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Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis

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Abstract

Regulatory T cells (T_{reg} cells) that express the transcription factor Foxp3 suppress the activity of other cells. Here we show that interleukin 10 (IL-10) produced by CD11b$^+$ myeloid cells in recombination-activating gene 1–deficient (*Rag1*$^{-/-}$) recipient mice was needed to prevent the colitis induced by transferred CD4$^+$ CD45RB^{hi} T cells. In *Il10* $^{-/-}$ *Rag1*$^{-/-}$ mice, T_{reg} cells failed to maintain Foxp3 expression and regulatory activity. The loss of Foxp3 expression occurred only in recipients with colitis, which indicates that the requirement for IL-10 is manifested in the presence of inflammation. IL-10 receptor–deficient (*Il10rb*$^{-/-}$) T_{reg} cells also failed to maintain Foxp3 expression, which suggested that host IL-10 acted directly on the T_{reg} cells. Our data indicate that IL-10 released from myeloid cells acts in a paracrine manner on T_{reg} cells to maintain Foxp3 expression.

CD4$^+$ regulatory T cells (T_{reg} cells) express the transcription factor Foxp3 (A002750), which is required for their suppressive function. A T cell–transfer model of colitis has been widely used to study the function of T_{reg} cells *in vivo*. When CD4$^+$CD45RB^{hi} T cells are transferred into immunodeficient mice, some of the transferred T cells secrete proinflammatory cytokines and induce an inflammatory bowel disease–like syndrome[1,2]. Cotransfer of sufficient numbers of T_{reg} cells can prevent or even cure this disease[3,4]. The transferred T_{reg} cell populations expand considerably *in vivo*, and most maintain Foxp3 expression[5,6].

Mice deficient in interleukin 10 (IL-10 (A001243); *Il10*$^{-/-}$ mice) or the IL-10 receptor β-chain (IL-10Rβ (A001245); *Il10rb*$^{-/-}$ mice) develop spontaneous inflammation of the large intestine, a process dominated by a T helper type 1 immune response[7,8]. Many cell types

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AUTHOR CONTRIBUTIONS
M.M. and M.K. designed experiments; M.M. did experiments; O.T. did histology and helped with cell preparation; R.M. and C.L.K. generated and provided IL-10 reporter mice; G.K. and H.C. helped with critical advice and discussions throughout; and M.M. and M.K. wrote the manuscript.

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can produce IL-10, however, and therefore the IL-10 source(s) needed to prevent inflammation must be identified. Much emphasis has been placed on the role of IL-10 released by $CD4^+$ T cells, and in fact mice with conditional deletion of IL-10 in the $CD4^+$ subset develop spontaneous inflammation of the intestine[9]. Mice with deletion of IL-10 solely in $Foxp3^+$ cells also develop inflammation in the intestine and elsewhere, although the pathogenesis is less intense than that in mice completely lacking IL-10 (ref. 10). Transgenic mice that overexpress IL-10 in intestinal epithelial cells are protected from colitis11, which suggests that IL-10 from nonlymphoid sources can be beneficial, although altered expression in the transgenic mice may not be physiologically relevant.

To further elucidate the cellular and molecular basis of the function of IL-10 in regulating colitis, we used the T cell–transfer model described above. We found that IL-10 from nonlymphoid cells, particularly $CD11b^+CD11c^+$ cells, had an unexpectedly important influence on the development of colitis. Furthermore, we provide evidence that this IL-10 acted in part on T_{reg} cells to maintain their expression of Foxp3, which was otherwise lost in inflammatory conditions after transfer.

RESULTS

Colitis prevention by *Il10*$^{-/-}$ T_{reg} cells

Several studies have suggested that IL-10 production by T_{reg} cells is required for the prevention of colitis[10,12,13]. However, some studies suggest that T_{reg} cell–derived IL-10 is less important than other studies do[14,15]. To assess the suppressive function of *Il10*$^{-/-}$$CD4^+CD25^+$ T_{reg} cells, we did both *in vitro* and *in vivo* experiments. *Il10*$^{-/-}$ $CD4^+CD25^+$ T_{reg} cells were as capable as wild-type cells of inhibiting the proliferation of $CD4^+CD45RB^{hi}$ T cells *in vitro* (data not shown). For *in vivo* analysis, we transferred sorted $CD4^+CD45RB^{hi}$ T cells together with sorted $CD4^+CD45RB^{lo}CD25^+$ T_{reg} cells from wild-type or *Il10*$^{-/-}$ mice into recombination-activation gene 1–deficient (*Rag1*$^{-/-}$) mice. As a positive control for colitis induction, we injected $CD4^+CD45RB^{hi}$ T cells without T_{reg} cells. Host mice given either wild-type or *Il10*$^{-/-}$ T_{reg} cells remained healthy without losing body weight (Fig. 1a) and survived for more than 4 months after transfer (data not shown). The average histology scores of recipients of wild-type or *Il10*$^{-/-}$ T_{reg} cells were similar and were indicative of a low degree of inflammation compared with that of mice that did not receive T_{reg} cells (Fig. 1b). These findings show that IL-10 derived from T_{reg} cells was dispensable for the prevention of colitis in our mice.

Host IL-10 is required for colitis suppression

The negative results of the analysis of T_{reg} cell–derived IL-10 raised the issue of the possible involvement of host-derived IL-10 in the inhibition of colonic inflammation. To address this possibility, we used *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice as recipients in transfer experiments. These mice did not spontaneously develop colitis (data not shown); however, both *Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients of $CD4^+CD45RB^{hi}$ T cells had lost approximately 20% of their initial weight at 8 weeks after donor cell injection (Supplementary Fig. 1a). The severity of colitis in *Rag1*$^{-/-}$ mice was similar to that in the *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts (Supplementary Fig. 1b), which suggested that host-derived IL-10 did not have a major effect on the colitis pathogenesis induced by the transferred T cell population.

Next we determined if host-derived IL-10 was essential for T_{reg} cell–mediated prevention of colitis. To more precisely define the transferred T_{reg} cell population, we used reporter mice with sequence encoding green fluorescent protein (GFP) inserted inframe into the *Foxp3* gene (*Foxp3*^{gfp})[16]. We selected the T_{reg} cell population from these mice on the basis of green fluorescence, as well as expression of CD25, CD4 and small amounts of CD45RB.

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The selected cells were up to 99% Foxp3$^+$ (Supplementary Fig. 2). We transferred CD45.1$^+$ CD4$^+$CD45RB^{hi} T cells together with CD45.2$^+$ *Foxp3*^{gfp} T$_{reg}$ cells into either *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts so that the two donor T cell populations could be distinguished on the basis of their CD45 alleles. As expected, *Rag1*$^{-/-}$ hosts that received CD4$^+$CD45RB^{hi} T lymphocytes and T$_{reg}$ cells did not lose weight (Fig. 2a). Unexpectedly, at 5 weeks after transfer, *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts injected with CD4$^+$CD45RB^{hi} T lymphocytes and T$_{reg}$ cells showed a weight loss of nearly 20% (Fig. 2a), similar to that induced by transfer of CD4$^+$CD45RB^{hi} T cells alone. Furthermore, *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients developed colonic inflammation characterized by the infiltration of mononuclear cells, loss of goblet cells and epithelial cell hyperplasia in both the distal colon (data not shown) and proximal colon (Fig. 2b,c). We obtained similar results with T$_{reg}$ cells isolated from wild-type mice and sorted on the basis of CD25, CD4 and CD45RB^{lo} expression alone (data not shown), which indicated that the results presented above were not related to the insertion of the gene encoding GFP into the *Foxp3* locus. These findings suggest that host-derived IL-10 is required for T$_{reg}$ cell–mediated prevention of colitis.

Lower Foxp3 expression in *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients

To determine if the T$_{reg}$ cells underwent population expansion and homed to different tissues in the absence of host IL-10, we collected lymphocytes from the lamina propria of the large intestine (LPL), spleen, peripheral lymph nodes (PLNs; inguinal and axillary) and mesenteric lymph nodes (MLNs); we separately analyzed by flow cytometry the CD45.1$^+$ progeny of CD4$^+$CD45RB^{hi} T cells and the CD45.2$^+$ progeny of T$_{reg}$ cells sorted from *Foxp3*^{gfp} mice. By 6 weeks after donor cell injection, the CD45.1$^+$ cell/CD45.2$^+$ cell ratios in *Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts were maintained at approximately the starting ratio of 4:1 in each of the organs analyzed (Fig. 3a). This suggested that the population expansion of *Foxp3*^{gfp} T$_{reg}$ cells *in vivo* was similar to that of the CD4$^+$CD45RB^{hi} T cells in *Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts.

Unexpectedly, however, T$_{reg}$ cells in *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts showed profound downregulation of the expression of Foxp3 protein (Fig. 3b,c) and Foxp3 mRNA (data not shown). In contrast, T$_{reg}$ cells transferred into *Rag1*$^{-/-}$ hosts maintained Foxp3 expression, although the percentage of Foxp3-expressing progeny of sorted T$_{reg}$ cells in the lamina propria was less than that in the lymphoid organs (Fig. 3b,c). The T$_{reg}$ cells obtained from *Il10*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ hosts expressed similar amounts of the immunomodulatory receptor GITR and cytokine receptor CD25 (data not shown). We detected some loss of Foxp3 expression, especially in LPL, as early as 2–3 weeks after transfer (Supplementary Fig. 3), the earliest times at which transferred T$_{reg}$ cells were readily detectable.

To determine if T$_{reg}$ cells from *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts had altered function, we assessed cytokine production. At 6 weeks after donor cell injection, CD4$^+$CD45RB^{hi} (CD45.1$^+$) T cells obtained from *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts produced interferon-γ (IFN-γ) when stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Fig. 4a). In addition, some of these cells produced IL-17 (Supplementary Fig. 4) and tumor necrosis factor (data not shown). In contrast, CD4$^+$CD45RB^{hi} T cells obtained from *Rag*$^{-/-}$ hosts did not produce IFN-γ or other proinflammatory cytokines after restimulation (Fig. 4a), which probably reflected the suppressive activity of the cotransferred T$_{reg}$ cells.

Notably, CD45.2$^+$ progeny of T$_{reg}$ cells obtained from all organs of *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts also produced IFN-γ after restimulation, although this was less evident in the spleen than in the mucosal immune system (Fig. 4b). These cells, however, did not produce IL-17 (Supplementary Fig. 4) or tumor necrosis factor (data not shown), which distinguishes their cytokine profile from that of the CD45.1$^+$ CD4$^+$CD45RB^{hi} T cells obtained from the same recipients. In agreement with their ability to produce IFN-γ, the CD45.2$^+$ progeny of T$_{reg}$

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cells transferred into *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients also expressed the transcription factor T-box 21 (also called T-bet), as detected by flow cytometry and RT-PCR (data not shown). Consistent with their maintenance of Foxp3 expression, the progeny of sorted T_{reg} cells obtained from *Rag1*$^{-/-}$ hosts did not produce IFN-γ (Fig. 4b), IL-17 or tumor necrosis factor and they did not express T-bet (data not shown).

The distinct cytokine profile of the cells that previously expressed Foxp3, compared with that of their $CD4^{+}CD45RB^{hi}$ counterparts, suggested that these cells were not derived from the progeny of a small number of contaminating $CD4^{+}CD45RB^{hi}$ To determine if contaminating $CD4^{+}$ T lymphocytes with an activated memory phenotype could have outgrown the transferred T_{reg} cells in IL-10-deficient conditions, we transferred $CD90.1^{+}$ $CD4^{+}CD45RB^{hi}$ cells together with $CD45.2^{+}$ T_{reg} cells and $CD45.1^{+}$ $CD44^{hi}CD62L^{-}Foxp3^{-}$ cells with an activated memory phenotype into *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts. The number of memory cells transferred was 3% the number of T_{reg} cells, a degree of impurity greater than that in the transferred T_{reg} cell populations that were enriched by flow cytometry. These memory cells did not outgrow the transferred T_{reg} cells in MLNs, perhaps outgrew them only slightly in spleen and were under-represented in lamina propria when analyzed 2 weeks after transfer (Supplementary Fig. 5). At this time, however, up to 50% of the original transferred T_{reg} cell population had lost Foxp3 expression.

To further define the function of the transferred T_{reg} cells, we assessed their suppressive activity *in vitro*. We sorted T_{reg} cells on the basis of CD45.2 expression from the MLNs of recipients at 6 weeks after donor cell injection. We cultured T_{reg} cells obtained from *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients with carboxyfluorescein succinimidyl ester (CFSE)-labeled splenic $CD45.1^{+}CD4^{+}CD25^{-}$ T cells obtained from wild-type mice. Whereas T_{reg} cells recovered from *Rag1*$^{-/-}$ hosts suppressed the proliferation of the CFSE-labeled naive $CD4^{+}$ T cells, T_{reg} cells isolated from *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients failed to suppress the proliferation of naive T cells (Fig. 4c). Collectively these findings show that IL-10 produced by cells in *Rag1*$^{-/-}$ mice is required for the maintenance of Foxp3 expression and the suppressive activity of transferred T_{reg} cells.

Il10rb$^{-/-}$ T_{reg} cells do not prevent colitis

IL-10 produced by cells in the *Rag1*$^{-/-}$ hosts may act directly on the transferred T_{reg} cells or may act on some other cell type, such as dendritic cells, which might be involved in stabilizing Foxp3 expression. To test for a direct effect of IL-10 on transferred T_{reg} cells, we compared the ability of T_{reg} cells from wild-type and *Il10rb*$^{-/-}$ mice to suppress colitis in *Rag1*$^{-/-}$ hosts, with *Foxp3*^{gfp} mice crossed to *Il10rb*$^{-/-}$ mice as a source of 'marked' T_{reg} cells. The percentage of *Foxp3*^{gfp} T_{reg} cells in 6- to 7-week-old *Il10rb*$^{-/-}$ mice, which were generally healthy, was similar to that in wild-type mice (Supplementary Fig. 6a). Furthermore, *Foxp3*^{gfp} T cells from *Il10rb*$^{-/-}$ mice were as capable as wild-type cells of inhibiting the proliferation of $CD4^{+}CD45RB^{hi}$ T cells *in vitro* (Supplementary Fig. 6b). However, *Rag1*$^{-/-}$ recipients of $CD4^{+}CD45RB^{hi}$ T cells and *Il10rb*$^{-/-}$ T cells had only approximately 20% of their initial starting weight by 4 weeks after donor cell injection (Fig. 5a). Moreover, these mice had severe colonic inflammation in both the distal colon (data not shown) and proximal colon (Fig. 5b,c). Similar to the outcome produced by transfer of wild-type T_{reg} cells into *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts, transferred *Il10rb*$^{-/-}$ T cells showed profound downregulation of Foxp3 expression (Fig. 5d,e). These findings suggest that IL-10 signaling is required, at least in part in a T_{reg} cell–intrinsic manner, for the maintenance of Foxp3 expression and suppressive function.

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Loss of Foxp3 expression requires inflammation

It may be problematic to compare Foxp3 expression in T_{reg} cells in *Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients, because only the latter recipients developed intestinal inflammation. It is possible that without IL-10 signaling, transferred T_{reg} cells are only marginally less effective, but that inflammation may amplify the loss of Foxp3 expression and T_{reg} cell function. Therefore, we did cotransfer experiments in which we injected equal numbers of allelically distinguishable wild-type (CD45.1^{+}) and *Il10rb*$^{-/-}$ (CD45.2^{+}) T_{reg} cells together with CD4^{+}CD45RB^{hi} (CD90.1^{+}) T cells into *Rag1*$^{-/-}$ hosts and monitored each subset over time (Supplementary Fig. 7a). At a ratio of 20:1 (CD4^{+}CD45RB^{hi} T cells/T_{reg} cells), as anticipated, all mice that received either type of T_{reg} cells or the mixture developed colitis (Supplementary Fig. 7b,c). At 6 weeks after transfer, wild-type and *Il10rb*$^{-/-}$ T_{reg} cells recovered from these mice were present at approximately the starting ratio of 1:1 (Fig. 6a). Notably, however, *Il10rb*$^{-/-}$ T_{reg} cells tended to lose Foxp3 expression more than the wild-type T_{reg} cells in the same hosts did (Fig. 6b,c). Also, there was a somewhat greater tendency for the wild-type T_{reg} cells to lose Foxp3 expression in these recipient mice than in recipient mice in which sufficient numbers of T_{reg} cells were present to prevent inflammation (Figs. 3b and 5d). These results show that inflammation itself was not the sole factor that caused the loss of Foxp3 expression, although it may have contributed to this. Furthermore, our results suggest that there is a cell-intrinsic effect of the interaction of IL-10 with its receptor in the maintenance of Foxp3 expression in inflammatory conditions.

We also investigated whether the lack of IL-10 signaling in T_{reg} cells caused the loss of Foxp3 expression when inflammation was not present. We transferred wild-type T_{reg} cells and *Il10rb*$^{-/-}$ T_{reg} cells (at a ratio of 1:1), along with CD4^{+}CD45RB^{hi} T cells, similar to the experiments described above but at a final ratio of 2:1 for CD4^{+}CD45RB^{hi} T cells/T_{reg} cells. In this case, the mice were completely protected from colitis (data not shown). In the absence of inflammation, the loss of Foxp3 expression by the transferred *Il10rb*$^{-/-}$ T_{reg} cells was blunted, and the maintenance of Foxp3 expression by cells in this population was similar to that of wild-type T_{reg} cells (Fig. 6d). In addition, when we transferred T_{reg} cells in the absence of CD4^{+}CD45RB^{hi} T cells, *Il10rb*$^{-/-}$ T_{reg} cells tended to not lose Foxp3 expression any more than wild-type T_{reg} cells did (data not shown). Collectively these data suggest that the loss of Foxp3 expression could be attributed to both the absence of IL-10 signaling in the T_{reg} cells and the presence of inflammatory signals and colitis in the hosts but that neither factor alone caused substantial loss of Foxp3. Therefore, unlike transforming growth factor-β, which is important for the homeostasis of natural T_{reg} cells[17–22], IL-10 is important for maintaining Foxp3 expression once the T_{reg} cells are differentiated and exposed to inflammation.

Myeloid cells in the mucosa produce IL-10

We next evaluated which cell types produced IL-10 in the *Rag1*$^{-/-}$ recipients. We used IL-10 reporter mice in which an internal ribosome entry site upstream and the gene encoding GFP were inserted in *Il10* (*Il10*^{gfp}; 'Vert-X' mice). There was good concordance between GFP fluorescence and IL-10 production when we activated T lymphocytes from these mice *in vivo* (Supplementary Fig. 8). We used cells from these mice as a source of CD4^{+}CD45RB^{hi} T lymphocytes and T_{reg} cells and also crossed these mice with *Rag1*$^{-/-}$ mice to track IL-10-producing nonlymphoid cells in the recipient mice.

As expected, before transfer, some CD4^{+}CD45RB^{lo}CD25^{+} T_{reg} cells from the spleen were GFP^{+} (an average of 4.0% ± 1.2%; Fig. 7a), as were some T_{reg} cells in MLNs (data not shown). In the CD4^{+}CD45RB^{hi} T cell population, in contrast, fewer cells expressed GFP (Fig. 7a). When we isolated lymphocytes from wild-type mice, stimulated them with PMA

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and ionomycin and analyzed IL-10 protein by intracellular cytokine staining, we found that IL-10$^+$ cells were confined to the T_{reg} cell population and constituted a minority of this group (data not shown).

Analysis of nonlymphoid cells in the *Rag1*$^{-/-}$ recipients indicated that before T cell transfer, 3.7% ± 0.9% of CD45$^+$ cells from LPL of *Il10*^{gfp}*Rag1*$^{-/-}$ mice expressed GFP (Fig. 7a); we detected a lower percentage of GFP$^+$CD45$^+$ cells in the MLNs and spleen, and GFP$^+$ cells were essentially undetectable in the CD45$^-$ population in LPL and MLNs (Fig. 7a). We also analyzed GFP expression in *Il10*^{gfp}*Rag1*$^{-/-}$ recipients 1 week after cotransfer of CD45.1$^+$ CD4$^+$CD45RB^{hi} T cells and CD45.2$^+$ *Il10*^{gfp} T_{reg} cells. We noted a much higher percentage of nonlymphoid cells in MLNs that were GFP$^+$ (an average of 7.8% ± 1.8% of the CD45$^+$ cells; Fig. 7b). Most of these GFP$^+$ cells (82.1% ± 5.2%) were CD11b$^+$, 62.1% ± 9.6% expressed both CD11b and CD11c, and most expressed the macrophage marker F4/80 (Supplementary Fig. 9). In contrast to cells in the MLNs, in the LPL, the percentage of GFP$^+$CD45$^+$ nonlymphoid cells was slightly lower by 1 week after transfer (an average of 2.7% ± 0.8%; Fig. 7a,b). The source of the greater number of GFP$^+$CD11b$^+$ cells in MLN after transfer could have been due in part to migration from the LPL, although we cannot exclude the possibility of cell division or recruitment from other sources. At 1 week after transfer, in the LPL there were very few GFP$^+$ cells in the CD45$^-$ population, and there was no higher GFP expression by gated CD45.2$^+$ *Il10*^{gfp} T_{reg} cells (an average of 3.0% ± 0.6%) than by the donor population of splenic T_{reg} cells (Fig. 7a,b).

To further define the kinetics of IL-10 production, we analyzed *Il10* mRNA by real-time PCR. Before donor cell injection, T_{reg} cells contained 40 times more *Il10* transcripts than did CD4$^+$CD45RB^{hi} T cells (Fig. 7c). These data are consistent with the greater percentage of GFP$^+$ cells in T_{reg} cells in the IL-10 reporter mice (Fig. 7a). Before donor cell injection, CD11c$^+$CD11b$^+$ cells of the LPL contained almost four times more *Il10* transcripts than did T_{reg} cells (Fig. 7c), whereas CD11c$^+$CD11b$^+$ cells of the MLNs had less *Il10* mRNA than did either T_{reg} cells or their counterparts in the LPL (Fig. 7c). At 1 week after injection of CD45.1$^+$ CD4$^+$CD45RB^{hi} T cells and CD45.2$^+$ *Il10*^{gfp} T_{reg} cells, however, there were substantial changes in the expression of *Il10* mRNA in the nonlymphoid cell populations in the intestine. *Il10* transcripts in the CD11c$^+$CD11b$^+$F4/80$^+$ cells from MLNs were 6.6-fold more abundant, whereas *Il10* mRNA in the same population from the LPL was slightly less abundant (Fig. 7c). These alterations in mRNA are in agreement with the changes in GFP$^+$ cells in the IL-10 reporter mice (Fig. 7b). The greater abundance of *Il10* mRNA in CD11c$^+$CD11b$^+$F4/80$^+$ cells in MLNs was transient, however, and peaked at 1 week after donor cell injection. *Il10* mRNA in T_{reg} cells increased much later, and the increase occurred selectively in the LPL (Fig. 7c). Those sorted CD11c$^+$ cells that did not express macrophage markers (CD11b and F4/80) and were probably dendritic cells did not induce *Il10* mRNA at any time after T cell transfer (Fig. 7c).

To determine if IL-10 production by myeloid cells was important for the maintenance of Foxp3 expression by T_{reg} cells, we transferred CD11c$^+$CD11b$^+$F4/80$^+$ cells from the LPL of *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice into *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients. When the myeloid cells were derived from *Rag1*$^{-/-}$ donors, the loss of Foxp3 expression by T_{reg} cells was less than that produced by transfer of the same population from *Il10*$^{-/-}$*Rag1*$^{-/-}$ donors (Fig. 8a). Furthermore, this effect was specific to the CD11b$^+$ cells, as transfer of the same number of CD11c$^+$CD11b$^-$F4/80$^-$ cells was not effective in stabilizing Foxp3 expression (Fig. 8b). We conclude that CD11b$^+$ myeloid cells, perhaps mostly macrophages, are key producers of IL-10 in the intestine at early times after T cell transfer and that this IL-10 is necessary for maintenance of Foxp3 expression in T_{reg} cells.

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DISCUSSION

The results from several studies have indicated that T cell–derived IL-10 is important for the prevention of colitis[9,10,12,23]. However, here we did not identify a major role for T_{reg} cell–derived IL-10 in the transfer model of colitis. The reasons for our discordant findings could reflect aspects of the endogenous flora in different colonies and/or the intensity of the pathogenesis. Regardless of the source of the discrepancy, our results have demonstrated that in some circumstances, T_{reg} cells can prevent colitis by means other than IL-10 secretion. Most notably, we made the unexpected observation that IL-10 produced by cells other than T lymphocytes was required for T_{reg} cell function, despite the ability of the donor T_{reg} cells to secrete IL-10. T_{reg} cells transferred into *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipient mice expanded in number *in vivo* and homed to various tissues, including the intestine. However, these cells failed to maintain Foxp3 expression and suppressive activity in the absence of IL-10 signaling.

Despite rigorous sorting of T_{reg} cells on the basis of Foxp3 expression, it was possible that a small population (<1%) of contaminating activated effector T cells outgrew the transferred Foxp3^{+} T_{reg} cells. However, the results from an experiment in which we deliberately transferred congenic activated memory cells together with T_{reg} cells demonstrated that these cells did not outgrow the T_{reg} cells in *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients. Therefore, loss of Foxp3 expression is the most plausible explanation for the ineffectiveness of T_{reg} cells in the absence of IL-10 signaling.

Studies have indicated that the phenotype and function of T_{reg} cells can be unstable. For example, a minority of T_{reg} cells lose Foxp3 expression *in vitro* when IL-6 is added[24]. Also, antibody ligation of T cell immunoglobulin mucin 1 causes loss of Foxp3 mRNA expression and T_{reg} cell function *in vitro*[25]. The effect of engagement of T cell immunoglobulin mucin 1 on Foxp3^{+} cells by its ligand Tim-4 *in vivo* remains to be analyzed. In addition, a sub-population of CD25^{-}Foxp3^{+} T_{reg} cells has a tendency to lose Foxp3 expression[26]. However, we excluded that subset from our analysis, as our T_{reg} cell populations were selected for high expression of CD25. Moreover, transferred T_{reg} cells can generate follicular helper T cells in the Peyer's patches under the influence of CD40 expression by B lymphocytes[27]. A study using a Foxp3 reporter lineage marker system has shown that there is some spontaneous loss of Foxp3 expression *in vivo*, which is enhanced in nonobese diabetic mice with autoimmune disease[28]. As in our experiments, these formerly T_{reg} cells secrete proinflammatory cytokines.

Many cell types, including mast cells, epithelial cells and dendritic cells, synthesize IL-10 (refs. 29–32). Our data have indicated that a population of CD11b^{+} myeloid cells in the LPL constitutively produced IL-10 and that a phenotypically similar population of IL-10-producing cells was greater in abundance in the MLNs early after T cell transfer. Many of the IL-10-producing myeloid cells were probably macrophages, given their expression of F4/80, although we did not exclude the possibility that other cell types were involved. Our findings are in agreement with the results of a study showing that IL-10 from lamina propria macrophages is important for the induction of Foxp3 expression[33]. However, that investigation focused on cells from the small intestine rather than the large intestine and studied induction rather than the maintenance of Foxp3 expression. Despite that, the concept is emerging that intestinal macrophages are important for supporting natural and induced T_{reg} cell function.

Why is T_{reg} cell–derived IL-10 not sufficient for the maintenance of Foxp3 expression in mice developing colitis? We suggest that the requirement for host cell–derived IL-10 is a matter of kinetics. At early time points, few T_{reg} cells were producing IL-10, although at 6

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weeks after transfer, T_{reg} cells in the intestine were the main IL-10 producers. Our data therefore suggest that T_{reg} cell–derived IL-10 cannot sustain Foxp3 expression because it is induced relatively late.

The outcome of the transfer of *Il10rb*$^{-/-}$ T_{reg} cells suggested that the IL-10 produced by cells in the *Rag1*$^{-/-}$ hosts acted in part directly on the T_{reg} cells to maintain Foxp3 expression, although these findings do not exclude the possibility that IL-10 serves additional functions by acting on other cell types that might influence T_{reg} cell function. A caveat to our conclusion is that IL-10Rβ can participate in the signals delivered by several other cytokines[34]. However, the concordance with the results obtained from transfer to *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients suggests that the ineffectiveness of the *Il10rb*$^{-/-}$ T_{reg} cells was due to the absence of T_{reg} cell IL-10 signaling.

Our data have indicated that colitis in the *Rag1*$^{-/-}$ recipients also contributed to the loss of T_{reg} cell Foxp3 expression. At relatively early times after transfer, when inflammation was less severe, the loss of Foxp3 was less pronounced and more localized to the intestine. However, Foxp3^{+} T lymphocytes developed in essentially normal numbers in *Il10rb*$^{-/-}$ mice. The *Il10rb*$^{-/-}$ mice we used were mainly on the colitis-resistant C57BL/6 background and were only 8–10 weeks old, and therefore they did not have signs of colitis (data not shown). The results from the transfer of mixed populations of T_{reg} cells into recipients that developed colitis indicated that colitis alone could not explain the loss of Foxp3. Furthermore, Foxp3 expression was not much lower in *Il10rb*$^{-/-}$ T_{reg} cells in recipients of mixed T_{reg} cell populations that did not develop colitis. We conclude, therefore, that the combined effects of the absence of IL-10 signaling and the inflammatory milieu in mice with severe colitis were responsible for loss of Foxp3 expression and T_{reg} cell function.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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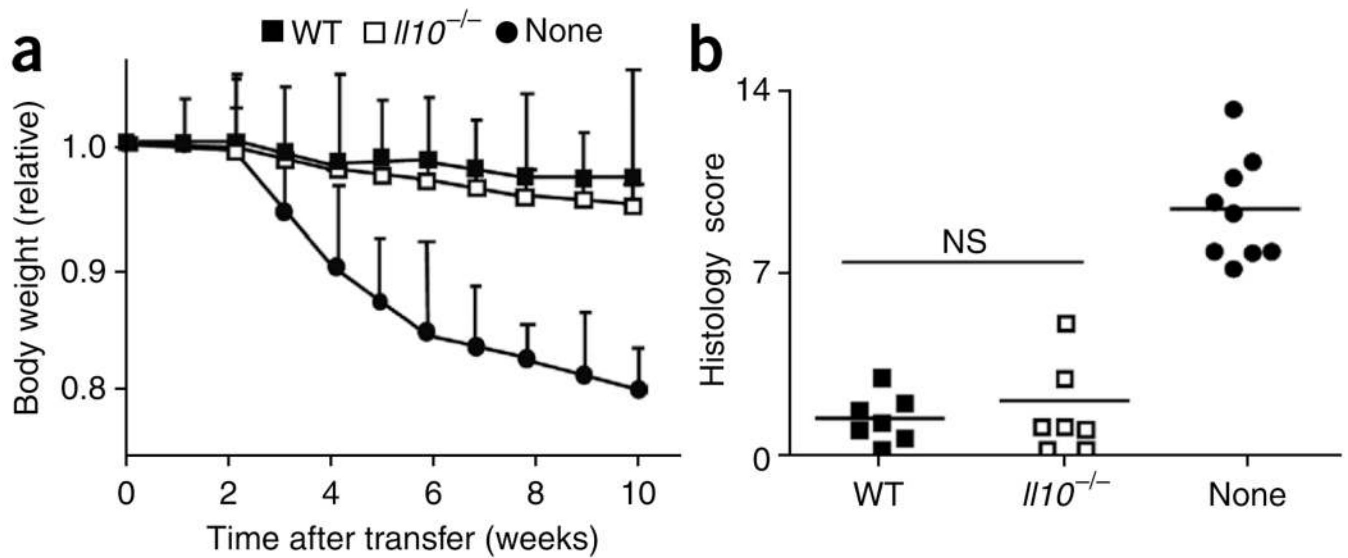

Figure 1.

IL-10-deficient T_{reg} cells prevent colitis. (**a**) Body weight of *Rag1*$^{-/-}$ mice given sorted *Il10*$^{-/-}$ or wild-type (WT) $CD4^{+}CD25^{+}$ T_{reg} cells, together with $CD4^{+}CD45RB^{hi}$ T cells, or of *Rag1*$^{-/-}$ mice given $CD4^{+}CD45RB^{hi}$ T cells alone (control; None), presented relative to initial body weight. Data are pooled from two independent experiments with six mice each (error bars, s.d.). (**b**) Histology scores of sections of the large intestine at 6 weeks after the cell transfer described in **a**. Each symbol represents an individual mouse; small horizontal lines indicate the mean. NS, not significant. Data are pooled from at least two independent experiments.

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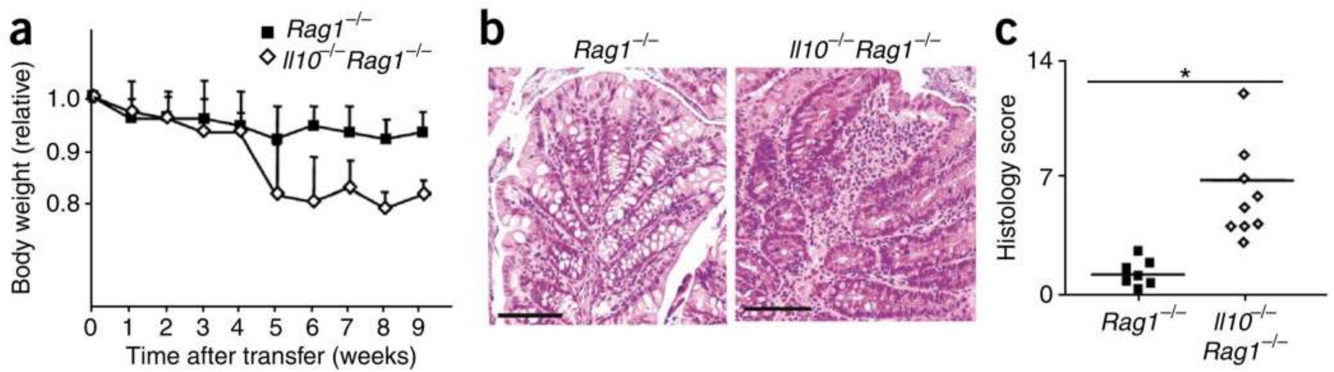

Figure 2.

Rag1$^{-/-}$ host IL-10 is required for T_{reg} cell function. (**a**) Body weight of *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts given $CD4^{+}CD45RB^{hi}$ T cells plus sorted *Foxp3*^{gfp} T_{reg} cells, presented relative to initial body weight. Data are pooled from two independent experiments with ten mice each (error bars, s.d.). (**b**) Proximal colon of *Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice at 6 weeks after the donor cell transfer described in **a**; sections are stained with hematoxylin and eosin. Original magnification, ×100; scale bars, 100 μm. Data are representative of one of three independent experiments. (**c**) Histology scores of sections of the large intestine at 6 weeks after the cell transfer described in **a**. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *$P < 0.001$ (two-tailed Student's *t*-test). Data are pooled from three independent experiments with a total of nine mice.

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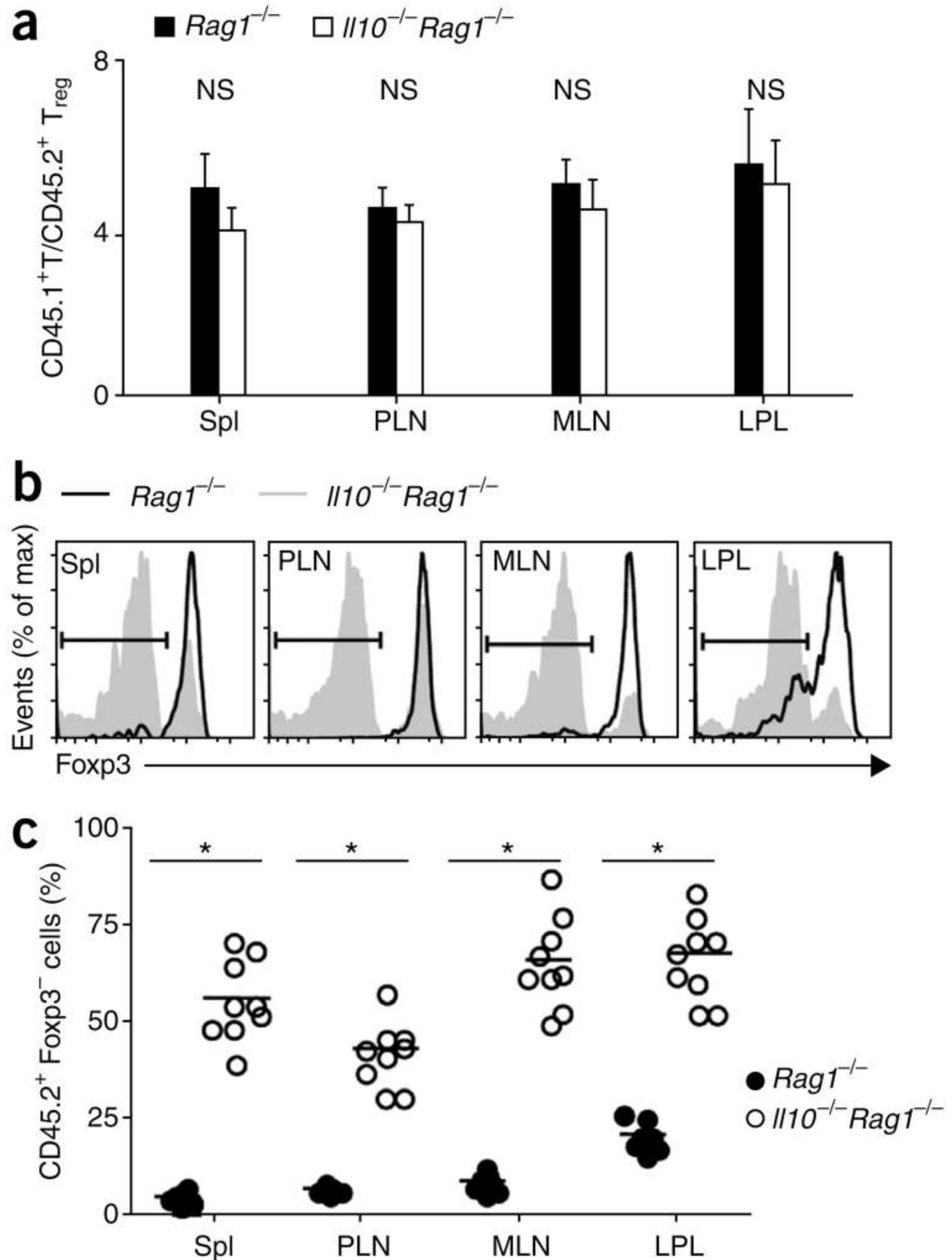

Figure 3.

Foxp3 is downregulated in *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients. (**a**) Composite ratios of CD45.1$^+$ to CD45.2$^+$ TCRβ$^+$CD4$^+$ cells in the spleen (Spl), PLNs, MLNs and LPL of *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipient mice at 6 weeks after injection of 4×10^5 CD4$^+$CD45RB^{hi} T cells derived from C57BL/6 (CD45.1$^+$) mice, plus 1×10^5 *Foxp3*^{gfp} (CD45.2$^+$) T_{reg} cells. (**b**) Foxp3 expression in the cells in **a**, gated on TCRβ$^+$CD4$^+$ CD45.2$^+$ cells. Bracketed lines indicate the Foxp3$^-$ population. max, maximum. (**c**) Foxp3$^-$ cells in the gated TCRβ$^+$CD4$^+$ CD45.2$^+$ populations in **b**. Each symbol represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.001$ (two-tailed Student's *t*-test). Data are pooled from three

independent experiments with a total of nine mice (**a** (mean and s.d.) and **c**) or are representative of one of three independent experiments (**b**).

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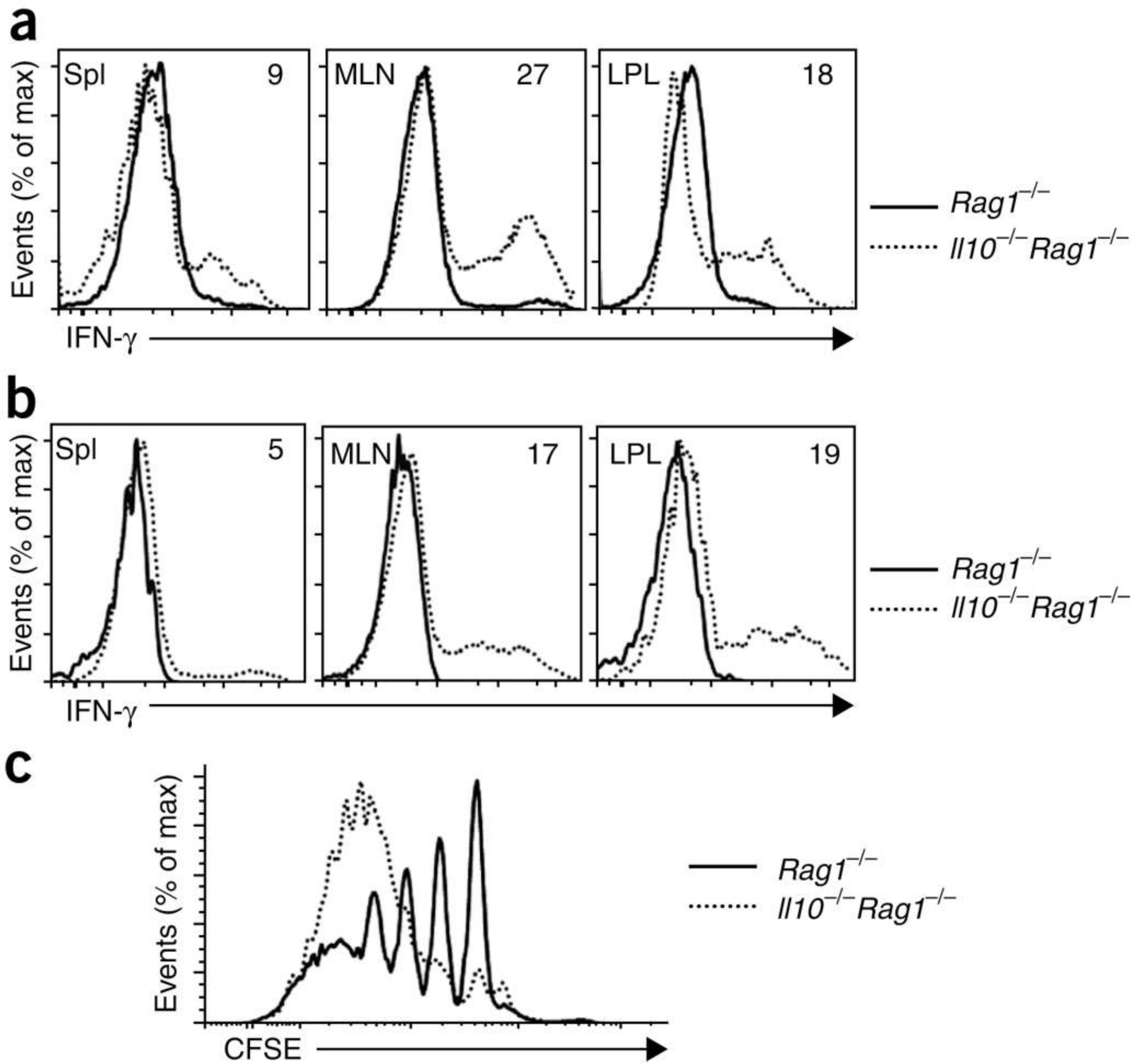

Figure 4.

Loss of function by T_{reg} cells from *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients. (**a,b**) Flow cytometry of intracellular IFN-γ in $CD4^+CD45RB^{hi}$ T cells derived from C57BL/6 ($CD45.1^+$) mice transferred with *Foxp3*^{gfp} ($CD45.2^+$) T_{reg} cells into *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ hosts; plots are gated on the $TCR\beta^+CD4^+$ $CD45.2^-$ progeny of donor $CD4^+CD45RB^{hi}$ T cells (**a**) and the $TCR\beta^+CD4^+$ $CD45.2^+$ progeny from donor *Foxp3*$^{gfp+}$ T_{reg} cells (**b**), isolated from spleen (Spl), MLNs and LPL in mice at 6 weeks after donor cell injection and then stimulated with PMA and ionomycin. Numbers in plots indicate percent IFN-γ-producing cells. (**c**) Suppressive function *in vitro* of sorted $TCR\beta^+CD4^+$ $CD45.2^+$ cells from MLNs of *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients of $CD45.1^+$ $CD4^+CD45RB^{hi}$ and $CD45.2^+$ $CD4^+CD25^+CD45RB^{lo}$ T_{reg} cell populations, cultured for 4 d together with CFSE-labeled

CD45.1$^+$ naive T cells; after stimulation of cultures, CFSE dilution was assessed by flow cytometry. Data are representative of one of three (**a,b**) or two (**c**) independent experiments.

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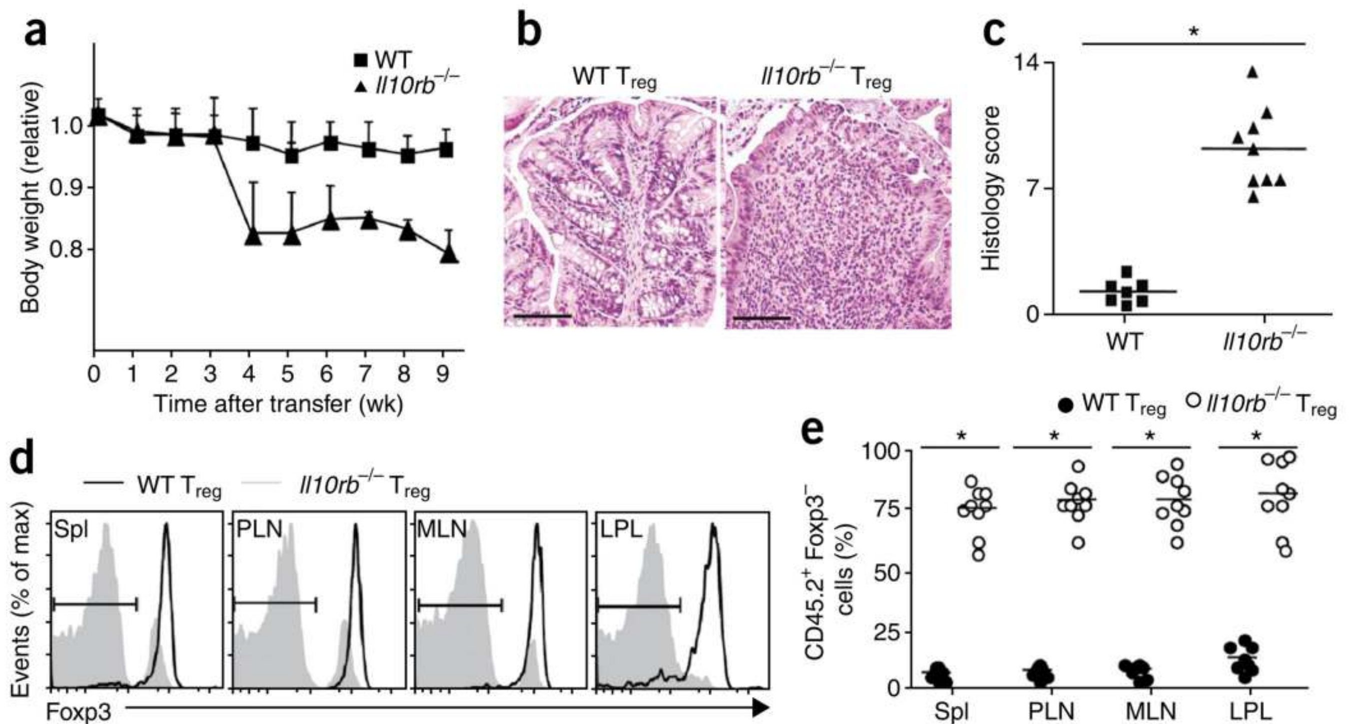

Figure 5.

Il10rb$^{-/-}$ T_{reg} cells fail to prevent colitis. (**a**) Body weight of *Rag1*$^{-/-}$ recipients of C57BL/6 (CD45.1^{+}) CD4^{+}CD45RB^{hi} T cells transferred together with wild-type or *Il10rb*$^{-/-}$ (CD45.2^{+}) T_{reg} cells, presented relative to initial body weight. Data are pooled from two independent experiments with a total of ten mice (error bars, s.d.). (**b**) Proximal colon of recipient mice at 6 weeks after injection of cells as described in **a**; sections are stained with hematoxylin and eosin. Original magnification, ×100; scale bars, 100 μm. Data are representative of one of three independent experiments. (**c**) Histology scores of sections of the large intestine at 6 weeks after the cell transfer described in **a**. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *$P < 0.001$ (two-tailed Student's *t*-test). Data are pooled from three independent experiments with a total of nine mice. (**d**) Foxp3 expression by cells isolated from the spleen, PLNs, MLNs and LPL of the recipient mice in **a**, with gating on TCRβ^{+}CD4^{+}CD45.2^{+} cells. Bracketed lines indicate the Foxp3^{-} population. Data are representative of one of three independent experiments with a total of nine mice. (**e**) Foxp3^{-} cells in the TCRβ^{+}CD4^{+} CD45.2^{+} T lymphocyte populations described in **d**. Each symbol represents an individual mouse; small horizontal bars indicate the mean. *$P < 0.001$ (two-tailed Student's *t*-test). Data are pooled from three independent experiments with a total of nine mice.

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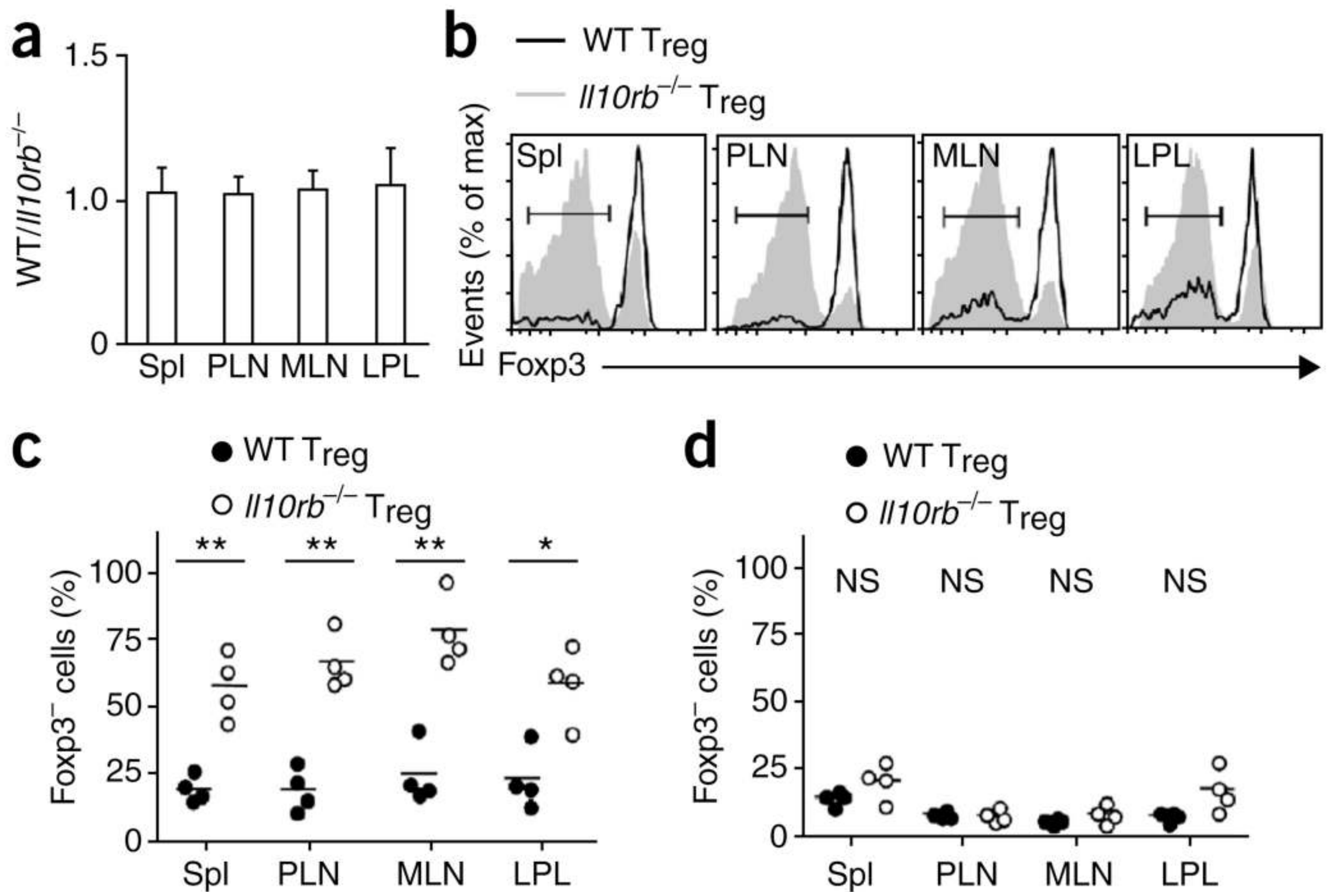

Figure 6.

Foxp3 is lost 'preferentially' by *Il10rb*$^{-/-}$ T_{reg} cells in mice with colitis. (**a**) Ratio of $CD45.1^+$ to $CD45.2^+$ $CD90.1^-CD4^+TCR\beta^+$ cells isolated from spleen, PLNs, MLNs and LPL of *Rag1*$^{-/-}$ recipients at 6 weeks after injection of 8×10^5 C57BL/6 ($CD90.1^+$) $CD4^+CD45RB^{hi}$ T cells, transferred with 2×10^4 wild-type ($CD45.1^+$) T_{reg} cells and 2×10^4 *Il10rb*$^{-/-}$ ($CD45.2^+$) T_{reg} cells. Data are pooled from two independent experiments with a total of four mice (mean and s.d.). (**b**) Flow cytometry of Foxp3 expression by cells isolated from a *Rag1*$^{-/-}$ recipient mouse as described in **a**, with gating on $CD90.1^-CD4^+TCR\beta^+CD45.2^-$ cells (wild-type T_{reg} cells) or $CD90.1^-CD4^+TCR\beta^+CD45.2^+$ cells (*Il10rb*$^{-/-}$ T_{reg} cells). Bracketed lines indicate the $Foxp3^-$ population. Data are representative of one of two independent experiments. (**c**) $Foxp3^-$ cells in the $CD90.1^-CD4^+TCR\beta^+CD45.2^-$ (wild-type T_{reg}) and $CD90.1^-CD4^+TCR\beta^+CD45.2^+$ (*Il10rb*$^{-/-}$ T_{reg}) populations isolated from *Rag1*$^{-/-}$ mice as described in **a**. Each symbol represents an individual mouse; small horizontal bars indicate the mean. Data are pooled from two independent experiments with a total of four mice. (**d**) $Foxp3^-$ cells in $CD90.1^-CD4^+TCR\beta^+CD45.2^-$ (wild-type T_{reg}) or $CD90.1^-CD4^+TCR\beta^+CD45.2^+$ (*Il10rb*$^{-/-}$ T_{reg}) populations isolated from *Rag1*$^{-/-}$ recipients of 4×10^5 C57BL/6 ($CD90.1^+$) $CD4^+CD45RB^{hi}$ T cells, transferred together with 1×10^5 wild-type ($CD45.1^+$) and 1×10^5 *Il10rb*$^{-/-}$ ($CD45.2^+$) T_{reg} cells. Each symbol represents an individual mouse; small horizontal bars indicate the mean. * $P < 0.01$; ** $P < 0.001$ (two-tailed Student's *t*-test). Data are pooled from two independent experiments with a total of four mice.

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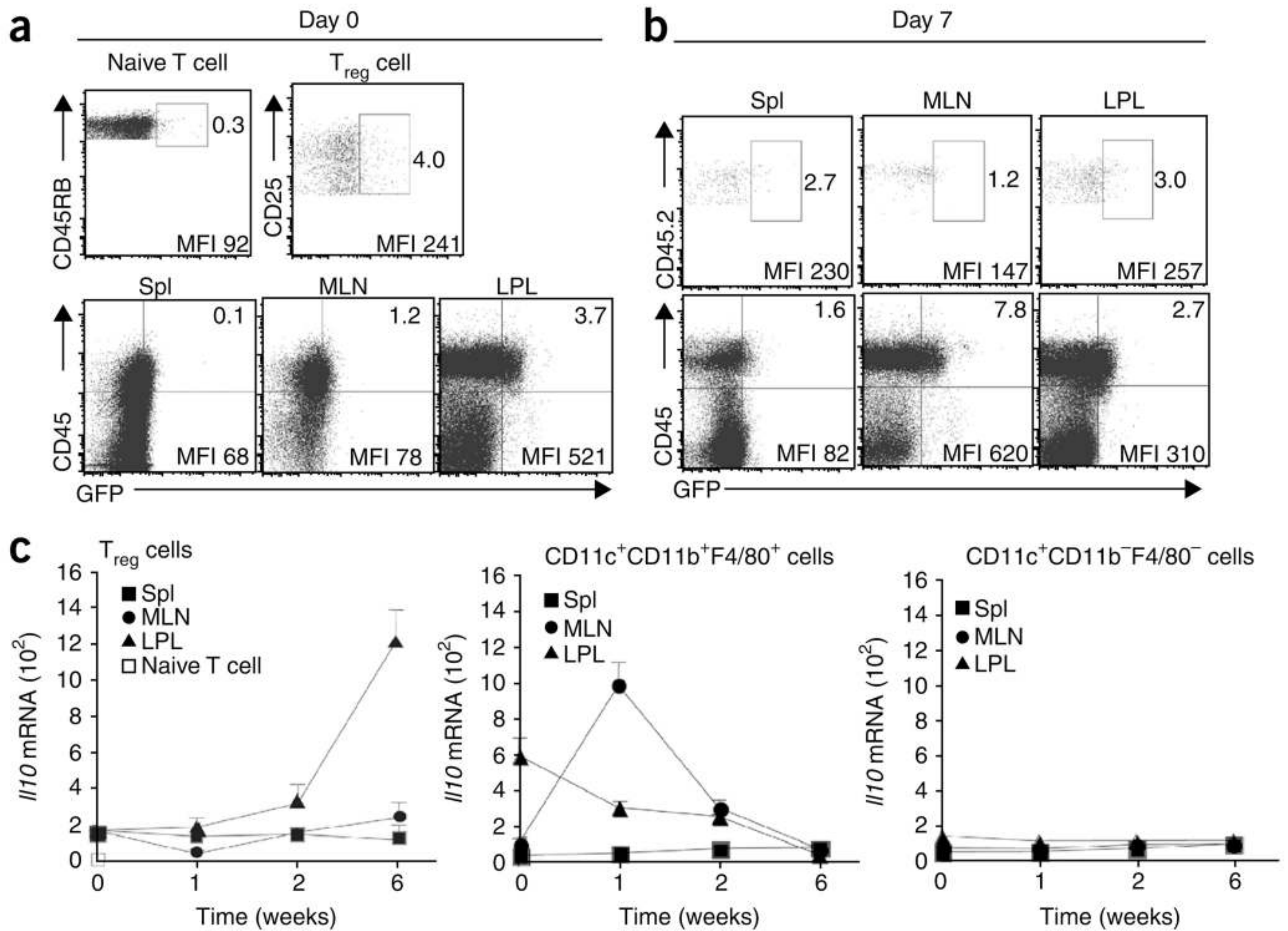

Figure 7.

Kinetics of IL-10 expression by T_{reg} cells and host cells. (**a**) GFP^+ cells in IL-10 reporter mice. Numbers adjacent to outlined areas (top row) indicate percent GFP^+ cells among gated naive splenocytes ($TCR\beta^+CD4^+CD45RB^{hi}$) and T_{reg} splenocytes ($TCR\beta^+CD4^+CD45RB^{lo}CD25^+$) from *Il10*^{gfp} mice; numbers in top right quadrants (bottom row) indicate percent $CD45^+GFP^+$ cells in tissues from *Il10*^{gfp}*Rag1*$^{-/-}$ mice. MFI, mean fluorescence intensity. (**b**) Flow cytometry of GFP^+ cells in tissues 7 d after transfer of a mixture of $CD45.1^+$ $CD4^+CD45RB^{hi}$ T cells and $CD45.2^+$ *Il10*^{gfp} T_{reg} cells (ratio, 4:1). Top row, T_{reg} cells gated as $CD45.2^+$ $TCR\beta^+CD4^+$ cells; bottom row, gated $TCR\beta^-CD4^-$ nonlymphoid cells. Numbers adjacent to outlined areas and in top right quadrants indicate percent GFP^+ cells. (**c**) Real-time PCR analysis of *Il10* mRNA in sorted T_{reg} cells (left), $CD11b^+$ cells (middle) and $CD11c^+$ dendritic cells (right) from various sites (keys) before transfer (0) or at 1, 2 and 6 weeks after transfer as in **b**. Data are from representative one of two independent experiments with six mice (**a,b**) or are pooled from two independent experiments with six mice (**c**; mean and s.d.).

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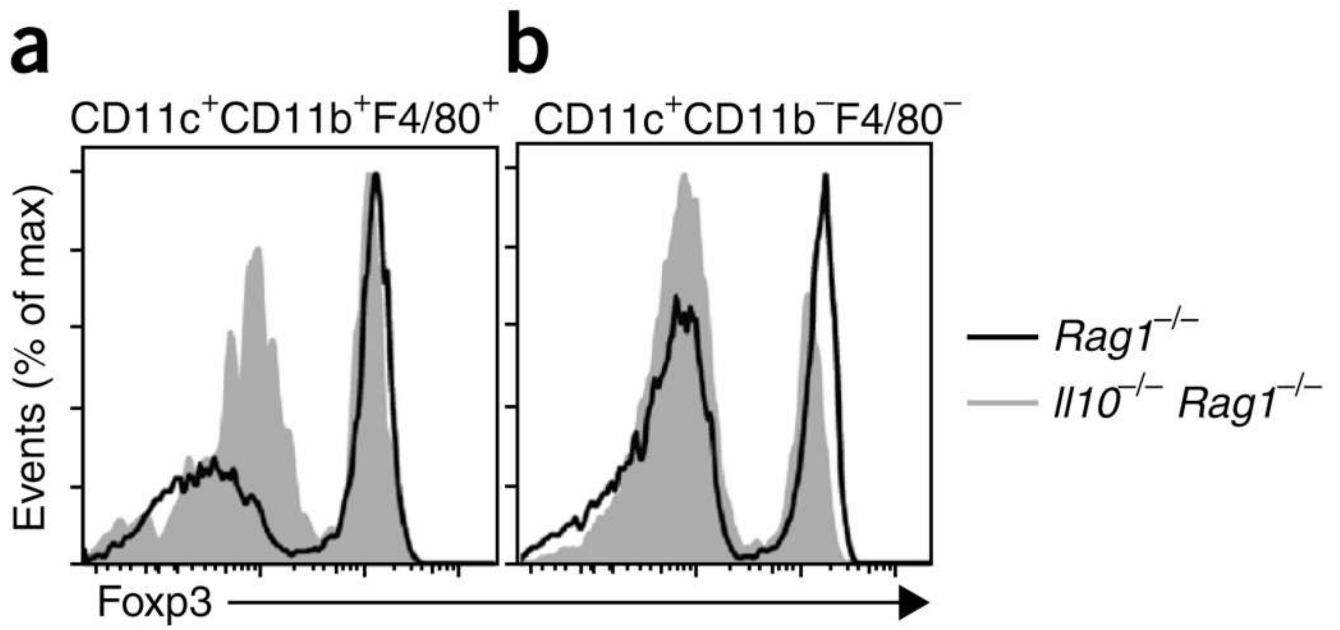

Figure 8.

IL-10-producing CD11b+ myeloid cells prevent the downregulation of Foxp3. Flow cytometry of Foxp3 expression 3 weeks after the injection of 5×10^6 *Rag1*−/− or *Il10*−/−*Rag1*−/− intestinal CD11c+CD11b+F4/80+ cells (**a**) or CD11c+CD11bF4/80− cells (**b**) into *Il10*−/−*Rag1*−/− recipients, transferred intravenously on days 0 and 7 (where 'day 0' is the day of T cell transfer) with 4×10^5 CD4+ CD45RB^{hi} (CD45.1+) cells in the presence of 1×10^5 (CD45.2+) T_{reg} cells from *Foxp3*^{gfp} mice. Plots are gated on TCRβ+CD4+CD45.2+ splenocytes. Data are representative of one of two independent experiments with a total of three mice.