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Immunotherapy with Costimulatory Dendritic Cells To Control Autoimmune Inflammation

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Costimulation-deficient dendritic cells (DCs) prevent autoimmune disease in mouse models. However, autoimmune-prone mice and humans fail to control expansion of peripheral autoreactive effector memory T cells (TEMs), which resist immunoregulation by costimulation-deficient DCs. In contrast, activation of DC costimulation may be coupled with regulatory capacity. To test whether costimulatory DCs control TEMs and attenuate established autoimmune disease, we used RelB-deficient mice, which have multiorgan inflammation, expanded peripheral autoreactive TEMs, and dysfunctional Foxp3+ regulatory T cells (Tregs) cells and conventional DCs. TEMs were regulated by Foxp3+ Tregs when costimulated by CD3/CD28-coated beads or wild-type DCs but not DCs deficient in RelB or CD80/CD86. After transfer, RelB and CD80/CD86-sufficient DCs restored tolerance and achieved a long-term cure of autoimmune disease through costimulation of TEMs and Foxp3+ Treg IFN-γ production, as well as induction of IDO by host APCs. IDO was required for regulation of TEMs and suppression of organ inflammation. Our data challenge the paradigm that costimulation-deficient DCs are required to regulate established autoimmune disease to avoid TEM activation and demonstrate cooperative cross-talk between costimulatory DCs, IFN-γ, and IDO-dependent immune regulation. IFN-γ and IDO activity may be good surrogate biomarkers measured against clinical efficacy in trials of autoimmune disease immunoregulation. The Journal of Immunology, 2011, 187: 4018–4030.

Dendritic cells (DCs) comprise a heterogeneous group of APCs with many specialized functions, including uptake, processing, and presentation of Ag and costimulation of naive T cells for immune priming. Moreover, mice in which DCs and Langerhans cells are extensively ablated develop severe autoimmune disease associated with myeloid proliferation and T and B lymphocyte activation, demonstrating the essential constitutive role that DCs play in peripheral self-tolerance (1). The mechanisms by which DCs maintain peripheral tolerance are still unclear, but they include central or peripheral clonal deletion, ignorance, anergy, and regulation, both through induction of functional regulatory T cells (Tregs) and through immunosuppressive mechanisms (2). The elucidation of tolerance mechanisms has important implications for development of Ag-specific immunotherapeutic strategies for autoimmune and allergic disease and allografting. Considerable evidence has demonstrated that resting or immature DCs promote peripheral tolerance constitutively, through the presentation of endogenous or apoptotic-derived self-Ag to naive CD4+ T cells, the cross-presentation of tissue-derived self-Ag to CD8+ T cells, and deletion or regulation of potentially self-reactive peripheral T cells (3–7). These principles have been used to demonstrate the usefulness of resting or costimulation-deficient DCs in tolerizing immunotherapeutic strategies. For example, transfer of hematopoietic stem cell precursors of resting DCs encoding self-Ag regulates Ag-specific CD4+ T cells in autoimmune-prone mice (8), and transfer of Ag-loaded DCs generated from mice deficient in the RelB subunit of NF-κB or CD40 regulates naive or effector CD4+ T cells in nonautoimmune-prone C57BL/6 mice through induction of Ag-specific Tregs (9, 10).

In contrast, activated or mature DCs costimulate T cells through cell surface CD80/CD86 and secreted proinflammatory cytokines. DC activation may also induce regulatory molecules, including programmed cell death 1 ligands 1 and 2, IL-10, and the enzyme IDO (11–13). Through catabolism of tryptophan, functional IDO promotes tolerance through Foxp3+ Treg differentiation and function, as well as inhibition of T cell proliferation (14, 15). The expression of IDO by costimulatory DCs is further reinforced under conditions of immune activation, after reverse signaling by T cells through GITR ligand or CD80/CD86 or stimulation by IFN-γ or TLR ligands. Recruitment of IDO+ macrophages, B cells, and stromal cells also occurs through this mechanism (16, 17).

Despite the evidence for use of costimulation-deficient DCs for Ag-specific prevention of autoimmune disease, autoimmune-prone mice and humans with central tolerance defects fail to control...
expansion of autoreactive effector memory T cells \( (T_{EM}) \) in the periphery, and these autoreactive T cells resist Ag-specific regulation using or targeting costimulation-deficient DCs. For example, in the NOD model of type 1 diabetes, the effectiveness of tolerizing immunotherapies, such as anti-CD40L, rapamycin, or DCs treated with NF-κB inhibitors, is greater when administered to young mice than to mice with advanced insulitis and expanded \( T_{EM} \) (18–23). Identification of immunotherapeutic DC strategies for autoreactive \( T_{EM} \), is critically important for clinical translation to control existing autoimmune disease, because it is very challenging to trial at-risk patients, and disease-specific \( T_{EM} \) and even subclinical organ inflammation can be identified in patients prior to symptoms (24, 25). Given this background, we hypothesized that, in contrast to naive T cells, immunotherapy using DCs with costimulatory capacity would be required for effective control of \( T_{EM} \) and attenuation of established autoimmune disease.

To test this, we made use of RelB-deficient mice, which have a central tolerance defect, expansion of autoreactive \( T_{EM} \) in the periphery, and multiorgan inflammatory disease, coupled with reduced numbers and costimulatory function of conventional DCs. Although microbial inflammatory or T cell-derived signals normally induce the nuclear translocation and transcriptional activity of RelB in DCs, inflammatory induction of MHC class II and costimulatory molecules is impaired in RelB−/− DCs (10, 26). T cell stimulatory function of RelB−/− DCs, including priming and cross-presentation of exogenous Ag, and control of infections are deficient (10, 27, 28). Mice lacking RelB develop thymic medullary atrophy (29, 30) and, consequently, have impaired thymic negative selection and T cell autoreactivity (31). Consistent with their central tolerance and DC defects, RelB−/− mice develop spontaneous Th2 T cell-dependent multisystem autoimmune, myeloproliferative, and allergic disease from birth, characterized by failure to thrive; splenomegaly (lymph nodes are absent); inflammatory disease of skin, eyes, lungs, liver, and pancreas; high levels of serum IgE; and death by ~3 mo of age (29, 30). T cell-deficient RelB−/− mice do not develop inflammatory disease, indicating a defect in the control of effector T cell or Treg functions in these mice either directly or as result of the lack of interaction with mature DCs with high RelB activity (32–34). We show in this article that \( T_{EM} \), in this model are efficiently regulated by Foxp3+ Tregs when costimulated by RelB-sufficient DCs but not DCs deficient in RelB or CD80/CD86. After transfer, a small number of RelB and CD80/CD86-sufficient DCs could restore tolerance and achieve a long-term cure of autoimmune disease in RelB−/− mice through costimulation of IFN-γ production by \( T_{EM} \) and Foxp3+ Tregs, leading to RelB-independent induction of IDO expression and function by host APCs. In turn, IDO was required for regulation of \( T_{EM} \) function and suppression of organ inflammation. Our data demonstrated the cooperative relationship between costimulatory DCs and IDO-dependent immune regulation through IFN-γ-dependent cross-talk.

Materials and Methods

**Mice**

Experiments were approved by the University of Queensland Animal Ethics Committee. All mice were maintained in specific pathogen-free conditions. Mice homozygous for an insertional mutation in the RelB gene (designated RelB−/−) on a C57BL/6 background, were generated as described (29) and used for experiments at 4–9 wk of age. RelB−/− mice display neither pathology nor immune dysfunction and were used as the source of RelB-deficient DCs. CD80/CD86−/− mice (35) were obtained from Jackson Laboratories. RelB−/− radiation chimeras, generated by transferring bone marrow from RelB−/− (or RelB−/− bone marrow for control chimeras) into lethally irradiated C57BL/6 hosts (Animal Resource Centre, Perth, Australia), were used after ~8 wk of reconstitution. For transfer studies,

RelB−/− mice were bred with B6.SJLPtprc (H-2b CD45.1) mice to generate RelB−/− (CD45.1, CD45.2) progeny. Five million splenic CD11c+ DCs from these mice were injected into RelB−/− recipients to track migration of RelB−/− DCs.

**Cell purification**

CD11c+ DCs were isolated from disaggregated, collagenase-treated spleens using CD11c-conjugated immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The negative fraction was immunomagnetically depleted of non-T cells and CD8+ cells. Enriched CD4+ T cells were fractionated into CD25+ and CD25− populations. CD4+CD25+ T cells were further purified using CD4-coated beads, Splenic CD11c+ DCs and CD4+CD25− and CD4+CD25+ T cells were >90% pure, by flow cytometry. CD4+ and CD4+ T cells were purified from enriched CD4+ T cells by staining with CD44-PE and then selecting with anti-PE beads.

**Suppression assay**

To assess suppressor function of CD4+CD25+ T cells, 25 × 10^5 purified splenic DCs were incubated with 50 × 10^5 CD4+CD25+ T cells and 50 × 10^5 CD4+CD25− T cells in the presence of 4 μg/ml anti-CD3 (clone 2KCl1; BD) for 3 d in 96-well round-bottom plates, followed by [3H]thymidine (1 μCi/well; Perkin Elmer, Wellesley, MA) overnight. Results are expressed as mean cpm ± SEM of triplicate wells.

**In vitro T cell cytokine production**

Purified T cells were plated at 5 × 10^5 cells/well and stimulated with immobilized anti-CD3 (BD Pharmingen, San Diego, CA) for 72 h. Supernatants were assayed for cytokine levels using mouse Th1/Th2 and inflammation kits (BD). For intracellular staining, T cells were stimulated with PMA/ionomycin in the presence of brefeldin and then stained for surface markers and intracellular cytokines.

**Flow cytometric analysis**

Organs were disaggregated and filtered, and RBCs were lysed. Cells were stained for 30 min on ice in blocking buffer. Ab clones included CD4 (GK1.1), Foxp3 (FJK-16s), CD44 (IM78.1), CD62L (MEL14), CD69 (H1.2F3), CD103 (2E7), GITR (DTA-1), CD11c (N418), CD45.1 (A20), CD40 (3/23), CD80 (16-10A1), and CD86 (GL-1).

**In vivo Ab and BrdU administration**

RelB−/− DC-treated or untreated RelB−/− mice were injected i.p. with 2 mg BrdU (Roche, Basel, Switzerland) every 12 h for 3 d. Splenocytes were harvested 3 d later, stained for surface CD4 and CD25, and then fixed, permeabilized, and stained with either FITC-labeled anti-BrdU (Serotec, Raleigh, NC) or isotype-control mAb.

**DC transfer**

RelB−/− splenic CD11c+ DCs were purified by MACS or sorted. Either 5 × 10^5 MACS-purified DCs (>90% CD11c+) or 2 × 10^5 sorted CD11c+ (>98% CD11c+) DCs were injected i.v. into each RelB−/− recipient. Recipients were between 5 and 8 wk old, and all showed signs of autoimmune disease before treatment. Mice were monitored every 5 d for weight gain, gait, agility, alopecia, progression of lacrimal disease/keratoconjunctivitis, dermatitis, joint swelling, and feeding. Some mice received BrdU before sacrifice. To test the role of CD80 and CD86, 1 × 10^5 MACS-purified DCs from CD80/CD86−/− mice or from B6.SJLPtprc mice were injected i.v. into each RelB−/− recipient. Transferred B6. SJLPtprc DCs were detected in similar number to transferred B6. SJLPtprc RelB−/− DCs 2 wk later.

**Inhibition of IDO with 1-methyl-dl-tryptophan**

1-methyl-dl-tryptophan (1-MT) was obtained from Sigma-Aldrich and prepared as a 20-mmol/l stock in 0.1 N NaOH, adjusted to pH 7.4, and stored at −20°C protected from light. RelB−/− mice treated or not with DCs were given 2 mg/ml 1-MT solution, supplemented with aspartame, in foil-wrapped standard autoclaved drinking water bottles. Mice drank an average of 5 ml/d, and water was replaced as needed for 3 wk.

**Histology**

Formalin-fixed liver, spleen, and pancreas from RelB−/− DC-treated and control RelB−/− mice, as well as RelB−/− mice, were sectioned and stained with H&E. BrdU incorporation in tissue sections was revealed with HRP-conjugated anti-mouse Ig (Silenus, Victoria, Australia) and...
diaminobenzidine (Biorche Medical, Concord, CA); hematoxylin identified cell nuclei. Images were acquired on a Nikon 50i microscope with NIS-Elements software (Nikon, Melville, NY) using a Nikon Plan Fluor 4×/0.13 2×—WD 17.2 or 40×/0.75 Ph2 DII, ×0.17 WD 0.66 objective at room temperature.

**Immunofluorescence microscopy**

Tissues were harvested and immersed in OCT freezing medium, and 10-μm sections were prepared using a microtome. Sections were fixed in ice-cold acetone, blocked with 5% BSA and 0.1% Tween-20, incubated with mAb in blocking buffer, washed, and mounted with fluorescent mounting medium. Images were acquired on an LSM-510 meta confocal microscope with Zen 2008 software (Carl Zeiss Micro Imaging, Gottingen, Germany) using a Zeiss Plan-Apochromatic 25×/0.1 1-mm Korr DiC M27 objective at room temperature. Ab clones included RelB (C-20), CD11c-allophycocyanin (N418), CD4-PE (RM4-5), Foxp3-Alexa Flour 488 (FJK-16s), and CD45.1-PE (A20). DAPI identified cell nuclei.

**Statistical analysis**

Unpaired, two-tailed Student t tests, α = 0.05, assessed whether the means of two normally distributed groups differed significantly. The Mann–Whitney U test (unpaired) was used when means were not normally distributed or for sample sizes < 10. One-way ANOVA analysis, with the Bonferroni multiple comparison posttest, was used to compare multiple means. All error bars represent SEM.

**Results**

**Reduced numbers and costimulatory capacity of splenic conventional DCs in RelB−/− mice**

We first characterized the effects of RelB deficiency on splenic DC numbers, cell surface phenotype, and response to microbial and T cell-derived signals. As previously described, the number of MHC class II⁺CD11c⁺ conventional DCs in RelB−/− mice was reduced compared with RelB⁺/⁺ mice (Fig. 1A, 1B), as the result of the specific reduction in CD4⁺CD8⁻ DCs (Fig. 1A, 1C) (33, 36, 37). Although DCs freshly isolated from spleens of RelB−/− mice expressed higher cell surface CD40 and MHC class II and equivalent levels of CD80 and CD86 relative to RelB⁺/⁺ DCs (Fig. 1A, 1D), after culture in medium or in the presence of anti-CD40 or LPS, expression of CD40, CD80, and CD86 increased significantly only if DCs expressed RelB, with the exception of a non-significant increase in CD86 by RelB−/− DCs stimulated with LPS. There was no difference in viability between cultured cells from each strain. Similarly, in contrast to RelB⁺/⁺ DCs, RelB−/− DCs secreted lower levels of IL-1β, IL-6, and IL-12p70 in response to anti-CD40 or LPS and no significant difference in IL-10 secretion (Fig. 1E), further demonstrating their reduced costimulatory capacity. Because these data are consistent with previous analyses of DCs from RelB−/− radiation chimeras, which have no inflammatory disease, this reduction in specific cytokine secretion by DCs is RelB intrinsic and not due to DC “exhaustion” in the face of chronic autoimmunity inflammation (34). In response to LPS, RelB−/− and RelB⁺/⁺ DCs produced similar levels of TNF. Thus, consistent with their prior activation upon exposure to chronic autoimmunity inflammatory stimuli, including TNF, splenic RelB−/− DCs resembled tolerant semi-mature DCs, which are resistant to the LPS- or anti-CD40-mediated upregulation of costimulatory capacity observed for RelB⁺/⁺ DCs (38, 39). This tolerant phenotype was evident whether splenic RelB−/− DCs were CD8⁺, CD4⁺, or double negative (data not shown). These data indicated that RelB−/− mice, which develop severe multi-system autoimmune disease, also have a reduction in numbers of splenic DCs and their capacity for upregulation of costimulatory molecules and cytokines upon activation. Therefore, they represent an ideal model in which to test the relationship between DC costimulatory function and autoimmune disease.

**Autoreactive RelB−/− T_EMs, are not suppressed by Foxp3⁺ Tregs unless costimulated by RelB⁺/⁺ DCs**

We next analyzed the function of T_EMs and Tregs as a result of interaction with RelB−/− DCs. RelB−/− mice were relatively lymphopenic (Fig. 2A), and the percentage of CD4⁺Foxp3⁺ Tregs was increased 2-fold in the spleen compared with RelB⁺/⁺ mice (data not shown), similar to other lymphopenic autoimmune mouse models (e.g., where TCR signaling is attenuated) (40). Hence, the numbers of splenic CD4⁺Foxp3⁺ T cells in RelB−/− mice were comparable to RelB⁺/⁺ mice (Fig. 2A). Splenic CD4⁺Foxp3⁺ T cells from RelB−/− mice were enriched in activated T_EMs, relative to RelB⁺/⁺ mice, with increased proportions of CD4⁺CD44hi, CD69⁺, and CD62L⁻ cells (Fig. 2B). Splenic CD4⁺Foxp3⁺ Tregs isolated from RelB−/− mice were also enriched in CD44⁺ and CD62L⁻ cells and expressed higher levels of activation markers associated with Treg function, including CD69, CD103, and GITR, than did RelB⁺/⁺ Tregs (Fig. 2E). When stimulated with anti-CD3 mAb, the RelB−/− CD4⁺CD44hi T_EMs were functional and secreted higher levels of IL-4, IL-5, and IL-10 and reduced levels of IFN-γ and IL-2 than did RelB⁺/⁺ CD4⁺CD44hi T_EMs (Fig. 2D). The proportion of CD4⁺Foxp3⁺ Tregs was similar in spleen, pancreas, liver, and lung of RelB−/− mice (Fig. 2E). The increased numbers of activated CD4⁺CD44hi Tregs preferentially secreting Th2-type cytokines in RelB−/− mice are consistent with peripheral expansion of autoreactive T_EMs, in the absence of effective regulation by Foxp3⁺ Tregs, and with the autoimmune inflammatory dermatitis and eosinophilic infiltrates, as described in lung and skin of these mice (41). A deficiency in Treg function is supported by the pathologic similarities (scaling eyelids and tail, splenomegaly, and lymphocytic infiltrates in the skin, liver, and lungs) between Foxp3-deficient mice and RelB−/− mice.

Therefore, we tested the capacity of RelB−/− splenic CD4⁺CD25⁻ T cells, which are enriched in T_EMs, to be suppressed by autologous Tregs in vitro in the presence of splenic DCs and anti-CD3. RelB−/− DCs failed to stimulate the proliferation of RelB−/− CD4⁺CD25⁻ T cells in the presence of anti-CD3, and RelB−/− CD4⁺CD25⁺ T cells could not be suppressed by CD4⁺CD25⁺ Tregs when stimulated by RelB−/− DCs (Fig. 3A). When stimulated by RelB⁺/⁺ DCs, RelB−/− CD4⁺CD25⁻ T cell proliferation increased; concomitantly, RelB−/− Tregs suppressed the proliferation of RelB−/− CD4⁺CD25⁻ T cells, suggesting that RelB−/− DCs fail to deliver the necessary signals to support suppression of T_EM proliferation by Tregs (Fig. 3A). Once stimulated by RelB⁺/⁺ DCs, RelB−/− Tregs suppressed RelB−/− CD4⁺CD25⁻ T cells even more effectively than did RelB⁺/⁺ Tregs, consistent with their elevated expression of Treg activation markers. Unlike RelB−/− CD4⁺CD25⁻ T cells, RelB⁺/⁺ CD4⁺CD25⁻ T cells were signaled to proliferate, albeit weakly, by RelB−/− DCs. In this case, RelB−/− Tregs again suppressed CD4⁺CD25⁻ T cell proliferation (Fig. 3A). Indeed, proliferation of RelB−/− CD4⁺CD25⁻ T cells was significantly reduced, compared with RelB⁺/⁺ CD4⁺CD25⁻ T cells, either in response to RelB−/− DCs (p < 0.05) or RelB⁺/⁺ DCs (p < 0.001) and anti-CD3 (Fig. 3A). The difference in proliferative response of RelB−/− and RelB⁺/⁺ CD4⁺CD25⁻ T cells is consistent with the activated T_EM phenotype of RelB−/− CD4⁺CD44hi T cells, which have been shown to produce low levels of IL-2 and to upregulate CD25 when stimulated, leading to a net consumption of IL-2 (42). Thus, responsiveness of effector T cells to suppression when weakly costimulated by RelB−/− DCs depends on the enrichment in autoreactive T_EMs because RelB−/− CD4⁺CD25⁻ T cells remained susceptible to suppression by RelB−/− Tregs. The data indicated that activated RelB−/− peripheral T_EMs, escape suppression by Foxp3⁺ Tregs if DC signaling
FIGURE 1. Reduced numbers and costimulatory capacity of splenic conventional DCs in RelB−/− mice. Collagenase-digested and disaggregated splenocytes from RelB+/− and RelB−/− mice were stained for MHCII⁺CD11c⁺ DCs (T, B, and NK cells excluded) and CD4 and CD8. A, Proportion of CD4⁺ and CD8⁺ splenic DCs. B, Number of splenic MHCII⁺CD11c⁺ DCs. C, Number of splenic DC subsets. Results are representative of four individual mice. D, Splenocytes were cultured with or without anti-CD40 or LPS for 24 h in medium, and MHCII⁺CD11c⁺ DCs were stained for CD40, CD80, and CD86 and analyzed by flow cytometry. Mean fluorescence intensity (MFI) before and after culture is shown. E, Cytokine production from CD11c bead-purified RelB+/− or RelB−/− DCs cultured for 72 h with or without anti-CD40 or LPS. Data show mean ± SEM of two separate experiments analyzing 5–10 individual mice. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 (B, C, t test; D, E, one-way ANOVA followed by Bonferroni post hoc test).
is inadequate because of a RelB deficit. In functional assays, the capacity of Tregs to suppress CD4+CD25+T cells was not restored by addition of anti–IL-6 or anti–IL-1 to the cultures (data not shown), indicating that the defect did not result from T cell resistance due to excessive proinflammatory cytokine secretion; rather, expression of RelB by DCs is required for appropriate signaling of RelB+/−/− TEMs (43, 44).

Therefore, we tested whether costimulation of T cells by anti-CD3/anti-CD28 mAb-coated beads could provide the necessary signal for suppression of RelB+/−/− TEMs when stimulated by RelB+/− DCs. The additional bead-derived costimulatory signals promoted the proliferative response and associated IL-2 production by RelB+/− CD4+CD25− T cells and made them more susceptible to suppression by RelB+/− Tregs (Fig. 3B).

Anti-CD3/CD28 mAb-coated beads enhanced production of both Th1 and Th2 cytokines by TEMs, but did not shift the cytokine profile from Th2 to Th1 (Fig. 3B). Thus, the failure of RelB+/− Tregs to suppress CD4+CD25− T cells when stimulated by RelB+/− DCs is not due to a failure to suppress the predominant Th2 cytokines secreted by RelB+/− TEMs. Rather, when adequately costimulated, RelB+/− TEmss secreted even higher levels of Th2 cytokines, as well as Th1 cytokines, and all cytokines became concomitantly susceptible to suppression. Addition of exogenous IL-2 to cultures of RelB+/− CD4+CD25− TEMs, or Tregs stimulated by RelB+/− DCs enhanced proliferation (p < 0.05; Fig. 3B), although to a lesser extent than did CD3/CD28 mAb-coated beads or RelB+/− DCs (p < 0.05; Fig. 3A, 3B), consistent with the conclusion that net IL-2 consumption underlies the T cell

**FIGURE 2.** Expansion of activated Th2 TEMs in periphery of RelB+/− mice. Collagenase-digested and disaggregated splenocytes from RelB+/+ and RelB−/− mice were stained for CD4-PE and Foxp3-FITC. A, CD4+ and Foxp3+CD4+ cells were enumerated by flow cytometry, and groups were compared. Each symbol represents an individual mouse. *p ≤ 0.05 (Mann–Whitney U test, tested over two experiments). Splenocytes were stained with T cell activation markers as shown, and CD4+Foxp3− (B) or CD4+Foxp3+ (C) T cells were gated; the proportion of CD4+ cells expressing Foxp3 was 14.2 ± 0.9% (mean ± SEM, n = 4) in RelB+/+ mice and 32.4 ± 1.2% (mean ± SEM, n = 7) in RelB−/− mice. D, CD4+CD44+ effector T cells purified from RelB+/+ or RelB−/− spleens were cultured for 3 d with immobilized anti-CD3. Cytokine production was measured in supernatants by cytokine bead array. Data are mean ± SEM of duplicate samples from individual mice and are representative of three separate experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; t test. E, Cells isolated from collagenase-digested tissues from RelB+/+ or RelB−/− mice were stained for CD4, CD44, and Foxp3 and analyzed by flow cytometry. The percentage of Foxp3+ T cells in the CD44+CD4+ population is indicated. Representative of seven mice in two separate experiments.
proliferation defect, at least in part, in the presence of costimulation-poor RelB−/− DCs (42).

Transfer of RelB+/− costimulatory DCs arrests autoimmune inflammatory disease in RelB−/− mice

To demonstrate the principle that increasing costimulation restores the capacity of Tregs to suppress TEMs mediating Th2 inflammation in vivo, we adoptively transferred RelB+/-DCs into RelB−/− recipients to promote suppression of inflammation by the Foxp3+ Tregs already present in the periphery in high numbers. To track the DCs, we crossed RelB−/− mice with CD45.1 congenic mice to generate RelB+/− DC donors expressing CD45.1 and CD45.2. CD11c+ splenic DCs purified from these mice were transferred i.v. into CD45.2 RelB−/− recipients between 5 and 8 wk of age, when all had developed autoimmune disease. Three days later, the mice were sacrificed, and the distribution and phenotype of donor DCs were analyzed. Donor CD45.1+CD11c+ DCs were found in the spleen, liver, and lung but not the thymus. CD80 and CD86 were expressed by a higher proportion of RelB+/- donor DCs than RelB−/− recipient DCs (Fig. 4A). CD40 was expressed by a lower proportion of RelB+/- donor DCs than recipient RelB−/− DCs (Fig. 4A), consistent with strain differences ex vivo (Fig. 1D). By immunofluorescence staining, Foxp3+ Tregs were found in close proximity to nuclear RelB+CD11c+ DCs in the splenic peri-arteriolar lymphoid sheath in RelB+/− mice (Fig. 4B). No RelB staining was observed in spleens from RelB−/− mice (data not shown). In RelB−/− mice treated with RelB+/− DCs, Foxp3+ Tregs contacted CD11c+ DCs. Although infrequent, CD45.1+ donor DCs also contacted Foxp3+ Tregs (Fig. 4C). After transfer, donor DCs expressed RelB in the cytoplasm and the nucleus (Fig. 4D). In suppression assays using CD45+CD25+ T cells and Tregs isolated from spleens of untreated (Fig. 5A, left) or RelB−/− DC-treated mice (Fig. 5A, right), Tregs from untreated RelB−/− mice were unable to suppress proliferation of CD45+CD25+ T cells were stimulated with CD11c+ splenic DCs purified from RelB−/− or RelB+/- mice and anti-CD3, with or without RelB−/− or RelB+/− CD4+CD25+ T cells. Mean (+ SEM) proliferative response is shown. A, RelB−/−CD4+CD25+ T cells were stimulated with RelB+/− splenic DCs and anti-CD3 in the absence or presence of 10 U/ml IL-2 or CD3/CD28 mAb-coated beads, with or without RelB−/− CD4+CD25+ T cells. Mean (+ SEM) proliferative response and cytokine production are shown. Results are representative of three separate experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; one-way ANOVA, followed by the Bonferroni post hoc test.
BrdU incorporation by CD4+CD25−RelB 

Conversely, untreated RelB and eye luster improved rapidly and remained at a healthy level mice receiving RelB3/5-reduced after transfer of RelB+/−transferred i.v. into RelB condition (Fig. 5D). These data were confirmed in vivo by analysis of BrdU incorporation by inflammatory cells observed in RelB−/−mice. In contrast, stained sections of pancreas from DC-treated RelB−/−mice showed minimal inflammation with normal acinar cell architecture and pancreatic and islet cell morphology, with only rare parenchymal cells incorporating BrdU. These findings suggested that the pancreas had undergone regeneration in the DC-treated mice. In stained liver sections, heavy peri-portal infiltration and BrdU incorporation by inflammatory cells observed in RelB−/−mice were no longer observed after transfer of RelB+/−DCs. In the spleen, extramedullary hematopoiesis with increased numbers of erythroblasts and megakaryocytes and inflammatory infiltration of the red pulp were present. In RelB−/−mice receiving RelB+/−DCs, infiltration and number of erythroblasts and megakaryocytes were reduced, resulting in larger areas of red pulp (Fig. 5D). Importantly, a single DC treatment afforded long-term immunoregulation, with mice surviving >1 y. Analysis of organs of 12-mo-old DC-treated RelB−/−mice revealed scattered benign infiltrates containing mostly lymphocytes (liver, Fig. 5E).

Given the capacity of anti-CD3/CD28 mAb-coated beads to restore suppression in vitro, as well as the increased levels of expression of CD80 and CD86 by RelB+/−DCs transferred to RelB−/−mice, we tested the capacity of DCs purified from spleens of CD80 and CD86 doubly deficient mice to suppress autoimmune inflammatory disease. Mice treated with DCs lacking CD80 and CD86 showed no improvement in appearance or weight infiltration of lymphoid and myeloid cells into the exocrine pancreas and islets, disruption of acinar cell architecture, atrophy of pancreatic and islet cells, and numerous proliferating cells in the inflammatory infiltrate that incorporated BrdU. In contrast, stained sections of pancreas from DC-treated RelB−/−mice showed minimal inflammation with normal acinar cell architecture and pancreatic and islet cell morphology, with only rare parenchymal cells incorporating BrdU. These findings suggested that the pancreas had undergone regeneration in the DC-treated mice. In stained liver sections, heavy peri-portal infiltration and BrdU incorporation by inflammatory cells observed in RelB−/−mice were no longer observed after transfer of RelB+/−DCs. In the spleen, extramedullary hematopoiesis with increased numbers of erythroblasts and megakaryocytes and inflammatory infiltration of the red pulp were present. In RelB−/−mice receiving RelB+/−DCs, infiltration and number of erythroblasts and megakaryocytes were reduced, resulting in larger areas of red pulp (Fig. 5D). Importantly, a single DC treatment afforded long-term immunoregulation, with mice surviving >1 y. Analysis of organs of 12-mo-old DC-treated RelB−/−mice revealed scattered benign infiltrates containing mostly lymphocytes (liver, Fig. 5E).
FIGURE 5. Transfer of RelB$^{−/−}$ DCs arrests autoimmune inflammatory disease. A, Suppression assays, as described for Fig. 2, were carried out using cells isolated from the spleens of RelB$^{−/−}$ mice or RelB$^{+/−}$ mice, which were treated for 7 d with RelB$^{−/−}$ CD11c$^{+}$ DCs. *p < 0.01; t test. B, RelB$^{−/−}$ mice were injected i.v. with either saline or with RelB$^{−/−}$ CD11c$^{+}$ DCs, along with 2 mg BrdU twice daily for 3 d. Harvested splenocytes were stained with anti-CD4, anti-CD25, along with 2 mg BrdU twice daily for 3 d. Harvested splenocytes were stained with anti-CD4, anti-CD25, anti-BrdU stain of organs collected from RelB$^{+/−}$ DCs. Arrows indicate inflammatory infiltrate; arrowhead (spleen) depicts megakaryocyte. Original magnification $×40$. Scale bar, 100 μm. E, H&E stain of liver isolated from RelB$^{+/−}$ mice treated with RelB$^{−/−}$ DCs. Arrows indicate inflammatory infiltrate; arrowhead (spleen) depicts megakaryocyte. Original magnification $×40$. Scale bar, 100 μm (upper panel, liver) and 20 μm (lower panel, infiltrate). Results are representative of three separate experiments.

Thus, costimulation by CD80 and CD86 is sufficient for memory CD4$^{+}$ T cell expansion and function

We next determined whether the reduction in organ inflammation after RelB$^{−/−}$ DC transfer was indeed associated with reduction in the number of functionally TEmg, Foxp3$^{−}$ CD4$^{+}$ T cells isolated from RelB$^{−/−}$ DC-treated, but not CD80/CD86-deficient DC-treated, mice expressed lower levels of CD44, CD69, and GITR and higher levels of CD62L compared with untreated RelB$^{−/−}$ mice, consistent with the reduced expansion of TEmg after RelB$^{−/−}$ DC transfer (Figs. 5R, 6A, 6B). In contrast, expression of activation markers by CD4$^{+}$Foxp3$^{−}$ Tregs did not change after DC treatment (Fig. 6A), and there was no change in the proportion of CD4$^{+}$ T cells expressing Foxp3 (untreated mice: 21.6 ± 6.2%, n = 8; DC-treated mice: 22.7 ± 6.7%, n = 5). Following DC transfer to RelB$^{−/−}$ mice, the percentage of TUNEL$^{+}$CD44$^{hi}$ TEmg was 4.7 after 3 d and 3.9 after 5 d, whereas 3.5% of CD44$^{hi}$ TEmg were TUNEL$^{+}$ in untreated RelB$^{−/−}$ mice. Thus, RelB$^{−/−}$ DCs with greater costimulatory capacity did not kill TEmg by activation-induced cell death (45). The myeloproliferative disease of RelB$^{−/−}$ mice is associated with the secretion of growth factors and chemokines by Th2 TEmg (46, 47). In RelB$^{−/−}$ mice, CD11b$^{+}$ myeloid cells comprise Gr1$^{−}$ inflammatory macrophages, eosinophils, and basophils and Gr1$^{+}$ monocytes, myeloid progenitors, and neutrophils (Fig. 6C). Associated with the reduction in Th2 effector function by TEmg, CD11b$^{+}$ myeloid cells were reduced in RelB$^{−/−}$ mice treated with RelB$^{−/−}$, but not CD80/CD86-deficient, DCs (Fig. 6C).

Consistent with increased costimulation by RelB$^{−/−}$ DCs, 7 d after RelB$^{−/−}$ DC transfer, cultures of splenic CD4$^{+}$CD25$^{−}$ T cells stimulated by autologous DCs and anti-CD3 produced higher levels of IFN-γ, IL-6, and TNF and lower levels of IL-5 when cells were derived from treated RelB$^{−/−}$ mice than from untreated mice (Fig. 7A). Similarly, CD4$^{+}$CD44$^{hi}$ splenic T cells stimulated with anti-CD3 produced lower levels of IL-5 when purified from RelB$^{−/−}$ DC-treated RelB$^{−/−}$ mice compared with untreated RelB$^{−/−}$ mice (Fig. 7B).

IDO is required for suppression of inflammation in RelB$^{−/−}$ mice

IDO is induced in response to a variety of signals, including IFN-γ and back-signaling of CD80/CD86 by CTLA4 (48). Given the low levels of IFN-γ in RelB$^{−/−}$ mice and the requirement for CD80/
CD86 expression for restoration of tolerance and suppression of T cell proliferation in RelB−/− recipients by RelB+/- DCs, we hypothesized that RelB+/- DC transfer promotes IDO activity. To test this, RelB−/− mice were treated with DCs or left untreated for 3 wk. Groups of mice were administered 1-MT or vehicle in the drinking water. Although the noncanonical NF-κB pathway (inhibitor of NFκB–inhibitor of NFκB–inducing kinase) signaling was shown to be required for CD40L and GITR-Ig–induced IDO expression (49, 50), basal and LPS/IFNγ–induced IDO expression by splenic CD11c+ DCs and MHC class II+CD11c− B cells from

**FIGURE 6.** DC costimulation by transferred DCs is required for control of peripheral inflammation. Splenocytes from untreated, RelB+/- DC-treated, or CD80−/−CD86−/− DC-treated RelB−/− mice were stained with T cell activation markers as shown. CD4+ Foxp3− or CD4+Foxp3+ T cells were gated (A), and the proportion of CD44hi cells (left panel) and CD69+ cells (right panel) among CD4+ T cells was determined (B). *p ≤ 0.05, **p < 0.01; one-way ANOVA, followed by the Bonferroni post hoc test. C, CD11b+Gr1+ and CD11b−Gr1− granulocytes from RelB+/- or RelB−/− mice were sorted, and cytospins were prepared and stained with Giemsa (left panel). Right panel, Percentage of CD11b+ cells isolated from spleens of untreated, RelB−/− DC-treated, or CD80−/−CD86−/− DC-treated RelB−/− mice, as determined by flow cytometry. Original magnification ×600. Data points represent individual mice analyzed over two separate experiments. **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA, followed by the Bonferroni post hoc test.

**FIGURE 7.** Transferred RelB+/- DCs reduce peripheral TEM-driven inflammation. A, Cytokine production from cultures of CD4+CD25− T cells and DCs purified from spleens of RelB+/- DC-treated or untreated RelB−/− mice, and anti-CD3 mAb. B, CD4+CD44hi T cells purified from DC-treated or untreated RelB−/− spleens were cultured for 72 h with immobilized anti-CD3, and cytokine levels were measured in supernatants by cytokine bead array. *p ≤ 0.05, **p ≤ 0.01; one-way ANOVA, followed by the Bonferroni post hoc test. Data represent the mean of duplicate samples from individual mice and are representative of three separate experiments. C, Cytokine levels were measured at sacrifice in the serum of untreated, RelB−/−, and RelB+/- DC-treated RelB−/− mice. Data represent measurements from individual mice analyzed in duplicate.
RelB+/− and RelB−/− mice was equivalent (Fig. 8A, 8B). These data indicated that IDO is not intrinsically deficient in RelB−/− mice and is inducible in response to LPS/IFN-γ. Consistent with our hypothesis, basal IDO expression was increased in RelB−/− mice treated with RelB+/- DCs relative to untreated controls (Fig. 8B). Although expressed by APCs in untreated RelB−/− mice, IDO did not effectively suppress autoimmune disease until further induced by RelB+/− DCs. IDO function was required for disease suppression and functional immune restoration, because RelB−/− mice treated with RelB+/− DCs had less liver damage (aspartate aminotransferase) and higher proportions of IFN-γ− effecter T cells and Tregs and lower proportions of IL-4+ and IL-5+ T cells. These proportions, as well as liver damage, were reversed by 1-MT in RelB−/− DC-treated mice (Fig. 8C, 8D). Taken together, our data indicated that DCs restore tolerance and achieve a long-term cure of autoimmune disease in RelB−/− mice through CD80/CD86 costimulation of T cell IFN-γ production by effecter T cells and Tregs, as well as induction of IDO expression and function by host APCs. This is required for regulation of effector T cell function and suppression of organ inflammation.

Discussion

Functional Foxp3+ Tregs are required to maintain peripheral tolerance, to prevent autoimmune disease, and to control inflammation through suppression of innate immunity and effecter T cells (51, 52). Although functional proliferating peripheral Foxp3+ Tregs infiltrate the inflamed organs and spleen of RelB−/− mice (in equivalent numbers to RelB+/− mice), they are incapable of controlling proliferation and cytokine production by autoreactive TEMs when signaled by semi-mature DCs deficient in RelB or CD80/CD86−/− DCs. In vitro, we showed that autoreactive CD44hi TEMs escape suppression by RelB−/− Tregs as a result of insufficient costimulation by RelB−/− DCs and a net consumption of IL-2, consistent with a previous study (42). It is noteworthy that patients with autoimmune disease show similar defects in TCR signaling and replicative capacity (53, 54). Remarkably, tolerance is restored in RelB+/− mice as a result of suppression of Th2-type autoreactive TEMs, in vivo by Tregs in an IDO-dependent manner after transfer of costimulatory DCs, even when severe peripheral organ inflammation is present. Consistent with this CD80/86-dependent costimulation, IL-2 increased in the serum of treated mice. Costimulation through CD28 is a well-described mechanism for increased IL-2 production by T cells (55). Moreover tolerance is long-lived, resulting in survival for ≥12 mo. Thus, DC-encoded costimulation restores tolerance through a process of self-perpetuating peripheral regulation.

IDO is an important mediator of tolerance, which is expressed by multiple APCs, including plasmacytoid DCs, macrophages, and B cells (16, 48, 56–58). In DCs, IDO is induced by IFN-γ and other T cell-derived signals, such as CD40L, GITR, and CTLA4 (15, 48, 50, 59). After CD40 stimulation of human DCs or GITR ligand stimulation of murine DCs, induced IDO was blocked by inhibition of alternate NF-κB pathway inhibitor of NFκB–inducing kinase signaling, which would be assumed to involve RelB (49, 50). As shown in this article, DC IDO expression and induction by LPS/IFN-γ may also occur in a RelB-independent fashion. In support of a signaling pathway independent of the alternate NF-κB pathway, IDO is induced through STAT1 and IRF1 signaling downstream of the IFN-γ receptor in DCs (60, 61). In the current study, IDO (although expressed) did not control autoimmune disease until induced by RelB+/− DCs. Subsequently, IDO function was required for disease suppression and functional immune restoration, including induction of IFN-γ+ T cells, and reduction of Th2 cells. Of interest, expression of IDO by host APCs and IFN-γ production by T cells were increased relative to untreated RelB−/− mice as long as 3 wk after DC transfer, when donor DCs were no longer detected (data not shown), and mice remained tolerant 12 mo later. Given that RelB−/− TEMs were resistant to suppression, and few T cells expressed IFN-γ prior to DC treatment, we concluded that RelB+/− DCs induced functional IFN-γ+ TEMs and Tregs as a result of CD80/CD86 costimulation, and this IFN-γ had a prolonged feed-forward effect on IDO expressed by DCs and other APCs in the host. This mechanism of tolerance differs from the conversion or expansion of Foxp3+ Tregs that is described in vivo after transfer or targeting of costimulation-deficient DCs (62, 63) or that occurs in vitro after stimulation of Foxp3+ T cells with mature CD80/CD86+ DCs (64, 65). In contrast, the proportion of Foxp3+ Tregs did not increase; instead, their proliferation was arrested after RelB+/− DC transfer to RelB−/− mice.

Our conclusions that IFN-γ and IDO are critical components of effective therapeutic immunoregulation of autoimmune disease are strongly supported by the literature in strains of mice prone to spontaneous Th1/Th17-mediated autoimmune disease. Although TEM and Treg IFN-γ production were increased relative to untreated RelB−/− mice as a result of suppression of Th2-type autoreactive TEMs, were shown to suppress established disease-relevant CD8+ TEMs, that were present in inflamed organs and spleen of RelB−/− mice (84). In that model, transfer of healthy Tregs attenuated disease in an IDO- and IFN-γ-dependent manner (73). In humans with type 1 diabetes, we showed that, similar to RelB−/− DCs, peripheral blood monocytes and DCs have a tolerant phenotype, with reduced p65 and RelB activity in response to LPS (74). Pancreatic function in patients with recent-onset diabetes is also improved after treatment with anti-CD3 mAb, which enhances Treg proliferation and function, but not by the use of immature or NK-cell–deficient DCs (21, 69–72). Recently, autotigeneic peptide–MHC–coated nanoparticles, despite paradoxical expansion of disease-relevant CD8+ TEMs, were shown to suppress established diabetes in an IDO- and IFN-γ–dependent pathway (75). In humans with type 1 diabetes, we showed that, similar to RelB−/− DCs, peripheral blood monocytes and DCs have a tolerant phenotype, with reduced p65 and RelB activity in response to LPS (74). Pancreatic function in patients with recent-onset diabetes is also improved after treatment with anti-CD3 Abs, leading to IFN-γ secretion by TEMs and Tregs (74–79). In the collagen-induced arthritis (CIA) model of rheumatoid arthritis, several tolerizing DC strategies have been reported (80, 81), including DCs generated in the presence of dexamethasone and vitamin D, followed by LPS stimulation, which suppressed established disease. Notably, these DCs induced higher T cell expression of IFN-γ compared with untreated mice with CIA (82). Furthermore, CD80/CD86-dependent suppression of established CIA was observed after injection of exosomes derived from IDO+ DCs (83). Therefore, the evidence in mice and humans is consistent with the idea that autoreactive TEMs can be regulated by either enhancing T cell signaling or by promoting IFN-γ–dependent cross-talk to IDO-producing costimulatory APCs. It will be of interest to determine whether DCs expressing CD80/CD86 suppress, rather than flare, autoimmune disease and the role played by IFN-γ and IDO in a human proof-of-concept study.

The current studies demonstrated that activated (RelB+CD80/CD86+) DCs are required to achieve long-term tolerance in an autoimmune model where DC numbers and costimulatory function are reduced but Th2 cytokine-producing and IL-2–consuming TEMs are abundant. Of interest, in the MRL/lpr model of lupus, Treg expansion declines over time as a result of deficient IL-2 production by TEMs. In vitro, deficient Treg proliferation was observed in the presence of DCs from MRL/lpr, but not MRL/+ mice (84). In that model, transfer of healthy Tregs attenuated...
FIGURE 8. IDO activity is required for DC control of organ inflammation. A, Splenocytes isolated from RelB \(^{+/−}\) mice or 8-wk-old RelB \(^{−/−}\) mice were cultured for 24 h with LPS and IFN-\(γ\) and stained for MHC class II and CD11c and intracellular IDO. IDO expression in freshly isolated and LPS/IFN-\(γ\)–stimulated DCs (blue) and MHC class II \(^{+}\) APCs (red) is shown. B, CD11c \(^{+}\) DCs freshly isolated from RelB \(^{+/−}\), RelB \(^{−/−}\), or DC-treated RelB \(^{−/−}\) mice or cultured for 24 h with LPS and IFN-\(γ\) were stained for intracellular IDO. The difference in mean fluorescence intensity (MFI) of IDO and isotype-stained samples is shown. C, RelB \(^{−/−}\) mice treated with or without DCs and 1-MT for 3 wk were sacrificed, and serum aspartate transaminase was measured (upper left panel). CD4 \(^{+}\) splenic T cells from those mice were cultured for 4 h with PMA/ionomycin and stained for Foxp3 and intracellular IFN-\(γ\), IL-4, and IL-5. Proportion of CD4 \(^{+}\)Foxp3 \(^{+}\) cells staining positive for IFN-\(γ\) (upper right panel), IL-4 (lower left panel), and IL-5 (lower right panel). D, Proportion of CD4 \(^{+}\)Foxp3 \(^{+}\) cells expressing IFN-\(γ\). Data points represent individual mice analyzed over two separate experiments. *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\); one-way ANOVA, followed by the Bonferroni post hoc test.
disease, presumably also associated with a feed-forward effect on host DCs. Unlike the MRL/lpr model, even ReIβ−/− Tregs from sick mice are functional ex vivo, and Treg numbers are maintained in vivo. The ReIβ− model identifies a feedback loop among costimulatory DCs, IFN-γ, and IDO that effectively restores regulation of T EMS by endogenous Foxp3+ Tregs. It challenges the current paradigm that DCs deficient in costimulation are required to regulate established autoimmune disease to avoid T EMS activation and disease flares. Furthermore, clinical trials of immunotherapies designed to restore tolerance, including anti-CD3, and various DC preparations are either in progress or planned in type 1 diabetes and rheumatoid arthritis (85, 86). The current studies suggested that host DC responsiveness to activation with TLRI ligands and IFN-γ effector and regulatory cell IFN-γ production, and IDO expression and activity would be important baseline disease biomarkers to consider in preclinical studies to determine likely responsiveness to immunoregulatory strategies. Furthermore, in addition to the analysis of Treg numbers and function, IFN-γ production and IDO activity would be valuable biomarkers of outcome measured against clinical efficacy in tolerance trials.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


