ONLINE METHODS

Mice. C57BL/6J, C57BL/6 CD45.1$^+$ congenic, C57BL/6 CD90.1$^+$ congenic, C57BL/6 *Rag1*$^{-/-}$ and C57BL/6 *Il10*$^{-/-}$ mice were from The Jackson Laboratory. *Il10rb*$^{-/-}$ mice on the C57BL/6 background, which lack IL-10Rβ, were generated as described[8] and were obtained from P. Allen with permission from Genentech. Foxp3-GFP (*Foxp3*^{gfp}) reporter mice, provided by A.Y. Rudensky[16], were backcrossed six generations to C57BL/6J mice. C57BL/6 IL-10–IRES–eGFP reporter mice (*Il10*^{gfp}; Vert-X) were generated by insertion of a *lox*P-flanked neomycin-resistance–internal ribosome entry site–enhanced GFP cassette between the endogenous stop and poly(A) sites of *Il10*, followed by excision of the neomycin-resistance gene by breeding with C57BL/6 Zp3-Cre mice (which express Cre recombinase driven by the promoter of the mouse gene encoding zona pellucida 3; this is useful for deleting a *lox*P-flanked sequence specifically in the female germline)[35]. For the generation of *Il10*^{gfp}*Rag1*$^{-/-}$ and *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice, *Rag1*$^{-/-}$ mice were crossed with the respective reporter-gene mice or *Il10*$^{-/-}$ mice, and the F_1 mice were interbred and typed. A similar strategy was followed for the generation of *Foxp3*^{gfp}*Il10rb*$^{-/-}$ mice. The Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology approved the studies.

Induction of colitis. Splenocyte samples were enriched for CD4$^+$ cells by positive selection on MACS columns with antibody to CD4 (anti-CD4) microbeads (Miltenyi Biotec). Cells were then stained with phycoerythrin–anti-CD45RB (16A), allophycocyanin–anti-CD25 (PC61), phycoerythrin-indotricarbocyanine–anti-CD4 (GK1.5) and 7-amino-actinomycin D (all from BD PharMingen) and were sorted into CD4$^+$CD25$^-$CD45RB^{hi} or CD4$^+$CD25$^+$CD45RB^{lo} populations. *Rag1*$^{-/-}$ recipients were injected intravenously with 4×10^5 CD4$^+$CD25$^-$CD45RB^{hi} cells in the presence or absence of 1×10^5 CD4$^+$CD25$^+$CD45RB^{lo} cells. Alternatively, T_{reg} cells were sorted from *Foxp3*^{gfp} mice on the basis of GFP expression and a CD4$^+$CD25$^+$CD45RB^{lo} surface phenotype. In some experiments, CD4$^+$CD25$^-$CD45RB^{hi} cells from CD90.1$^+$ congenic mice were injected into *Rag1*$^{-/-}$ recipients together with wild-type (CD45.1$^+$) T_{reg} cells or *Foxp3*^{gfp}*Il10rb*$^{-/-}$ (CD45.2$^+$) T_{reg} cells, or a 1:1 mixture of the two types of T_{reg} cells .

Assessment of colitis. Recipient mice were weighed weekly and were monitored for signs of illness. Cryosections were cut from intestinal tissue samples, fixed in paraformaldehyde, stained with hematoxylin and eosin and assigned scores as described[36]. All samples were coded and assigned scores by researchers 'blinded' to the experimental conditions.

Flow cytometry. Mucosal lymphocytes were isolated as follows: the intestine was opened longitudinally, was washed thoroughly with Hank's balanced-salt solution (HBSS) and was incubated with shaking for 30 min. RPMI-1640 medium, supplemented with 5% (vol/vol) FBS and 1 mM dithiothreitol, was added at 37 °C, followed by vortexing for 30 s. Supernatants were collected and rinsed with HBSS and were purified by centrifugation on a 44–70% discontinuous Percol gradient for isolation of the intraepithelial lymphocytes. The remaining tissue was minced and was incubated for 20 min at 37 °C in RPMI-1640 medium with 5% (vol/vol) FBS and 1.5 mg/ml of collagenase IV. Aliquots were collected for isolation of the LPL. Isolated splenocytes and cells from MLNs, PLNs and LPL were incubated with anti-FcγRII/III for blockade of nonspecific binding of labeled monoclonal antibodies and were then stained with fluorescein isothiocyanate–anti-CD90.1 (HIS51), allophycocyanin-indotricarbocyanine–anti-TCRβ (H57-597), peridinin chlorophyll protein–cyanine 5.5–anti-CD45.2 (104), phycoerythrin–anti-CD25 (PC61) and phycoerythrin-indotricarbocyanine–anti-CD4 (L3T4; all from eBioscience). After surface molecules were stained, cells were incubated for 18 h at 4 °C in the dark in Fix/Perm Working solution (eBioscience). Cells were then washed in Permeabilization buffer (eBioscience) and were stained with allophycocyanin–anti-Foxp3 (FJK-16s; eBioscience).

Intracellular cytokine staining. Cells were stimulated with PMA and ionomycin for 2 h before the addition of Golgistop (BD PharMingen) and then were cultured for an additional 2 h. Cells were made permeable and then were stained with anti-IFN-γ (XMG1.2) and anti-IL-17 (eBio17B7; both from eBioscience).

T_{reg} cell functional assay. Cells were isolated from the MLNs of *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipient mice at 6 weeks after transfer of CD45.1$^+$ CD4$^+$CD45RB^{hi} and CD45.2$^+$ CD4$^+$CD25$^+$CD45RB^{lo} cell populations and then were stained with allophycocyanin-indotricarbocyanine–anti-TCRβ (H57-597) and peridinin chlorophyll protein–cyanine 5.5–anti-CD45.2 (104). Cell sorting was used to isolate the TCRβ$^+$CD45.2$^+$ progeny of the donor T_{reg} cell population. Naive CD4$^+$CD25$^-$ T cells were negatively selected from the spleens of C57BL/6J mice with magnetic beads (>95% purity by flow cytometry; Miltenyi Biotec). T_{reg} cells sorted by flow cytometry (2.5×10^4 cells) were cultured with CD4$^+$CD25$^-$ T cells purified by magnetic-activated cell sorting and labeled with CFSE (1×10^5 cells). Cells were stimulated for 96 h with T cell–depleted irradiated spleen cell samples from *Il10*$^{-/-}$ mice (5×10^5 cells) and anti-CD3ε (1 μg/ml). CFSE dilution was assessed by flow cytometry.

Quantitative RT-PCR. Total RNA was isolated from sorted CD45.1$^+$ naive T cell populations, CD45.2$^+$ T_{reg} cell populations, CD11c$^+$CD11b$^+$F4/80$^+$ cell populations and CD11c$^+$CD11b$^-$F4/80$^-$ cell populations from *Rag1*$^{-/-}$ recipients with TRIzol according to the manufacturer's instructions (Invitrogen Life Technologies) and were treated with DNase I (Ambion) for removal of contaminating DNA, then cDNA was synthesized from 1 μg total RNA with an iScript cDNA Synthesis kit according to the instructions provided by the manufacturer (Bio-Rad). RNA was extracted from as little as 1×10^4 cells. The cDNA was amplified by real-time PCR with SYBR Green (Bio-Rad Laboratories) and the following primers: IL-10 sense, 5′-CCCTTTGCTATGGTGTCCTT-3′ and antisense, 5′-TGGTTTCTCTTCCCAAGACC-3′; GAPDH (glyceraldehyde phosphate dehydrogenase) sense, 5′-TGGCAAAGTGGAGATTGTTGCC-3′ and antisense, 5′-AAGATGGTGATGGGCTTCCCG-3′. Target gene expression was assessed by the comparative cycling threshold (C_T) method with expression of GAPDH as a control, as described in the instructions of the iCycler (Bio-Rad; Hercurus).

Adoptive transfer of CD11b$^+$ or CD11b$^-$ myeloid cells. Small and large intestines from *Rag1*$^{-/-}$ or *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice were removed, opened longitudinally and washed of fecal contents. Intestines were shaken for 20 min in HBSS with 5% (vol/vol) FBS and 2 mM EDTA. This process was repeated two additional times. Cell suspensions were passed through a strainer and the remaining intestinal tissues were washed and then minced, followed by shaking for 20 min at 37 °C in HBSS supplemented with 5% (vol/vol) FBS and type VIII collagenase (1.5 mg/ml; Sigma). Cell suspensions were collected and pelleted by centrifugation. Suspensions were enriched for CD11b$^+$ or CD11c$^+$ cells by positive selection with anti-CD11b or anti-CD11c microbeads, respectively. Enriched intestinal cells were then stained with phycoerythrin-indotricarbocyanine–anti-CD11b (M1/70; PharMingen), phycoerythrin–anti-CD11c (HL3; PharMingen) and allyphycocyanin–anti-F4/80 (BM8; eBioscience), then were sorted as CD11c$^+$CD11b$^+$F4/80$^+$ and CD11c$^+$CD11b$^-$F4/80$^-$ cells. The sorted cells (5×10^6) were then transferred on days 0 and 7 into *Il10*$^{-/-}$*Rag1*$^{-/-}$ recipients injected intravenously with CD4$^+$CD25$^-$CD45RB^{hi} cells (4×10^5) in the presence of GFP$^+$CD4$^+$CD25$^+$CD45RB^{lo} cells (1×10^5).

Statistics. The statistical significance of differences was calculated with the Student's *t*-test (two-tailed). Differences with a *P* value of less than 0.05 were considered statistically significant.

35. Madan, R. *et al.* Non-redudant roles for B cell-derived IL-10 in immune counter-regulation. *J. Immunol.* **183**, 2312–2320 (2009).
36. Kim, G., Levin, M., Schoenberger, S.P., Sharpe, A. & Kronenberg, M. Paradoxical effect of reduced costimulation in T cell-mediated colitis. *J. Immunol.* **178**, 5563–5570 (2007).