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The T Cell Response to IL-10 Alters Cellular Dynamics and Paradoxically Promotes Central Nervous System Autoimmunity

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IL-10 is a critical anti-inflammatory cytokine, the deficiency of which leads to spontaneous autoimmunity. However, therapeutically administered or ectopically expressed IL-10 can either suppress or promote disease. Distinct lineage-specific activities may explain the contradictory effects of IL-10. To dissect the T cell-specific response to IL-10 during organ-specific autoimmunity, we generated mice with a selective deletion of IL-10Ra in T cells and analyzed its effects in an autoimmune model, experimental allergic encephalomyelitis (EAE). Surprisingly, the T cell response to IL-10 increased EAE severity. This did not result from altered T cell functional potential; T cell cytokine profile was preserved. IL-10 also diminished the proliferation of T cells in situ within the target organ, an effect that would be expected to restrain disease. However, IL-10 acted cell autonomously to sustain the autoreactive T cells essential for immunopathogenesis, promoting their accumulation and distorting the regulatory and effector T cell balance. Indeed, in chimeric mice and after adoptive transfer, wild type T cells showed a competitive advantage over cells deficient in IL-10Ra. Therefore, T cell specific actions of IL-10 can support autoimmune inflammation, and this appears to result from an overall increase in the long term fitness of pathologic T cells. Lineage-restricted, disease-promoting activities of IL-10 should be considered in the therapeutic manipulation of the IL-10 pathway.

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B cells were isolated by flow cytometric sorting from IL-10R for quantitative analysis of lineage specific deletion, CD4 T, CD8 T, and 5
9
Tyrc-2J Southern. Embryonic stem cells were injected into C57BL/6J-ES26.2 embryonic stem cell line, with correct targeting ascertained by
the comparative Ct approach (2
normalized to Ct data from simultaneously analyzed WT CD4 T cells using
reverse primer (primer set 2) using real-time PCR. Cycle threshold (Ct)
ACTFLPe
nase target-flanked neo r gene introduced into intron 3 and loxp sites added
9205Dym/J
a
exon 3. Exon 3 incorporates much of the IL-10 interacting domain,
and its deletion leads to an additional frameshift in downstream sequence.

Materials and Methods

IL-10Ra conditionally deficient mice

A 12.6-kb segment incorporating IL-10Ra exon 1-5 was isolated from a C57BL/6-derived bacterial artificial chromosome, and an FLP recombinase target-flanked neo gene introduced into intron 3 and loxp sites added to the 5′ end of exon 3 by homologous recombination into the C57BL/6-derived ES26.2 embryonic stem cell line, with correct targeting ascertained by Southern. Embryonic stem cells were injected into C57BL/6-J-Tg(Tcratm1Mom)
DD
gd
Ct).

Mice and reagents

Myelin oligodendrocyte glycoprotein (MOG)25-35 peptide (MEVG-WYRSPESPRVLYRNGK) was synthesized and HPLC purified by the St. Jude Hartwell Center for Biotechnology. C57BL/6J, B6.Cg-Tg
ACTFLPe
9205Dym/J
Ptprca Pepcb
and EAE was induced.

Histopathology scoring

Mice were sacrificed, lymph nodes (LN) and spleens were removed, the circulation was perfused with 4˚C PBS, and the brains and spinal cords were removed. CNS lymphocytes were isolated by density centrifugation and either perfused with 4˚C PBS or fixed for histopathology. Histopathologic examination of H&E sections was performed in a blinded fashion, with sections evaluated in staggered order.

Cytokine production

Lymphocytes were stimulated with PMA (50 ng/ml) and ionomycin (1
m
H37RA
Mycobacterium tuberculosis
l CFA containing 0.4 mg
b
m
C57BL/6J mice. Wild type (WT) mice controls consisted of IL-10R+/J mice or, when needed, due to needs for congenic strains, matched mice lacking the IL-10Ra
a
allele. Studies were approved by the St. Jude Institutional Animal Care and Use Committee.

IL-10Ra
a
mouse phenotyping and quantitative PCR

To generate mice, genomic DNA was amplified with primers specific for Cre (5′-GGGCCATGGCATCATTATCTGAGGATC-3′ and 5′-TCGCTCGAGGTGATCGCCATCTTCCAGCAG-3′), and WT IL-10Ra alleles (5′-CTCCATACAGGAGGCTTGGG-3′ and 5′-GCTAAGGCTCCCTGGGACGCC-3′), and GFP (5′-TGCTCTCGAGCAGGACAAGCAGTTGTTCA-3′ and 5′-GCCAGGAGTTTCAAGGAAAG-3′). For quantitative analysis of lineage specific deletion, CD4+ T cells, CD8 T cells, and B cells were isolated by flow cytometric sorting from IL-10Ra
a
and WT (IL-10Ra+/J Cre- mice) mice, and genomic DNA isolated. IL-10Ra exon 3 was amplified using forward primer 5′-CCCCCCTGAAGAGCTCATCA-3′ and reverse primer 5′-CCACTTGAGGACCGAGCTTCAGT-3′ (primer set 1) or forward primer 5′-AACCTGGAATTACGATCATC-3′ and the same reverse primer (primer set 2) using real-time PCR. Cycle threshold (Ct) data were compared with GAPDH as an internal control, and this was normalized to Ct data from simultaneously analyzed WT CD4 T cells using the comparative Ct approach (2

Flow cytometry and cell sorting

Analytic cytometry was performed on a Becton-Dickinson FACSCalibur or LSRII and analyzed with FlowJo software (Tree Star). Sorting was performed with a MoFlo (Dako) or Reflection (Cytometry) cytometer. Abs targeting CD4, TCRβ, CD11c, CD11b, CD205, CD80, CD86, Gr-1, F4/80, CD5, CD44, CD25, CD19, TCRαβ, NK1.1, 45Rb, CD20L, CCR2, CCR5, CD11, CXCR3, VLA-4, CD69, CD45.1, CD45.2, IL-10R, and RatIgG2b were from BD Biosciences. Anti-Thy1.1 and Foxp3 were from eBioscience.

Intracellular staining

For cytokine intracellular staining (ICS), isolated lymphocytes were washed and stimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for 4 h in the presence of 10 µg/ml Brefeldin A (Sigma), labeled with CD4 and TCRβ Abs, fixed and permeabilized (Fixation Buffer, eBiosience), and then labeled with anti-IFNG, IL-10, and IL-17 Abs. ICS for Foxp3 was performed using the eBioscience Foxp3 staining kit. For phospho-Stat3, d4 activated T cells were stimulated with 10 ng/ml Rl-10 or medium only for 40 min at 37˚C, fixed with 1% formaldehyde for 10 min at 37˚C, washed, permeabilized with ice-cold 90% methanol for 30 min on ice, labeled with anti-P-Stat3 (Y705; Cell Signaling), washed, and stained with F(ab′)2 goat anti-rabbit IgG (Invitrogen).

EAE induction and clinical evaluation

EAE was induced by s.c. immunization with 100 µg MOG35-55 peptide in 100 µl CFA containing 0.4 mg
b
m
acclimated to for 24 h, and then harvested 18 h later on a Filtermat for scintillation counting. Samples were analyzed in triplicate.

Cell isolation and enumeration

Mice were sacrificed, lymph nodes (LN) and spleens were removed, the circulation was perfused with 4˚C PBS, and the brains and spinal cords were removed. CNS lymphocytes were isolated by density centrifugation as described (31), counted on a hemacytometer, and quantitatively analyzed by flow cytometry.

T cell proliferation

Spleenic, draining LN, and CNS cells were isolated, and total cells or magnetic bead-isolated CD4 T cells were stimulated as indicated at 5 × 106 to 1 × 107 cells per well. Cells were pulsed with 1 µCi [3H]-thymidine after 72 h, and then harvested 18 h later on a Filtermat for scintillation counting. Samples were analyzed in triplicate.

Cytokine production

Lymphocytes were stimulated as above. Culture supernatants were collected at 24 or 48 h and analyzed for IL-2, IL-4, IL-10, IFNG, and IL-17 by Milliplex multiplex bead assay.

Proliferation suppression assay

Flow cytometrically isolated native T cells (CD4+CD45RbhiCD44hi) were seeded at 5 × 105 cells per well. Isolated GFP-Foxp3+ regulatory T cells (Tregs) were added at the indicated ratio with 0.5 µg/ml anti-CD3 and irradiated TCRαβ-splenich APCs; 1 µCi [3H]-thymidine added after 48 h and incorporation measured at 60–72 h.

In vivo proliferation

Mice were injected i.p. with 150 µl BrdU (10 mg/ml) in sterile 1× DPBS. After 16–20 h, lymphocytes were isolated, stained with Abs to cell surface markers, fixed and permeabilized with Cytofix/Cytoperm Buffer (BD Biosciences), treated with DNase (300 µg/ml) at 37˚C for 1 h, and then stained with anti-BrdU-APC (3D, BD Biosciences), and analyzed by flow cytometry.

In vivo Treg depletion

Mice were injected i.p. with 1 mg purified PC61 mAb or isotype control (IgG1) at day −3. On day 0, depletion of CD25+ T cells was confirmed by analyzing CD4+CD25+ and CD4+Foxp3+ cells in peripheral blood samples, and EAE was induced.
Concentrations of IL-2, IFNG, IL-17, IL-4, and IL-10 were measured in cell-free supernatant. (CD4 T cells. Quantitative PCR using either of two oligonucleotide primer sets. Quantities calculated by the anti-CD3. Cells were pulsed at 72 h with [3H]-thymidine and incorporation measured 18 h later. The modest difference in proliferation seen at the 0.2 concentration was not seen in additional experiments. (splenic cells from WT and IL-10R T del or WT splenocytes and LN cells. DNA was isolated and the quantity of IL-10R exon 3 measured by quantitative PCR using either of two oligonucleotide primer sets. Quantities calculated by the ΔΔCt method and plotted relative to the quantity observed in WT CD4 T cells. (WT or IL-10R±T del T cells, stimulated for 4 d with anti-CD3/28, were stained with IL-10R-specific Ab. Histograms show staining intensity for IL-10R (light gray line) or isotype control (dark filled) for each population. (D) T cells were activated as in (C) and then treated with IL-10. Phosphorylated Stat3 was measured by intracellular staining of IL-10-treated cells (light gray line) or control cells untreated with IL-10 (dark filled) for each population. (E) Equal numbers of purified CD4 T lymphocytes from IL-10R T del or WT mices were stimulated with the 1 μg/ml anti-CD28 and the indicated concentration of anti-CD3. Cells were pulsed at 72 h with [3H]-thymidine and incorporation measured 18 h later. The modest difference in proliferation seen at the 0.2 μg/ml concentration was not seen in additional experiments. (F) Purified CD4 T cells were stimulated with anti-CD28 with or without 2 μg/ml anti-CD3. At 48 h, concentrations of IL-2, IFNG, IL-17, IL-4, and IL-10 were measured in cell-free supernatant. (G) CD4*GFP-Foxp3+ Tregs were flow-cytometrically purified from IL-10R T del or WT splenocytes and LN cells and cocultured at the indicated Treg:target cell ratio with WT CD4*GFP-Foxp3+ target cells in the presence of anti-CD3/CD28. Proliferation was measured by [3H]-thymidine incorporation. Representative of two or more experiments.

**FIGURE 1.** Conditional deletion of the mouse IL-10Rα locus. (A) The upper diagram maps the construct used for homologous recombination. An Frt-flanked neo cassette is located in intron 2, and loxP sites inserted flanking exon 3. The lower diagram shows the IL-10Rα locus after deletion of the neo cassette in recombinant mice, leaving a minimally altered gene containing a single Frt site and loxP sites surrounding exon 3. (B) CD4 and CD8 T cells, and B cells were flow cytometrically sorted from IL-10R T del or WT splenocytes and LN cells. DNA was isolated and the quantity of IL-10R exon 3 measured by quantitative PCR using either of two oligonucleotide primer sets. Quantities calculated by the ΔΔCt method and plotted relative to the quantity observed in WT CD4 T cells. (C) WT or IL-10R T del T cells, stimulated for 4 d with anti-CD3/28, were stained with IL-10R-specific Ab. Histograms show staining intensity for IL-10R (light gray line) or isotype control (dark filled) for each population. (D) T cells were activated as in (C) and then treated with IL-10. Phosphorylated Stat3 was measured by intracellular staining of IL-10-treated cells (light gray line) or control cells untreated with IL-10 (dark filled) for each population. (E) Equal numbers of purified CD4 T lymphocytes from IL-10R T del or WT mice were stimulated with the 1 μg/ml anti-CD28 and the indicated concentration of anti-CD3. Cells were pulsed at 72 h with [3H]-thymidine and incorporation measured 18 h later. The modest difference in proliferation seen at the 0.2 μg/ml concentration was not seen in additional experiments. (F) Purified CD4 T cells were stimulated with anti-CD28 with or without 2 μg/ml anti-CD3. At 48 h, concentrations of IL-2, IFNG, IL-17, IL-4, and IL-10 were measured in cell-free supernatant. (G) CD4*GFP-Foxp3+ Tregs were flow-cytometrically purified from IL-10R T del or WT splenocytes and LN cells and cocultured at the indicated Treg:target cell ratio with WT CD4*GFP-Foxp3+ target cells in the presence of anti-CD3/CD28. Proliferation was measured by [3H]-thymidine incorporation. Representative of two or more experiments.

**Hematopoietic chimeras and lymphocyte cotransfers**

For hematopoietic chimeras, bone marrow cells were obtained from femurs and tibias of WT and IL-10R T del mice expressing the indicated congenic markers, washed, and mixed in equal quantities; 10×10^6 cells were injected i.v. into lethally irradiated (900 rad) TCR gd T del or WT mice. For hematopoietic chimeras, single-cell suspensions of erythrocyte-lysed lymph node and splenic cells from WT and IL-10R T del mice expressing the indicated congenic markers were prepared. Cells were mixed in approximately equal quantities and injected i.v. into CD45.1-congenic mice.

**Statistics**

Standard deviations or errors and confidence intervals were calculated with Microsoft Excel or PRISM software. Significance between two groups was calculated with two-tailed t test. When more than two groups were present in an assay, significance was determined with one-way, two-tailed ANOVA using a Bonferroni correction for multiple comparisons. A p value < 0.05 was considered significant. For multiple comparisons, significance is only shown relative to the control group unless otherwise indicated.

**Results**

**Characterization of mice with T cell deficiency in IL-10 response**

We generated C57BL/6 mice bearing a floxed IL-10Rα exon 3 (IL-10Rαfl/fl; Fig. 1A) and bred these with mice expressing CRE selectively on T cells (CD4-Cre). Sorted IL-10R T del T but not B cells showed loss of genomic IL-10Rα exon 3 by quantitative PCR (Fig. 1B). IL-10R expression, measured by surface staining, was absent from IL-10R T del T cells, but expressed at WT levels on B cells, macrophage, and dendritic cells (Fig. 1C and data not shown). IL-10 signals through STAT3 (1), and IL-10Rα T del T cell STAT3 phosphorylation was absent to IL-10 (Fig. 1D).

IL-10R T del mice did not display spontaneous systemic disease, clinically or at necropsy. No differences were observed in their proportions of DN1-4, DP, or SP thymocyte subsets, CD4*, CD8*, CD4*Foxp3+ (Treg), NK-T, and memory/naive TCRβ* cells, TCRγδ* cells, NK cells, CD19* B220*, CD5*, and regulatory CD5*CD1d* B cells, myeloid, lymphoid, and plasmacytoid dendritic cells, macrophage, or granulocytes, either in LN or spleen (Supplemental Fig. 1). To functionally assess the IL-10Rα T del T lymphocytes, CD4* T cells were purified and stimulated with titrations of anti-CD3. Proliferative response was equivalent to that of cells from matched CD4*Cre IL-10Rα T del controls (WT; Fig. 1E).

No differences in the production of representative Th0 (IL-2), Th1 (IFNG), Th2 (IL-4), Th1/Th2 (IL-10), or Th17 (IL-17) cytokines were identified (Fig. 1F). The IL-10Rα T del mice were further bred with GFP-Foxp3 knock-in mice for intravital demarcation of Tregs.
Flow cytometrically purified IL-10RαTdel and WT Tregs demonstrated equivalent capacity to suppress naïve T cell proliferation (Fig. 1G). Therefore, unmanipulated IL-10RαTdel mice appear normal through clinical observation, gross and histologic analysis, phenotypic characterization, and in vitro functional assessments.

**Diminished EAE severity in IL-10RαTdel mice**

Considering the documented role of IL-10 in restraining EAE and other forms of autoimmunity, and of T-specific IL-10 in suppressing intestinal inflammation, we anticipated that IL-10RαTdel mice would develop more severe EAE than WT controls. To the contrary, after immunization with MOG35–55, the mice showed consistently less severe disease (Fig. 2A). Cumulative and peak disease were decreased in the IL-10RαTdel cohorts, although not time to initial disease symptoms (Fig. 2B, Supplemental Table I). All mortality was in the WT group. Therefore, although globally immunoregulatory, IL-10 acts focally on T cells to promote MOG-induced EAE.

**Diminished infiltrates and increased Treg:Teff ratios in IL-10RαTdel mice**

Pathologically distinct forms of EAE can be distinguished historically. To determine whether the altered disease severity was associated with an altered immunopathologic process, we reviewed the histopathology of 6 WT and 7 IL-10RαTdel mice with diverse disease scores. Forebrain, brain stem, and cervical, thoracic, and lumbar spinal cord were assessed for gliosis, parenchymal vacuolation and demyelination, neutrophil infiltrate, and mononuclear cuffing. Linear regression modeling of histologic scores showed a positive correlation with clinical disease (Fig. 3A–E). However, slope and intercept for individual parameters and cumulative score did not differ between the WT and IL-10RαTdel CNS. Therefore, WT and IL-10RαTdel mice develop a qualitatively similar disease that differs in magnitude of severity.

To quantify differences in the T cell response, CD4+ Teff and Foxp3+ Tregs were enumerated in diseased CNS and spleen 14 and 21 d after induction. At the earlier time point, a significantly increased number of Teff was present in WT compared with IL-10RαTdel CNS, consistent with their more severe disease (Fig. 4A). This increase, however, was not associated with a parallel increase of CNS-infiltrating Tregs, resulting in a diminished Treg:Teff ratio in WT compared with IL-10RαTdel CNS. We and others have demonstrated that Tregs are potently inhibitory in MOG-induced EAE (28, 31, 32), and the Treg:Teff ratio is a critical indicator of activity. Therefore, T cell response to IL-10 augments early Teff infiltration in the CNS while decreasing the proportion of Tregs.

At day 21, the magnitude of the infiltrate in both WT and IL-10RαTdel mice was substantially increased (Fig. 4B). An increase in both Teff and Treg numbers was evident in WT mice; however, the growth of the Treg population exceeded that of Teff, leading to an increased Treg:Teff ratio compared with day 14. A rapid expansion of CNS Tregs during EAE has been previously described (33). In IL-10RαTdel mice, infiltrating Teff and Treg numbers were overall decreased relative to WT mice, reflecting their milder disease. The percentage of Tregs was increased relative to day 14; however, the magnitude of this increase was not as pronounced as in the WT mice, and the proportion of Tregs did not significantly differ from WT mice as it had at the earlier time point. Therefore, the kinetics of CNS T cell accumulation differs between WT and IL-10RαTdel mice, with IL-10RαTdel mice showing overall

![FIGURE 2. EAE development in IL-10RαTdel and WT mice. (A) Clinical disease course is shown. Disease was induced with MOG35–55 at day 0. Mean clinical score ± SEM is plotted; n = 5 per cohort. (B) A summary of results from eight independent experiments, with 5–14 mice per cohort in individual experiments, in is shown. Measures of disease activity include mean integrated disease score (area under the curve [AUC]), mean peak disease score (Max Dis), and mean time to initial symptoms (Init Dis) for individual experiments. Ratio of value for IL-10RαTdel to WT mice is plotted. Mean of values is indicated by the central line, and 95% confidence interval for means is indicated by brackets to the right of this. Additional data are provided in Supplemental Table I.](http://www.jimmunol.org/)

![FIGURE 3. Histologic analysis of CNS in IL-10RαTdel and WT mice with EAE. Forebrains, brain stems, and cervical, thoracic, and lumbar spinal cords from mice 20–22 d after disease induction were blindly assessed and scored for gliosis, parenchymal vacuolation and demyelination, neutrophil infiltrate, and mononuclear cuffing. Cumulative histologic scores across location and parameter (A) or for individual parameters across location (B–E) were tallied and are plotted. Linear regression modeling is overlayed and p values for slope and intercept comparing IL-10RαTdel and WT mice are indicated.](http://www.jimmunol.org/)
decreased total T cell accumulation early and late and an increased proportion of Tregs early in disease.

Similar cytokine profiles and migratory phenotype of WT and IL-10Rα Tdel T cells

A mixed Th1 and Th17 response fosters the development and progression of MOG-induced EAE (26, 34). Blockade of Th17 differentiation ameliorates disease, and direct actions of IL-10 have been shown to suppress Th17 cells (22, 23). An increased Th17 response would have been expected to increase disease severity in IL-10Rα Tdel mice, whereas disease was decreased. Nevertheless, to evaluate for altered Teff subset differentiation, we measured CD4+ T cell cytokine profiles directly by intracytoplasmic staining. Similar proportions of CNS or splenic CD4+ T cells producing IFNG, IL-17, and IL-10 were detected in WT and IL-10Rα Tdel mice (Fig. 5A–C). MOG-induced cytokine production, including IL-2, IL-4, IL-10, IFNG, and IL-17, was also similar in WT and IL-10Rα Tdel T cells (Fig. 5D). Therefore, the actions of IL-10 on T cells did not detectably skew subset responses during EAE.

As a supplemental explanation for the diminished number of T cells in CNS infiltrates of IL-10Rα Tdel compared with WT mice (Fig. 4), we considered the possibility that they had decreased migratory potential. To examine this possibility, levels of CCR2, CCR5, CXCR3, CD11a, and VLA-4, markers associated with T cell CNS migration, were measured on draining LN, splenic, and CNS T cells in mice with EAE (Supplemental Fig. 2). No differences were seen between WT and IL-10Rα Tdel populations. Therefore, defective response to IL-10 does not affect phenotypic markers associated with CNS localization.

Ex vivo IL-10Rα Tdel T cell proliferation

To further define the role of IL-10 in shaping response magnitude, IL-10Rα Tdel and WT splenocytes were isolated from mice with EAE, stimulated with MOG 35–55, and their autoantigen-specific proliferative response measured. At 14 d after disease induction, responses were similar, indicating that an equivalent proportion of T cells was capable of recognizing and being effectively stimulated by Ags (Fig. 5E). By 21 d, however, the T cell responses diverged, with less proliferation seen among IL-10Rα Tdel splenocytes (Fig. 5F). Therefore, the peripheral T cell response to IL-10 varies with time after disease induction. IL-10 helps to sustain the autoantigen-specific response later in disease.

MOG-specific T cells are concentrated in the CNS of mice with EAE. Although fewer T cells were present in the CNS infiltrates of IL-10Rα Tdel compared with WT mice (Fig. 4), on a per cell basis, purified CD4+ T cells from WT or IL-10Rα Tdel mice demonstrated...
Representative plots from the staining performed in (A) 3,3,2. For proliferation studies, splenocytes were isolated at 14 d ([E] 10R stimulated with the indicated concentration of MOG 35–55 peptide. Ag-specific proliferation was measured at 72 h by [3H]-thymidine incorporation. Means ± 1 SD are plotted for individual mice. **p < 0.01, ***p < 0.001 for IL-10Rα Tdel mice. To further examine this, we measured in situ T cell proliferation by BrdU labeling. The decreased absolute T cell numbers in the CNS of IL-10Rα Tdel mice would be expected to be associated with a decreased proportion of cycling (BrdU+) cells. In the spleen, only a small proportion of T cells will be MOG specific. At day 14, WT and IL-10Rα Tdel splenic CD4+ T cells showed similar BrdU incorporation, both for Foxp3+ and Foxp3− cells (Fig. 6A, 6B). Autoantigen-specific cells will, however, concentrate in the CNS. In this study, IL-10Rα Tdel Tregs and Teffs showed significantly increased BrdU uptake compared with WT, indicating that, in contrast with our prediction, these were proliferating more rapidly at the site of autoimmunity. Analysis of total numbers of BrdU+ cells in the CNS did not show any difference between IL-10Rα Tdel and WT mice, either for Teff or Tregs (data not shown). Therefore, despite an early increase in proliferation rate in IL-10Rα Tdel mice in situ and absolute numbers of proliferating T cells that did not differ compared with WT mice, there is a diminished accumulation of CNS T cells in the IL-10Rα Tdel mice.

**FIGURE 5.** Cytokine profiles and proliferation of IL-10Rα Tdel and WT T cells. (A–C) Intracellular cytokine staining. CNS (A) or splenic (B) T cells were isolated from mice with EAE (day 21), and intracellular staining for IFNG, IL-17, and IL-10 performed directly ex vivo after a brief stimulation with PMA-ionomycin. Percent of cells positive for the indicated cytokines is plotted. Significant differences were not observed between the IL-10Rα Tdel and WT cells here or in similar analyses at day 14–15 (not shown). Disease scores of mice analyzed in the plot were: WT: 3,3,2,2,1; IL-10Rα Tdel: 2,2,1,1,0.0. (C) Representative plots of the staining performed in (A) and (B) are shown. (D) Ag-induced cytokine production. Splenic T lymphocytes were purified from mice with EAE (day 16). Equal numbers were cultured without or with 100 μg/ml MOG35–55 and secreted IL-2, IFNG, IL-17, IL-4, and IL-10 measured at 48 h. No significant differences were seen between the IL-10Rα Tdel and WT cells. Disease scores of mice analyzed in the plot were: WT: 3,3,3; IL-10Rα Tdel: 3,3,2. For proliferation studies, splenocytes were isolated at 14 d ([E], early disease; disease scores of analyzed mice in the plot were WT: 2,1,1,1,; and IL-10Rα Tdel: 2,1,1,1 or 21 d ([F], progressive disease; disease scores of analyzed mice in the plot were WT: 3,3,3, and IL-10Rα Tdel: 3,3,2) after induction and stimulated with the indicated concentration of MOG35–55 peptide. Ag-specific proliferation was measured at 72 h by [3H]-thymidine incorporation. Means ± 1 SD are plotted for individual mice. (G) Alternatively, CD4+ T cells were purified from the CNS of mice with EAE (day 21), and equal numbers were stimulated with no or 100 μg/ml MOG35–55 in the presence of irradiated splenic APCs from nonimmune WT mice. Proliferation was measured as above. Mean proliferation of individual mice is plotted. Results were not significantly different for IL-10Rα Tdel versus WT mice. Data are representative of two or more experiments. **p < 0.01, ***p < 0.001 for IL-10Rα Tdel versus WT cohorts (F) or unstimulated versus stimulated (G).

**Increased in situ Treg and Teff proliferation in IL-10Rα Tdel mice**

The diminished late in vitro MOG-specific T cell proliferative response of IL-10Rα Tdel compared with WT T cells together with the decreased number of infiltrating T cells in the IL-10Rα Tdel CNS may indicate a diminished expansion of autoreactive T cells in situ in the IL-10Rα Tdel mice. To further examine this, we measured in situ T cell proliferation by BrdU labeling. The decreased absolute T cell numbers in the CNS of IL-10Rα Tdel mice would be expected to be associated with a decreased proportion of cycling (BrdU+) cells. In the spleen, only a small proportion of T cells will be MOG specific. At day 14, WT and IL-10Rα Tdel splenic CD4+ T cells showed similar BrdU incorporation, both for Foxp3+ and Foxp3− cells (Fig. 6A, 6B). Autoantigen-specific cells will, however, concentrate in the CNS. In this study, IL-10Rα Tdel Tregs and Teffs showed significantly increased BrdU uptake compared with WT, indicating that, in contrast with our prediction, these were proliferating more rapidly at the site of autoimmunity. Analysis of total numbers of BrdU+ cells in the CNS did not show any difference between IL-10Rα Tdel and WT mice, either for Teff or Tregs (data not shown). Therefore, despite an early increase in proliferation rate in IL-10Rα Tdel mice in situ and absolute numbers of proliferating T cells that did not differ compared with WT mice, there is a diminished accumulation of CNS T cells in the IL-10Rα Tdel mice.

By d21, as clinical disease crested, rates of BrdU incorporation were diminished and similar between the two cohorts in both the CNS and spleen (Fig. 6C). Therefore, in early but not late EAE, CNS-infiltrating IL-10Rα Tdel Teff and Tregs are cycling more rapidly than WT cells. This increased proliferation is consistent with the documented antiproliferative effects of IL-10 in vitro (35) and after anti-CD3 treatment in vivo (22), however, is incongruously associated with diminished total CNS IL-10Rα Tdel Teff numbers and an increased Treg proportion that would be expected to suppress proliferation (Fig. 4). It is also incongruously associated with the diminished proliferative response to MOG among IL-10Rα Tdel splenocytes at day 21 in vitro (Fig. 5F).
Retained IL-10RaTdel disease resistance after Treg depletion

One possible explanation for this inconsistency is that the increased proportion of Treg in the IL-10RaTdel CNS did not act by limiting Teff proliferation, but instead promoted Teff elimination either directly by cytolysis or indirectly by decreasing overall inflammation. To evaluate the independent effects of IL-10 on Teff cells, we depleted Tregs by treatment with the CD25-specific PC61 Ab prior to EAE induction. Consistent with prior observations (36), PC61 eliminated virtually all CD25+ and most Foxp3+ cells (Supplemental Fig. 3A–C). As previously described, Treg-depleted mice developed more severe EAE than did mice treated with control Ab (Fig. 7A); however, this was similarly true for IL-10RaTdel and WT mice. Indeed, IL-10RaTdel mice treated with PC61 demonstrated substantially less severe disease than WT mice treated with even control Ab. Like in Fig. 2, both cumulative and maximal disease scores were greater in WT than IL-10RaTdel mice (Fig. 7B). Furthermore, the extent of this increase was similar in PC61 and control Ab-treated cohorts. This finding supports the presence of a Treg-independent effect in governing disease susceptibility.

Competitive analysis of Treg and Teff cell dynamics

To isolate the cell-intrinsic effects of IL-10 on T cells, we competitively analyzed mixed populations of IL-10RaTdel and WT T cells. Because the cells share an identical environment, we could thereby exclude indirect effects of T cell IL-10 signaling that otherwise may influence outcome. We generated chimeric mice in which CD45.2+ GFP-Foxp3 IL-10RaTdel bone marrow cells were mixed with equal numbers of CD45.2+ GFP-Foxp3 WT bone marrow cells and transplanted into lethally irradiated TCRα2/2 hosts. This allowed delineation of the origins of the T cell populations with the CD45.2 congenic marker. EAE development in the chimeric mice was modestly delayed compared with unmanipulated mice, with first symptoms at 16–20 d after induction. Cell populations were monitored by blood sampling and after sacrifice in the spleen and CNS.

Similar proportions of IL-10RaTdel and WT CD4+ T cells, Foxp3+ or Foxp3−, were present in the blood at different time points prior to the onset of clinical symptoms (days 0, 8, and 15; Fig. 8A–C). However, in chimeric mice with symptomatic disease, a significant increase in WT versus IL-10RaTdel Teff cells was observed in the spleen and CNS.

FIGURE 6. In situ proliferation of Tregs and Teff in IL-10RaTdel and WT mice. (A) A pulse of BrdU was administered to IL-10RaTdel and WT mice with EAE (day 14). At 20 h, CNS and splenic T cells were isolated, and BrdU incorporation was measured by ICS. Percent BrdU positive cells among CD4+TCR+Foxp3− or CD4+TCR+Foxp3+ populations is shown. Graphs show results from individual mice (dots) and mean values (bars). (B) Sample flow cytometry plots of BrdU staining from individual mice in (A). (C) Mice were analyzed 21 d after EAE induction as in (A). Data are cumulative from two experiments. Scores of analyzed mice were: day 14 WT: 3,3,3,3,3,3,3,3,2,2; day 14 IL-10RaTdel: 4,3,2,2,1,1,1,1,1; day 21 WT: 3,3,3,2,2,2,2,1,1,1,1; day 21 IL-10RaTdel: 3,2,2,1,1,1,1,1,1. ***p < 0.001.

FIGURE 7. EAE in Treg-depleted IL-10RaTdel and WT mice. WT or IL-10RaTdel mice were treated with PC61 anti-CD25 Ab or isotype control on day −3 prior to EAE induction and elimination of Treg confirmed in the blood on day 0. (A) EAE was induced in WT and IL-10RaTdel mice and clinical disease monitored. Clinical score tracked longitudinally in mice treated with PC61 or control Ab showed that PC61 treatment led to increased disease severity in both cohorts of mice. (B) The ratio of mean cumulative disease scores (area under the curve [AUC]), maximal disease scores (Max), or time to initial disease (Init) for IL-10RaTdel and WT mice from three independent experiments is plotted. Although PC61 treatment led to overall more severe disease, the ratio of each parameter between IL-10RaTdel and WT mice was not significantly altered by PC61 treatment.
observed in the CNS. A similar trend was apparent for Tregs, although this did not achieve significance. Foxp3<sup>+</sup> Teff from diseased mice were further segregated into memory or activated (CD44<sup>hi</sup>) and naive (CD44<sup>lo</sup>CD62L<sup>hi</sup>) populations. The memory or activated populations were selectively skewed toward WT T cells, and this was true in both the spleen and CNS (Fig. 8D). In contrast, the naive populations showed a WT:IL-10R<sup>−/−</sup> ratio similar to that of cells prior to immunization (preimmune blood, 1.2 ± 0.4; naive spleen, 1.2 ± 0.4; memory spleen, 2.1 ± 0.9; naive CNS, 1.4 ± 0.7; memory CNS, 2.7 ± 1.5). Therefore, activated but not naive WT T cells outcompete corresponding IL-10R<sup>−/−</sup> T cells in the chimeric mice.

As an alternative approach to verify these findings and competitively evaluate the cell-intrinsic effect of IL-10 on T cell responses, one excluding thymic generation of new T cells, mature WT and IL-10R<sup>−/−</sup> cells were mixed and cotransferred into WT recipients. Host and transferred populations could be distinguished by congenic markers (host, CD45.1<sup>+</sup>CD45.2<sup>+</sup>Thy1.1<sup>+</sup>; WT, CD45.1<sup>+</sup>CD45.2<sup>−</sup>Thy1.1<sup>+</sup>). EAE was induced and cells were monitored in the blood or, after sacrifice, in organs. Owing to the small numbers of transferred cells detected, event numbers precluded effective monitoring of transferred Tregs; however, Foxp3<sup>+</sup> T cells could be readily quantified. Prior to disease induction (day 0), an approximately equal number of WT and IL-10R<sup>−/−</sup> CD4<sup>+</sup> T cells was seen in the blood (Fig. 8E, 8F). This ratio was maintained at day 7. By day 16, after EAE development, a significantly increased proportion of WT T cells was observed in the spleen. A more dramatic increase was evident within the CNS (WT/IL-10R<sup>−/−</sup> ratio: preimmune blood day 0, 0.68 ± 0.29; day 7 blood, 0.83 ± 0.03; day 16 spleen, 1.7 ± 0.3; day 16 CNS, 8.7 ± 1.2). Skewing of the splenic response toward WT cells was specific to the activated or memory population (Fig. 8G; naive day 16, 1.1 ± 0.1; memory day 16, 2.3 ± 0.4). A corresponding analysis of differential skewing among naive and activated or memory populations was not possible in the CNS because of the paucity of naive T cells at this location (>90% memory) and the small number of transferred cells detected. Nevertheless, these results confirm findings in the bone marrow chimeras and indicate that IL-10 signaling provides a cell-intrinsic competitive advantage to WT Teff cells during EAE.

**Discussion**

IL-10 controls inflammation, whether infectious, autoimmune, or toxic. Its production, proportionate to the level of inflammation, provides negative feedback against excessive responses (37). IL-10 lacks recognized proinflammatory activities in T cells. Despite this, we show that IL-10 promotes autoimmune inflammation in EAE. Our data support a cell-intrinsic role for IL-10 in the long-
IL-10 can regulate genes inhibiting cell cycle progression. In accordance with this, we observe that WT T cells proliferate less rapidly than IL-10R^Kdelt^ cells in the CNS in vivo. Implicitly, IL-10 is acting to restrain autoreactive T cell expansion. The decreased rate of cell cycling in the WT CNS contrasts with the equivalent proliferation of CNS T cells measured in vitro. This indicates that environmental conditions present in the WT CNS inhibit WT T cell cycling, and that these conditions are not present when T cells are purified and restimulated with Ag and feeder cells in culture. The decreased cell cycling in the CNS of WT mice is also incongruously associated with an increased accumulation of CNS-infiltrating T cells and more severe clinical disease. This cannot be explained solely by the decreased proportion of Tregs seen early in disease in WT mice. IL-10R^Kdelt^ mice depleted of Tregs still showed less severe EAE than similarly treated WT mice. More severe disease would be expected if the effects of IL-10 were wholly Treg dependent. Moreover, WT T cells outcompete IL-10R^Kdelt^ T cells in mixed chimeras, both bone marrow chimeras and after the cotransfer of mature WT and IL-10R^Kdelt^ T cells, indicating that the effect of IL-10 on T cell dominance is cell intrinsic. Therefore, IL-10 supports the Treg response in EAE despite its suppression of T cell proliferation in situ, and this is independent of altered Treg quantities or function.

Our data are also inconsistent with IL-10 altering the functional profile of autoreactive T cells. A classical pattern of clinical and histologic disease was seen in the IL-10R^Kdelt^ mice. Histologic patterns may be altered by perturbations in T cell profiles (38). Cytokine production by responding T cells was also unaffected. Likewise, expression of relevant chemokine receptors and addressins failed to differentiate WT and IL-10R^Kdelt^ populations, implying that the cells had similar capacity to localize to the inflamed CNS.

The balanced inhibition of T cell subsets in this study contrasts with what has been observed in studies of mucosal immunity in mice bearing T cells expressing a dominant negative IL-10R or with a selective deficiency of IL-10R on Tregs (22–24). In those Tregs, a specific effect on Th17 accumulation was documented. Indeed, we have observed a similar skewing of the IL-10R^Kdelt^ T cells in the CNS in vivo. Implicitly, IL-10 acts to restrain autoreactive T cell expansion and promoting their persistence, when integrated over time, determines outcome. Cues that differentially alter IL10's influence on proliferation inhibition and T cell survival may thereby alter the net effect of IL-10 on T cell-mediated immune responses.

Presumably, increased attrition of cells unable to respond to IL-10 results from their apoptotic death. Apoptotic cells are rapidly cleared in vivo, and measuring ongoing rates of apoptosis in situ is technically difficult. Nevertheless, we observed diminished MOG-induced proliferative response among peripheral IL-10R^Kdelt^ splenocytes and a selective competitive advantage among memory-activated WT T cells in mixed chimeras, indicating that the autoreactive T cells are not sustained in the absence of T-specific IL-10 responsiveness. Cytokine withdrawal or activation-induced apoptosis would seem the most likely causes. Further breeding of IL-10R^Kdelt^ mice onto backgrounds with altered apoptosis susceptibilities will be important to directly evaluate the hypothesis that IL-10 alters cell death rates and to interrogate mechanism.

Global IL-10 deficiency leads to increased EAE severity (27). That T-specific IL-10 unresponsiveness attenuates disease indicates that the effect of IL-10 on T cells is counterbalanced by more potent activities on other cell types. Indeed, myeloid-derived APCs and effector cells express higher levels of IL-10R than do T cells, and these and the glial population that more directly mediates myelin damage would seem probable IL-10 targets. Dissecting responsible cell types is possible using conditional systems, such as that described here, which were not previously available. Nevertheless, that T cell directed IL-10 has an ultimately pathologic effect does emphasize the importance of directed targeting by therapeutically administered IL-10, and might explain the conflicting results on the efficacy of pharmacologically administered IL-10. Our results argue that it is the balance of opposing effects of IL-10 both within individual cell types, where cell preservation may coincide with decreased proliferation, as well as across lineages that defines ultimate outcome.

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Disclosures
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References


