (A–C), representing at least three independent experiments plotting the results from individual animals. Bars represent means. *$p < 0.05$, as determined by *t*-test.

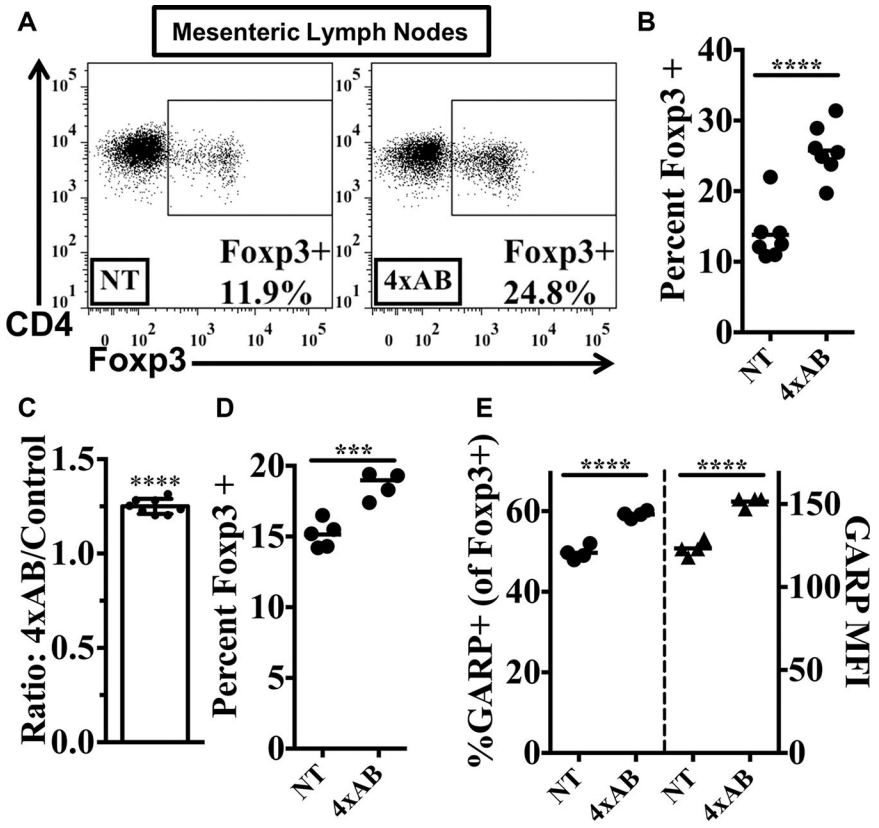

Figure 5.

Transient depletion of the normal bacterial flora enhances de novo Treg-cell induction during oral tolerance. Animals were given multiple antibiotic water (4xAB) then switched to OVA water as detailed in experimental procedures. OT-II ($CD4^+CD45.1^+$ $Rag1^{-/-}$) cells were transferred on day 0. (A) Representative dot plot from antibiotic-treated animals (4xAB) or control animals (NT) gated on transferred OT-II cells ($CD4^+TCR\beta^+CD45.1^+CD45.2^-$), as indicated in Supporting Information Figure 1A. (B) Plot of the results from individual animals from a single experiment, representative of four independent experiments. (C) Ratio of the percent $Foxp3^+$ T cells from 4xAB pretreated animals to control treated animals 7 days after removal of the antibiotics (recipient animals from (A)). For (C), each dot represents the pooled result of each experiment of eight. Statistics are from a ratio paired *t*-test. (D) Representative Foxp3 expression by recipient $CD4^+$ cells ($CD4^+TCR\beta^+CD45.1^-CD45.2^+$) from one experiment from (C). (E) Percent $GARP^+$ (left) and GARP MFI (right) of $CD4^+Foxp3^+$ cells from day 6 MLN (D). (A–E) All samples were derived from the MLN. $^{***}p < 0.001$, $^{****}p < 0.0001$, as determined by *t*-test.