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Leukemia inhibitory factor inhibits plasmacytoid dendritic cell function and development

Renata Sesti-Costa[*,†], **Luisa Cervantes-Barragan**[*,‡], **Melissa K. Swiecki**[*,§], **José Luís Fachi**[*], **Marina Cella**[*], **Susan Gilfillan**[*], **João Santana Silva**[†], **Marco Colonna**[*]

[*]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA.

[†]Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.

[‡]Present Address: Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA.

[§]Present Address: Janssen Pharmaceuticals, Raritan, NJ, USA.

Abstract

Plasmacytoid dendritic cells (pDCs) produce abundant type I IFNs (IFN-I) in response to viral nucleic acids. Generation of pDCs from bone marrow (BM) DC progenitors and their maintenance is driven by the transcription factor E2–2 and inhibited by its repressor Id2. Here, we find that mouse pDCs selectively express the receptor for leukemia inhibitory factor (LIF) that signals through STAT3. Stimulation of pDCs with LIF inhibited IFN-I, TNF and IL-6 responses to CpG and induced expression of the STAT3 targets SOCS3 and Bcl3 that inhibit IFN-I and NF-kB signaling. Moreover, although STAT3 has been also reported to induce E2–2, LIF paradoxically induced its repressor Id2. A late stage BM DC progenitor expressed low amounts of LIFR and developed into pDCs less efficiently after being exposed to LIF, consistent with the induction of Id2. Conversely, pDC development and serum IFN-I responses to Lymphocytic Choriomeningitis Virus infection were augmented in newly generated mice lacking LIFR in either $CD11c^+$ or hematopoietic cells. Thus, a LIF-driven STAT3 pathway induces SOCS3, Bcl3 and Id2 that render pDCs and late DC progenitors refractory to physiological stimuli controlling pDC functions and development. This pathway can be potentially exploited to prevent inappropriate secretion of IFN-I in autoimmune diseases or promote IFN-I secretion during viral infections.

Keywords

Plasmacytoid dendritic cells; leukemia inhibitory factor; IFN-α; LCMV; development

Correspondence: Marco Colonna: (+1) 314-362-0367; mcolonna@wustl.edu.

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INTRODUCTION

Plasmacytoid dendritic cells (pDCs) specialize in abundant production of type I IFNs (IFN-I), i.e. IFN-α and IFN-β (1, 2), and hence directly contribute to control of viral replication and indirectly enhance virus-specific T cell responses (3–7). Secreted IFN-I also binds to the IFN-I receptor (IFNAR) on pDCs, thereby completing an autocrine loop that sustains IFN-I production (8). pDCs sense viral RNA and DNA or their synthetic surrogate oligoribonucleotides (ORN) and oligodeoxyribonucleotides (ODN) through endosomal TLR7 and TLR9, respectively (9, 10). These TLRs trigger MyD88-dependent IRF7 and NF-kB pathways that culminate in production of IFN-I and pro-inflammatory cytokines, respectively (11–13). However, inappropriate pDC recognition of endogenous nucleic acids elicits excessive IFN-I (11, 14–17) that can contribute to the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE). Thus, pDC development and function must be tightly regulated to maintain homeostasis.

PDCs can derive from both the lymphoid (18) and myeloid (2, 19) developmental pathways. In the latter, pDCs originate in the bone marrow (BM) from the common DC progenitor (CDP), which can generate both pDCs and conventional DCs (cDCs) (20, 21). CDPs express FMS-like tyrosine kinase 3 (Flt3), a receptor tyrosine kinase that triggers a signaling network through PI3K and the Ras cascade, which activate the Akt, ERK and STAT pathways (22). Engagement of Flt3 by its ligand (Flt3L) drives both pDC and cDC development. However, pDC generation requires higher concentrations of Flt3L and concurrent IFN-I signaling (23). Moreover, whether CDPs generate pDCs or cDCs hinges on the balance between expression of the transcription factor E2–2 (encoded by *Tcf4*) and the transcriptional repressor inhibitor of DNA binding 2 (Id2) (2). E2–2 is a basic helix-loop-helix transcription factor that commits CDP to the pDC lineage by inducing a set of pDC-specific target genes that include *Tlr7*, *Tlr9*, and *Irf7* (24–26). Moreover, E2–2 maintains the identity of mature pDCs, as ablation of *E2–2* in pDCs induces their differentiation into cDC-like cells (24, 25). In contrast, Id2 propels CDP differentiation into the cDC lineage by binding E2–2 so that this key transcription factor can no longer associate with DNA (27). Id2 is required for the generation of resident $CD8\alpha^+$ cDCs, migratory $CD103^+$ cDCs, and epidermal Langerhans cells (28, 29), while it opposes the generation of pDCs (19, 27, 29).

Leukemia inhibitory factor (LIF) is a member of the IL-6 cytokine family that is well known for promoting the pluripotency and self-renewal of embryonic stem cells and inhibiting their differentiation (30–32). It binds to a receptor complex formed by a ligand-specific subunit, the LIF receptor (LIFR), and the gp130 signaling subunit, which is shared by all members of the IL-6 family (33). The LIFR-gp130 complex recruits and phosphorylates JAKs, which in turn phosphorylate STAT3 and other STAT family members. Phospho-STAT3 translocates to the nucleus to direct the transcription of a wide range of target genes, including a JAK-STAT inhibitor known as suppressor of cytokine signaling 3 (SOCS3) that provides a negative feedback loop. LIF signaling also activates MAPKs, src family kinases, CREB, ribosomal s6 kinase and PI3K (34–37). In addition to its known impact on embryonic stem cells, LIF has been shown to have immunoregulatory functions in various contexts. During autoimmune neuroinflammation, LIF induces an anti-inflammatory phenotype in macrophages (38), suppresses differentiation of pathogenic Th17 (39), augments Treg numbers (40) and

mediates neuroprotective and neuroregenerative effects (41–43). In an animal model of stroke, LIF attenuates the IL-12–IFN-γ-mediated inflammatory response to vessel occlusion (44). Similarly, LIF promotes tolerance by inducing $Foxp3^+$ Tregs in transplantation models (45). Moreover, LIF facilitates the acquisition of an M2 phenotype by macrophages and the recruitment of myeloid-derived suppressor cells into the tumor microenvironment (46, 47). Finally, LIF ameliorates cutaneous inflammation (48) and attenuates endotoxin shock through induction of IL-10 (49).

This study was prompted by the observation that LIFR is expressed on pDCs and, to a lesser extent, on a late stage BM DC progenitor with both cDC and pDC potential, but not on cDCs. Exposure of mature pDCs to LIF *in vitro* induced STAT3 phosphorylation, and inhibited IFN-α, TNF and IL-6 responses to CpG oligodexyribonucleotides (ODNs). Analysis of the transcriptional profile of pDCs showed that LIF induces the expression of *Socs3* and *Bcl3*, which are known STAT3 targets that inhibit the IFN-I and NF-kB pathways. Although STAT3 signaling has been shown to induce E2–2 (50), LIF paradoxically induced expression of the E2–2 antagonist Id2, which is known to inhibit pDC development and maintenance. Consistent with this, LIF drove BM cultures with Flt3L towards the generation of $CD11b^+$ cDCs rather than pDCs. In mice with impaired LIFR expression in the $CD11c^+$ lineage, BM cultures with Flt3L produced more pDCs and were less sensitive to LIF suppression than were BM cultures from control mice. Moreover, mice with defective LIFR in the $CD11c^+$ lineage had higher IFN-α levels in the serum than did control mice after Lymphocytic Choriomeningitis Virus (LCMV) infection. We conclude that LIFR provides a new paradigm of pDC inhibition based on biased STAT3 signaling. On one hand, the LIF–STAT3 pathway effectively induces blockers of IFN-I and NF-kB; on the other hand, the same pathway fails to induce the master pDC transcription factor E2–2 that is required for pDC development, but rather allows induction of its inhibitor, Id2. Overall, our results suggest that STAT3 signaling could be potentially exploited for modulation of pDCs in diseases.

MATERIALS AND METHODS

Generation of LIFR conditional mice

We generated $Itgax^{Cre}Lif^{-/flox}$ by crossing $Lifr^{-/+}$ with $Itgax^{Cre}Lif^{flox/flox}$ mice. $Itgax^{Cre}$ mice were obtained from Jackson. To generate the $Lifr^{-/+}$ and $LIFr^{flox/flox}$ mice, three $LIFr^{tm1a(EUCOMM)Hmgu}$ ES cell clones (ES line JM8A3.N1) were obtained from EuComm (European Conditional Mouse Mutagenesis Consortium); two correctly targeted clones, confirmed by Southern blot analysis, were introduced into B6-albino ($C57BL/6J\text{-}Tyr^{c-2J}/J$) eight-cell embryos by laser-assisted injection. Male chimeras were initially bred to B6-albino mice to assess germline transmission; those transmitting were bred to CMV-Cre transgenic mice (B6.C-Tg(CMV-Cre)1Cgn/J; >99% C57BL/6) to delete exon 5 of *Lifr* (transcript variant 1) and the neomycin-resistance cassette; this null LacZ allele is $Lifr^{tm1b}$ in the official EuComm nomenclature and referred to simply as $Lifr^{-}$ in this paper. To delete the lacZ–neomycin-resistance cassette and generate mice with a *lox*P-flanked *Lifr* allele (EuComm $Lifr^{tm1c}$ = $Lifr^{flox}$ in this paper), chimeras were bred to CAG-FLPe C57BL/6

mice. The CMV-Cre and FLPe transgenes were bred out of the lines before intercrossing and/or breeding to *Itgax*^{Cre} or *Vav1*^{Cre} mice.

Infection

Mice were infected with 3×10^5 PFU of LCMV Armstrong i.p. Serum was harvested for IFN-α measurement.

Differentiation of DCs, sorting and treatment

Bone marrow cells were obtained by flushing femurs and tibias with RPMI, followed by incubation with red blood cells lysis buffer (Sigma) for 5 min. After wash, cells were cultured with RPMI containing 10% FCS, 1% kanamycin, sodium pyruvate, nonessential amino acids, L-glutamine, β-mercaptoethanol and Flt3L (10ng/ml) for 7–10 days in the presence or absence of LIF (10ng/ml). Medium with added LIF but without Flt3L was refreshed on day 3 and day 6. pDCs and cDCs were sorted from Flt3L BM cultures by FACS as CD11c^{int}SiglecH$^+$B220$^+$ and CD11c$^+$CD24$^+$Sirp-α$^+$ cells, respectively. Sorting of pDCs from BM was performed by magnetic beads using negative selection kit (Miltenyi Biotec), followed by sorting of CD11c^{int}B220$^+$SiglecH$^+$ cells by FACS. The purity of FACS sorting was routinely > 92%. In some experiments, staining of LIFR was added to sort LIFR$^+$ and LIFR$^-$ pDCs. pDCs were treated with LIF (10ng/ml) for 1 or 24h and stimulated with CpG-A (2216) or CpG-B (1826), both 6μg/ml, for 18–24h. Common macrophages/dendritic cells precursors (MDP), common dendritic cells progenitors (CDP) and pre-DC were sorted from BM by FACS for staining of lin$^-$Flt3$^+$ckit^{high}CD115$^+$, lin$^-$Flt3$^+$ckit^{low}CD115$^{+/-}$ and lin$^-$Flt3$^+$CD11c$^+$, respectively. Cells were cultured with RPMI 10% FCS and Flt3L for 2 or 4 days in the presence or not of LIF (10ng/ml).

Flow cytometry

Single-cell suspensions were treated with monoclonal antibody 197 (anti-mFcgRI) for 15 min to block Fc receptors and stained with fluorescent monoclonal antibodies against B220 (RA3–6B2), SiglecH (551), CD11c (N418), CD11b (M1/70), Sca-1 (D7), LIFR (#673602), CD24 (M1/69), Ly6C (HK1.4), IA/IE (2G9 or M5/114.14.2), CX3CR1 (SA011F11), CD172a (P84), CD8a (53–6.7), CD115 (AFS98), CD117 (ACK2) and CD135 (A2F10). The antibodies against CD3 (145.2c11), CD19 (1D3), NK1.1 (PK136), Ly6G (1A8), CD11b (M1/70) and TER-119 (TER-119) were used for linage staining. Intracellular staining was performed on fixed and permeabilized cells with antibodies against Ki-67 (B56), phosphorylated STAT3 (p-STAT3), STAT5 (p-STAT5) and TLR9 (M9.D6). Apoptosis was quantified by staining with annexin-V and 7AAD kit. All antibodies were purchased from BD, eBioscience, BioLegend or R&D Systems. Data were acquired on a FACSCantoII (BD) and analyzed using FlowJo software (Tree Star, Inc.).

RNA isolation and qRT-PCR

RNA was extracted from cells using RNeasy micro kit (QIAGEN) and cDNA synthesis was carried out with SuperScript III first strand (Invitrogen) according to the manufacturer's instructions. qRT-PCR reactions were performed with SYBR Green Supermix (BioRad) using the following primers: *Id2* forward: 5'-AAAACAGCCTGTCGGACCAC-3'; *Id2*

reverse: 5'-CTGGGCACCAGTTCCTTGAG-3'; *Bcl3* forward: 5'-CCTTTGATGCCCATTTACTCTA-3'; *Bcl3* reverse: 5'-AGCGGCTATGTTATTCTGGAC-3'; *Socs3* forward: 5'- ATGGTCACCCACAGCAAG-3'; *Socs3* reverse: 5'-TCAAGTGGAGCATCATACTG-3'; *Dgkz* forward: 5'-CTGCCCCAAGGTGAAGAGCTG-3'; *Dgkz* reverse: 5'-GCTGTCTCCTGGTCCTCACGT-3'; *Slpi* forward: 5'-GGCCTTTTACCTTTCACGGTG-3'; *Slpi* reverse: 5'- TACGGCATTGTGGCTTCTCAA-3'; *Ier3* forward: 5'- AAGGGTGCTCTACCCTCGAGT-3'; *Ier3* reverse: 5'-GCAGAAATGGGCTCAGGTGT-3'; *Lifr* forward: 5'-GGGCCCACGAAATACAGAATA-3'; *Lifr* reverse: 5'-CGGTAGGTCTCGTTTGTAAGTC-3'. Amplification cycles were acquired using ABI7000 (Applied Biosystems), and the expression of target mRNA was calculated and normalized to the expression of *gapdh* gene using the ΔCT method.

Microarray

Sorted pDCs or cDCs were treated with LIF and stimulated or not with CpG. After RNA extraction, RNA amplification and hybridization to the Mouse Gene 1.0 ST arrays (Affymetrix) were carried out. Data were processed and normalized using RMA (robust multichip average) and analyzed with GenePattern software (Broad Institute). Heat maps were generated with Gene-E. Microarray data have been deposited in NCBI's Gene Expression Omnibus repository and are accessible through accession number GSE138018.

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138018.

ELISA and Cytometric Bead Array

Serum and culture supernatant were collected and stored at −20°C until analysis. IL-6 and TNF levels were measured by cytometric bead array (BD Biosciences). IFN-α was quantified by ELISA (PBL Interferon).

Statistical analysis

Data are from at least two independent experiments, unless otherwise stated, and were analyzed by Student's-t or Mann-Whitney tests. Error bars represent ± SEM and $p \leq 0.05$ was considered significant.

RESULTS

PDC but not cDCs express LIFR

We examined the Immgen expression profiles of mouse DCs to identify genes specifically expressed by pDCs. We noted that *Lifr* mRNA was highly expressed in pDCs from spleen and skin-draining lymph nodes, while expression was minimal in $CD4^+$ and $CD8\alpha^+$ cDCs from the thymus, spleen, skin-draining and mesenteric lymph nodes. In addition, pDCs had the highest *Lifr* expression among all immune cells (http://www.immgen.org/databrowser/index.html). To corroborate this observation, we assessed the expression pattern of LIFR in primary DCs by flow cytometry. LIFR was highly expressed on cell surface of CD11c

$^{+}$B220^{+}SiglecH^{+} pDCs isolated from BM, spleen and lymph nodes, while it was not expressed on CD11c^{+}SiglecH^{-} cDCs isolated from any of those tissues (Fig. 1A)

Like their primary counterparts, pDCs derived from BM cells cultured *in vitro* with Flt3L expressed *Lifr* mRNA (Fig. 1B). Moreover, stimulation of Flt3L-cultured pDCs with CpG-A and CpG-B markedly downregulated or completely abolished *Lifr* mRNA expression. In contrast, cDCs derived from Flt3L cultures did not express *Lifr* mRNA either before or after CpG stimulation. We confirmed that CpG-A-induced downregulation of LIFR at the protein level in primary pDCs isolated from BM and spleen after mice had been injected i.v with CpG-A (Fig. 1C). LIFR downregulation was detected as soon as 6h after CpG treatment and reached its nadir after 24h. We next verified that LIFR on pDCs was functional. A well-characterized intracellular signal of LIFR-gp130 heterodimer is STAT3 phosphorylation (51). We observed phospho-STAT3 in pDCs as soon as 15 minutes after incubation with LIF (Fig. 1D). Altogether, these data show that pDCs, in contrast to cDCs, express functional LIFR.

LIF suppresses pDC functions

To determine the role of LIFR in pDCs, we treated spleen cells or pDCs sorted from BM with LIF for 1hr followed by stimulation with CpG-A. As expected, spleen cells and sorted pDCs produced high amounts of IFN-α after CpG-A stimulation. However, pre-treatment with LIF dramatically suppressed IFN-α production by these cells (Fig. 2A). Additionally, *in vivo* treatment of mice with LIF attenuated the increase of serum IFN-α induced by i.v. injection of CpG-A (Fig. 2B). While CpG-A triggers IFN-I production through an IRF7-dependent pathway in early endosomes, CpG-B induces production of proinflammatory cytokines through a NF-κB-dependent pathway in late endosomes (9, 10, 12, 13). Therefore, we asked whether LIF also affects the NF-κB pathway in pDCs. We stimulated pDCs with CpG-B in the presence or absence of LIF and found that LIF suppressed TNF and IL-6 production by pDCs (Fig. 2C). LIF had no impact on the expression of TLR9 (Fig. 2D). However, pDCs treated with LIF did have a phenotype slightly biased towards cDC-like cells; they displayed more B220 and CD11c on the cell surface than did untreated cells (Fig. 2E). Taken together, these data show that LIF inhibits pDC production of IFN-α and inflammatory cytokines in response to CpG and slightly modifies their cell surface phenotype without affecting TLR9 expression.

pDCs modify their transcriptional profile in response to LIF

To investigate the mechanisms by which LIF suppresses pDC functions, we performed microarray analysis of primary pDCs sorted from BM that were incubated with a) medium; b) LIF; c) CpG; d) LIF plus CpG (Fig. 2F, G). Key results were validated by qPCR (Fig. 2H). One of the most up-regulated genes after treatment with LIF alone was *Socs3,* a known target of STAT3, which inhibits JAK/STAT signaling by various cytokine receptors (52). SOCS3 has been reported to inhibit IFN-β during vesicular stomatitis virus (VSV), influenza A virus (53) and HIV infections (54, 55), as well as during obesity, where SOCS3 is induced by the receptor for leptin (56). LIF also augmented the expression of *Bcl3,* another known target of STAT3 that inhibits NF-kB. Exposure of pDCs to LIF also induced other genes reportedly involved in inhibition of NF-κB signaling, such as immediate early response 3

(*Ier3*) and secretory leukocyte peptidase inhibitor (*Slpi*) (57–59) (Fig. 2F, H). LIF also downregulated the expression of diacylglycerol kinase zeta (*Dgkz*); lack of *Dgkz* results in impaired induction of TLR-dependent proinflammatory cytokines in cDCs and macrophages (60) (Fig. 2F, H). Thus, exposure of pDCs to LIF alone induced a coordinated gene expression program that inhibits secretion of IFN-I and inflammatory cytokines. Accordingly, the transcriptional profile of pDC treated with LIF and CpG showed a marked reduction in the expression of all IFN-α genes compared to pDC treated with CpG (Fig. 2G).

Transcriptome analysis also showed that LIF induced upregulation of *Id2*, which inhibits E2–2 and hence both development and maintenance of pDCs. This result was surprising, as LIF and Flt3 share a STAT3 signaling pathway that has been shown to induce E2–2 and inhibit Id2 (50). We postulate that LIF-mediated induction of SOCS3 results in a negative feedback loop that blocks further STAT3 signaling, leaving alternative signaling pathways that induce Id2 unopposed, such as IL-2-, IL-12- and IL-21-induced STAT5 pathways (61).

LIF impairs pDC development in FLT3L cultures

Given that Id2 drives CDP differentiation towards cDCs (2), we hypothesized that LIF could affect pDC development. To test this hypothesis, we examined the output of BM cells cultured *in vitro* with Flt3L with or without LIF. While cultures with Flt3L alone contained about 30% of SiglecH^{+}B220^{+}CD11c^{+} pDCs, almost ten-fold fewer pDCs were obtained in the presence of LIF (Fig. 3A). In contrast, LIF promoted the differentiation of CD11c^{+}B220^{-}SiglecH^{-} cDCs, particularly the CD11b^{+}CD24^{-} subset (Fig. 3A). Phenotypic analysis of differentiating DCs at different time points during BM cultures with Flt3L showed that addition of LIF promptly induced dowregulation of SiglecH and upregulation CD11b by day 2, resulting in the appearance of B220^{+}CD11b^{+}SiglecH^{-} cells early during differentiation. These changes were followed by an increase in CD11c expression by day 4 and finally by downregulation of B220 by day 6 (Fig. 3B). Ultimately, LIF promoted the development of CD11c^{+} cDCs with a SiglecH^{-}B220^{-}Ly6C^{-}MHCII^{hi}CX3CR1^{+}CD11b^{+} phenotype (Fig. 3C). Based on forward scatter (FSC) and side scatter (SSC) parameters, these cells were also larger and more granular than those in control cultures without LIF (Fig. 3D). Phenotypic analysis of proliferating Ki67^{+} cells corroborated that cells expanding in BM cultures containing LIF expressed more CD11b than those grown in the absence of LIF, consistent with the expansion of CD11b^{+} cDC at the expense of pDCs (Fig. 3E). We excluded the possibility that LIF selectively induces apoptosis of developing pDCs, since the percentages of annexin V^{+} cells in the presence of LIF were similar or even lower than in the absence of LIF (Fig. S1). Moreover, non-DC populations, such as NK cells, neutrophils and macrophages, were not affected by LIF (Fig. S2). Altogether, these data show that LIF skews differentiation of DCs towards CD11b^{+} cDCs at the expense of pDCs.

LIF impacts DC progenitors

We noticed that the earlier LIF was added to BM cultures, the stronger was its inhibitory effect on pDC development (Fig. 4A). Thus, we hypothesized that LIF acted on DC progenitors by deviating their differentiation away from pDCs towards cDCs. Immgen expression profiles of mouse DCs depicted essentially no expression of *Lifr* mRNA in

macrophage-DC progenitors (MDPs) and minimal expression in common DC progenitors (CDPs) in comparison to mature pDCs (Fig. 4B). Consistent with this, no LIFR protein was detected on MDPs (62, 63) or CDPs (20, 21, 64) by flow cytometric analysis (Fig. 4C). However, LIFR receptor was detected on a post-CDP $CD11c^+$ progenitor that maintains both pDC and cDC potential (65) (Fig. 4C). Therefore, we sorted MDPs, CDPs and the post-CDP $CD11c^+$ progenitor and assessed the generation of cDCs versus pDCs in BM cultures with Flt3L with or without LIF. In the absence of LIF, all progenitors produced pDCs, with the late stage post-CDP progenitor yielding pDCs more rapidly (2 days of culture) than CDPs and MDPs (4 days of culture). LIF deviated DC differentiation from all three progenitors towards the generation of $CD11b^+$ cDCs, whereas pDC development was suppressed (Fig. 4D, E). We conclude that LIF biases differentiation of a late stage $CD11c^+$ post-CDP progenitor towards cDCs at the expense of pDCs.

LIFR deficiency enhances pDCs development and function

We next sought to validate the role of LIFR in pDC function and development *in vivo* in mice lacking LIFR. $Lifr^{-/-}$ mice die during or soon after birth due to disruptions of the placental architecture that result in poor intrauterine nutrition as well as pleiotropic defects involving the bone, nervous system and metabolism (65); therefore, we generated $Itgax^{Cre}Lifr^{flox/flox}$ mice, which lack LIFR in $CD11c^+$ cells. Previous work has shown that $Itgax^{Cre}$ mediated recombination is ~60–80% efficient in pDCs (66). Excision of the floxed exon 5 was even less efficient in the $Itgax^{Cre}Lif^{flox/flox}$mice, perhaps because the *Lifr* locus is relatively inaccessible. To increase the efficacy of $Itgax^{Cre}$-mediated deletion, we generated $Itgax^{Cre}Lif^{-/flox}$ by crossing $Lifr^{-/+}$ with $Itgax^{Cre}Lifr^{flox/flox}$ mice, so that *Itgax*-Cre had only to excise exon 5 of *Lifr* from one floxed allele rather than two. We found that primary pDCs isolated from the BM or spleen of $Itgax^{Cre}Lifr^{-/flox}$ mice expressed intermediate or essentially no LIFR, reflecting incomplete excision of floxed *Lifr* by $Itgax^{Cre}$ (Fig. 5A). WT mice expressed relatively high levels of LIFR on the cell surface, whereas those from heterozygous $Lifr^{-/flox}$ mice expressed about half as much.

Using $Itgax^{Cre}Lifr^{-/flox}$ mice, we examined the impact of LIF on pDC development *in vitro*. BM cells from $Itgax^{Cre}Lifr^{-/flox}$ mice cultured with Flt3L contained slightly more pDC (Fig. 5B) and less $CD11b^+$ cDC (Fig. 5C) than BM cells from $Lifr^{-/flox}$ or WT mice. Moreover, BM cells from $Itgax^{Cre}Lifr^{-/flox}$ mice were more resiliant to the pDC suppressive activity of LIF than $Lifr^{-/flox}$ or WT BM cells, yielding more pDCs and less cDCs in the presence of LIF (Fig. 5B,C). We also isolated pDCs with the highest and lowest levels of LIFR expression from $Lifr^{-/flox}$ mice and demonstrated that $LIFR^{lo}$ pDCs produce more IFN-α upon CpG-A stimulation than do $LIFR^{hi}$ pDCs (Fig. 5D). Finally, we demonstrated that $Itgax^{Cre}Lifr^{-/flox}$ mice had more IFN-α in the serum than did $Lifr^{-/flox}$ mice after LCMV infection (Fig. 5E).

Since some pDCs in $Itgax^{Cre}Lifr^{-/flox}$ mice clearly express LIFR, we crossed $Lifr^{flox/flox}$ with $Vav1^{Cre}$ mice to obtain more efficient deletion of *Lifr* in pDCs and DC progenitors. Efficient depletion of LIFR in pDCs and, as expected, all hematopoietic cells was evident in $Vav1^{Cre}Lifr^{flox/flox}$ mice (Fig. 6A). However, there was no impact on the number of pDCs in spleen, bone marrow, lymph nodes, or within intraepithelial cells (IELs) (Fig. 6B). Thus, DC

development is not affected by the absence of LIFR *in vivo*, suggesting that either DC progenitors are under other influences that may compensate the absence of LIFR, or that homeostatic pDC development is not regulated by LIF *in vivo*. However, *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice infected with LCMV produced more IFN-α in the serum than did *Lifr*$^{flox/flox}$ littermates (Fig. 6C), corroborating the data obtained from *Itgax*^{Cre}*Lifr*$^{-/flox}$ mice showing that LIF inhibits the function of mature pDCs *in vivo*.

We sought to evalute LIFR signaling in Flt3L cultures of BM cells from *Lifr*$^{flox/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice. We first confirmed that LIF-induced STAT3 phosphorylation can be detected by intracellular staining in mature pDCs enriched from *Lifr*$^{flox/flox}$ but not from *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice, corroborating complete deletion of LIFR in pDCs from these mice (Fig. 6D). However, we noticed that the cell fixation required for intracellular detection of phospho-STATs affected various cell surface markers used to identify pDCs, such as SiglecH, that became undetectable after fixation (Fig. 6D). Therefore, BM cultures stained for intracellular content of phospho-STATs were counterstained with CD11b and B220, which were not affected by methanol fixation (Fig. 6E). In BM cultures from *Lifr*$^{flox/flox}$ mice, LIF induced a clear STAT3 phosphorylation in CD11b^{+}B220^{+} cells, which include post-CDP progenitors with pDC potential, as well as CD11b^{-}B220^{+} cells, which encompass mature pDCs (Fig. 6E). A more modest phosphorylation was detected in the other BM subsets. In BM cultures from *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice, no STAT3 phosphorylation was observed at any time, consistent with LIFR deletion (Fig. 6E). In parallel with STAT3 phosphorylation, *Id2* mRNA expression significantly increased after 4 and 6 days of cultures with LIF, while *E2–2* mRNA expression declined (Fig. 6F), confirming the results obtained with primary pDCs. Although Id2 has been previously shown to be induced by STAT5 signaling (50), no STAT5 phosphorylation was detected at any time in BM cultures stimulated with LIF (Fig. 6E). We conclude that LIFR signaling during pDC development is similar to that observed in mature pDCs.

DISCUSSION

Here we found that pDCs are unique among DCs for the expression of LIFR, which inhibits their IFN-I and cytokine production and modifies their phenotype towards a cDC-like type. Incubation of pDC with LIF inhibited both IRF7-dependent IFN-α and NF-κB-dependent TNF and IL-6 secretion in response to CpG and LCMV. Comparison of IFN-α production by LIFR^{lo} and LIFR^{hi} pDCs isolated from *Lifr*$^{+/-}$ mice corroborated a marked inhibitory effect of LIFR on pDCs. Conversely, *Itgax*^{Cre}*Lifr*$^{-/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice, which lack LIFR in the CD11c lineage and in hematopoietic cells, respectively, had higher levels of IFN-α in the serum after LCMV infection *in vivo* than did control mice. Mechanistically, LIFR activated the STAT3 pathway, which induced the expression of target genes known to inhibit IFNAR and NF-kB signaling. Among these, SOCS3 is a suppressor of the JAK-STAT pathway that negatively regulates signaling of proinflammatory cytokines (52) and inhibits the TBK-IRF3-IFN-I pathway during vesicular stomatitis virus (VSV) and influenza A virus infections (53). Additional STAT3-induced molecules, such as *Bcl3*, inhibit NF-kB activation. Although the STAT3 pathway has been shown to induce E2–2, the pDC master transcription factor, pDCs exposed to LIF paradoxically expressed the E2–2 antagonist Id2, which can inhibit development of pDCs and destabilize their phenotype and functions.

Our study also shows that signaling through LIFR inhibits pDC development in BM cultures. LIFR was expressed on a late $CD11c^+$ post-CDP progenitor that maintains potential for pDCs and cDCs, whereas only a small amount of *Lifr* mRNA was detected in CDPs. Addition of LIF to BM cells or purified DC progenitors cultured in Flt3L biased DC differentiation towards $CD11b^+$ cDCs rather than pDCs. Conversely, BM cells from $Itgax^{Cre}Lifr^{-/flox}$ mice that lack LIFR in the $CD11c^+$ lineage spontaneously generated more pDCs and fewer $CD11b^+$ cells than did BM cells from control mice and were more refractory to suppression mediated by addition of LIF to the cultures. pDC numbers were not affected in either $Itgax^{Cre}Lifr^{-/flox}$ or $Vav1^{Cre}Lifr^{flox/flox}$ mice, suggesting that DC progenitors are under other influences that may compensate for the absence of LIFR *in vivo*, or that homeostatic pDC development is not controlled by LIF. It remains possible that BM levels of LIF increase in pathological settings, which might impact pDC development. LIFR-mediated inhibition of pDC development *in vitro* was paralleled by the induction of Id2. It has been shown that overexpression of Id2 inhibits pDC development (67), while pDCs numbers are elevated in $Id2^{-/-}$ mice (29). In addition, cells overexpressing Id2 spontaneously differentiate into a $CX_3CR1^+CD8\alpha^+$ cDC-like population (25, 68). Similarly, diminished E2–2 activity reportedly impedes pDC development and facilitates the emergence of a non-canonical DC subset (69) that is heterogeneous and encompasses pDC-like and cDC-like cells (70–72). Thus, expression of Id2 and inhibition of E2–2 in various contexts induce an alternative fate for pDC-committed cells. In the current study, addition of LIF to Flt3L-cultured BM cells induced the differentiation of $CX_3CR1^+CD8\alpha^-CD11b^+$ cDC-like cells.

The induction of Id2 by LIF was unexpected. Previous studies have shown that Flt3, like LIF, signals through STAT3 (73), which promotes the expression of E2–2 in CDPs; E2–2 propels their differentiation into pDCs (2) and sustains pDC phenotype and function (50). We postulate that the paradoxical induction of Id2 by LIF may be a consequence of SOCS3 induction. By modulating JAK–STAT signaling, SOCS3 inhibits Flt3 and IFNAR signaling. However, since pDC generation requires Flt3 and is bolstered considerably by IFN-I (23), curtailing Flt3 and IFNAR signaling may attenuate pDC development from post-CDP progenitors. Furthermore, alternative signaling pathways initiated by other cytokines, such as IL-2, IL-12 and IL-21 (61), may be left unopposed, leading to induction of Id2 expression and consequent bias of post-CDPs towards differentiation of cDC-like cells.

Examination of human databases did not reveal expression of LIFR in human pDCs or their progenitors. It remains to be seen whether LIFR is expressed during activation or in pathological contexts, such as pDC neoplasms with leukemic presentation (74) or pDCs infiltrating solid tumors. Regardless of whether LIFR is only expressed in mouse pDCs and their progenitors, our study reveals a novel pDC inhibitory mechanism based on biased STAT3 signaling that could be exploited to modulate pDCs in human. Several inhibitory mechanisms of pDCs have been previously reported. In mice, the cell surface receptors SiglecH and CD300c inhibit type I IFN production when crosslinked with an antibody as a surrogate ligand (75, 76). In humans, the cell surface receptors ILT7 and BDCA-2 perform similar functions (77, 78). Accordingly, treatment of patients with systemic lupus erythematosus with a humanized monoclonal antibody that binds to BDCA-2 improved skin lesions by attenuating IFN-I production (79). The protein tyrosine phosphatase receptor sigma, PTPRS, was also identified as a pDC-specific inhibitory receptor that can prevent

intestinal inflammation in both humans and mice (80). Our study extends these studies demonstrating a novel pathway for pDC modulation based on enhanced STAT3-SOCS3-Id2 expression levels that can be potentially exploited to prevent inappropriate secretion of IFN-I in autoimmune diseases or promote IFN-I secretion during viral infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

pDCs express LIFR

LIF inhibits pDC production of type I IFN

LIF biases DC development towards cDC at the expenses of pDCs

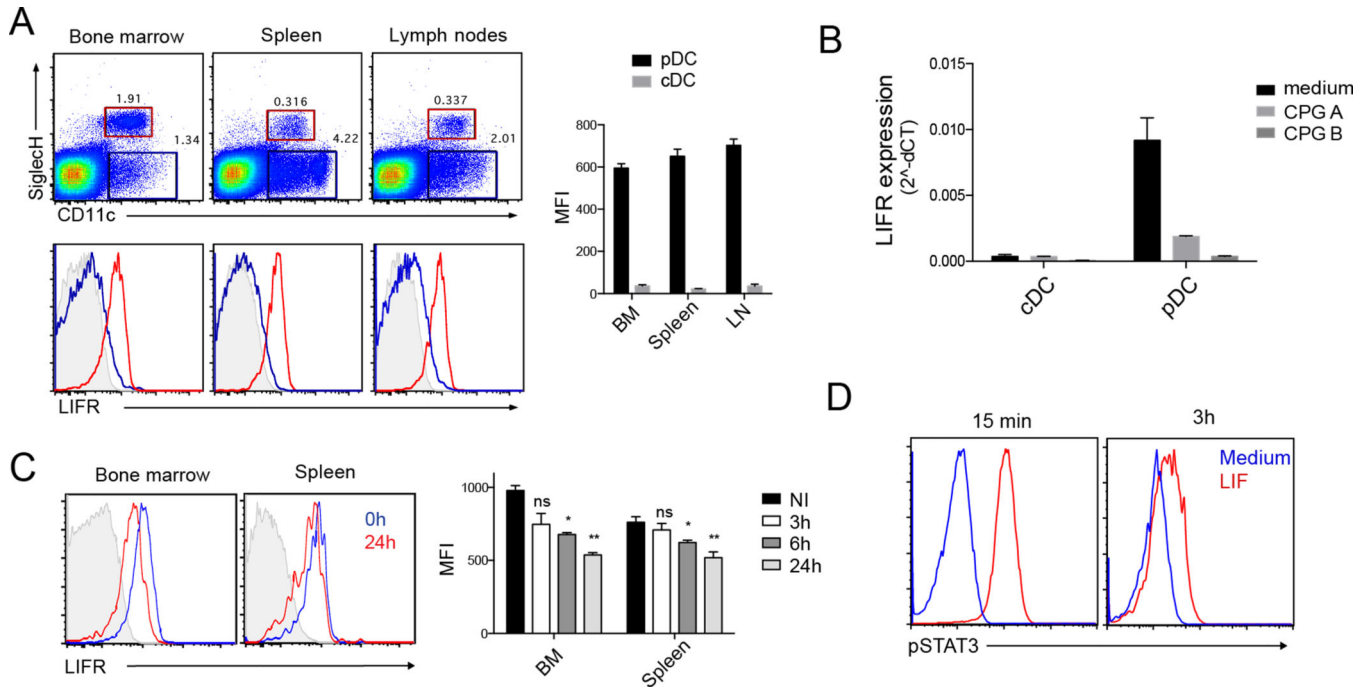

Figure 1. pDCs express LIFR.

(**A**) Expression of LIFR by pDCs (SiglecH^{+}CD11c^{int}, red gates and histograms) and cDC (SiglecH^{-}CD11c^{+}, blue gates and histograms) from BM, spleen and lymph nodes. Gray profiles represent staining with isotype control antibody. MFI of LIFR is indicated on the right (n = 3 mice). (**B**) BM cells were cultured in the presence of Flt3L. On day 7, pDCs (SiglecH^{+}B220^{+}CD11c^{int}) and cDCs (CD11c^{+}CD24^{+}Sirpα^{+}) were sorted and stimulated with CpG-A or CpG-B. After 24h the expression of LIFR mRNA was measured by qPCR. (**C**) C57BL/6 mice were injected i.v with CpG-A and BM and spleen pDCs were analyzed for LIFR expression by flow cytometry at the indicated times after injection. Blue and red histograms on the left represent cells from non-injected (NI) and CpG-A injected mice at 24h respectively (n = 3 or 2 mice per group). Anova analysis with Bonferroni's multiple comparison test was performed. The differences between pDCs from NI and CpG-A injected mice at each time point are shown. *<0.05 and **<0.01. (**D**) pDCs were sorted from BM, incubated with LIF and analyzed for intracellular content of phosphorylated STAT3 (pSTAT3) by flow cytometry at the indicated times. Blue and red histograms represent unstimulated and stimulated cells, respectively. The data are representative of at least three independent experiments.

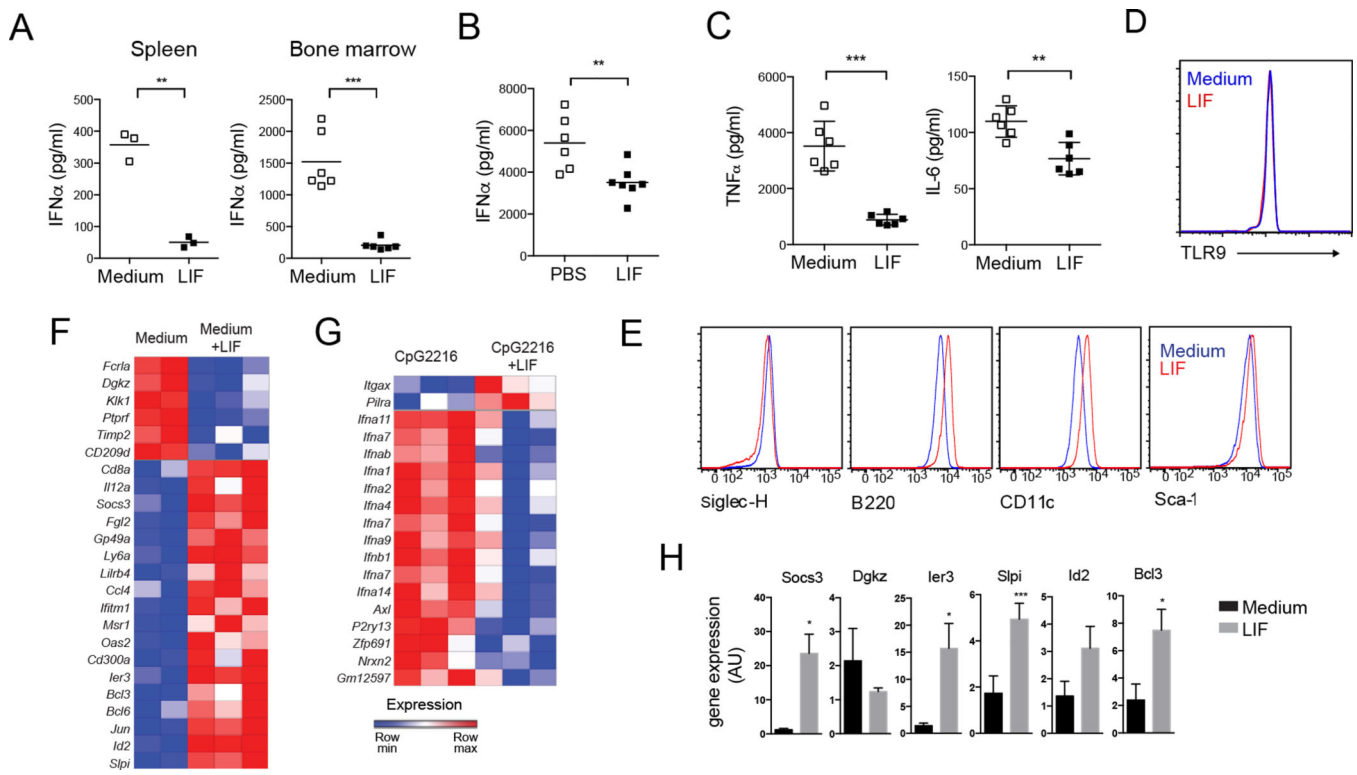

Figure 2. LIF inhibits pDC function.

(**A**) Spleen cells (left) and BM-sorted pDCs (right) were incubated with LIF for 1h and then left unstimulated or stimulated with CpG-A for an additional 19h. IFN-α was measured in the supernatant (n = 3–6 mice). (**B**) C57BL/6 mice were treated with LIF and then injected i.v with CpG-A. IFN-α was measured in serum after 6 hours (n = 6–7 per group). (**C**) Spleen cells and BM-sorted pDCs were incubated with LIF for 1h and then left unstimulated or stimulated with CpG-B for an additional 19h. TNF and IL-6 were measured in the supernatant (n = 3–6 mice). (**D-H**) PDCs were sorted from BM and incubated with or without LIF for 20h. (**D**) TLR9 expression was analyzed by flow cytometry. Blue and red histograms represent untreated and treated pDCs, respectively. (**E**) Expression of pDCs markers by flow cytometry. Blue and red histograms represent untreated and treated pDCs, respectively. (**F, G**) Heatmaps showing gene expression changes induced by LIF with or without CpG. pDCs were sorted from BM and incubated with medium containing or not LIF in the absence or presence (**G**) of CpG 2216. (**H**) Q-PCR showing genes up-regulated or down-regulated by LIF. The data are representative of two to three independent experiments.

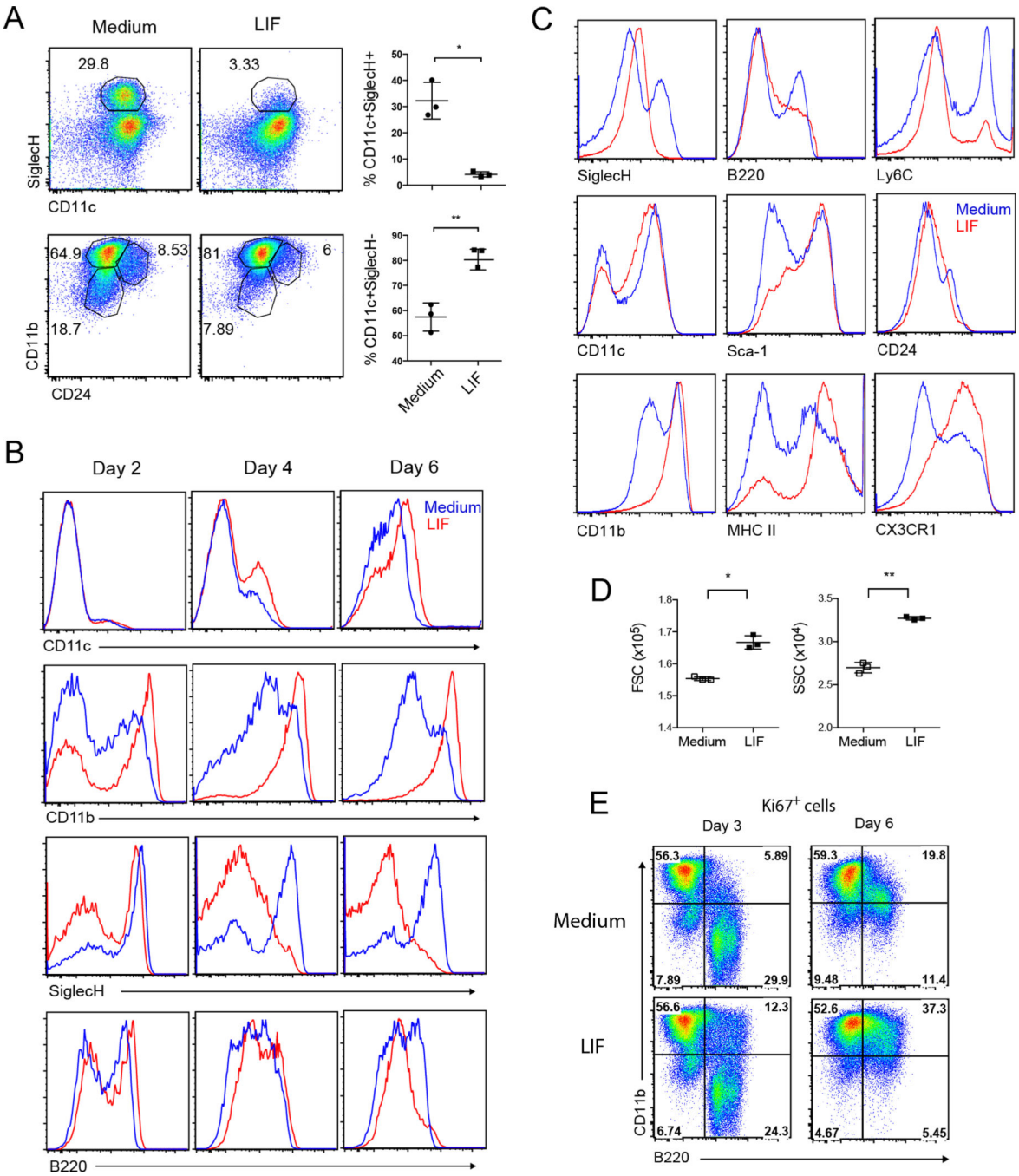

Figure 3. LIF impairs pDCs development *in vitro*.

BM cells were incubated with medium containing Flt3L for 2, 4, 6 or 9 days with or without LIF. Medium was replaced on days 3.5 and 7. (**A**) Flow cytometry showing the percentages of $CD11c^{+}SiglecH^{+}$ pDCs (top panels) and $CD11c^{+}SiglecH^{-}$ cDCs (bottom panels) at day 9. cDCs are further divided based on their expression of CD11b and CD24. (**B**) Kinetics of CD11c, CD11b, B220 and SiglecH expression by BM cells in culture with or without LIF for 2, 4 or 6 days. (**C**) Phenotyping of cells at day 6 based on pDCs and cDCs surface markers. (**D**) Forward scatter (FSC) and side scatter (SSC) of cells after 6 days of culture

with or without LIF. (**E**) Flow cytometry showing CD11b expression of proliferating cells marked by Ki67. BM cells were incubated with medium containing Flt3L with or without LIF for the indicated times. The data are representative of at least three independent experiments.

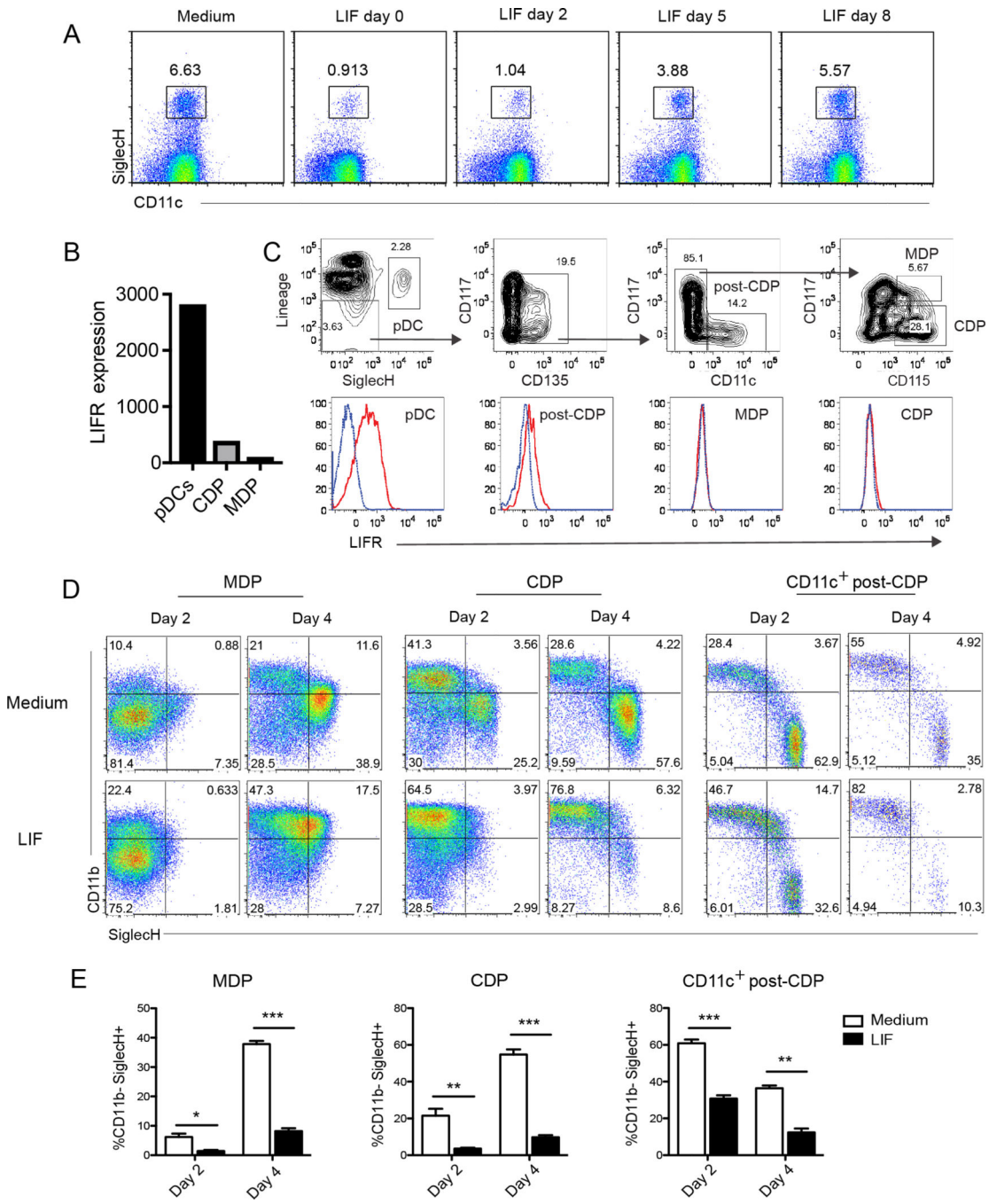

Figure 4. LIF acts on a late DC progenitor.

(**A**) LIF impairs pDC development in culture. BM cells were incubated with medium containing Flt3L for 10 days and LIF was added to the culture on day 0, 2, 5 or 8. The percentages of differentiated pDCs were ascertained by flow cytometry. (**B**) *Lifr* mRNA expression in pDCs, CDPs, and MDPs from Immgen database. (**C**) LIFR expression on pDCs ($lin^+SiglecH^+$), post-CDPs with pDC and cDC potential ($lin^-Flt3^+CD11c^+$), MDPs ($lin^-Flt3^+ckit^{hi}M\text{-}CSFR^+$), and CDPs ($lin^-Flt3^+ckit^{lo}M\text{-}CSFR^+$). Red histograms, LIFR; blue histograms, isotype control. (**D-E**) Sorted MDPs ($lin^-Flt3^+ckit^{hi}M\text{-}CSFR^+$), CDPs ($lin^-$

$Flt3^+ckit^{lo}$) and post-CDPs ($lin^-Flt3^+CD11c^+$) were incubated with medium containing Flt3L with or without LIF. Development of pDCs and $CD11b^+$ cDCs was assessed by flow cytometry on days 2 and 4 of culture. (**D**) Representative dot plots and (**E**) statistical analysis of $SiglecH^+CD11b^-$ pDC generation. Two-way ANOVA with Bonferroni's post-test was performed. Statistical differences are shown as *<0.05; **<0.01; and ***<0.001. The data are representative of two to three independent experiments.

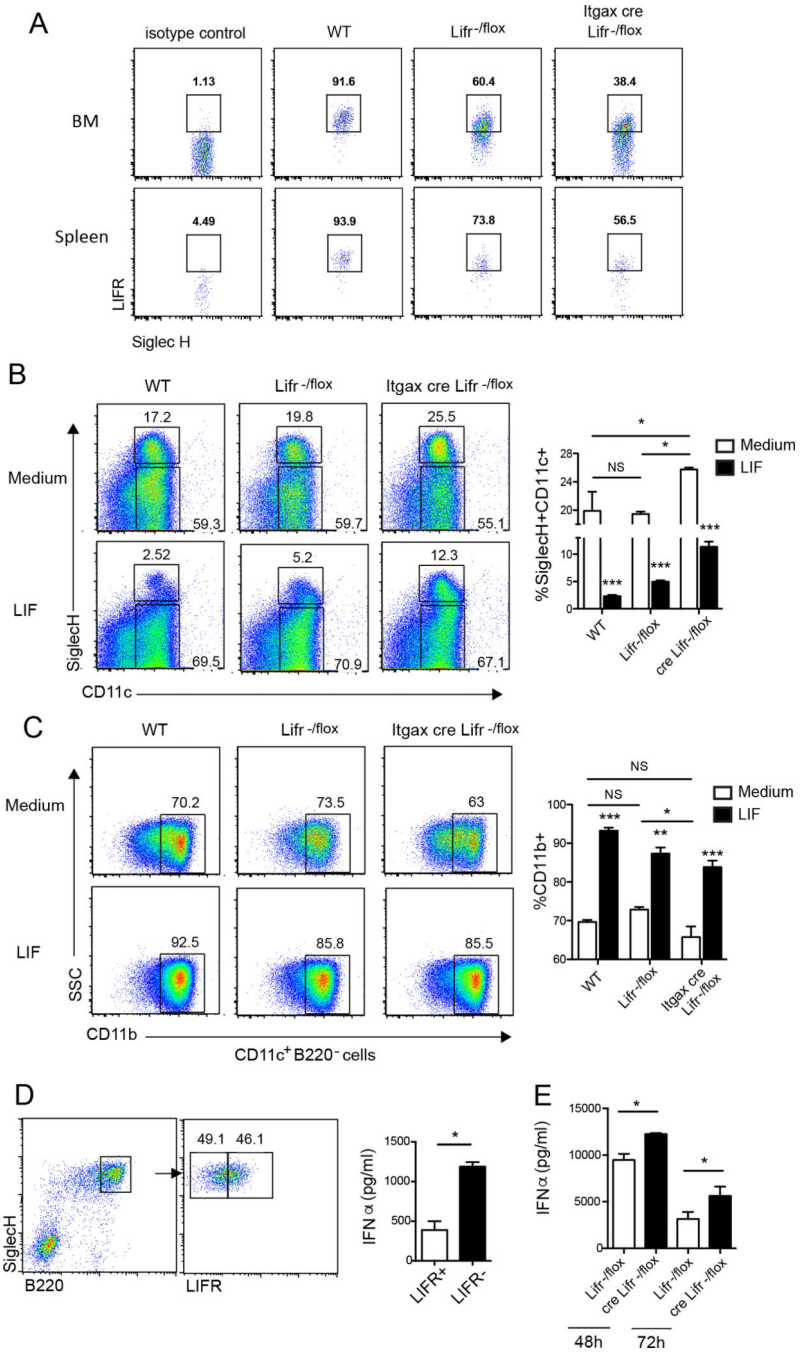

Figure 5. Conditional targeting of LIFR in the CD11c^{+} lineage augments pDCs development and function.

(**A**) Flow cytometry showing the expression of LIFR by pDCs from BM and spleen of WT, *Lifr*$^{-/flox}$ and *Itgax*^{Cre}*Lifr*$^{-/flox}$ mice. (**B**, **C**) BM cells of WT, *Lifr*$^{-/flox}$ and *Itgax*^{Cre}*Lifr*$^{-/flox}$ mice were incubated with medium containing Flt3L with or without LIF for 7 days. Development of pDCs (B) and CD11b^{+} cDCs (C) was assessed by flow cytometry. Two-way ANOVA with Bonferroni's post-test was performed. Statistical differences are shown as *<0.05; **<0.01; and ***<0.001. (**D**) LIFR^{hi} and LIFR^{lo} pDCs were sorted from BM of *Lifr*$^{-/+}$ mice and incubated with CpG-A for 24h for IFN-α measurement on the supernatant

by ELISA. Dots represent gate strategy for cells sorting. (**E**) WT, *Lifr*$^{-/flox}$ and *Itgax*^{Cre}*Lifr*$^{-/flox}$ mice were infected with LCMV Armstrong and IFN-α was quantified by ELISA in the serum 48h and 72h later. The data are representative of two to three independent experiments.

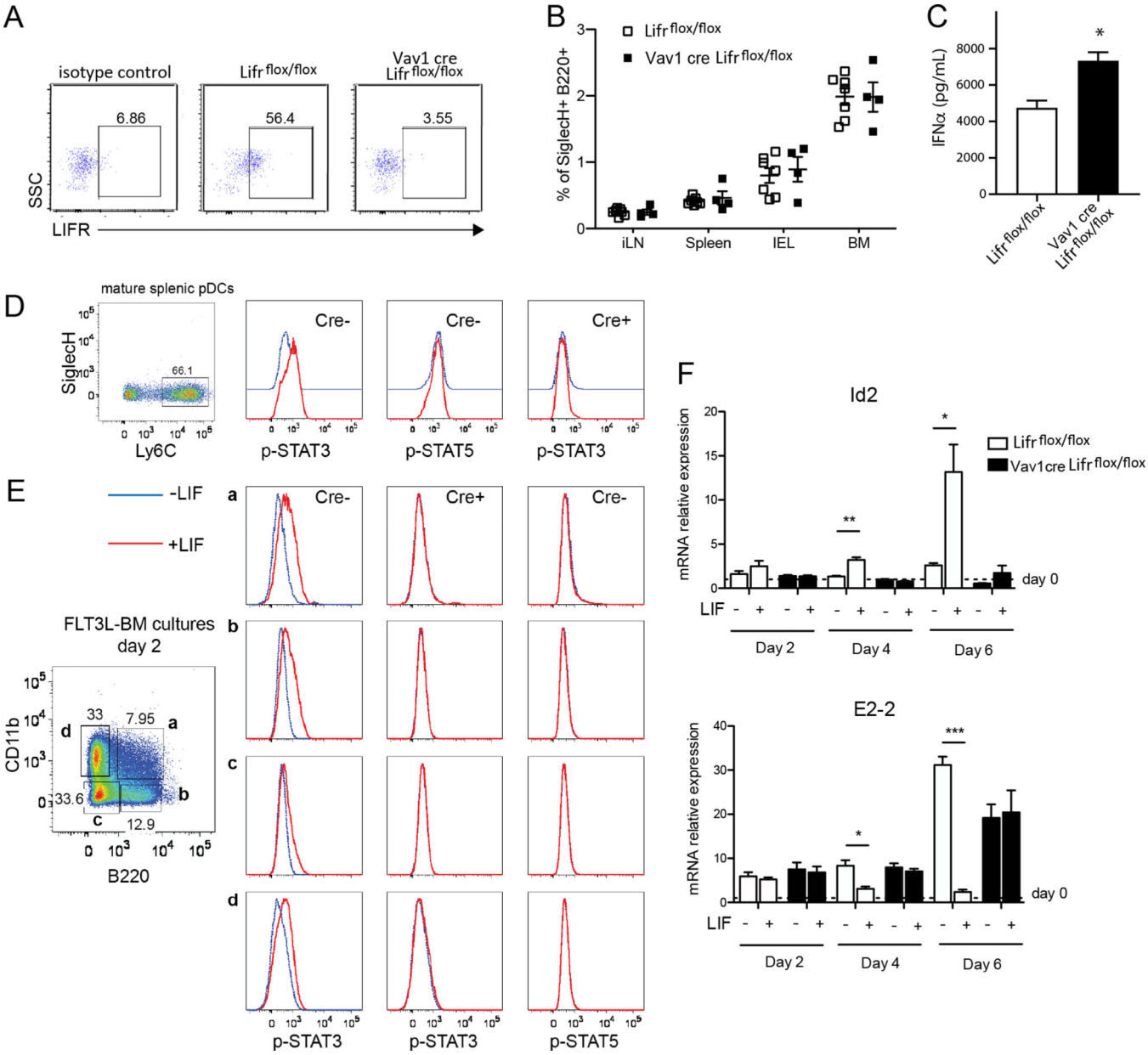

Figure 6. Conditional targeting of LIFR in the hematopoietic lineage impacts mature pDC functions and LIFR signaling in BM cultures.

(**A**) Flow cytometry showing the expression of LIFR on pDCs from BM of *Lifr*$^{flox/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice. (**B**) Percentages of SiglecH^{+}B220^{+} cells in lymph nodes (iLN), spleen, bone marrow (BM) and within intestinal intraepithelial cells (IELs) from *Lifr*$^{flox/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice ascertained by flow cytometry. (**C**) *Lifr*$^{flox/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice were infected with LCMV Armstrong and IFN-α was quantified by ELISA in the serum 48h later. NI: non-infected. Student's t test was performed. (**D**) Mature pDCs enriched from spleens of *Lifr*$^{flox/flox}$ and *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice were pre-stained for SiglecH and Ly6C, stimulated with LIF, methanol fixed, permeabilized and stained for intracellular phospho-STAT3. Of note, SiglecH is no longer detectable after cell fixation,

while the alternative pDC marker Ly6c remains viable. (**E**) BM cells from *Lifr*$^{flox/flox}$ *or* *Vav1*^{Cre}*Lifr*$^{flox/flox}$ mice were cultured with Flt3L for 2 days and stimulated with LIF for 15 min or left unstimulated. Cells were stained for B220 and CD11b, methanol fixed, permeabilized and stained for phospho-STAT3 or phospho-STAT5. Histograms for phospho-STAT3 or phospho-STAT5 (red) and isotype control (blue) are shown for each BM cell subset defined by B220 and CD11b. (**F**) Q-PCR for Id2 and E2–2 mRNA in BM cultures ± LIF at the indicated times. Student's t test was performed to compare cells cultured with and without LIF. Statistical differences are shown as *p<0.05; **p<0.01; and ***p<0.001. The data are representative of at least two independent experiments.