A type I IFN–Flt3 ligand axis augments plasmacytoid dendritic cell development from common lymphoid progenitors

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During infections and inflammation, plasmacytoid dendritic cells (pDCs) are the most potent type I interferon (IFN-I)–producing cells. However, the developmental origin of pDCs and the signals dictating pDC generation remain incompletely understood. Here, we report a synergistic role for IFN-I and Flt3 ligand (FL) in pDC development from common lymphoid progenitors (CLPs). Both conventional DCs (cDCs) and pDCs were generated from CLPs in response to FL, whereas pDC generation required higher concentrations of FL and concurrent IFN-I signaling. An absence of IFN-I receptor, impairment of IFN-I signaling, or neutralization of IFN-I significantly impeded pDC development from CLPs. Furthermore, FL induced IFN-I expression in CLPs, which in turn induced Flt3 up-regulation that facilitated survival and proliferation of CLPs, as well as their differentiation into pDCs. Collectively, these results define a critical role for the FL/IFN-I/Flt3 axis in pDC differentiation from CLPs.

Plasmacytoid DCs (pDCs) are a subset of DCs that are capable of producing large quantities of type I IFN (IFN-I) upon stimulation (Liu, 2005). pDCs are short-lived and require constant replenishment from their precursors in the BM. DCs, including pDCs, are generated from Flt3-expressing progenitors of myeloid lineages, including common myeloid progenitor (CMP), macrophage/DC progenitor, and common dendritic progenitor (CDP), and of lymphoid lineages such as common lymphoid progenitor (CLP; D’Amico and Wu, 2003; Karsunky et al., 2003; Shigematsu et al., 2004; Naik et al., 2007; Onai et al., 2007; Liu et al., 2009). Targeted deletion of Flt3 or FL in mice leads to reduced numbers of DC progenitors and impaired DC development (McKenna et al., 2000; Waskow et al., 2008). Ectopic expression of Flt3 on Flt3-megakaryocyte/erythrocyte progenitors permits them to generate DCs (Onai et al., 2006). These results suggest that the Flt3 ligand (FL) is one of the most physiological relevant cytokines for maintaining homeostasis of steady-state DCs.

IFN-I, a pluripotent cytokine, regulates different aspects of DC biology. Mouse pDCs depend on IFN-I for migration and clustering in the marginal zone of the spleen (Asselin-Paturel et al., 2005). pDCs developed in the presence of IFN-I fail to produce IFN-I in response to CpG (Li et al., 2011). IFN-I induced during systemic viral infections and inflammation trigger apoptosis of pDCs and cDCs, resulting in decline of the populations in the spleen (Swiecki et al., 2011). Therefore, IFN-I appears to govern migration, function, apoptosis, and homeostasis of DCs. Whereas cDCs and pDCs can be generated from myeloid or lymphoid lineages, the developmental origin and signals controlling pDC development remain elusive. Here, we showed that FL controlled the developmental program of cDCs and pDCs from CLPs in a dose-dependent manner. FL also induced IFN-I in CLPs, resulting in up-regulation of Flt3 that facilitated survival,
RESULTS AND DISCUSSION

pDC development is impaired in the absence of IFN-I signaling

We have previously shown that Stat2 hypomorphic mutant mice (Stat2<sup>m/m</sup>) are hyporesponsive to IFN-I and have reduced numbers of DCs, particularly pDCs, in the spleen (Chen et al., 2009). Because STAT2 is predominantly activated by type I and III IFN, we used Ifnar1<sup>−/−</sup> mice to investigate whether the phenotype was due to impaired IFN-I signaling. Indeed, Ifnar1<sup>−/−</sup> mice showed significantly lower percentages and numbers of pDCs in the BM and spleen compared with WT mice (Fig. 1, A–C). A similar phenotype was observed in Stat1<sup>−/−</sup> and Stat2<sup>m/m</sup> mice (unpublished data), suggesting that IFN-I receptor and its downstream signals are involved in pDC homeostasis.

Because pDCs can be generated from myeloid or lymphoid progenitors, we next examined whether the generation of pDC from CDPs (myeloid) or CLPs (lymphoid) is affected by IFN-I signaling. In an AC-6.21 feeder system, FL primarily supported cDC development from CDPs of both WT and Ifnar1<sup>−/−</sup> mice (unpublished data). In sharp contrast to the development from CDPs, FL showed poor ability to support cDC development from WT CLPs. Instead, pDCs were preferentially generated (Fig. 1, D and E). However, this process was reversed by the absence of IFN-I signaling, as Ifnar1<sup>−/−</sup> CLPs predominantly developed cDCs but not pDCs. The total numbers of DCs generated from Ifnar1<sup>−/−</sup> CLPs were also lower than those from WT CLPs. Therefore, the numbers of Ifnar1<sup>−/−</sup> CLP-derived cDCs were actually comparable to those from WT CLPs, whereas the numbers of Ifnar1<sup>−/−</sup> CLP-derived pDCs were markedly reduced (Fig. 1 F). A similar result was also observed using BM cells as feeder (unpublished data). We next performed competitive adoptive transfer to examine whether the developmental defect is cell intrinsic. As shown in Fig. 1 (G–I), the reconstitution rate of WT and Ifnar1<sup>−/−</sup> CLPs in the BM was comparable. However, the percentages of Ifnar1<sup>−/−</sup> CLP-derived pDCs remained significantly lower than those from WT CLPs. Conversely, the percentages of Ifnar1<sup>−/−</sup> CLP-derived cDCs were higher than those from WT CLPs. A similar phenotype was observed in proliferation, and differentiation of CLPs. These results establish a critical role for IFN-I in FL-dependent pDC development from CLPs.
that exogenous IFN-I might facilitate pDC generation from CLPs. Indeed, the addition of exogenous IFN-α promoted pDC development even at low-dose FL (50 ng/ml) treatment compared with control (Fig. 3, H–J). Conversely, neutralization of IFNAR1 abrogated pDC formation upon stimulation with 100 ng/ml of FL (Fig. 3, K–M), mimicking the phenotype seen in Ifnar1−/− CLPs under the same culture conditions (Fig. 1 D). Together, these results suggest that FL induces IFN-I, which in turn facilitates pDC generation from CLPs.

**FL enhances Flt3 expression on CLPs in an IFN-I–dependent mechanism**

It was proposed that FL induces Flt3 expression on progenitors through up-regulation of PU.1, which in turn controls DC development (Merad, 2010). Because IFN-I accentuated FL-dependent pDC differentiation, we examined whether IFN-I could induce Flt3 expression. IFN-α4 treatment in BM cells significantly augmented Flt3 expression on CLPs (Fig. 4, A–C). The effect of IFN on CLPs was direct, as the same phenomenon was observed when treating CLPs with IFN-α4 (Fig. 4 D).

In vivo administration of IFN-α4 also enhanced Flt3 levels on WT CLPs, as opposed to empty vector (EV) control (Fig. 4, E–G). It has been reported that IFN-α can activate dormant HSCs, inducing their proliferation and reestablishing homeostasis (Essers et al., 2009). Although it is still not clear if IFN-α will enhance the production of Flt3+ CLPs, in vivo delivery of IFN-α4 did not alter the percentages or numbers of CLPs in the BM, at least, within 24 h (Fig. 4 H–I). The requirement of IFN-I for FL-dependent Flt3 induction was also investigated. In vitro FL treatment markedly enhanced Flt3 expressions on WT CLPs, whereas IFNAR1 deficiency or neutralization of IFNAR1 blocked this process (Fig. 4, J–L, and not depicted).

**FL induces IFN-α in CLPs, which promotes FL–dependent pDC development**

We have demonstrated that IFN-I activity was involved in FL-dependent DC development from CLPs. One possibility is that FL induces IFN-I in CLPs. Indeed, FL induced IFN-α expression and production in CLPs in vitro in a dose- and time-dependent manner, which was blocked by neutralization of FL3 (Fig. 3, A–C). In vivo FL delivery also induced IFN-α in CLPs (Fig. 3 D). Moreover, IFN-I downstream genes, including BST2, Ifit3, and Oas2 were up-regulated in FL–treated CLPs (Fig. 3, E–G). Given that IFN-α is induced by FL in CLPs, we reasoned that exogenous IFN-I might facilitate pDC generation from CLPs.
The effect of IFN-I on DC development, maturation, and function has been controversial (Honda et al., 2003; Hahm et al., 2005; Li et al., 2011; Swiecki et al., 2011). Measles virus and lymphocytic choriomeningitis virus infection–induced IFN-I blocks FL-mediated DC development (Hahm et al., 2005). Moreover, IFN-I also contributes to systemic viral infection–induced decline of pDC numbers (Swiecki et al., 2011). In contrast, IFN-I promotes pDC development in the Peyer’s patches in the gut (Li et al., 2011). Our results also support the beneficial effect of IFN-I for pDC development.

**Figure 2.** *Ifnar1<sup>−/−</sup>* CLPs display reduced Flt3 expression and impaired survival, proliferation, and differentiation in response to FL. (A) CLPs were isolated from WT or *Ifnar1<sup>−/−</sup>* mice and Flt3 expression was analyzed by flow cytometry (*n* = 7). Results represent five experiments. (B) BM was stimulated with or without 100 ng/ml of FL for the indicated times, cells were gated on lin<sup>−</sup> Flt3<sup>+</sup> cells, and expression of pAkt was analyzed by flow cytometry (*n* = 3). One experiment out of three is shown. (C) CLPs were stimulated with 100 ng/ml of FL for the indicated times and stained for Annexin V (*n* = 4). Results represent four experiments. (D) Same as in C, except CLPs were CFSE labeled before FL stimulation for the indicated times. (E) Mean percentages of CFSE<sup>lo</sup> population are shown (*n* = 4). Results represent two experiments. (F) CLPs that had been transduced with MIGR1 or MIGR1-Flt3 were co-cultured with AC-6.21 supplied with 100 ng/ml of FL, gated on CD11c<sup>+</sup>GFP<sup>+</sup> cells, and analyzed for DC populations. Mean percentages of GFP<sup>+</sup> cDCs (G) and pDCs (H) are shown (*n* = 4). Results represent four experiments. (I) CLPs were co-cultured with AC-6.21 supplied with the indicated doses of FL, gated on CD11c<sup>+</sup>, and analyzed for DC populations. Mean percentages (J and L) and numbers (K and M) of DCs are shown (*n* = 3–9). Results represent five experiments. Same as in I, except 400 ng/ml of FL was supplied. CLP-derived cDCs or pDCs were sorted, and expression of Tcf4 (N), Rag1 (O), or Id2 (P; *n* = 3) was evaluated by RT-qPCR. One experiment out of three is shown.
Therefore, targeting the production or actions of IFN-I may provide a therapeutic approach to reduce pDC numbers and ameliorate the autoimmune diseases.

MATERIALS AND METHODS

Mice. All mice used in this study were from the C57BL/6 background. Mice were bred and kept under specific pathogen–free conditions. C57BL/6 (CD45.2) wild-type mice were purchased from the National Laboratory Animal Center, Taiwan. *Ifnar1*−/− mice (provided by M. Karin, University of California, San Diego, CA, through G.-Y. Yu at National Health Research Institute, Taipei, Taiwan), which have been backcrossed to C57BL/6 for at least six generations. C57BL/6-Thy1.1 (CD45.1) congenic mice and CD45.1×CD45.2 mice were rederived and bred at the Laboratory Animal Center of National Taiwan University College of Medicine. Procedures and the use of the animals were reviewed and approved by the Institutional Animal Care and Use Committee.

Several possibilities may account for these seemingly contradictory results. First, IFN-I may exert differential effects on different subsets of DCs or DC progenitors through altered intracellular signaling networks (Longman et al., 2007). Second, IFN-I may trigger opposite effects in a dose-dependent manner (Dalod et al., 2002; Gautier et al., 2005). FL and IFN-I are both induced by chemo- or radiotherapy, inflammation, and viral infections (Levy et al., 2011; Stirewalt and Radich, 2003). Therefore, the FL–IFN-I–Flt3 axis can function under physiological settings, triggering the amplification of DC compartments, particularly pDCs, thereby enhancing immunity. On the other hand, dysregulated pDC activation and IFN-α production is strongly linked to the development of systemic lupus erythematosus and psoriasis (Gilliet et al., 2008). Therefore, targeting the production or actions of IFN-I may provide a therapeutic approach to reduce pDC numbers and ameliorate the autoimmune diseases.
Figure 4. IFN-I is required for FL-dependent up-regulation of Flt3 on CLPs. (A) BM cells stimulated with or without IFN-α4 (1,000 U/ml) for 24 h and Flt3⁺ CLPs were enumerated by flow cytometry. Mean percentages of Flt3⁺ CLPs (B) and MFI of Flt3 on CLPs (C) are shown (n = 3). Results represent three experiments. (D) Same as in A, except sorted CLPs were stimulated for the indicated times. (E) EV or IFN-α4–expressing vector (1 µg each) was delivered in vivo using the HGT method for 24 h, and Flt3 expression on CLPs was evaluated by flow cytometry. Mean percentages of Flt3⁺ CLPs (F) and MFI of Flt3 on CLPs (G) are shown (n = 3). Results represent two experiments. (H and I) Same as in E, except percentages (H) and numbers (I) of CLPs were evaluated by flow cytometry. (J) Same as in A, except BM was stimulated with or without 100 ng/ml of FL. Mean percentages of Flt3⁺ CLPs (K) and MFI of Flt3 on CLPs (L) are shown (n = 3). Results represent two experiments. (M) Same as in E, except EV or FL-expressing vector (5 µg each) was delivered into mice. Mean percentages of Flt3⁺ CLPs (N) and MFI of Flt3 on CLPs (O) are shown (n = 3). (Results represent three experiments.) (P) A model for FL/IFN-I/Flt3 axis in promoting pDC development from CLPs. High-dose FL triggers the production of IFN-I (1), which facilitates FL-stimulated up-regulation of Flt3 (2), resulting in increased survival, proliferation, and differentiation into CLPs. It remains to be determined whether IFN-I is directly involved in these processes.
Antibodies and flow cytometry. Staining was performed at 4°C with Fc block (hybridoma 2-4G2) in FACS staining buffer (0.5% FBS and 0.1% NaN₃ in PBS). The following antibodies were purchased from eBioscience: biotin-anti-CD1c (N418), PE-anti-CD11b (M1/70), PE-anti-CD3 (17A2), PE-anti-CD8 (53-6-7), PE-anti-G1 (RB6-8C5), PE-anti-CD19 (eBio1D3), PE-anti-TER119 (E2E), PE-anti-MHCII (NIMP-R), PE-anti-Thy-1.1 (HS51), PE-anti-NK1.1 (PK13E), APC-anti-B20 (RA3-6B2), PerCP-eFlour780-anti-Siglec-H (eBio440), PerCP-eFlour780-anti-c-Kit (2B8), APC-anti-M-CSFR (AF598), PE-Cy7-anti-IL-7Ra (A7R34), biotin-anti-Flt3 (A2F10), FITC-anti-SCA-1 (D7), APC-Cy7-Streptavidin, FITC-anti-BrdU, and anti-IFNAR1 (MAR1-5A3). Purified anti-Flt3 (A2F10), anti-B20 (927), and FITC-Annexin V were purchased from BioLegend. FITC-anti-IFN-α (RMMa-1) was purchased from PBL Biomedical Laboratories. Flow data were analyzed with Flowjo (Tree Star) or Kaluza (Beckman Coulter) software.

Progenitor analysis and cell sorting. After lysis of RBCs, BM cells were stained with PE-conjugated lineage markers, including anti-CD3, anti-CD8, anti-B20, anti-CD19, anti-CD11b, anti-Ge-1, anti-TER119, anti-Thy1.1, anti-NK1.1, and anti-MHC II. CLPs defined as Lin−c−kit+ScA−1−M-CSFR−IL−7Ra− (Kondo et al., 1997), were analyzed with the BD FACS Canto II or sorted with the BD FACSAria III.

In vitro DC development. In vitro DC development was performed as previously described (Chicha et al., 2004; Naik et al., 2005) with minor modifications. For AC-6.21 (Whitlock et al., 1987) feeder system, AC-6.21 (kindly provided by I. Weissman, Stanford University, Stanford, CA) were seeded at 5.9 × 10⁴ into 12-well plates 1 d before to reach a confluency of 80%, followed by γ-irradiation with 3,000 rad (30 Gy). Sorted CLPs at 500 cells/well were co-cultured with the AC-6.21 cells supplied with the indicated doses of huFL-Ig for 20 d. FL was produced in-house using the FL expression system provided by M. Manz (University Hospital Zürich, Zürich, Switzerland; Onai et al., 2006). Progeny cells from AC-6.21 feeder system were stained with antibodies to CD11c, CD11b, and CD200. CDcs and pDCs are CD11c+CD11b+CD200+ and CD11c−CD11b+CD200−, respectively. For IFN-α treatment, different doses of mouse IFN-α (Merck) or IFN-α4 (produced in-house) were added into the co-culture system supplied with 50 ng/ml of FL. For antibody-blocking experiments, the indicated doses of anti-IFNAR1 (MAR-1-5A3; eBioscience), anti-Flt3 (A2F10, eBioscience), or their isotype antibodies was added.

Competitive adoptive transfer. 10,000 CLPs sorted from C57BL/6 (CD45.1) and C57BL/6 (CD45.2)–Bflt1−/− mice were co-transfered intravenously with 2 × 10⁶ C57BL/6 (CD45.1xCD45.2) BM cells into C57BL/6 (CD45.1xCD45.2) mice that had been lethally irradiated with 600 rad (6 Gy) twice at 4-h intervals. 12 d later, BM cells were stained and gated on the CLP-derived cells (CD45.1+ or CD45.2+) and analyzed for pDCs (CD11c+CD11b+CD200+Slecle−H+) or cDCs (CD11c−CD11b+CD200−).

Apoptosis and proliferation assays. For apoptosis assay, sorted CLPs co-cultured with AC-6.21 were treated with 100 ng/ml FL for the indicated times, followed by staining with FITC-Annexin V (BioLegend) according to the manufacturer’s instructions. For proliferation assay, WT BM cells (CD45.1) and Bflt1−/− (CD45.2) were mixed first, and then stained with 5 µg CFSE (Invitrogen), followed by sorting out CFSE+ CLPs, co-culturing with AC-6.21, and stimulation with 100 ng/ml of FL. The cells were collected and analyzed with flow cytometry at the indicated times.

Hydrodynamic gene transfer (HGT). Mice were injected intravenously via tail veins with plasmid DNA in 10% body-weight sterile PBS. DNA injection was completed within 5 s. For IFN-α4 treatment, 0.25–1 µg pcDNA3.1 EV or the vector expressing mIFN-α4 (provided by L.-H. Hwang, National Yang-Ming University, Taipei, Taiwan) was injected. For FL treatment, 2.5–5 µg of pcDNA3 EV or the vector expressing mFL (provided by T.-C. Wu, Johns Hopkins University, Baltimore, MD) was injected.

Intracellular staining. For intracellular pAkt staining, BM cells treated with FL were fixed with 1% paraformaldehyde for 10 min, followed by permeabilization with 90% methanol for 30 min at room temperature. After the removal of methanol, the cells were resuspended and stained with antibodies to lineage markers, Flt3, and pAkt (pSer473; Cell Signaling Technology). F(ab)’2 anti-rabbit IgG-FITC (eBioscience) was used for secondary labeling. For intracellular IFN-α staining, 3–5 × 10⁴ sorted CLPs were stimulated with FL in vitro for 20 h. Monensin (2 µM, eBioscience) was added into the culture for an additional 4 h before performing intracellular staining. For in vivo treatment, CLPs were sorted directly into 1% paraformaldehyde after HGT with mFL expressing placid for the indicated times. The treated cells were fixed in BD Cytofix/Cytoperm solution. Staining was performed in BD Perm/Wash buffer using FITC-anti-mouse IFN-α antibody (RMMa-1; PBL Biomedical Laboratories).

RT-qPCR. For gene expression analysis, sorted CLPs (5 × 10⁴/treatment) were seeded onto AC-6.21 feeder 1 d before the treatments. mRNA of the treated CLPs was prepared with TurboCapture kit (QIAGEN), followed by cDNA synthesis via HiScript I reverse transcription (BIONOVAS) using random primer. The cDNA was then subjected to RT-qPCR, using primers to Ift4, Ifit3, Oas2, and Actb. 50,000 splenic cDCs and pDCs or CLP-derived cDCs and pDCs using AC-6.21 feeder co-culture system were sorted and subjected to RT-qPCR for Ifit4, Rat1, Id2, and Actb. Data were normalized to Actb. All the primer sequences are listed in Table S1.

Retroviral transduction. Retroviral transduction of CLPs was prepared as previously described (Onai et al., 2006). In brief, MigR1 or MigR1-mFlt3 (provided by S. Nutt, Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) plasmids were co-transfected with two plasmids expressing retroviral gag-pol and env, respectively, into 293T cells using the calcium phosphate precipitation method. The viral supernatants were collected 2 d later, concentrated, and then spin-infected GP+E86 cells in DMEM containing 10% FBS and 8 µg/ml polybrene (Sigma-Aldrich) at 1,100 g for 50 min. After viral transduction for 48 h, GFP+GP+E86 cells were sorted. For infection of CLPs, sorted WT and Bflt1−/− CLPs were co-cultured with γ-irradiated (2,000 rad) GFP+GP+E86 cells (1.5 × 10⁵ cells/well of 24-well plate) for 18 h in the presence of 4 µg/ml polybrene and 100 ng/ml FL Transduced CLPs were subjected to in vitro AC-6.21 feeder co-culture system as described in the In vitro DC development section.

Statistical analyses. All values are shown as mean ± SD. Two-tailed, unpaired Student’s t test was used to assess statistical significance: *, P < 0.05; **, P < 0.01; and ***, P < 0.005.

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