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The Cytokines IL-21 and GM-CSF have Opposing Regulatory Roles in the Apoptosis of Conventional Dendritic Cells

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Abstract

Interleukin-21 (IL-21) has broad actions on T- and B-cells, but its actions in innate immunity are poorly understood. Here we show that IL-21 induced apoptosis of conventional dendritic cells (cDCs) via STAT3 and Bim, and this was inhibited by granulocyte-macrophage colony-stimulating factor (GM-CSF). ChIP-Seq analysis revealed genome-wide binding competition between GM-CSF-induced STAT5 and IL-21-induced STAT3. Expression of IL-21 *in vivo* decreased cDC numbers, and this was prevented by GM-CSF. Moreover, repetitive α-galactosylceramide injection of mice induced IL-21 but decreased GM-CSF production by natural killer T (NKT) cells, correlating with decreased cDC numbers. Furthermore, adoptive-transfer of wild-type $CD4^+$ T cells caused more severe colitis with increased DCs and interferon (IFN)-γ producing $CD4^+$ T cells in $Il21r^{-/-}Rag2^{-/-}$ mice (which lack T cells and have IL-21-unresponsive DCs) than in $Rag2^{-/-}$ mice. Thus, IL-21 and GM-CSF exhibit cross-regulatory actions on gene regulation and apoptosis, regulating cDC numbers and thereby the magnitude of the immune response.

Keywords

IL-21; GM-CSF; apoptosis; dendritic cells

Interleukin-21 (IL-21) is a pleiotropic type I cytokine that signals via IL-21R and the common cytokine receptor γ chain, γ_c (Spolski and Leonard, 2008). γ_c is also an essential component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (Leonard and Spolski, 2005) and is mutated in humans with X-linked severe combined immunodeficiency

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(XSCID) (Noguchi et al., 1993). IL-21 is primarily produced by $CD4^+$ T cells and natural killer T (NKT) cells, but it acts on multiple lineages, including T cells, B cells, natural killer (NK) cells, NKT cells, and bone marrow-derived dendritic cells (BMDCs) (Spolski and Leonard, 2008). Among its actions, IL-21 is a co-mitogen for T cells, cooperates with IL-7 or IL-15 to expand $CD8^+$ T cells (Zeng et al., 2005), promotes the differentiation of T follicular helper (Tfh) cells (Nurieva et al., 2008) and Th17 cells (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007), and has potent anti-tumor activity (Hinrichs et al., 2008; Ma et al., 2003; Wang et al., 2003; Zeng et al., 2005). IL-21 also promotes B cell differentiation to Ig-producing plasma cells through its induction of BLIMP1 (Kwon et al., 2009; Ozaki et al., 2004), a transcription factor critical for plasma cell formation (Martins and Calame, 2008). Interestingly, IL-21 also induces BCL6 (Ozaki et al., 2004) and drives T follicular cell differentiation (Nurieva et al., 2008; Vogelzang et al., 2008). IL-21 cooperates with IL-4 for Ig class switching particularly to IgG1 and IgG3 (Ozaki et al., 2002; Pene et al., 2004), and defective signaling by IL-21 and IL-4 appears to explain the B cell defect in humans with XSCID (Ozaki et al., 2002). IL-21 signaling is mediated in part via its activation of JAK1 and JAK3, which in turn activate STAT3, which is a major mediator of IL-21 signaling (Spolski and Leonard, 2008).

As compared to its actions on T and B cells, the role(s) of IL-21 in innate immunity are less well studied. IL-21 increases cytotoxic activity of NK and NKT cells, but its proliferative effect on these cells is stage-dependent (Spolski and Leonard, 2008). Previously, it was shown that the maturation and subsequent activation of granulocyte- macrophage colony-stimulating factor (GM-CSF)-induced bone marrow-derived DCs (GMCSF-DCs) are inhibited by IL-21, with diminished production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Brandt et al., 2003). We now show that IL-21 potently induces apoptosis of conventional DCs (cDCs) but only has a modest effect on plasmacytoid DCs (pDCs). The apoptotic effect on cDCs depended on STAT3 and Bim and was reversed by GM-CSF. These opposing actions of IL-21 and GM-CSF on cDCs might therefore influence the maintenance of immune tolerance versus the activation of an effective immune response.

RESULTS

IL-21 induces apoptosis of splenic but not GM-CSF-induced DCs

Previously, *in vitro* activation and maturation of GMCSF-DCs was shown to be inhibited by IL-21 (Brandt et al., 2003). To investigate the actions of IL-21 on these GM-CSF-DCs as well as on cDCs and pDCs, which develop from precursors distinct from monocytes (Liu et al., 2009; Naik et al., 2006) and in which the effect of IL-21 has not been reported, we first compared the effect of IL-21 on cell viability of splenic DCs (which include cDCs and pDCs) and GMCSF-DCs. IL-21 alone or in combination with LPS or CpG did not alter the viability of GMCSF-DCs (Figure 1A). In contrast, it potently decreased viability of splenic DCs (Figure 1B). Interestingly, the addition of LPS or CpG, which signal via toll-like receptors (TLRs) (Medzhitov, 2001) slightly diminished this effect (Figure 1B).

Splenic DCs can be divided into $B220^-CD11c^{hi}$ cDCs and $B220^+CD11c^{int}$ pDCs. cDCs reside in lymphoid tissues and process self-antigen to maintain immune tolerance, and after encountering foreign antigens, they mature and initiate immune responses (Shortman and Naik, 2007), whereas pDCs trigger innate immunity by producing type I IFNs upon stimulation by viruses or TLR ligands (Asselin-Paturel et al., 2001). Interestingly, whereas IL-21 decreased the percentage (Figure 1C, panel vi vs. i) and absolute number (Figure 1D) of cDCs, it increased the relative percentage of pDCs. GM-CSF almost completely inhibited this effect (Figure 1C, vii vs. vi and Figure 1D), whereas IL-3, which like GM-CSF shares the common β chain, β_c, as part of its receptor, as well as Flt3L and IL-4 had only modest effects (Figure 1C, panels viii-x vs. vi, and Figure 1D). Correspondingly, IL-21 decreased

viability and induced apoptosis of cDCs and this was reversed by GM-CSF (Figure 1E). IL-3 partially reversed IL-21-induced apoptosis (Figure 1E), consistent with its modest effect on viability (Figure 1D). In contrast to its potent pro-apoptotic effects on cDCs, IL-21 had a weaker effect on pDC apoptosis (Figure 1E). Splenic cDCs can be subdivided based on their CD8 expression (Liu and Nussenzweig, 2010). Both $CD8^+$ and $CD8^-$ cDCs express IL-21R in the steady state and it was induced by *in vivo* stimulation with LPS (Figures S1A-S1C). Moreover, IL-21-induced apoptosis of both subpopulations of cDCs, and this was reversed by GM-CSF (Figures S1D and S1E).

MHC class II and CD80 expression correlates with DC maturation, and IL-21 can diminish MHC class II expression on GMCSF-DCs (Brandt et al., 2003). Interestingly, IL-21 had only modestly affected MHC class II and CD80 expression on cDCs but had a potent effect on pDCs (Figure 1F, left panels, ii vs. i). GM-CSF had no obvious effect on MHC class II expression (panels iii and iv) and did not affect its induction by IL-21 (panels v and vi vs. i and ii). GM-CSF appeared to cooperate with IL-21 for CD80 induction on cDCs and pDCs with somewhat higher MFIs with both cytokines than with either alone (Figure 1F, right panels i-vi). Thus, in cDCs, GM-CSF reversed IL-21-induced apoptosis but not its effect on MHC class II and CD80 expression, suggesting that apoptosis and maturation marker expression by IL-21 involve different pathways.

IL-21 and GM-CSF have distinctive and overlapping effects on gene regulation

To study the mechanisms of IL-21-induced apoptosis of cDCs and its prevention by GM-CSF, we used microarray analysis. IL-21 induced 209 and repressed 85 genes ≥ 1.5-fold at 5 h in splenic DCs (Figure 2A and Table S1), whereas GM-CSF induced 412 and repressed 269 genes at this time point (Figure 2A and Table S2). Both cytokines similarly regulated certain genes, including inducing of *Socs1* and *Socs3* and repressing *Icosl* and *Il12b* (Figure 2B), while some genes were differentially regulated; for example, IL-21 induced *Il10* but not *Ccl17* expression in splenic DCs (Figure 2C), whereas GM-CSF induced *Ccl17* but not *Il10* (Figure 2C). Thus, IL-21 and GM-CSF have overlapping and distinctive actions on splenic DCs.

GM-CSF prevents IL-21-induced apoptosis by inhibiting Bim

We sought to identify genes induced by IL-21 but where the induction was suppressed by GM-CSF, to potentially explain the effects of these cytokines on apoptosis. GM-CSF inhibited expression of 33 of the 209 genes induced by IL-21, including *Il21r* and *Bcl2l11* (which encodes Bim) (Tables S3 and S4). Because Bcl-2 family proteins are implicated in IL-21-mediated apoptosis in B cells (Jin et al., 2004; Mehta et al., 2003; Ozaki et al., 2004); we focused on Bim. Consistent with the microarray data, IL-21 did not significantly alter expression of Bcl-2, Bclx, or Bax (Figure 2D), but it significantly induced Bim mRNA (Figure 2D), including both BimEL and BimL isoforms (O'Connor et al., 1998) (Figure 2E), and GM-CSF inhibited this induction (Figures 2D and 2E). Moreover, the number of cDCs was increased in *Bcl2l11*$^{-/-}$ mice (Figure 2F) and *Bcl2l11*$^{-/-}$ cDCs exhibited lower IL-21-induced death (Figures 2G and 2H). Thus, GM-CSF inhibits IL-21-induced apoptosis by decreasing the induction of Bim. Similar to the ability of IL-21 to induce IL-21R in T cells (Zeng et al., 2005), IL-21 induced *Il21r* expression in DCs, and this was inhibited by GM-CSF (Figure 2I), providing another mechanism for the effect of GM-CSF. In contrast, IL-21 did not affect expression of the genes encoding GM-CSF receptor α (*Csf2ra*) and β_c (*Csf2rb*) (Figure 2I). Thus, Bim is critical for IL-21-induced apoptosis of cDCs and its induction is inhibited by GM-CSF.

IL-21-mediated apoptosis of cDCs requires STAT3

In T and B cells, IL-21 activates STAT1, STAT3, and STAT5, with STAT3 activation being the most potent and sustained (Konforte and Paige, 2006; Zeng et al., 2007). Similarly, in cDCs IL-21 induced phosphorylation of STAT1, STAT3, and weakly STAT5 within 10 min. By 90 min, phosphorylation of STAT1 and STAT5 decreased but substantial STAT3 phosphorylation persisted (Figure 3A). GM-CSF had only transient effects on STAT1 and STAT3 phosphorylation, but as expected, it induced sustained phosphorylation of STAT5 (Figure 3A). Although GM-CSF inhibited IL-21-induced *Il21r* expression (Figure 2I), it did not diminish IL-21-induced STAT3 phosphorylation (Figure 3A), suggesting that GM-CSF does not affect proximal IL-21 signaling. Consistent with IL-21 potently activating STAT3, after deletion of *Stat3* in DCs by crossing *Stat3*-floxed mice to CD11c-*Cre* transgenic mice, IL-21 no longer increased apoptosis or diminished viable cells (Figures 3B and 3C), nor did it induce *Il21r* or *Bcl2l11* (Figure 3D). However, STAT3 probably is not involved in DC development and maturation, as the number of splenocytes, cDCs, and pDCs were at most minimally changed (Figures S2A–S2C), and expression of CD80, CD86, ICOSL, and MHC class II was not altered (Figure S2D) in mice in which *Stat3* was conditionally deleted in DCs using CD11c-*Cre*. Although IL-21-induced apoptosis in mantle cell lymphoma depends on STAT1 (Gelebart et al., 2009), the weak induction of STAT1 by IL-21 in DCs suggested that STAT1 might not contribute to IL-21-induced apoptosis, and indeed, *Stat1*$^{-/-}$ mice had essentially normal numbers of splenocytes, cDCs, and pDCs (Figures S2E–S2G), and the lack of STAT1 did not prevent IL-21-induced cell death (Figure 3E) nor substantially alter IL-21-induced *Il21r* or *Bcl2l11* expression (Figure 3F). Although IL-21 induced transient phosphorylation of STAT5, cDCs from *Stat5a*$^{-/-}$ mice exhibited more potent IL-21-induced apoptosis and cell death (Figures 3G and 3H) as well as higher *Il21r* and *Bcl2l11* expression (Figure 3I), suggesting that STAT5 negatively regulates IL-21's action. Indeed, GM-CSF's potent activation of STAT5, a known survival factor (Debierre-Grockiego, 2004), might explain the inhibitory effect of GM-CSF on IL-21-induced apoptosis. Indeed, suppression of *Bcl2l11* and *Il21r* by GM-CSF was diminished in the absence of STAT5A (Figure 3I), underscoring the ability of STAT5 to counteract IL-21's effects of DCs. These data indicate that STAT3 rather than STAT1 or STAT5 mediates IL-21-induced apoptosis of cDCs.

IL-21-induced STAT3 and GM-CSF-induced STAT5 compete for DNA binding

We next used ChIP-Seq to examine STAT3 and STAT5 genome-wide binding patterns in DCs *in vivo*. IL-21-induced STAT3 and GM-CSF-induced STAT5 each broadly bound to intergenic regions of DNA, introns, 5′UTRs, and promoter regions, and this distribution was not altered when IL-21 and GM-CSF were combined (Figure 4A). However, GM-CSF reduced the number of IL-21-induced STAT3 binding sites by ~ 50% from 3,564 to 1,742, and IL-21 lowered GM-CSF-induced STAT5 binding from 30,623 to 19,033 (Figure 4B). In the gene bodies of *Bcl2l11* and *Il21r*, GM-CSF induced the STAT5 binding at the same locus where IL-21 induced STAT3 binding, and when IL-21 and GM-CSF were combined, the number of ChIP-Seq tags decreased (Figure 4C), suggesting a competition between STAT5 and STAT3 as one explanation for the ability of GM-CSF to inhibit IL-21's effect on DCs.

Cross negative regulatory effect of IL-21 and GM-CSF on DCs *in vivo*

To study the effects of IL-21 and GM-CSF on DCs *in vivo*, we used hydrodynamic injection (Liu et al., 1999) of *Il21* and *Csf2* (encodes GM-CSF) expression plasmids. GM-CSF expression expands monocyte-derived pro-inflammatory DCs (mo-DCs) *in vivo* (Naik et al., 2006), which resemble *in vitro* cultured GMCSF-DCs. We distinguished cDCs and mo-DCs by CD64 expression, which is expressed in mo-DCs ($CD64^+$ $CD11c^{hi}$ but not cDCs ($CD64^-$ $CD11c^{hi}$) (Langlet et al., 2012). IL-21 significantly reduced the number of cDCs (Figures 5A and S3A) but not pDCs ($CD64^-$ $CD11c^{int}$ Siglec H^+) (Figures 5B and S3A), consistent

with our *in vitro* observations, and GM-CSF partially inhibited the effect of IL-21 on cDCs (Figures 5A and S3A). Interestingly, the number of mo-DCs expanded by GM-CSF was significantly diminished by IL-21 (Figures 5C and S3A), confirming a previous *in vitro* study (Brandt et al., 2003). Thus, IL-21 and GM-CSF have cross-regulatory effects.

We next sought to confirm that IL-21-induced cDC death *in vivo* was also dependent on STAT3-mediated induction of Bim. After hydrodynamic injection of *Il21* plasmid, *Bcl2l11* and *Il21r* expression was increased in splenic DCs (Figure 5D) and this induction was abolished in the absence of STAT3 (Figure 5E). As expected, IL-21-induced *Bcl2l11* expression and cDC death were not observed in $Il21r^{-/-}$ mice (Figures 5F-5H), and the number of cDCs was not substantially decreased by expressing IL-21 in animals lacking *Bcl2l11* (Figures 5I and 5J), suggesting that Bim is a critical mediator of cDC death. Interestingly, IL-21-induced *Il21r* expression was higher in $Bcl2l11^{-/-}$ than in wild-type (WT) mice (Figure 5K). Consistent with $Stat5a^{-/-}$ mice having enhanced IL-21-induced effects on cDCs *in vitro* (Figures 3G-3I), injection of *Il21* plasmid more strongly induced *Bcl2l11* expression in $Stat5a^{-/-}$ than in WT mice (Figure 5L), suggesting that STAT5A negatively regulated *Bcl2l11* expression. Correspondingly, IL-21 induced more cDC death in $Stat5a^{-/-}$ than in WT mice (Figures 5M and 5N). These data indicate that IL-21-induced cDC death *in vivo* requires expression of IL-21 receptor, STAT3 and Bim, and that STAT5 is a negative regulator of IL-21's effects.

To study the effects of IL-21 on $CD8^+$ and $CD8^-$ cDC subsets *in vivo*, we purified these subsets after injection of *Il21* plasmid (Figure S3B). IL-21 preferentially induced *Il21r* and *Bcl2l11* expression in $CD8^-$ cDCs (Figure S3C) and correspondingly induced death of $CD8^-$ but not $CD8^+$ cDCs *in vivo* (Figure S3D). Thus, although IL-21 induces apoptosis of both subtypes of splenic cDCs with similar potency *in vitro*, the $CD8^-$ cDCs are more sensitive to IL-21 *in vivo*.

Repetitive stimulation of NKT cells leads to the IL-21-dependent DC death

Activation of NKT cells by glycolipid α-galactosylceramide (α-GalCer) induces production of IL-21 (Coquet et al., 2007), which can induce Bε cell apoptosis and suppress IgE responses (Harada et al., 2006). Whereas a single injection of α-GalCer into mice activates DCs as reflected by increased MHC class II and IL-12 expression, repetitive challenges of α-GalCer (three injections at intervals of 3–4 days) induces regulatory properties of DCs, including production of IL-10 (Kojo et al., 2005). To study the physiological role of IL-21 on DCs, we injected α-GalCer once (GC1) or three times (GC3) into WT mice, and as previously reported (Kojo et al., 2005), repetitive challenge of NKT cells diminished *Ifng* but increased *Il10* expression (Figure 6A). Interestingly, a single injection of α-GalCer led to rapid induction (4 h) of *Csf2*, but this was greatly reduced with repetitive challenge (4 h after the last injection). In contrast, *Il21* expression was induced late (48 h) after a single challenge, but induction was higher and more rapid after repetitive challenge (Figure 6A). Thus, repetitive stimulation of NKT cells potently induced IL-21 but diminished GM-CSF production. Importantly, repetitive injection of α-GalCer not only induced suppressive properties of DCs, as reflected by expression of *Il10* but not *Il12b*, but it also induced *Bcl2l11* and *Il21r* expression (Figure 6B) and decreased the number of $CD8^-$ but not $CD8^+$ splenic DCs (Figures 6C and 6D), consistent with the actions of IL-21 *in vivo* (Figure S3D), and the decrease of $CD8^-$ DCs was attenuated in the absence of IL-21 receptor or Bim (Figures 6E-6H). Thus, repetitive antigen stimulation of NKT cells leads to increased production of IL-21, which in turn regulates DC numbers via the induction of Bim.

IL-21 inhibits GM-CSF production

Because GM-CSF inhibited the apoptotic effect of IL-21 on cDC, and both GM-CSF and IL-21 are produced by activated $CD4^+$ T cells, we asked if IL-21 inhibited GM-CSF production. Indeed, after TCR stimulation of $CD4^+$ T cells for 4 hours, expression of *Csf2* was induced, but this was suppressed by IL-21 (Figure 7A; left). Interestingly, deletion of *Stat3* in $CD4^+$ T cells increased TCR-induced *Csf2* expression and abrogated IL-21-mediated suppression (Figure 7A; left), suggesting that STAT3 inhibits *Csf2* expression. Consistent with prior reports, IL-21 induced *Il21* mRNA expression in WT (Korn et al., 2007; Nurieva et al., 2008; Suto et al., 2008) but not in *Stat3*-deficient T cells (Figure 7A; right) indicating that IL-21 auto-regulation required STAT3. Because IL-21-mediated STAT3 activation requires JAK3 (Leonard and Spolski, 2005), we examined the effect of a JAK3 inhibitor, CP-690550 (Changelian et al., 2003), on *Csf2* expression. CP-690550 reduced TCR-induced *Csf2* expression, but it abolished IL-21-mediated suppression of *Csf2* (Figure 7B), confirming a role for the JAK3-STAT3 pathway for this effect. When we studied GM-CSF production after 4 days of $CD4^+$ T cell differentiation in the presence of IL-21, both mRNA and protein levels of GM-CSF were reduced even in *Stat3*-deficient cells (Figures 7C and S4A), suggesting that IL-21 could also suppress GM-CSF production independent of STAT3. Because IFN-γ, IL-12, and IL-27 can negatively regulate GM-CSF production in $CD4^+$ T cells (Codarri et al., 2011), we isolated T cells from WT mice or mice in which *Stat3* was deleted in T cells using CD4-*Cre* and differentiated them in the presence of IL-21 with or without a mixture of blocking antibodies for IFN-γ, IL-12 and IL-27. These antibodies indeed increased production of GM-CSF, but IL-21 inhibited GM-CSF expression in the presence of these antibodies, even in the absence of STAT3 (Figure 7D). Corresponding to the increased *Csf2* mRNA in the absence of STAT3 (Figure 7A), GM-CSF production increased in $Stat3^{-/-}$ mice 4 days after TCR stimulation (Figures 7D and S4B). Thus, IL-21 can suppress GM-CSF production by a mechanism independent of IFN-γ, IL-12, and IL-27 as well as STAT3. Furthermore, GM-CSF not only can block the actions of IL-21 in cDCs, but IL-21 can diminish the production of GM-CSF, indicating cross-regulatory effects of these two cytokines.

IL-21 decreases DC numbers in T cell adoptive transfer-mediated colitis

To elucidate the functional role of IL-21 on DCs in a disease setting, we used a T cell adoptive transfer colitis model. We crossed $Il21r^{-/-}$ mice with $Rag2^{-/-}$ mice to generate mice lacking expression of both *Il21r* and *Rag2* and adoptively transferred naïve ($CD25^-$ $CD45RB^{hi}$) WT $CD4^+$ T cells into $Il21r^{-/-}Rag2^{-/-}$ or $Rag2^{-/-}$ mice. We found that $Il21r^{-/-}Rag2^{-/-}$ mice had earlier weight loss than $Rag2^{-/-}$ mice (Figure 7E), and their colons had greater infiltration of mononuclear cells (Figure 7F, right panels), inflammation, thickened walls, and epithelial damage (Figure 7G). The morphology of colons from $Il21r^{-/-}Rag2^{-/-}$ mice that did not receive WT $CD4^+$ T cells was normal (Figure 7F, left panels); thus, the inflammation depended on donor T cells. When naïve $CD4^+$ T cells isolated from *Il21*-mCherry transgenic reporter mice (Wang et al., 2011) were injected into $Rag2^{-/-}$ and $Il21r^{-/-}Rag2^{-/-}$ mice, the percentage of T cells that expressed mCherry tended to be higher in $Il21r^{-/-}Rag2^{-/-}$ than in $Rag2^{-/-}$ mice, but the difference was not statistically significant (Figure 7H). GM-CSF was also produced by these cells but its expression level was much lower than IL-21 (Figure 7H), suggesting that IL-21 might be critical for the observed pathology. Consistent with the actions of IL-21 on DCs, colons from $Il21r^{-/-}Rag2^{-/-}$ mice had more DCs than those from $Rag2^{-/-}$ mice (Figure 7I). DC-T cell interactions are critical for T cell expansion, differentiation, and function, and consistent with more severe disease in the $Il21r^{-/-}Rag2^{-/-}$ mice, after adoptive transfer these animals had increased $CD4^+$ T cells in colon (Figure 7J) and mesenteric lymph nodes (Figure 7K). After stimulation with PMA + ionomycin, $CD4^+$ T cells from $Il21r^{-/-}Rag2^{-/-}$ mice expressed less IL-17 but more IFN-γ than did cells from $Rag2^{-/-}$ mice (Figure 7L),

indicating increased Th1 differentiation, consistent with the importance of IFN-γ in colitis progression (Bouma and Strober, 2003). These data suggested a physiological significance of IL-21 on DC turnover and T cell-mediated pathology.

DISCUSSION

In this study, we unexpectedly found that IL-21 has markedly different effects on cDCs and pDCs, inducing a greater decrease in viability and corresponding increase in apoptosis of cDCs than in pDCs, while more potently inducing MHC II expression on pDCs than on cDCs. IL-21 was previously reported to inhibit LPS-induced production of IL-12, TNF-α, and IL-6 in GMCSF-DCs (Brandt et al., 2003). Our demonstration that IL-21-induced apoptosis of cDCs is prevented by GM-CSF reveals at least partially opposing biological actions by these cytokines. Indeed, the inhibitory action of GM-CSF helps to explain the lack of IL-21-induced cell death in GMCSF-DCs. By microarray analysis, we found that GM-CSF inhibited a subset of the genes induced by IL-21, including *Il21r* and *Bcl2l11*, whereas other genes were induced (e.g., *Socs3, Socs1*) or inhibited (e.g., *Icosl*, *Il12b*) by both IL-21 and GM-CSF, revealing that IL-21 and GM-CSF have overlapping but also distinctive effects. ChIP-Seq analysis for STAT3 and STAT5 in splenic DCs treated with IL-21 and GM-CSF revealed that the number of STAT3 sites and the intensity of binding were reduced in the presence of GM-CSF, suggesting that binding competition between STAT3 and STAT5 may be a mechanism by which GM-CSF might inhibit IL-21-induced gene expression and biological actions.

We show that IL-21 induces apoptosis of cDCs via STAT3-dependent activation of Bim, even though IL-21-activated STAT3 is generally viewed as a positive regulator of T cell development and function (e.g., deletion of STAT3 in $CD4^+$ T cells prevents the development of inflammatory bowel disease (Durant et al., 2010)). However, several studies showed that STAT3 activation negatively regulates DC function (Cheng et al., 2003; Melillo et al., 2010; Nefedova et al., 2005), and inhibiting STAT3 can increase the ability of DCs to stimulate allogeneic or antigen-specific T cells (Nefedova et al., 2005). Importantly, mice in which *Stat3* was conditionally deleted using CD11c-*Cre* develop cervical lymphadenopathy and mild ileocolitis, and DCs from these mice have enhanced immune activity (Melillo et al., 2010). Our findings may explain these observations, with IL-21-induced STAT3-dependent apoptosis of cDCs providing a mechanism for limiting immune responses. Indeed, apoptosis of DCs is critical for preventing autoimmunity upon challenge by pathogens, and mice bearing apoptosis-resistant DCs exhibit chronic lymphocyte activation and systemic autoimmune disease (Chen et al., 2006). The production of IL-21 may be a way to eliminate DCs, thus maintaining immune tolerance. Importantly, however, IL-21 can positively regulate autoimmunity in the context of type 1 diabetes (McGuire et al., 2011; Spolski et al., 2008; Sutherland et al., 2009) and systemic lupus erythematosus (Bubier et al., 2009; Herber et al., 2007; Ozaki et al., 2004), indicating critical roles for IL-21 *in vivo*. Given that DCs might receive multiple cytokine signals simultaneously, the ability of GM-CSF to inhibit IL-21-induced apoptosis was striking. Indeed, cDCs produce IL-6 after IL-21 stimulation (Spolski et al., 2012) and the induction is not inhibited by GM-CSF (Table S3), suggesting that GM-CSF might switch the role of IL-21 to cDCs from anti-inflammatory to pro-inflammatory. Moreover, we showed not only that GM-CSF inhibits IL-21-mediated apoptosis, but that IL-21 inhibits the production of GM-CSF, indicating a fine-tuning of actions via a cross-negative regulation. Importantly, repetitive injection of mice with α-GalCer induced IL-21 and decreased GM-CSF production by NKT cells, as well as increased *Il21r* and *Bcl2l11* expression by cDCs, correlating with a reduction in the number of these cells, and furthermore data in the colitis model revealed greater numbers of colonic DCs in $Rag2^{-/-}Il21r^{-/-}$ than in $Rag2^{-/-}$ mice, with an associated increase in pathology.

Previously, it was shown that IL-21 cooperates with IL-15 to enhance $CD8^+$ T cell expansion and function (Zeng et al., 2005) and with IL-4 to induce antigen-specific IgG production (Ozaki et al., 2002). Our current study identifies distinctive roles for IL-21 depending on the type of DCs and reveals that GM-CSF can potently inhibit IL-21-mediated effects on cDCs. Given the critical roles served by DCs in immune responses, our elucidation of the actions of IL-21 and GM-CSF on DCs have implications for the rational design of strategies for modulating immune responses and new treatments of immune-related diseases.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, *Rag2*$^{-/-}$, *Bcl2l11*$^{-/-}$ (Bouillet et al., 1999) and CD11c-*Cre* (Caton et al., 2007) mice were from The Jackson Laboratory. *Il21r*$^{-/-}$ (Ozaki et al., 2002) and *Stat5a*$^{-/-}$ mice (Liu et al., 1997; Rochman et al., 2010) have been described. In some experiments, control mice were from Taconic. *Il21*-mCherry reporter transgenic mice were described (Wang et al., 2011). STAT3-deficient DCs or $CD4^+$ T cells were generated by crossing *Stat3*-floxed mice (Lee et al., 2002) with CD11c-*Cre* or CD4-*Cre* mice, respectively. *Stat1*$^{-/-}$ and control mice on a 129S6 background were from Taconic Farms. *Il21r*$^{-/-}$ and *Rag2*$^{-/-}$ mice were bred to generate *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice. All protocols were approved by the NHLBI Animal Care and Use Committee and followed NIH guidelines for using animals in intramural research.

Isolation of splenic DCs

Mouse spleens were injected with 1 ml of 1 mg/ml collagenase D and 20 μg/ml DNase I (Roche), cut and incubated in collagenase solution at 37°C for 20 min. After passage through a cell strainer, red blood cells were lysed using ACK lysis buffer, and remaining cells incubated with Fc block (BD Biosciences) at 4°C for 10 min. $CD11c^+$ cells were positively selected with pan-DC or CD11c microbeads (Miltenyi Biotec). Purity of $CD11c^+$ DCs was 93–95%. pDCs ($CD11c^{lo}B220^+PDCA\text{-}1^+$) were 3–10% of DCs isolated with pan-DC microbeads.

In vivo administration of α-GalCer and the isolation of splenic NKT cells

Mice were injected i.p. with 2 μg of α-GalCer (Enzo Life Sciences) either once (GC1) or three times at intervals of 3 days (GC3). Four or 48 h after the last injection, spleens from 3 mice were pooled and NKT cells were isolated with $NK1.1^+$ iNKT cell isolation kit (Miltenyi Biotec).

Generation of GMCSF-DCs

Bone marrow cells from mouse femurs and tibias were cultured for 8 days in RPMI-1640 medium containing 10% FBS, 200 μM L-glutamine, 10 IU/ml penicillin, 100 μg/ml streptomycin, 55 μM β-mercaptoethanol, and 20 ng/ml GM-CSF (Peprotech), with medium changed every 3 days. $CD11c^+$ DCs were > 90% pure.

Cell viability and apoptosis studies

Splenic DCs or GMCSF-DCs were rested at 37°C for 1 h and treated with cytokines for 6, 16, and 24 h. The number of DCs was counted, and cDCs and pDCs were distinguished by staining for B220, CD8, and CD11c. For apoptosis, cells were stained with Annexin V and 7-AAD (BD Biosciences). Data in viability studies are mean ± SEM from ≥ 3 experiments.

FACS analysis of phosphorylated STAT proteins

To reduce the background p-STAT signal, splenic DCs in complete medium were rested at 37°C for 16 h, stained with anti-CD11c-APC and anti-B220-FITC at 37°C for 10 min, stimulated with cytokines for indicated time, fixed with 2% paraformaldehyde at 37°C for 10 min, and permeabilized in 90% ice-cold methanol for 30 min. After two washes with FACS buffer (PBS with 1% FBS), cells were stained with p-STAT1 (Y701)-PE, p-STAT3 (Y705)-PE, or p-STAT5 (Y694)-Alexa Fluor 647 antibodies at RT for 1 h, and analyzed on a FACSCalibur.

RNA analysis

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). First-strand cDNAs were synthesized using the Omniscript reverse transcription kit (Qiagen) and oligo(dT). Quantitative RT-PCR was performed on a 7900H sequence detection system (Applied Biosystems). Real-time primers and TaqMan probes were from Applied Biosystems. Expression was normalized to *Rpl7*.

Immunoblot analysis

Purified splenic DCs were rested 1 h, then left unstimulated or stimulated with IL-21 and/or GM-CSF for 8 h, lysed in RIPA buffer (Thermo Scientific) for 15 min, and 5 μg cell lysate protein was resolved by 4–12% SDS-PAGE and immunoblotted with anti-Bim (Millipore) or anti-β-actin (Santa Cruz Biotechnology).

Th cell polarization

Naïve ($CD62L^+$ $CD44^-$ $CD25^-$) $CD4^+$ T cells were negatively selected from splenic $CD4^+$ T cells, followed by positively selecting $CD62L^+$ cells (Miltenyi Biotec), cultured for 4 days with 5 μg/ml plate-bound anti-CD3 + 2 μg/ml anti-CD28 in the presence of IL-21 (R & D Systems), washed, and re-stimulated with 50 ng/ml PMA + 1 μM ionomycin for 4 h to measure mRNA expression, or 2 μg/ml anti-CD3 for 24 h to measure GM-CSF protein by ELISA (BD Biosciences).

Hydrodynamic injected-mediated expression of cytokines *in vivo*

Experiments were done as described (Liu et al., 1999). Control, *Csf2* (from Howard Young, NIH), and *Il21* (Invivogen) expression plasmids were prepared by EndoFree Plasmid Mega Kit (Qiagen). Mice were injected i.v. with 10 μg plasmid DNA in 1.5 ml PBS. Splenic DCs were analyzed 24 to 72 h post-injection.

Adoptive transfer of naïve $CD4^+$ T cells and cell isolation from colons

Splenic $CD4^+$ T cells were isolated by negative selection using $CD4^+$ T cell isolation kit (Miltenyi Biotec), stained with antibodies to CD4, CD25, and CD45RB, and $CD4^+CD25^-CD45RB^{hi}$ (brightest 40%) cells sorted by flow cytometry. 4×10^5 cells were injected i.p. into $Rag2^{-/-}$ or $Il21r^{-/-}Rag2^{-/-}$ mice. Mice were weighed periodically and analyzed when the $Il21r^{-/-}Rag2^{-/-}$ mice showed significant weight loss. Total colon cells (without lymphocyte enrichment) were isolated as described (Kitamura et al., 2010).

Microarray and data analysis

1 μg total RNA was incubated with oligo dT/T7 primers and reverse transcribed into doubled stranded cDNA. *In vitro* transcription and biotin labeling were performed with T7 RNA polymerase at 37°C for 16 h (Affymetrix IVT labeling kit). 20 μg of this biotin-labeled RNA was fragmented to ~200 bp by incubating in 200 mM Tris-acetate pH 8.2, 500 mM potassium acetate and 500 mM magnesium acetate for 35 min at 94°C and then

hybridized to Affymetrix U133 plus 2.0 chips for 16 h, washed and stained on an Affymetrix fluidics station. Affymetrix GCOS version 1.4 was used to calculate the signal intensity and percent present calls. Signal intensity values for probe sets were transformed using an adaptive variance-stabilizing, quantile-normalizing transformation and data from the chips were subjected to a principal component analysis (PCA) to detect outliers, and fold-cut off and false discovery rate (FDR) analysis filters were applied. To bring together sets of samples and genes with similar expression patterns, two-way hierarchical clustering was run using the JMP5.1 statistical software package (SAS Institute) and the Ward method.

ChIP-Seq analysis

Splenic DCs were rested for 1 h, treated with cytokines for 2 h, and fixed with 1% formaldehyde at 37°C for 10 min. Chromatin from 5×10^7 cells was sonicated for 20 cycles of 12 sec on/48 sec off, into 200–500 bp fragments, DNA ends were repaired using polynucleotide kinase and Klenow enzyme, and treated with Taq polymerase to generate a protruding 3′ "A" nucleotide. A pair of Solexa PE adaptors (Illumina) was ligated and DNA amplified using the adaptor primers (PE 1.0 and 2.0, Illumina) for 19 cycles. DNA fragments of ~250–450 bp were purified from agarose gels and used for cluster generation and sequencing (Solexa 1G Genome Analyzer). Anti-STAT3 antibody (13–7000, Invitrogen) and anti-STAT5 (PA-ST5B, R & D Systems) were used. Sequenced short reads (typically 25 bp) were obtained with the Solexa Analysis Pipeline and mapped to the mouse genome (mm8/NCBI36, 2006 Assembly), retaining only uniquely matched reads. To eliminate bias caused by PCR amplification, redundant reads at specific genomic coordinates were also removed. The Pipeline output was converted to browser-extensible data (BED) files for viewing on the UCSC genome browser (http://genome.ucsc.edu/). BED files represent the genomic coordinates of each read; to make graph files, we mapped reads into non-overlapping 200 bp windows. The location of a tag on +/− strand was shifted by up to ±100 bp from its 5′ start to represent the center of the DNA fragment associated with the read. Reads in each 200 bp summary window were counted.

Genomic regions enriched for GM-CSF-induced STAT5B and IL-21-induced STAT3 binding were detected using the Model-based Analysis of ChIP-Seq (MACS, version 1.3.7.1) algorithm (Zhang et al., 2008), using *gsize* = 2.04e+09, *mfold* = 15, *tsize* = 25, *bw* = 300, *pvalue* = 1.0e−05. Final peaks were filtered by FDR = 0.02. Our ChIP-Seq data were saturating (i.e., adding more reads would not detect more peaks). To determine STAT3 and STAT5B binding site distribution, based on UCSC genes annotations database, we divided the mouse genome into 6 regions: promoters (from transcriptional start sites [TSS] to 5kb 5′ of TSS), 5′ UTR (5′ untranslated region, from TSS to coding region start), exons, introns, 3′ UTR, and intergenic regions.

Statistical analysis

Statistical comparison between samples was done by student's *t* test. *, $P < 0.05$; **, $P < 0.01$. NS, not statistically different.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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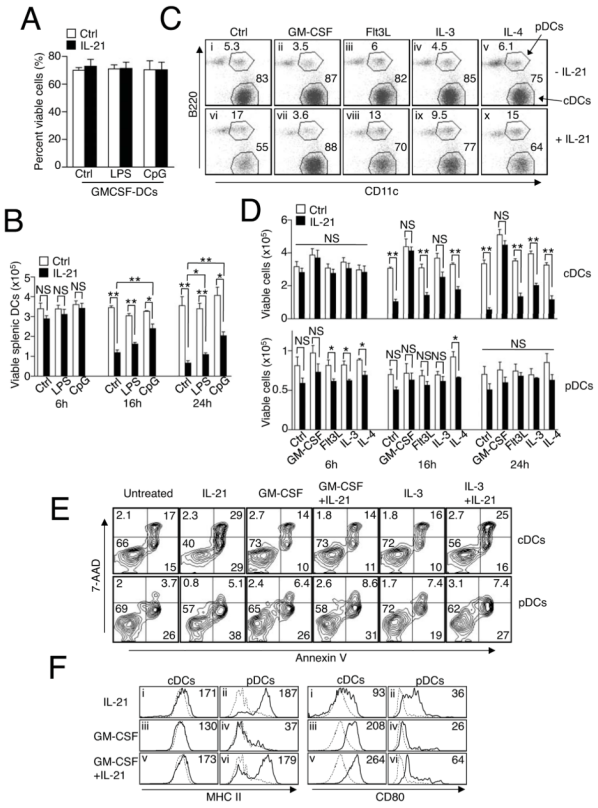

Figure 1. see also Figure S1. IL-21 strongly induces apoptosis of splenic cDCs and modestly affects pDCs, with reversal of these effects by GM-CSF

(A and B) Effect of IL-21 on viability of DCs. (A) GMCSF-DCs were rested 1 h, stimulated with 1 μg/ml LPS and 200 nM CpG-B with or without 100 ng/ml IL-21 for 24 h, and percent viable cells determined by flow cytometry. (B) Splenic DCs were treated as in (A) for 6, 16, or 24 h, and the number of DCs determined (means ± SEM from 3 experiments). (C) Splenic DCs were rested 1 h and not stimulated or stimulated with 20 ng/ml GM-CSF, 100 ng/ml Flt3L, 20 ng/ml IL-3, 20 ng/ml IL-4 with or without 100 ng/ml IL-21 for 24 h. cDCs and pDCs were identified by CD11c and B220 staining. (D) Splenic DCs were treated as in (C) for 6, 16, or 24 h, and viable cells counted by trypan blue exclusion; numbers of cDCs and pDCs were calculated by multiplying total cells by percentages of cDCs and pDCs from (C). Shown are means ± SEM from 3 experiments. (E) Splenic DCs were rested 1 h, stimulated with indicated cytokines for 12 h, and % apoptotic cells determined by Annexin V and 7-AAD staining. Data are representative of 3 experiments. (F) Effect of IL-21 and GM-CSF on MHC class II and CD80 expression for cDCs and pDCs. Splenic DCs were treated as in (C). Data are representative of 3 experiments. Solid line, mean florescence intensity (MFI) after cytokine treatment. Dotted line, MFI of untreated control for MHC II expression: cDCs = 145, pDCs = 34; for CD80 expression: cDCs = 86, pDCs = 10.

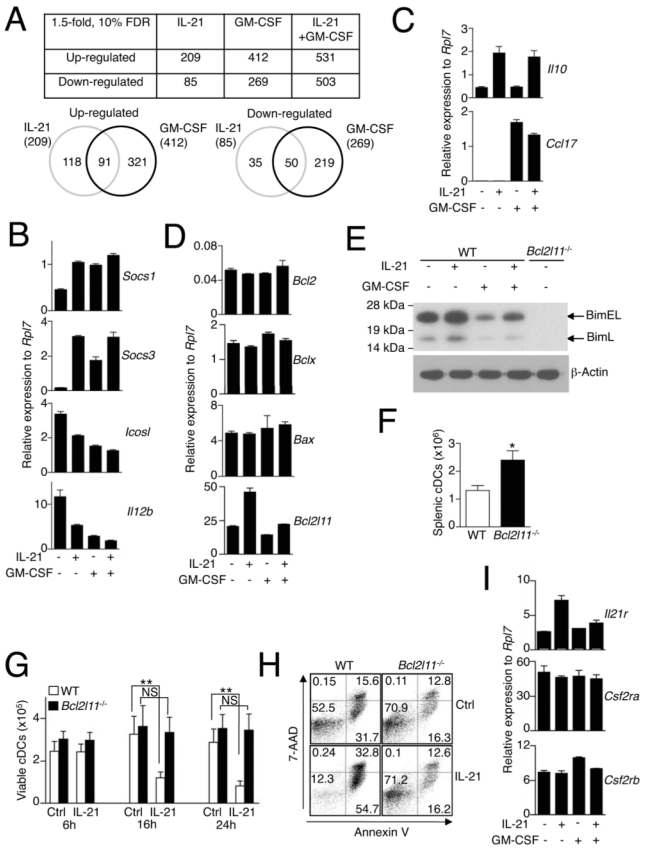

Figure 2. see also Tables S1-S4. IL-21 and GM-CSF have both distinctive and overlapping gene regulation profiles

(A; top) Number of genes induced or repressed by IL-21, GM-CSF, or IL-21 + GM-CSF (≥1.5-fold induction or repression with 10% FDR. (A; bottom) Venn diagrams showing numbers of genes induced or repressed by IL-21 and/or GM-CSF. (B and C) IL-21 induces *Socs1*, *Socs3*, and *Il10*, suppresses *Icosl* and *Il12b,* but does not affect *Ccl17* expression. Splenic DCs were rested 1 h, treated with IL-21 and/or GM-CSF for 5 h, and mRNA expression of indicated genes analyzed by RT-PCR. Shown is expression relative to *Rpl7*; data are representative of 3 experiments. (D and E) IL-21 induced Bim but this was inhibited by GM-CSF. (D) Splenic DCs were rested 1 h, treated with IL-21 and/or GM-CSF for 5 h, and mRNA expression of *Bcl2*, *Bclx*, *Bax*, and *Bcl2l11* analyzed by RT-PCR. Shown is expression relative to *Rpl7*. Data are representative of 3 experiments. (E) Splenic DCs were treated as in (D) for 8 h, and Bim levels determined by western blotting; β-actin was a loading control. Data are representative of 3 experiments. (F–H) *Bcl211*$^{-/-}$ cDCs are resistant to IL-21-induced apoptosis. (F) Total splenic cDCs were higher in *Bcl2l11*$^{-/-}$ mice. cDCs were identified by CD11c and B220 staining. Data are representative of 3 experiments. (G) Splenic DCs were not treated or were treated with IL-21 for 6, 16, or 24 h and viable cells determined. (H) Splenic DCs were treated with IL-21 for 20 h and % apoptotic cells determined. (I) IL-21 induces *Il21r* expression but this is inhibited by GM-CSF. mRNA expression of *Il21r, Csf2a, and Csf2b* were measured as in (B). Data are representative of 3 experiments. In B–D, F–G, I, shown are means ± SEM.

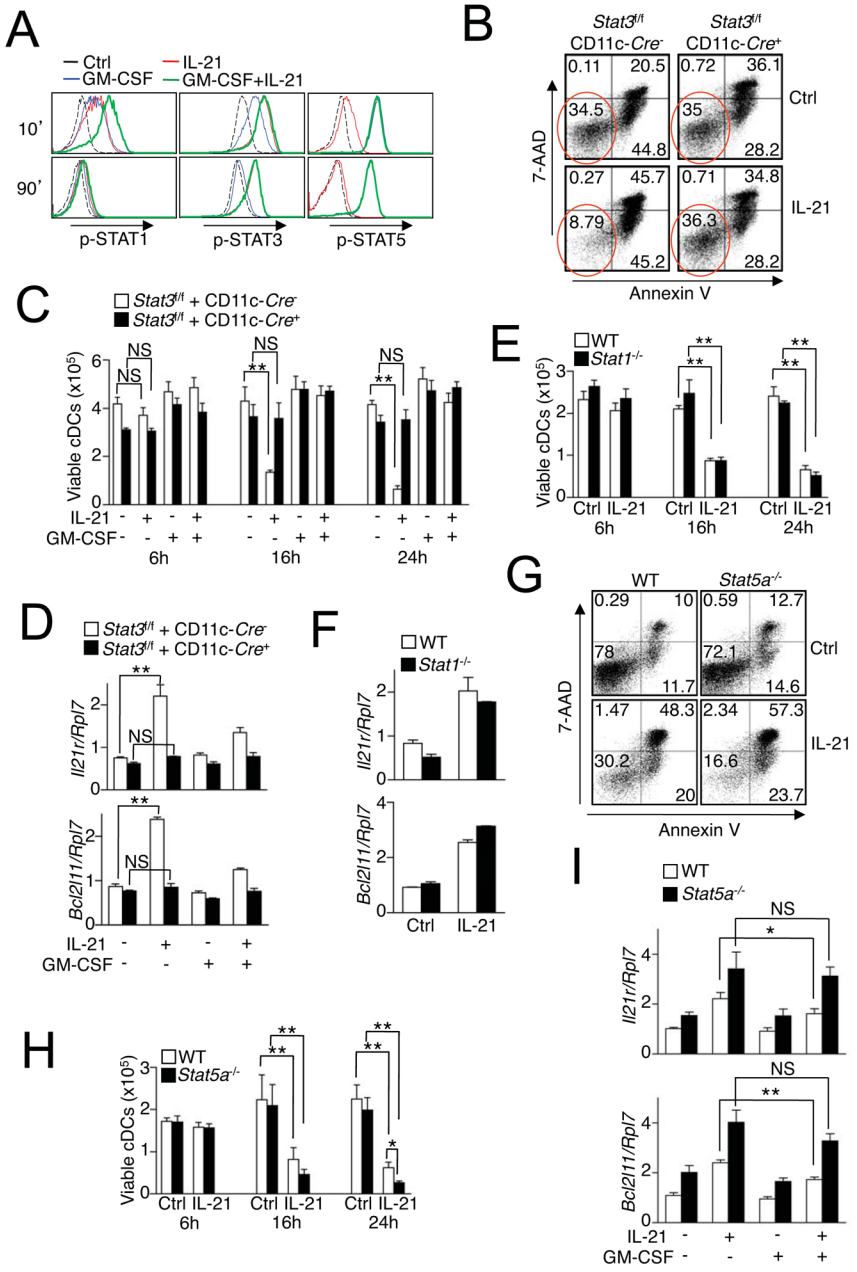

Figure 3. see also Figure S2. IL-21-induced, STAT3-dependent apoptosis and induction of *Bcl2l11* and *Il21r* expression

(A) IL-21 activates STAT proteins in splenic DCs. Splenic DCs were rested 16 h and stimulated with indicated cytokines for 10 or 90 min. cDCs were gated as $CD11c^{hi}$ $B220^{-}$ cells. Phospho-STAT1, STAT3, and STAT5 were evaluated by flow cytometry. Data are representative of 3 experiments. (B–D) STAT3 is critical for IL-21-induced apoptosis and for *Bcl2l11* and *Il21r* expression. (B) *Stat3*-CD11c-*Cre* conditionally-deficient mice and WT littermates were treated as in Figure 2H, and % apoptotic cells determined. (C) *Stat3*-CD11c-*Cre* conditionally-deficient mice and WT littermates were treated with IL-21 and/or GM-CSF for 6, 16, or 24 h, and viable cells determined. Data are representative of 3 experiments. (D) *Stat3*-CD11c-*Cre* conditionally-deficient mice and WT littermates were

treated as in Figure 2D, *Il21r* (top) and *Bcl2l11* (bottom) levels were measured relative to *Rpl7*. Data are representative of 3 experiments. (E and F) STAT1 is not required for IL-21-induced apoptosis, *Bcl2l11* and *Il21r* expression. (E) DCs from *Stat1*$^{-/-}$ and WT littermates were treated as in Figure 2G and the number of viable cells counted. (F) *Il21r* (top) and *Bcl2l11* (bottom) mRNA was measured as in (D). Data are representative of 3 experiments. (G–I) STAT5A is important of DC survival, *Bcl2l11* and *Il21r* suppression. (G) DCs from *Stat5a*$^{-/-}$ mice and WT littermates were treated as in (B) and % apoptotic cells determined. (H) *Stat5a*$^{-/-}$ and WT mice were treated as in (E), and the number of viable cells determined. (I) DCs from *Stat5a*$^{-/-}$ mice and WT littermates were rested 1 h and then treated with IL-21 and/or GM-CSF for 5 h. mRNA expression of *Il21r* (top) and *Bcl2l11* (bottom) were measured as in (D). Data are representative of 3 experiments. In C–F, H–I, shown are means ± SEM.

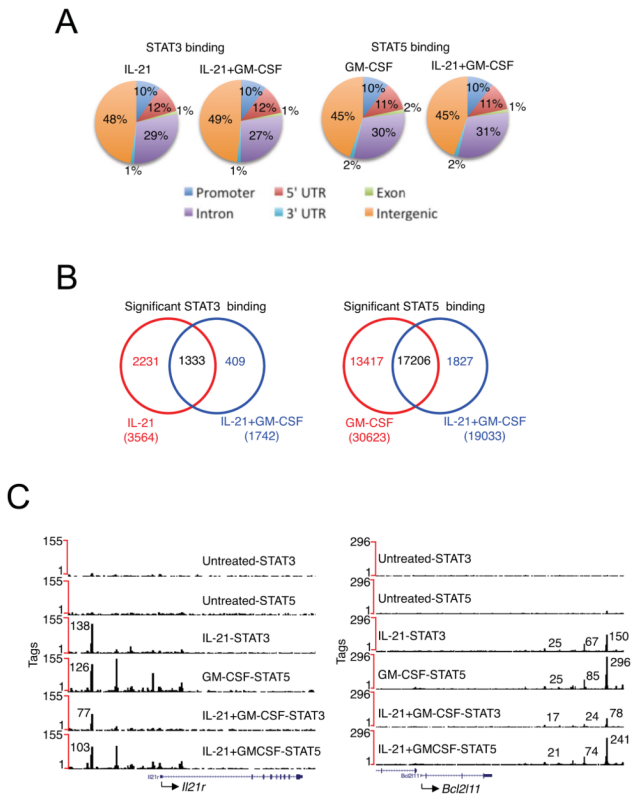

Figure 4. IL-21-induced STAT3 and GM-CSF-induced STAT5 compete for DNA binding

(A) Distribution of STAT3 and STAT5 binding sites from ChIP-Seq analysis of splenic DCs treated with IL-21 and/or GM-CSF for 2 h. (B) IL-21-induced STAT3 (left) and GM-CSF-induced STAT5 (right) binding sites were lower in DCs treated with GM-CSF + IL-21. (C) STAT3 binding to *Il21r* and *Bcl2l11* genes was induced by IL-21 but inhibited by GM-CSF. Tag numbers for peaks are indicated. Untreated control showed minimal STAT3 binding. Data are representative of 2 experiments.

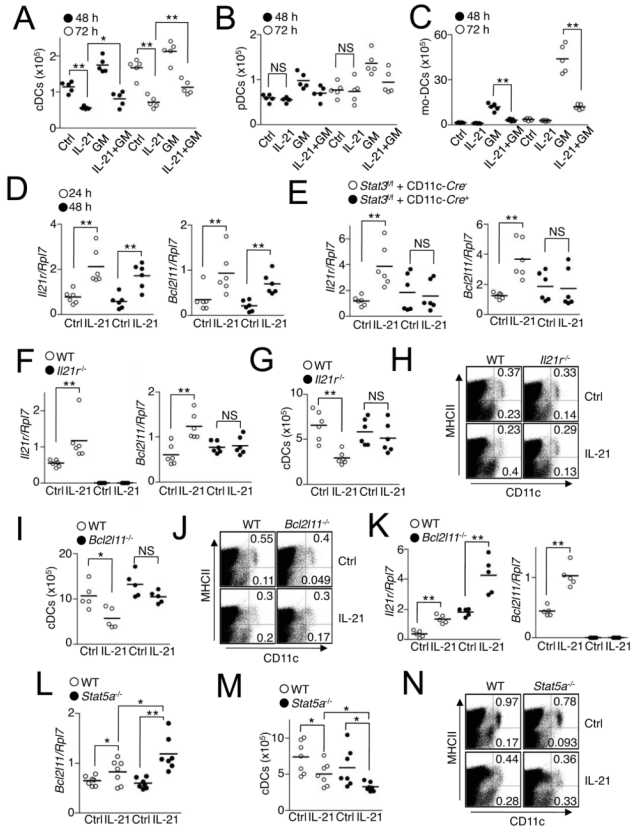

Figure 5. see also Figure S3. IL-21-induced cDC death *in vivo* requires STAT3-dependent Bim expression

(A–C) WT mice were injected i.v. with 10 μg of control (Ctrl), *Il21* (IL-21), and/or *Csf2* (GM) plasmids, and 48 or 72 h later numbers of cDCs (A), pDCs (B), and mo-DCs (C) were analyzed. Data are representative of 3 experiments with a total of 15 mice/group. (D) WT mice were injected i.v. with control or *Il21* plasmids as in (A). After 24 or 48 h, splenic DCs were isolated and *Il21r* (left) and *Bcl2l11* (right) mRNA levels assessed. (E) *Stat3*-CD11c-*Cre* conditionally-deficient mice and WT littermates were treated as in (A) for 24 h, splenic DCs isolated, and *Il21r* (left) and *Bcl2l11* (right) mRNA levels assessed. (F–H) *Il21r*$^{-/-}$ and WT littermates were treated as in (A) for 48 h, splenic DCs were isolated, and *Il21r* (left) and *Bcl2l11* (right) mRNA levels were assessed (F). Absolute number (G) and relative percentage based on MHCII and CD11c expression (H) of cDCs were determined. (I–K) *Bcl2l11*$^{-/-}$ mice and WT littermates were treated as in (A) for 48 h, splenic DCs were isolated and absolute number (I) and relative percentage (J) of cDCs were determined. *Il21r* (left), and *Bcl2l11* (right) mRNA levels were assessed (K). (L–N) *Stat5a*$^{-/-}$ and WT control mice were treated as in (A) for 48 h, splenic DCs were isolated and *Bcl2l11* mRNA was assessed (L). Absolute number (M) and relative percentage (N) of cDCs were determined. In D–N, data from 2 experiments were combined, with a total of 5–6 mice/group.

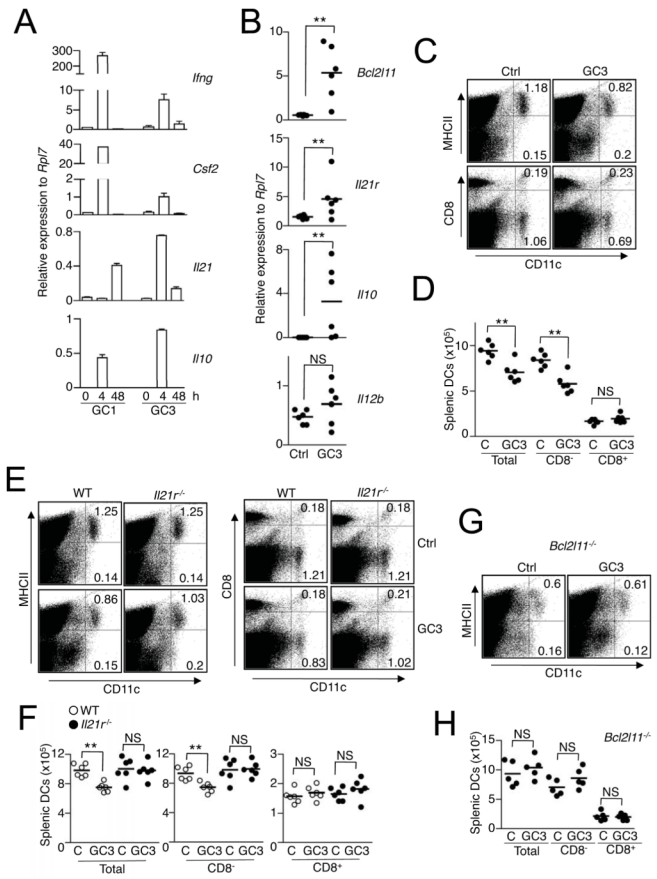

Figure 6. IL-21 produced by NKT cells induces DC death

(A) Mice were injected with α-GalCer once (GC1) or three times (GC3), splenic NKT cells (pooled from 3 mice) were isolated 4 or 48 h after the last injection, and mRNA expression of indicated genes was analyzed. Expression levels are relative to *Rpl7* (mean ± SEM) and representative of 3 experiments. (B) Mice were injected with α-GalCer three times, splenic DCs isolated 4 h after the last injection, and mRNA expression of indicated genes analyzed relative to *Rpl7*. (C and D) Mice were challenged as in (B), total spleen cells harvested 48 h after the last injection, and relative percentage (C) and absolute number (D) of total, $CD8^-$ or $CD8^+$ cDCs determined. Data are from 2 experiments with a total of 6 mice/group. (E and F) WT or *Il21r*$^{-/-}$ mice were treated as in (B), total spleen cells were harvested 48 h after the last injection, and relative percentage (E) and absolute number (F) of total, $CD8^-$ or $CD8^+$ cDCs determined. Data are from 2 experiments with a total of 6 mice/group. (G and H) WT or *Bcl2l11*$^{-/-}$ mice were treated as in (B), total spleen cells were harvested 48 h after the last injection, and the relative percentage of cDCs (G) and number of total cDCs and $CD8^-$ or $CD8^+$ subpopulations of cDCs (H) determined. Data are from 2 experiments with a total of 5 mice/group.

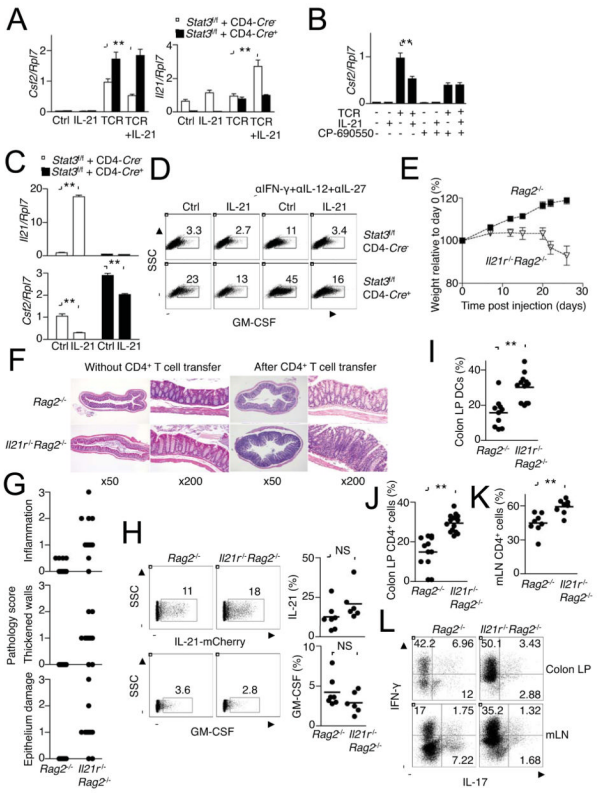

Figure 7. see also Figure S4. Reciprocal production of IL-21 and GM-CSF and its physiological significance

(A–D) IL-21 inhibits GM-CSF production in CD4$^+$ T cells. (A) CD4$^+$ T cells from WT or *Stat3*-CD4-*Cre* conditionally deficient mice were treated with 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 without or with 100 ng/ml IL-21 for 4 h. mRNA expression of *Csf2* (left) and *Il21* (right) was measured. Data are representative of 3 experiments. (B) CD4$^+$ T cells from WT mice were treated as in (A) with or without 10 μM of CP-690550, and *Csf2* mRNA expression was measured. Data are representative of 3 experiments. (C and D) CD4$^+$ T cell differentiation with IL-21 diminishes GM-CSF production independent of STAT3, IFN-γ, IL-12, and IL-27 signaling. (C) Naïve CD4$^+$ T cells from WT or *Stat3*-CD4-*Cre* conditionally-deficient mice were cultured with 5 μg/ml plate-bound anti-CD3 and 2 μg/ml anti-CD28 without or with 100 ng/ml IL-21 for 4 days, then washed and re-stimulated with PMA + ionomycin for 4 h, and IL-21 (*Il21*) or GM-CSF (*Csf2*) mRNA measured. Data are representative of 3 experiments. (D) CD4$^+$ T cells were differentiated as in (C) without or with 10 μg/ml of anti-IFN-γ, anti-IL-12 and anti-IL-27 for 4 days. Cells were washed and re-stimulated with PMA + ionomycin and golgiplug for 4 h, and intracellular GM-CSF was analyzed by flow cytometry. Data are representative of 3 experiments. In A–C, shown are means ± SEM. (E–L) Naïve (CD25$^-$ CD45RB^{hi}) CD4$^+$ T cells from C57BL/6 mice were injected into *Rag2*$^{-/-}$ or *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice. (E) *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice had earlier weight loss than *Rag2*$^{-/-}$ mice. Data are representative of 4 experiments with > 5 mice/experiment. (F) Colons were removed when the *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice showed significant weight loss, fixed with 10% formalin, and paraffin sections stained with hematoxylin and eosin. Colons from un-transferred mice were controls. (G) Colon pathology scores were based on severity of mononuclear cell inflammation, intestinal wall thickening, including infiltration to the muscularis, and epithelial damage, including edema, degeneration, and necrosis. Score: 0 = normal, 0.5 = very mild, 1 = mild, 2 = moderate, 3 = severe. (H) Naïve CD4$^+$ T cells from *Il21*-mCherry reporter transgenic mice were injected into *Rag2*$^{-/-}$ and *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice,

colon cells isolated and expression of IL-21 (based on mCherry) and GM-CSF (by intracellular staining) analyzed by flow cytometry. Graphs are representative of 5 mice/group. (I) Colon cells from *Rag2*$^{-/-}$ and *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice were isolated when *Il21r*$^{-/-}$*Rag2*$^{-/-}$ mice showed significant weight loss, and % DCs determined by flow cytometry. (J) Relative percentage of CD4^{+} T cells in colon and (K) mesenteric lymph nodes (mLN) was determined by flow cytometry. (L) Colon and mLN cells were stimulated with 50 ng/ml PMA + 1 μg/ml ionomycin and golgiplug for 4 h. Intracellular IFN-γ and IL-17 in CD4^{+} T cells were analyzed by flow cytometry. Graphs are representative of ≥ 8 mice/group.