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Cutting Edge: In the Absence of TGF-β Signaling in T Cells, Fewer CD103+ Regulatory T Cells Develop, but Exuberant IFN-γ Production Renders Mice More Susceptible to Helminth Infection

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Multiple factors control susceptibility of C57BL/6 mice to infection with the helminth *Heligmosomoides polygyrus*, including TGF-β signaling, which inhibits immunity in vivo. However, mice expressing a T cell-specific dominant-negative TGF-β receptor II (TGF-βRII DN) show dampened Th2 immunity and diminished resistance to infection. Interestingly, *H. polygyrus*-infected TGF-βRII DN mice show greater frequencies of CD4⁺Foxp3⁺Helios⁺ Tregs than infected wild-type mice, but levels of CD103 are greatly reduced on both these cells and on the CD4⁺Foxp3⁺Helios⁺ population. Although Th9 and Th17 levels are comparable between infected TGF-βRII DN and wild-type mice, the former develop exaggerated CD4⁺ and CD8⁺ T cell IFN-γ responses. Increased susceptibility conferred by TGF-βRII DN expression was lost in IFN-γ-deficient mice, although they remained unable to completely clear infection. Hence, overexpression of IFN-γ negatively modulates immunity, and the presence of Helios⁺ Tregs may maintain susceptibility on the C57BL/6 background. The Journal of Immunology, 2012, 189: 1113–1117.

Immunity to gastrointestinal helminth infection is mediated by Th2-dependent mechanisms (1, 2), which are impaired by regulatory T cells (Tregs) (3) and cross-regulated by conventional IFN-γ Th1 effector populations (4). In the case of the murine nematode parasite *Heligmosomoides polygyrus*, immunity is boosted by interference with TGF-β signaling associated with the induction and activation of Foxp3⁺ Tregs (3), a well-established property of this cytokine (5). *H. polygyrus* is a broadly immunomodulatory parasite that can alleviate colitis in the absence of IL-10 (6) but not when T cell responsiveness to TGF-β is abrogated (7). TGF-β also participates in generating IL-9- (8) and IL-17-producing (9) Th subsets in the presence of IL-4 and IL-6, respectively, although in the setting of *H. polygyrus* infection, both IL-9–dependent mast cell responses (10) and Th17 cells (11) have previously been reported to be blocked.

Because TGF-β signaling was found to promote Tregs and prolong *H. polygyrus* infection, it was surprising that mice whose T cells expressed a dominant-negative TGF-β receptor II (TGF-βRII DN) were not more resistant to this parasite (7). Our laboratory not only confirmed this phenotype but also established that TGF-βRII DN mice are in fact more susceptible to infection than wild-type animals. However, TGF-β can actively downregulate multiple cell types (5, 12), and among the conspicuous phenotypes of TGF-βRII DN mice is potentiation of IFN-γ and Th1 responses (7, 13). We therefore investigated, using mice lacking IFN-γ, whether ablation of TGF-β signaling increased susceptibility because of uninhibited Th1 cytokine release.

Our results show that when IFN-γ stimulation is abrogated in the absence of TGF-β signaling in T cells, the increased fecundity of *H. polygyrus* within the host is lost. Infected TGF-βRII DN and wild-type mice show equivalent differentiation of IL-9– or IL-17–producing CD4⁺ T cells and display no differences in mast cell expansion following infection. Our data therefore support the hypothesis that the increased susceptibility of TGF-βRII DN mice to *H. polygyrus* is due to elevated IFN-γ release and not to a loss of Th9 or Th17 effector responses.

In addition, TGF-β plays a central role in the induction and maintenance of Tregs, particularly in the periphery (5, 14). Because of the importance of Tregs in modulating responses to pathogens in general (15), and *H. polygyrus* in particular (3), we also investigated the balance of Treg frequencies and subsets in the presence or absence of TGF-β signaling and the consequent outcome of infection. These studies show that within the Foxp3⁺ Treg population of TGF-βRII DN mice, CD103 expression is low on both Helios⁺ and Helios⁻ cells, but a compensatory increase in Helios⁺ Tregs may account for the continuing susceptibility of mice expressing the mutated receptor.
Materials and Methods

Animals and parasites
C57BL/6, TGF-ßRII DN (T cell-specific TGF-ßRII DN (16)), IFN-γ−/−, and doubly transgenic mice were housed in individually ventilated cages. Both transgenic lines were on a C57BL/6 background. Mice were infected by oral gavage with 200 H. polygyrus bakeri third-stage larvae, obtained from fecal cultures (3); 14 and 28 d later, small intestinal adult worms and fecal pellet eggs were enumerated.

Restimulation, flow cytometry, and cytokine measurements
Mesenteric lymph node cells (MLNC) were stained directly ex vivo (for Foxp3) and Helios measurements) or restimulated with 0.5 μg/ml PMA and 1 μg/ml ionomycin for 3.5 h, with 10 μg/ml brefeldin A included for the final 2.5 h (for intracellular cytokine measurements). Cells were stained with Abs to surface CD4 (RM4-5; BD), CD8α (53-6.7; BioLegend), TCR-ß (H57-597; eBioscience), and CD103 conjugated to biotin (M290; BD Pharmingen), followed by PerCP-cytochrome (BD). Cells were fixed according to the manufacturer’s instructions with Cytofix/Cytoperm (BD) or Fix/Perm (for Foxp3 and Helios staining; eBioscience) and then stained with Abs to intracellular IFN-γ (XMG1.2; BioLegend), IL-9 (RM09A; BioLegend), IL-13 (eBio 13A; eBioscience), IL-17A (TG11-18H10.1; BioLegend), Foxp3 (FJK-16B; eBioscience), and Helios (22F6; BioLegend). Cells were analyzed using FACSCanto or LSRII flow cytometers (BD) and FlowJo software (Tree Star). Serum cytokines were assayed by CBA flex set (BD) with a minimum detection limit of 2.5 pg/ml.

Histology
Transverse sections of jejunum were fixed in 4% formaldehyde and stained with H&E and toluidine blue. Mast cell counts per micrometer of villus crypt were recorded.

Statistical analysis
Statistical tests were applied according to data normality and group numbers. Normally distributed two-way comparisons used unpaired t tests, and multiple comparisons used one-way ANOVA, followed by Tukey’s test. If normality was not achieved, Mann–Whitney (for two-way comparisons) and Kruskal–Wallis tests (for multiple comparisons, followed by Dunn’s test) were used. Data from multiple experiments were pooled only where no statistical differences existed between separate data sets.

Results and Discussion

The C57BL/6 mouse strain has a high level of susceptibility to the gastrointestinal helminth H. polygyrus (17), but immunity can be enhanced by pharmaceutical inhibition of TGF-ß signaling (3). Because TGF-ßRII DN mice have deficient TGF-ß signaling in T cells, they may be expected to be more resistant to H. polygyrus than their wild-type littermates and may lack inducible Tregs to inhibit effector responses against the worm. Surprisingly, however, H. polygyrus shows height-enened fecundity in TGF-ßRII DN mice (Fig. 1A), and mice have similar adult worm burdens to wild-type mice (Fig. 1B), consistent with an earlier report (7). Furthermore, TGF-ßRII DN mice show diminished Th2 cytokine responses, failing to generate a significant population of IL-13+CD4+ T cells in the MLNC (Fig. 1C). Although a degree of IL-4 responsiveness was maintained in TGF-ßRII DN mice (data not shown), we also found that serum IL-5 responses to infection were absent in all but one gene-targeted animal (Fig. 1D). The IL-5 serum response at day 7 of infection in wild-type mice was transient and had returned to naive levels by day 14 of infection (data not shown). No IL-4 or IL-13 was detectable in the sera of either mouse strain. Reduced IL-10 production measured by Ag-specific recall responses to H. polygyrus excretory–secretory (HES) Ags in vitro was also found in TGF-ßRII DN mice (data not shown), consistent with its role in promoting Th2 responsiveness in gastrointestinal helminth infections (2) and with a report that IL-10 release by lamina propria cells is inhibited in H. polygyrus-infected TGF-ßRII DN mice (7). Hence, T cell-specific ablation of TGF-ß signaling does not recapitulate the effects of global pharmaceutical inhibition (3), and the phenotype of the TGF-ßRII DN mice does not equate to the Th2-boosting effects of broader interference with Treg function in nematode infection (18–20).

Because TGF-ß signaling promotes Treg differentiation, particularly in the periphery, we next examined Treg fre-
frequencies in H. polygyrus-infected TGF-βRII DN mice. Surprisingly, we found a significantly higher proportion of CD4⁺Foxp3⁺ T cells in MLNC of infected TGF-βRII DN mice compared with wild-type C57BL/6 animals (Fig. 2A). The increased frequency of Foxp3⁺ cells was accounted for by a greater proportion of CD4⁺ cells expressing the transcription factor Helios (Fig. 2B), which is associated with thymic or natural Tregs (21), whereas the frequencies of Foxp3⁺Helios⁻ T cells (considered to be peripherally induced Tregs, known to be more dependent on TGF-β signaling) were not significantly different between the two genotypes (data not shown). Although the Treg compartment was not thus numerically diminished in TGF-βRII DN mice, their expression of CD103 [an activation/memory marker known to be inducible by TGF-β (22)] was substantially reduced (Fig. 2C, 2D), with low levels in both Helios⁺ and Helios⁻ subsets (data not shown).

We next addressed the question of whether a loss of TGF-β signaling impacts on other effector functions in the immune response. Because TGF-β signaling promotes differentiation of Th17 cells in the presence of IL-6 (9), and Th9 in the presence of IL-4 (8), we investigated the generation of these cell types following infection. Few Th17 cells identified by intracellular IL-17A staining develop in the MLNC in either genotype (Fig. 3A), suggesting that the conditions for optimal Th17 expansion are not generated at this site during H. polygyrus infection.

The frequency of CD4⁺ T cells producing IL-9 was, however, altered in TGF-βRII DN mice, with a significantly greater, rather than lower, proportion of IL-9⁺ T cells compared with wild-type mice (Fig. 3B). IL-9 is important for mast cell survival and proliferation (23), and mast cells have been suggested as an effector population for H. polygyrus expulsion (10, 24, 25). We therefore quantified the extent of jejunal mast cells but found their numbers increased significantly and equivalently after H. polygyrus infection in both C57BL/6 and TGF-βRII DN mice (Fig. 3C).

By day 14 postinfection, effector responses in wild-type mice are predominantly Th2 type (26). TGF-βRII DN mice, however, display strong Th1 IFN-γ production (Fig. 3). Notably, a high proportion of splenic CD4⁺ and CD8⁺ T cells develop into IFN-γ⁺ cells at this site during H. polygyrus infection in both C57BL/6 and TGF-βRII DN mice (Fig. 3D).

**FIGURE 3.** TGF-βRII DN mice have exaggerated IFN-γ responses yet Th17, Th9, and mast cell responses are not compromised. (A, B, E, F) MLNC were isolated from 14-d H. polygyrus-infected C57BL/6 and TGF-βRII DN mice, stimulated with PMA/ionomycin, and stained for flow cytometry. (A and B) Data are pooled from three experiments, with 6- to 18-wk-old mice age matched between groups. Percentage of IL-17A⁺ cells (A) or percentage of IL-9⁺ cells (B) of CD4⁺ lymphocytes. (C) Jejunums were sectioned and stained for mast cells with toluidine blue. The number of mast cells per micrometer of villus crypt is shown in naïve (C) and 14-d H. polygyrus infected (D) mice. Data are shown from 7- to 9-wk-old mice and are representative of two experiments each with four to five mice per group for infected mice; naïve mice were included in one of these experiments. (D) Levels of circulating IFN-γ in the sera of naïve (C) or 7-d H. polygyrus-infected C57BL/6 and TGF-βRII DN mice (E). Data are pooled from two experiments with 7- to 12-wk-old mice. (E and F) Percentage of IFN-γ⁺ cells among CD4⁺ (E) or CD8α⁺ (F) TCR-β⁺ lymphocytes. Data shown are from 7- to 9-wk-old mice and are representative of four experiments each with two to five mice per group. (A) was analyzed by unpaired t test; (B) and (D) by Mann–Whitney; (C) by Kruskal–Wallis; and (E) and (F) by unpaired t test between H. polygyrus-infected groups. Abbreviations used as in Fig. 1.

* *p < 0.05, **p < 0.001.

**FIGURE 4.** Increased susceptibility of TGF-βRII DN mice is reversed in the absence of IFN-γ. (A) Fecal egg counts after 14 d of infection in C57BL/6, TGF-βRII DN, TGF-βRII DN IFN-γ⁻/⁻, and IFN-γ⁻/⁻ mice. Data are pooled from three experiments with 6- to 14-wk-old mice age matched between groups. (B) Adult worm counts from the same experiments after 28 d of infection. (C and D) Levels of circulating IFN-γ (C) and IL-5 (D) in the same experiments after 7 d of infection. Data are pooled from two experiments with 7- to 14-wk-old mice. (E) Percentage of Foxp3⁺ T cells among total CD4⁺ lymphocytes. After 28 d of infection, MLNC were stained directly ex vivo. Data are pooled from two experiments with 7- to 14-wk-old mice. (F) Percentage of CD103 among Foxp3⁺CD4⁺ lymphocytes, in the same experiments as (E). (A), (B), (E), and (F) were analyzed by ANOVA; (C) and (D) by Kruskal–Wallis test. B6, C57BL/6; DN, TGF-βRII DN; DN IFN-γ⁻/⁻. TGF-βRII DN IFN-γ⁻/⁻; γ⁻/⁻; IFN-γ⁻/⁻; H. p., H. polygyrus infected.

* *p < 0.05, **p < 0.01, ***p < 0.001.
CD103 is important for effector T cell migration and retention and therefore do not correspond to the susceptibility of the mouse showing elevated serum levels of IFN-γ.

To investigate whether the substantial IFN-γ in TGF-βRII DN mice inhibits Th2 cytokines required to control H. polygyrus, we bred double-transgenic mice with the TGF-βRII DN mutation together with the IFN-γ−/− genotype on the C57BL/6 background (TGF-βRII DN IFN-γ−/− mice).

After 14 d of infection, TGF-βRII DN mice lacking IFN-γ had a lower fecal egg burden than IFN-γ-sufficient TGF-βRII DN mice (Fig. 4A) and, by day 28, lower worm counts (Fig. 4B). However, the double-transgenic mice were not able to fully clear infection. Thus, although overexpression of IFN-γ is responsible for the heightened susceptibility of TGF-βRII DN mice, IFN-γ itself is not solely responsible for the failure of mice to expel the parasite. In this manner, control of H. polygyrus appears to be more complex than Trichus muris, in which neutralization of IFN-γ is sufficient to convert a susceptible genotype to a resistant phenotype (4). Hence, the reported greater susceptibility of TGF-βRII DN mice to T. muris may be due to high intrinsic IFN-γ in this model rather than lack of Th9-driven mast cell responses (8).

Serum cytokine analysis confirmed the absence of IFN-γ in gene-targeted mice (Fig. 4C) and showed that serum IL-5 levels were restored partially (Fig. 4D). However, intracellular staining of MLNC showed broadly similar levels of Th2 cytokine production by day 28 of infection in C57BL/6 and double-transgenic genotypes (data not shown), indicating that the suppression of Th2 responses is only partly relieved in the absence of both IFN-γ and TGF-β signaling in T cells. HES-specific IgG1 responses were equivalent in all strains at day 28 of infection.

Analysis of Treg populations showed that the proportion of Foxp3+CD4+ T cells are increased in TGF-βRII DN mice, irrespective of their IFN-γ status (Fig. 4E). Moreover, CD103 expression is reduced on both Helios+ and Helios− Foxp3+CD4+ T cells in both IFN-γ−/− and −/− sufficient TGF-βRII DN mice (Fig. 4F), confirming that CD103 expression is regulated by TGF-β signaling (27). Levels of CD103 therefore do not correspond to the susceptibility of the mouse strain, suggesting that CD103 is not required for functional suppression of the antihelminthic response. However, because CD103 is important for effector T cell migration and retention in the gut (28), and because the TGF-βRII DN Foxp3− effector population also fails to express high CD103 levels (Fig. 2C, data not shown), the susceptibility of this genotype could reflect a diminished presence of effector cells at the site of infection.

Overall, these data argue that neither Th1 nor TGF-β–induced adaptive Tregs are essential for repression of the protective Th2 response to H. polygyrus. Several interesting alternatives can now be considered. First, the greater expansion of natural Tregs in TGF-βRII DN mice may account for their continued susceptibility. The outgrowth of natural or Helios− Tregs in vivo may result from a homeostatic compensation for the paucity of CD103+ adaptive Tregs (modulated by cytokines such as IL-2) and/or outgrowth to control a greater mucosal inflammatory response in the absence of TGF-β–inducible CD103+ adaptive Tregs (29). Second, although Th2 effectors would be inured from TGF-β–mediated inhibition (30), Tregs operate through other suppressive pathways including coinhibitors such as CTLA-4 and programmed cell death-1 known to be important in other helminth systems (18). Thirdly, many other regulatory subsets are known to arise in H. polygyrus infection including DCs, macrophages and regulatory B cells (reviewed in Ref. 2), which may account for the susceptibility of these mice. Finally, it should noted that significant nonlymphoid populations are responsive to TGF-β, and the efficacy of global TGF-β inhibition (3) and the TGF-β–dependent effects in H. polygyrus-infected RAG-deficient hosts (6), implies that there are critical non-T cell targets of this suppressive cytokine.

In conclusion, although inducible Tregs control mucosal inflammation (29), our data support the idea that control of protective immunity in the intestinal setting may be regulated by natural and not inducible Tregs. This scenario has been suggested by recent studies of the IL-6−/− deficient BALB/c mouse, which are highly resistant to H. polygyrus infection (K.A. Smith and R.M. Maizels, submitted for publication). This intriguing and unexpected division of labor between Treg subsets remains to be further explored.

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References


