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Dendritic Cell (DC)-Specific Targeting Reveals Stat3 as a Negative Regulator of DC Function

Jessica A. Melillo,*,‡ Li Song,‡ Govind Bhagat,‡ Ana Belen Blazquez,§ Courtney R. Plumlee,*,‡ Carolyn Lee,‡ Cecilia Berin,‡ Boris Reizis,‡ and Christian Schindler‡,§

Dendritic cells (DCs) must achieve a critical balance between activation and tolerance, a process influenced by cytokines and growth factors. IL-10, which transduces signals through Stat3, has emerged as one important negative regulator of DC activation. To directly examine the role Stat3 plays in regulating DC activity, the Stat3 gene was targeted for deletion with a CD11c-cre transgene. Stat3 CKO mice developed cervical lymphadenopathy as well as a mild ileocolitis that persisted throughout life and was associated with impaired weight gain. Consistent with this, Stat3-deficient DCs demonstrated enhanced immune activity, including increased cytokine production, Ag-dependent T-cell activation and resistance to IL-10–mediated suppression. These results reveal a cell-intrinsic negative regulatory role of Stat3 in DCs and link increased DC activation with perturbed immune homeostasis and chronic mucosal inflammation. The Journal of Immunology, 2010, 184: 2638–2645.

Dendritic cells (DCs) play an important role in host immunity through their ability to distinguish between self-Ags and those associated with microbes (reviewed in Refs. 1–3). Microbial associated Ags potently activate DCs through engagement of innate pattern recognition receptors, including TLRs. Activation is associated with an up-regulation in the expression of MHC class II (MHC-II) and costimulatory molecules (e.g., CD80 and CD86), which in turn promote enhanced T cell activation. This process is also regulated by inflammatory cytokines, such as IFN-Is, TNF, IL-6, IL-10, IL-12, and IL-23, serving both to regulate DC activity and direct effector T cell differentiation [e.g., IFN-γ–producing Th1 cells, IL-4–producing Th2 cells, or IL-17–producing Th17 cells (4, 5)]. By contrast, self-Ags fail to activate DCs, endorsing a tolerant state. Failure of negative regulation can lead to aberrant DC activation and loss of tolerance (2, 6, 7). However, a direct causal relationship between exuberant DC activation and inflammatory disease has been more difficult to demonstrate in vivo.

Conventional DCs (cDCs), phenotypically marked by the expression of CD11c, are found throughout the host (1, 2). They can be further divided into CD8± DCs, which cross-present endogenous Ags on MHC class I, and CD8– DCs, which prefer to present exogenous Ags on MHC-II (8). Plasmacytoid DCs (pDCs; CD11c+, mPDCA-1+, and B220+) represent a functionally distinct lineage that also arises from a Flt3+ and M-CSFR+ common DC progenitor (1, 2). In contrast to cDCs, pDCs can be found in the bone marrow BM and secrete prodigious quantities of IFN-Is. The transcription factor Stat3 has recently been ascribed an important role in Flt3-mediated DC progenitor proliferation, especially for pDCs, whereas Stat5 directs a phenotypically dominant GM-CSF–driven expansion of cDCs (9–11).

STATs play a critical role in directing the biological responses for members of the four-helix bundle family of cytokines (12). Of these, Stat3 is unique for its capacity to direct the anti-inflammatory activities of IL-10 and related cytokines (13). To overcome the embryonic lethal phenotype associated with the targeted deletion of the Stat3 gene, several conditional knockout models have been generated (14). This includes deletion in myeloid (i.e., LysM-Cre) as well as additional hematopoietic lineages (9, 15–19). Notably, each of these conditional deletions has been associated with a severe colitis, analogous to what had been observed in IL-10–deficient mice (20). In the myeloid deletion model, the inflammatory process is associated with increased IL-12 production and enhanced Th1 T cell activity, raising the possibility of aberrant DC activity (15, 21, 22). Moreover, a potential role for Stat3 as a cell intrinsic negative regulator of DC activity has been observed in several experimental systems (23–25). However, the complex phenotypes associated with hematopoietic deletion of Stat3 have precluded a more careful analysis of the specific role Stat3 plays in regulating DC activity.

To clarify the role Stat3 plays in DC function, the Stat3 gene was targeted for deletion with a DC-specific CD11c-cre transgene (26–28). Underscoring the value of this strategy, Stat3 CKO mice developed a significantly less severe colitis than observed with other targeting strategies (9, 16–19). In addition, CKO mice exhibited cervical lymphadenopathy, increased peribronchiolar inflammation, as well as impaired weight gain and fertility that were associated with ileocolitis. The constellation of intestinal, peribronchiolar, and likely nasopharyngeal inflammation suggests that DC-specific deletion of Stat3 is associated with impaired mucosal tolerance. Consistent with this, Stat3 CKO DCs, which developed normally, were resistant to IL-10 suppression and exhibited both an enhanced ability to secrete inflammatory cytokines and stimulate T cells. These observations underscore an important role for...
Stat3 in antagonizing DC activity and directly link DC hyperactivity with inflammatory disease.

**Materials and Methods**

**Mice**

Stat3<sup>flk/flk</sup> mice, in a 129/C57BL/6J mixed background (16), were crossed seven generations onto C57BL/6J background, and then with CD11c-BAC-Cre transgenic mice (26). The genotype of Stat3<sup>flk/flk</sup> mice was confirmed by PCR (16). OT-I and OT-II TCR transgenic mice were obtained from The Jackson Laboratory (Bangor, ME). The Columbia University Institutional Animal Care and Use Committee approved the murine studies.

**Cell culture**

DCs were either isolated from the spleen, mesenteric lymph node (MLN), or derived from BM. Purified DCs were cultured in 24-well plates at 0.5 × 10<sup>6</sup> cells/ml in RPMI 1640 medium (Life Technologies; Grand Island, NY), with 10% FCS (HyClone, Logan, UT), penicillin/streptomycin (Life Technologies), and 2-ME (Life Technologies). For BM-derived DCs, BM cells were plated in 24 well plates (0.5 × 10<sup>6</sup> cells/ml) in RPMI 1640 medium, 10% FCS, penicillin/streptomycin, 2-ME, and either 1/50 GM-CSF or 1/500 IL-4 conditioned medium from L929 cells as previously described (26). Primary DCs were recovered by digestion with collagenase D (1 mg/ml; Roche Diagnostics), DNase (0.1 mg/ml, 37˚C, 30 min; Roche Diagnostics), and RBC depletion (Sigma-Aldrich, St. Louis, MO). Single-cell suspensions were prepared from the spleen, BM, and LNs of CKO mice were evaluated by flow cytometry. To examine the effect of Stat3 deletion on DC homeostasis, cells from the spleen, BM, and LNs of CKO mice were evaluated by immunophenotyping (Fig. 1, Table I). No significant differences were detected between Stat3<sup>flk/flk</sup> and Stat3<sup>−/−</sup> mice.

**Flow cytometry**

Cells were stained with different fluorochrome-coupled Abs. Anti-CD11c (HL3), anti-CD3e (145-2C11), anti-CD86 (GL1), anti-CD4 (L3T4), anti-CD40, anti-CD80 (16-10A1), anti-CD8 (53-6.7), streptavidin-perCP, and streptavidin-allophycocyanin were from BD Pharmingen (San Diego, CA). Anti-CD11b (M1/70), anti-B220 (RA3-6B2), and anti-CD19 (MB19-1) were from Santa Cruz, CA). DNA was recovered from FACS-purified leukocyte populations and amplified with specific primers by quantitative-PCR (Q-PCR) (ABI Prism 7700 with SYBR green master mix; Applied Biosystems, Foster City, CA). Primers were annealed at 60˚C and run 40 cycles. Stat3 primers included 5′-CCA AGT TCA TCT GTG TGA CAC C-3′ (common); 5′-ATC GGC AGG TCA ATG GTA TTG G-3′ (wild-type [WT]); and 5′-GAG TCA GGG ATC CAT AAC TCC-3′ (flx). Control cyclinHl A primers included 5′-CTG AGC ACT GGA GAG AAA GG-3′ and 5′-CTT GCT GTT GGT CTT GCC ATT CC-3′. Cre primers included 5′-GGA CAT GTT CAG GAT CGT CAC GGA TGC-3′ and 3′-CTG TGC TGG TGC CAC AAC CAT G-3′. A standard and a standard curve were generated by plotting log of DNA concentration versus Ct values from 1.5 serial dilutions of DNA for each primer set SDS1.9.1 software.

**Cytokine profiling**

Cultured DCs were stimulated with LPS (1 μg/ml, 12 h; from Escherichia coli) and IFN-γ (10 ng/ml), or CpG-oligodeoxynucleotides (1 μM, 12 h; Invitrogen, Frederick, MD), including D19 (A/D-type), 1668 (K-type), and D (negative control). Cytokines were measured by bead array (IL-6, TNF, and IL-12; BioSource International [Camarillo, CA] on Luminex [Austin, TX]), ELISA (IFN-γ, IL-17, IL-4, IL-13, IL-23 [Ebioscience, San Diego, CA] and IFN-α [PBL]), or intracytoplasmic staining (i.e., anti-CD4-FITC, anti-IFN-γ-allophycocyanin, anti-IL-4-PE, or IL-12-allophycocyanin; BD Pharmingen). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). Cell sorting was carried out on LSRII (BD Biosciences, Franklin Lakes, NJ). Data were acquired with 50-75% purity (BD Pharmingen).

**T cells analysis**

A total of 3 × 10<sup>5</sup> OVA-specific CD4<sup>+</sup> T cells (anti-CD4 MACS beads; Miltenyi Biotech) from OT-II TCR transgenic spleens were cultured with 3 × 10<sup>5</sup> WT CD11c<sup>+</sup> DCs that had been loaded with increasing concentrations of OVA (Worthington Biochemical, Lakewood, NJ) or OVA233-339 peptide, as indicated. T-cell proliferation was measured by [3H]thymidine incorporation (16 h, 37˚C, PerkinElmer Life Sciences, Shelton, CT). In some studies, cytokine production was measured by intracellular staining after 5 d of OVA stimulation, followed by restimulation with plate bound anti-mouse CD3 (5 μg/ml, 6 h; 145-2C11; BD Pharmingen). For Ag presentation by purified MLN-DCs, 0.25–1 × 10<sup>5</sup> OVA<sub>233-339</sub>-loaded (100 and 1000 ng/ml) primary DCs were incubated with 10<sup>5</sup> OT-II CD4<sup>+</sup> splenic T cells. Cytokine accumulation (72 h) was evaluated by ELISA (Ebioscience) 3 d after restimulation with 5 μg/ml anti-CD3 and 3 μg/ml anti-CD28 (Ebioscience). Alternatively, 0.25–1 × 10<sup>5</sup> OVA-stimulated Stat3<sup>−/−</sup> BMDCs were incubated with 10<sup>5</sup> OT-I CD8<sup>+</sup> splenic T cells.

**Histopathology**

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections (3 μm) were stained with H&E. The severity of inflammation was scored using a four-tiered scale (see Fig. 3).

**Statistics**

Statistical significance was estimated by calculating two-tailed p values in unpaired Student t test from independent studies (n = 3; unless otherwise stated).

**Results**

**CD11c-Cre directs efficient and DC-specific Stat3 deletion**

To test the hypothesis that Stat3 is an important regulator of DC function, mice carrying a loxP-flanked Stat3 allele (Stat3<sup>flk</sup> [16]) were crossed with CD11c-Cre transgenic mice (26). To evaluate Stat3 deletion specificity and efficiency in Stat3<sup>flk/flk</sup> × CD11c-Cre mice, genomic DNA from purified leukocyte populations (i.e., cDCs, pDCs, macrophages, T cells, and B cells) was analyzed by Q-PCR (Fig. 1A). Consistent with previous studies with the CD11c-Cre transgene, the Stat3<sup>flk</sup> allele was deleted ~94% of cDCs and 89% of pDCs from CKO mice (26–28). This contrasted a relatively low deletion efficiency in CKO monocytes and lymphocytes (4 and 13%, respectively; Fig. 1A), as well as DX5<sup>+</sup> NK cells (~10% [26]). Q-PCR analysis of day 8 BM-derived DCs (BMDCs) (~75% pure; Supplemental Fig. 1) revealed ~70% deletion efficiency in CKO cells. Consistent with this, the level of Stat3 protein expression was also dramatically reduced in CKO BMDCs, but not BM-derived macrophages (Supplemental Fig. 2) (30). No compensatory increase in Stat1 was observed (data not shown [16]). Likewise, little if any activated (i.e., phosphorylated) Stat3 could be detected in IL-12-stimulated Stat3 CKO BMDCs (Supplemental Fig. 2). These results correlated well with Southern blotting studies (data not shown) and demonstrate that the CD11c-Cre transgene directs both the efficient and specific deletion of Stat3 in DCs, consistent with studies on other LoxP flanked loci (26–28).

**cDC and pDC populations in Stat3 CKO mice**

To examine the effect of Stat3 deletion on DC homeostasis, cells from the spleen, BM, and LNs of CKO mice were evaluated by immunophenotyping (Fig. 1, Table I). No significant differences...
were observed in the splenic cDCs (CD11c<sup>hi</sup> and MHC-II<sup>hi</sup>), including CD8<sup>+</sup> and CD8<sup>−</sup> populations (Fig. 1B). Likewise, there were no significant differences in the expression of activation markers (e.g., CD80, CD86, CD40, and MHC-II; data not shown). Moreover, circulating lymphocyte populations were not affected by DC-specific Stat3 deletion (data not shown) (Table I). There was, however, a consistent ∼2-fold reduction in the number of pDCs (CD11c<sup>lo</sup>, mPDCA<sup>-</sup>, and B220<sup>+</sup>), in the spleen (0.48 ± 0.07 versus 0.83 ± 0.06%), and in the BM (0.78 ± 0.34 versus 1.65 ± 0.30%) of CKO mice (Fig. 1C), likely reflecting the ∼50% deletion efficiency directed by the CD11c-Cre transgene in developing pDCs (M. Caton and B. Reizis, unpublished observation) (26). Yet, the number of pDCs found in “normal” LNs and the lower gastrointestinal tract was similar, likely reflecting a compensatory recruitment (Figs. 1C, 3D) (33). In addition, CKO mice were noted to develop persistently enlarged cervical LNs (c-LNs). This was associated with a 2-fold increase in pDCs but did not represent an absolute increase in the tissue concentration of these cells (see Fig. 1C, Table I). In addition, the enlarged c-LNs featured more lymphoid follicles, with a corresponding increase in lymphocytes (B cells > T cells) (Supplemental Fig. 4, Table I). Stat3 deletion was also not associated with a significant change in the capacity of splenic or BM pDCs to secrete IFN-α (see Supplemental Fig. 3).

To determine whether the absence of Stat3 affected DC development in vitro, WT and CKO BM cells were cultured in media supplemented with GM-CSF or Flt3 ligand to generate cDCs and pDCs, respectively (Supplemental Fig. 1) (26, 34). Consistent with the relatively late onset of Cre recombination associated with in vitro DC development (26), no differences were observed in the ratio of these two populations (data not shown). Overall, these studies reveal a relatively normal DC compartment in CKO mice, as well as cultured DCs, affording the opportunity to carry out functional studies on Stat3-deficient DCs in the absence of any confounding developmental or systemic defects.

**Stat3 CKO mice exhibit a mild inflammatory phenotype**

Although Stat3 CKO mice were born at the expected Mendelian ratio, they exhibited isolated cervical lymphadenopathy (see above; see also Supplemental Fig. 4), developed infertility and failed to accumulate body fat with increasing age (Supplemental Fig. 5). To determine whether these latter two phenotypes could be attributed to a chronic inflammatory state, serum cytokine levels were evaluated at 24 wk of age. Stat3 CKO mice revealed a significant and consistent 1.5- to 2-fold increase in levels of circulating TNFα (i.e., cachexin [35]) and IFN-γ, as well as less significant increases in IL-12 and IL-6 (see Fig. 2). No significant differences in circulating levels of IL-1β, IL-10, or Th2-type cytokines (i.e., IL-4 and IL-5) (see Fig. 2; data not shown) were noted. These data highlight a mild proinflammatory state in CKO mice, which may account for their reduced fertility and body fat.

To determine the etiology of chronic inflammation in Stat3 CKO mice, a histopathologic survey was carried out. Specific pathology was not evident in most CKO organs systems including, heart, pancreas, liver, kidneys, thymus, non–c-LNs, and spleen. However, four WT and eight CKO mice (n = 16 for each group) exhibited peribronchial thickening and inflammation. The inflammatory infiltrate consisted largely of lymphocytes as well as some monocytes (data not shown).

The most striking pathology in Stat3 CKO mice was an inflammatory process that extended throughout the lower intestinal tract. To evaluate this more carefully, a blinded histopathologic survey was carried out in WT and CKO GI tracts (n = 5 for both genotypes at 4, 12, and 24 wk and n = 3 at 36 wk; see Fig. 3A, 3B). This revealed mild to moderate inflammation that was most evident in the lamina propria of the large intestine and cecum, an area with a large Ag load and abundant DCs (21). A more detailed analysis of the leukocytes accumulating in the lamina propria (i.e., LPLs) revealed a significant increase in CD8<sup>+</sup> cells in CKO mice (p < 0.05, 0.01, and 0.01 for small intestine, cecum, and colon, respectively), but no differences in the limited number of recovered CD4<sup>+</sup> cells, especially in regions where inflammation was prevalent (i.e., cecum and colon) (Fig. 3C, 3E). In addition, no significant differences were observed in the intracytoplasmic accumulation of IFN-γ or IL-17 in CD4<sup>+</sup> cells (data not shown). Likewise, there were no significant differences in the DCs recovered from the cecum and colon (Fig. 3D, 3E). Intriguingly, there was a decrease in B cells isolated from cecum and colon of CKO mice (Fig. 3C), which may reflect a decrease in the number of observed Peyer’s patches (data not shown). Finally, there was a trend toward increasing inflammation as mice aged, which did not achieve significance in our study.

A more careful analysis of the inflammatory infiltrate revealed a variable lymphocytic cryptitis, expansion of intercryptal spaces secondary to inflammatory cells and occasional crypt abscesses, which were composed of clusters of neutrophils within crypt lumens (see inset in Fig. 3B, CKO large intestine). Although some mucin depletion (decreased goblet cell mass) was evident in areas

**FIGURE 1.** DC populations in Stat3 CKO null mice. A, Stat3 is efficiently deleted in CKO DCs. WT and Stat3 CKO splenocytes were FACS sorted into the following: cDCs (CD11c<sup>hi</sup> and CD11b<sup>+</sup>); pDCs (CD11c<sup>lo</sup> and mPDCA<sup>+</sup>); macrophages (CD11c<sup>-</sup> and CD11b<sup>+</sup>); T cells (CD3<sup>+</sup>); and B cells (CD19<sup>+</sup>). DNA was evaluated for presence of WT or deleted Stat3gene by Q-PCR. Results are representative of two independent studies and were confirmed by Southern blotting. B, Circulating cDC populations are normal in CKO mice. WT and CKO splenic DCs (CD11c<sup>+</sup> and MHC-II<sup>+</sup>) were evaluated for mDCs (CD11c<sup>lo</sup>, MHCII<sup>+</sup>, and CD11b<sup>+</sup>) and lymphoid DCs (CD11c<sup>hi</sup>, MHC-II<sup>-</sup>, and CD8<sup>+</sup>) populations. Analysis is representative of more than three independent studies. C, Circulating pDCs are reduced in CKO mice. Analysis of WT and CKO cDCd (CD11c<sup>lo</sup> and mPDCA<sup>+</sup>) and pDCs (CD11c<sup>lo</sup> and mPDCA<sup>+</sup>) populations from spleen, BM, normal-sized LNs (see text), and enlarged CKO c-LNs (see Supplemental Fig. 3, Table I). Analysis is representative of more than three independent studies. The ∼2-fold decrease in CKO pDCs was significant in spleen (p < 0.004) and BM (p < 0.03).
of inflammation, granulomas were not observed. Disease was most apparent in the cecum (four of five at 4 wk and all older CKO mice) and the large intestine (all CKO mice), where inflammation was patchy, with intervening normal mucosa. The small intestine, especially in the distal ileum, was largely spared of disease until 36 wk of age. Overall, the pattern of inflammation bore similarities to Crohn’s disease (37). Not only was the extent of colonic inflammation considerably less severe than that observed in other Stat3 CKO models, but it also extended into the distal small intestine (16–18, 38, 39).

**Functional characterization of Stat3 CKO DCs**

To determine whether the increased inflammation observed in CKO mice could be attributed to augmented DC activity, the capacity of WT and Stat3 CKO BMDCs to be activated by TLR agonists was evaluated. Although CKO BMDCs developed normally (Supplemental Fig. 1), they appeared to mature a little more rapidly in several independent experiments when interrogated for CD40, CD80, CD86, and MHC-II expression (data not shown). As anticipated, stimulation of WT BMDCs with synthetic TLR9 agonists (i.e., D19 and 1668 CpG oligonucleotides) or the TLR4 agonist LPS stimulated robust secretion of IL-6, TNF and IL-12, as well as more modest increases in IL-10, IL-23, and IFN-κ (see Fig. 4A, Supplemental Fig. 3) (26, 40). Other TLR ligands (e.g., poly[I]•poly[C] and imiquimod) were considerably less effective in inducing cytokine expression. Intriguingly, Stat3 CKO DCs produced significantly higher levels of IL-12 and IL-23 after stimulation with the two most potent agents, 1668 and LPS (Fig. 4A). There was also a trend toward higher TNF and IL-6 but not IFN-κ secretion in CpG-treated CKO DCs (Fig. 4A, Supplemental Fig. 3) (data not shown).

To determine whether endogenous CKO DCs exhibited the same pattern of enhanced cytokine secretion, splenic DCs were examined after the mice had been injected with LPS (9, 26). Consistent with in vitro observations, splenic CD11c+ DCs from both WT and CKO mice exhibited comparable levels of CD86 and MHC-II (Supplemental Fig. 6A). More importantly, Stat3-deficient splenic DCs produced significantly more IL-12 in response to LPS than WT DCs, paralleling results with cultured DCs (Fig. 4B).

Next, the ability of CKO DCs to present Ag to T cells was evaluated. At lower doses, WT and CKO OVA-loaded BMDCs were equivalent in their capacity to stimulate proliferation and IFN-γ secretion in naive OT-II transgenic CD4+ T cells (Fig. 4C, Supplemental Fig. 6B). Consistent with elevated levels of IL-12 secreted by CKO DCs (Fig. 4A, 4B), these cells exhibited a significant increase in their capacity to stimulate T cell proliferation and IFN-γ secretion when loaded with higher doses of OVA (i.e., >0.1 mg/ml) (see Fig. 4C, Supplemental Fig. 6B). In contrast, however, OVA-dependent induction of IL-4 and IL-17 secretion was considerably more modest, suggesting that corresponding effector populations may not contribute to the inflammatory response in Stat3 CKO mice (Supplemental Fig. 6) (data not shown). A similar increased capacity to promote OT-II cell polarization toward IFN-γ–producing Th1, but not other effectors, was observed when DCs were harvested from the MLNs of CKO mice and loaded with OVA peptide (OVAP) (Fig. 4D, Supplemental Fig. 6D). Likewise, OVAP-loaded MLN CKO DCs exhibited an enhanced trend toward promoting IFN-γ production in OT-I T cells (see Supplemental Fig. 6E). Collectively, these data reveal that Stat3-deficient DCs are hyperresponsive to TLR stimulation, thereby directing a more exuberant activation of proinflammatory effector T cells.

IL-10, which transduces critical signals through Stat3, is an important negative regulator of DC activity (5, 41). To determine whether Stat3 mediates the inhibitory effect of IL-10 on these cells, WT and CKO DCs were incubated with IL-10 prior to LPS stimulation. As anticipated, IL-10 pretreatment blocked the robust LPS-dependent upregulation of MHC-II and CD86 (see Fig. 5A).
In contrast, MHC-II and CD86 expression was insensitive to IL-10 pretreatment in Stat3 CKO DCs (Fig. 5A). Similarly, IL-10 failed to block LPS-dependent cytokine secretion in Stat3 CKO DCs (see Fig. 5B). A similar autocrine phenomenon may also account for the enhanced levels of cytokines observed in CKO serum and in CpG-stimulated DCs (Figs. 2, 4A). These observations are consistent with previous studies on Stat3-deficient macrophages (16), underscoring an important role for Stat3 in directing the anti-inflammatory activity of IL-10 on DCs. These studies raise the possibility that a loss in autocrine IL-10 response, or another analogous Stat3-dependent negative regulator, may account for the hyperactivity observed in Stat3 CKO DCs.

Discussion
The development of a more specific CD11c-Cre transgene has provided an opportunity to explore the role Stat3 plays in regulating DC activity in vivo. Previous efforts, exploiting panhematopoietic or inducible Stat3 deletion, yielded complex phenotypes. Specifically, deletion of Stat3 in hematopoietic progenitors led to a severely contracted cDC compartment and a substantial block in pDC development, with the rapid emergence of a lethal ileocolitis (9, 39). Defects in DC development were attributed to impaired Flt3-Stat3–dependent signaling (9, 10). Inducible Stat3 deletion has been associated with systemic granulocytosis and a comparable aggressive colitis marked by a neutrophil predominant infiltrate (L. Song and C. Schindler, unpublished observation) (17, 18). This inflammatory process featured a disrupted epithelial barrier, reminiscent of the pathophysiology reported for T-bet/RAG2 double-knockout mice (42). Analogously, deletion of Stat3 in regulatory T cells was associated with an aggressive, albeit Th17-dependent destructive colitis (19). In contrast, the colitis that developed in mice with a myeloid-specific (i.e., LysM-Cre transgene targeting polymorphonuclear neutrophils/C25).
The increase in IL-12 production was quantified by mean fluorescence intensity (CFP; 1 μM), D19 (A/D-type Cpg; 1 μM), 1668 (K-type Cpg; 1 μM), and LPS (1000 ng/ml). Twelve-hour supernatants were evaluated for cytokine production by ELISA (IL-23 and IL-6). Values of each cytokine were compared with WT and CKO GM-CSF DCs were stimulated with LPS (100 ng/ml, 12 h) either before or after a 12-h pretreatment with IL-10 (10 ng/ml) Bar graph quantifies significant differences (graphed as percent change) in the LPS-stimulated CD69 and MHC-II expression in WT (versus CKO) CD11c+ cells. IL-12−pretreated DCs (p values of 0.0001 and 0.008, respectively). Results are representative of greater than three independent experiments. B, CKO DC cytokine secretion is not suppressed by IL-10. Twelve-hour LPS culture supernatants were collected from DCs treated as outlined in A and evaluated for cytokine production by bead array. Each data point is representative of four independent samples.

Inhibitory activity of IL-10 on Stat3 CKO DCs. A, CKO DC activation is not sensitive to IL-10 suppression. Day 9 WT and CKO GM-CSF DCs were stimulated with LPS (100 ng/ml, 12 h) either before or after a 12-h pretreatment with IL-10 (10 ng/ml) Bar graph quantifies significant decreases (graphed as percent change) in the LPS-stimulated CD69 and MHC-II expression in WT (versus CKO) CD11c+, IL-12−pretreated DCs (p values of 0.0001 and 0.008, respectively). Results are representative of greater than three independent experiments. B, CKO DC cytokine secretion is not suppressed by IL-10. Twelve-hour LPS culture supernatants were collected from DCs treated as outlined in A and evaluated for cytokine production by bead array. Each data point is representative of four independent samples.
Indeed, previous studies have highlighted an important role for lamina propria DCs in sampling commensal bacterial and food Ags to promote tolerance (21, 22). Similarly, deletion of the transcription factor T-bet in the DCs of RAG2−/− mice was associated with enhanced DC-dependent TNF secretion (42). Although these mice developed an aggressive, granulocyte predominant colitis that was quite distinct from the Crohn’s-like disease found in Stat3 CKO mice; both studies underscore an important role for DCs in promoting mucosal tolerance. Intriguingly, recent evidence that Stat3 may control DC plasticity (i.e., inflammatory versus tolerogenic states) through a capacity to regulate the expression of IDO has provided additional insight into how Stat3 may control mucosal tolerance (46, 47). In addition, our data support a role for Stat3 as a negative transcriptional regulator, serving to antagonize aberrant or exuberant DC activity through ligand(s) from the IL-10 family of cytokines (see below) (48). This process may also be associated with changes in regulatory T cell activity (7). Hence, loss of Stat3 is associated with an increase in inflammatory cytokines, including TNF (i.e., cachexin), which we speculate is responsible for the cachexia and infertility observed in CKO mice (21, 35). Although the number of infiltrating CD8+ and not CD4+ T cells correlated most closely with disease, analysis of these CD4+ T cells failed to detect significant differences in their cytokine signatures (e.g., IFN-γ versus, IL-4 versus IL-17; data not shown). The observed lymphocytic infiltration may reflect changes in the CKO DC-dependent CD4+ T cell activation, or these CD8+ T cells may directly be modulated by altered DC function. Consistent with the latter possibility, there was a trend toward higher OT-I-dependent IFN-γ secretion when these cells were stimulated with SIINFEKL-loaded CKO MLN DCs (Supplemental Fig. 6F). The current data did not evaluate the potentially pathogenic, compensatory, or regulatory roles changing B cell and/or CD8+ T cell populations may have played in the development of enterocolitis. Finally, it seems likely that malabsorption did not contribute significantly to weight loss in Stat3 CKO mice, because the onset of small intestinal inflammation was both relatively modest and post adolescence.

Consistent with a direct effect of a loss in Stat3, CKO DCs exhibited an augmented proinflammatory capacity. There were significant increases in TLR-dependent IL-12 and IL-23 secretion in these DCs, supporting recent evidence that Stat3 negatively regulated the IL-12-p40 promoter (49). Similar results were observed in these DCs, supporting recent evidence that Stat3 negatively regulates the IL-12-p40 promoter (49). Similar results were observed in these DCs, supporting recent evidence that Stat3 negatively regulates the IL-12-p40 promoter (49).

Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental Figures

Figure 1S  DC populations in Stat3 CKO null mice.

(A) CKO DCs mature normally in GM-CSF cultures. WT and CKO DCs were evaluated for CD11b and CD11c surface expression by FACS after 6 and 10 days of culture in GM-CSF conditioned media.

(B) CKO DCs mature normally in Flt-3L cultures. WT and CKO DCs were evaluated for CD11b and B220 expression after 10 days of culture in Flt3L conditioned media.

Figure 2S  Stat3 protein is absent in CKO DCs.

Day 11 BMDCs or day 8 BM macrophages (BMMs), from wild type (WT) and Stat3 conditional knockout (CKO) mice, were stimulated with IL-10 (10 ng/ml; 30 min) or IFN-α (1000 U/ml; 30 min) and evaluated for Stat3 activation by phospho-immunoblotting (phospho-Stat3; Cell Signaling Tech.). Filters were then reprobed with a total Stat3 antibody (Cell Signaling Tech.). Data is representative of at least three independent experiments.

Figure 3S  WT and Stat3 CKO pDCs are equivalent in their capacity to secrete IFN-α.

WT and CKO CD11c+ (Miltenyi Biotech) splenocytes (left panel) or bone marrow cells (right panel) were stimulated with CpG, as in Figure 4, and evaluated for IFN-α production by ELISA (PBL).

Figure 4S  CKO mice exhibit cervical lymphadenopathy.

(A) B-cells are modestly increased in CKO LNs. Lymphocytes harvested from WT (3.3 x 10^6 ± 2.5 x 10^5) and CKO (9.4 x 10^6 ± 2.2 x 10^5) mice cervical lymph nodes were stained with antibodies specific for CD3 (T-cells) and B220 (B-cells), and then evaluated by FACS. Analysis is representative of > 3 independent studies.

(B) Enlarged CKO cervical LNs feature an increased number of secondary lymphoid follicles. H & E stained sections of WT and enlarged cervical lymph nodes (LN) in Stat3 CKO mice. Data is representative of four independent samples.
Stat3 is a critical regulator of DC activity

(C) Increased PNA positive follicles in cervical CKO LNs. Paraffin sections of cervical LNs were stained with PNA (1:300; Vector BA-0074) to detect germinal centers and anti-syndecan-1 (1:200; Pharmingen 553714) for plasma cells (n=3) (51). Briefly, EDTA based antigen retrieval was performed. Endogenous peroxidase activity was blocked with 3% H2O2 in CH3OH for 10 min and endogenous biotin was blocked with egg white and BSA. PNA was visualized by Streptavidin-HRP (Jackson ImmunoResearch) and AEC (dark red; Vector labs). Incubation with an antibody for Syndecan-1 was followed by staining with rabbit anti-PE and goat anti rabbit-AP antibodies, and then visualized with the chromogen NBT/BCIP (dark blue).

Figure 5S Decreased weight gain in Stat3 CKO mice.
Weight (grams) is shown for male mice at weaning (4 weeks), 6 months and 7 months (n=5 for CKO and n=6 for WT).

Figure 6S Activation of WT and CKO DCs.
(A) CKO DCs are activated normally. WT (n = 5) and CKO (n = 8) mice were injected with LPS (250 ng) or PBS (from Fig. 5B). 3 h later splenocytes were harvested, fixed, stained (α-CD11c-APC, α-CD86-PE, α-MHC-II I-A\(^b\)-biotin, Streptavidin-PerCP, as indicated) and analyzed by FACS.
(B) Increased T-cell proliferation by CKO DCs. Purified CD4\(^+\) OT-II splenocytes were added to OVA loaded GM-CSF cultured WT and CKO DCs from Figure 4C for 3 days and evaluated for their ability to proliferate (\(^3\)H-Tdr incorporation; 16 h) in response to antigen. P values between WT and CKO were significant for \(^3\)H-Tdr incorporations at 0.5 mg/ml (p < 0.01) and 1 mg/ml (p < 0.03).
(C) CKO DCs stimulate a modest increase in IL-17 secretion. Purified CD4\(^+\) OT-II splenocytes were cultured with OVA loaded WT and CKO DCs from Figure 4C and evaluated for their capacity to secrete IL-17 by ELISA (BioSource).
(D) CKO DC exhibited a modest change in their capacity to stimulate T-cells. OT-II T cells (10\(^5\) cells per well) were stimulated with CD11c\(^+\) cells isolated from the MLN of WT (n=3) and CKO (n=5) mice, in the presence or absence of the peptide OVA\(^{323-339}\), at DC:T-cell ratio of 1:10 and 1:40, re-stimulated with anti-CD3 and anti-CD28 and
Stat3 is a critical regulator of DC activity

evaluated for cytokine production by ELISA as outlined in Fig. 4D. Results are expressed as means (±SME) of six independent experiments. *p<0.05.

(E) CKO DCs stimulate a modest increase of IFN-γ secretion in CD8 cells. Purified CD8⁺ OT-I splenocytes were cultured with pooled (n=3), SIINFEKL loaded, WT and CKO m-DCs, as in Figure 4C, at a ratio of 1:10 and 1:20. Culture supernatants were collected as above and evaluated for IFN-γ secretion by ELISA (BioSource).
Figure 1S (Melillo et al.)

A

GM-CSF Culture

CKO

WT

D6

67.33 + 3.90

66.53 + 3.09

D10

92.30 + 0.62

90.50 + 2.60

CD11b

CD11c

B

FLT-3L Culture

CKO

WT

D10

45.70 + 2.34

42.87 + 1.86

CD11b

B220
Figure 2S (Melillo et al.)

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WB: PY-Stat3

WT CKO IL-10

WT CKO IL-10

pSt3

St3
Figure 3S (Melillo et al.)

CD11c\(^+\) Splenocytes

Bone Marrow Cells

IFN-\(\alpha\) (pg/ml)

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Figure 4S (Melillo et al.)

A

WT

Cervical Lymph Nodes

B220

CD3

56.1±2.9

37.8±3.9

43.1±0.9

50.9±1.2

B

WT

Cervical Lymph Nodes

H & E

C

WT

PNA / Syndecan1

CKO
Figure 5S (Melillo et al.)

Average Male Weights

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grams

WT

CKO
Figure 6S (Melillo et al.)

**A** CD86 Expression

**B** $^{3}$H Thymidine (cpm)

**C** IL-17 secretion

**D** IL-17 secretion

**E** IFN-γ secretion (pg/ml)