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Organ-Specific Cellular Requirements for In Vivo Dendritic Cell Generation

Tewfik Miloud,* Nathalie Fiegler,* Janine Suffner,* Günter J. Hämmerling,*† and Natalio Garbi*†‡

Bone marrow-derived dendritic cell (DC) precursors seed peripheral organs, where they encounter diverse cellular environments during their final differentiation into DCs. Flt3 ligand (Flt3-L) is critical for instructing DC generation throughout different organs. However, it remains unknown which cells produce Flt3-L and, importantly, which cellular source drives DC development in such a variety of organs. Using a novel BAC transgenic Flt3-L reporter mouse strain coexpressing enhanced GFP and luciferase, we show ubiquitous Flt3-L expression in organs and cell types. These results were further confirmed at the protein level. Although Flt3-L was produced by immune and nonimmune cells, the source required for development of the DC compartment clearly differed among organs. In lymphoid organs such as the spleen and bone marrow, Flt3-L production by hematopoietic cells was critical for generation of normal DC numbers. This was unexpected for the spleen because both immune and nonimmune cells equally contributed to the Flt3-L content in that organ. Thus, localized production rather than the total tissue content of Flt3-L in spleen dictated normal splenic DC development. No differences were observed in the number of DC precursors, suggesting that the immune source of Flt3-L promoted pre-cDC differentiation in spleen. In contrast, DC generation in the lung, kidney, and pancreas was mostly driven by nonhematopoietic cells producing Flt3-L, with little contribution by immune cells. These findings demonstrate a high degree of flexibility in Flt3-L–dependent DC generation to adapt this process to organ-specific cellular environments encountered by DC precursors during their final differentiation. The Journal of Immunology, 2012, 188: 000–000.
developmentally related to CD8+ cDCs in lymphoid organs in that they also arise from pre-cDCs and require Flt3-L and similar transcription factors for their generation (10, 11). Flt3-L induces mTOR activation (32) and is required both during the differentiation of early hematopoietic precursors in the bone marrow (33) and during the final steps of DC differentiation in peripheral organs (16, 29). Earlier studies have shown that Flt3-L is produced by a variety of different organs and cell lines (34–39), but little is known about its in vivo distribution.

In this study, we report on the cellular requirements for Flt3-L–dependent generation of DCs in lymphoid and nonlymphoid organs. Using a novel bacterial artificial chromosome (BAC) transgenic reporter mouse to characterize Flt3-L production, we find widespread expression in a large variety of organs and cell types. However, there are specific cellular requirements for relevant Flt3-L provision in different organs, which reflect their particular characteristics. For example, Flt3-L production by hematopoietic cells is necessary for DC development in the spleen, whereas production by nonhematopoietic cells is required in nonlymphoid organs. We also demonstrate that the cellular source rather than the organ content of Flt3-L is dominant for DC generation in spleen and bone marrow, suggesting that DC development in those organs proceeds within specific cellular environments where close interactions between Flt3-L–producing cells and DC precursors take place.

Materials and Methods

Mice and cell depletion in vivo

C57BL/6N mice (B6), congenic B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice (Charles River), flt3l+/- mice (Taconic), and enhanced GFP (eGFP)–expressing mice (16) were bred at the German Cancer Research Center in specific pathogen–free conditions. Experiments were conducted according to institutional guidelines and regulations (Zentrals Tierlabor, German Cancer Research Center).

Flt3-L reporter BAC transgenic mice were generated as previously reported (16, 40). eGFP and CBGr99 luciferase cDNA were separated by a 2A sequence and inserted at the start codon of the flt3I gene in the BAC RP23-226f6 (Children’s Hospital Oakland Research Institute) by homologous recombineering using Escherichia coli DH10B. The SmI- and PvuI-linearized construct was injected into the pronuclei of fertilized C57BL/6 mouse eggs. The resulting transgenic mouse lines were designated FL-GL (eGFP and Luciferase expression under the control of the flt3I promoter). Six transgenic lines were obtained yielding similar results. The data reported in this article were obtained using line #8. The BAC RP23-226f6 contains the 190.9 kbp spanning region 52269801-52480735bp of mouse chromosome 7, with the flt3I start codon at position 69353 bp.

To deplete DCs in vivo in an inducible fashion, CD11c–DOG mice (41) were injected i.p. with 8 ng/g body weight diphtheria toxin (DT; Sigma) in PBS. Splenic cDC depletion was typically 90–97%.

To deplete Gr-1+ monocytes and granulocytes, mice were injected i.v. with 400 µg anti–Gr-1 depleting Ab (RB6-8C5). NK cells were depleted by i.p. injection of 1 mg anti-NK1.1 depleting Ab (PK136) (16) at the indicated time points.

Bioluminescence imaging

In vivo bioluminescence imaging was performed as described previously (42). Mice were injected i.p with D-Luciferin (150 mg/kg of body weight); 5 min later mice were imaged using an IVIS 100 charge-coupled device imaging system (Xenogen) for 1 min. Further confirmation was obtained by dissecting mice and incubating organs in 1 mg/ml D-Luciferin prior to imaging. Image data were analyzed with Living Image software (Xenogen).

Generation of bone marrow chimera mice

BM chimera mice were made as previously described by transferring 2 × 10^6 Thy1.2+–depleted donor bone marrow cells into 10 Gy-irradiated recipient B6 or CD45.1 flt3l+/- mice (16). Donor BM consisted of cells from CD45.1, eGFP, or flt3l+/- mice. CD45 congenic markers and eGFP allowed us to confirm engraftment of donor BM. Mice were administered antibiotics in drinking water until experiments were performed 11 wk after reconstitution.

Flow cytometric analysis and cell sorting

One to 5 × 10^6 cells were stained in 200 µl Dulbecco-modified PBS containing 3% FCS and 5% Sandoglobulin (human poly-Ig; CSL Behring). The following biotinylated or fluorochrome-labeled Abs were obtained from BD Biosciences, eBioscience, or CalTAG: CD45.1 (A20), CD45.2 (104), CD11c (H35–7.2), I-A/I-E (K74), I-Ab (KH7), CD11b (M1/70), Gr-1 (RB6-8C5), DX5, CD117 (2B8), IgE (RME-1), CD35 (SC12), CD31 (MEC13.3), Ep-CAM (G8.8), and anti-erythocyte (Ter119). Propidium iodide was used in all analyses as a viability dye. Cell populations were identified as follows: leukocytes, CD45+; cDCs, CD11c+ MHC-II+; plasmacytoid DCs (pDCs), CD11c+PDCA-1+; NK cells, NK1.1+CD3+; NK cells, CD11b+CD11c+; T cells, CD4+ CD3+; B cells, CD19+; granulocytes, Gr1+; CD11b+SSC+; monocytes, Gr1+CD11b+SSC+; basophils, Ter119+CD3+ CD19+; DX5+CD117+ IgE+; mast cells, Ter119+CD3+ CD19+ DX5+CD117+ IgE+; endothelial cells, CD19+ CD3+ NK1.1+ Gr-1+ CD4+ CD11c+ CD31+; stromal cells, CD19+ CD3+ NK1.1+ Gr-1+ CD4+ CD11c+ CD117+ CD31+; epithelial cells, CD19+ CD3+ NK1.1 Gr-1+ CD4+ CD11c+ EpCAM+; macrophage and DC precursors (MDPs), CD3+ CD19+ NK1.1 Ter119+ Gr-1+ CD45R+ CD11b+ sca-1+ CD11c+ CD11bhi+ CD135+ (16); common DC precursors (CDPs), CD3+ CD19+ NK1.1 Ter119+ Gr-1+ CD45R+ CD11b+ sca-1+ CD11c+ CD135+CD150+ (16); pre-cDCs, CD3+ CD19+ NK1.1+ Ter119+ Gr-1+ CD45R+ CD11c+ MHC-II+ CD135+CD150- (16); common DC precursors (CDPs), CD3+ CD19+ NK1.1+ Ter119+ Gr-1+ CD45R+ CD11b+ sca-1+ CD11c+ CD135+CD150+ (16); pre-cDCs, CD3+ CD19+ NK1.1+ Ter119+ Gr-1+ CD45R+ CD11c+ MHC-II+ CD135+CD150- (16). Labeled cells were measured on a FACS Canto II (BD Biosciences) and analyzed using FlowJo 6.4.7 (Tree Star) software Diva 6.2 software. Cell sorting was performed on a FACSAria (BD Biosciences).

Quantification of cell types

Organs were digested in Dulbecco-modified PBS containing 1 mg/ml Collagenase IV (Sigma) and 50 U/ml DNase I (Roche) for 30 min at 37°C in mild shaking (41). After lysing RBCs in ammonium-chloride-potassium buffer, cells were stained for flow cytometric analysis as described above. Leukocytes in nonlymphoid organs were gated based on CD45 expression and alveolar macrophages, and other highly autofluorescent cells were excluded using a blank fluorescence channel. The number of cells of interest per lymphoid organ was calculated based on their percentage and the total viable count obtained by trypan blue exclusion. For nonlymphoid organs, cells were enumerated by flow cytometry by adding a known amount of Calibrate FITC beads (BD Biosciences).

Flt3-L ELISA

Mouse serum was collected from tail blood. Organs and cells were lysed immediately after collection using 1% Nonidet P-40 (Fluka) as previously described (16, 43). The Flt3-L amount in the postnuclear fraction was quantified using the Quantikine mouse Flt3-L immunoassay (R&D Systems) following the manufacturer’s instructions.

Real-time qPCR

Real-time quantitative PCR was performed as described previously (16). RNA from sample homogenates was prepared using the RNAeasy Mini Kit (Qiagen), and RNA was translated into single-stranded cDNA using the Superscript cDNA Synthesis Kit (Invitrogen) and random hexamers (Amersham Biosciences). Gene expression levels were determined using real-time quantitative PCR TaqMan technology and SYBR green incorporation (Applied Biosystems). The murine flt3I primers used were: 5’- GGCTGGTAGAGACAGGCAA-3’, 5’- CTCACAGGCCGTTCGATC- TT-3’. The egfp primers used were: 5’-TATATCATGCGCCAGAAGC-3’, 5’- GTGGTGCCGGATCTGAGT-3’. The hyprt primers used were: 5’- ACACCTGTAATTTACTGCGAACA-3’, 5’- TGGAAAAAGCCTAATAA-CAGCTA-3’. PCR was performed on 43 ng cDNA template per 25 µl reaction volume for all samples following instructions provided by the manufacturer.

Statistical analysis

Comparisons between two samples were performed with Student t test. Comparisons between three or more samples were performed with one-way ANOVA using the Tukey or Dunnett test for multiple comparisons. Data were analyzed using Prism 5 (GraphPad) and statistical significance was set at p < 0.05. Data are expressed as mean values ± SEM.

Results

Expression analysis of Flt3-L in different organs and cell types

Flt3-L is critical for the differentiation of DC populations in lymphoid and nonlymphoid organs (10, 16, 27, 29). However, little
is known about the source of Flt3-L in tissues and primary cells. To investigate its distribution in vivo, we have generated a BAC transgenic Flt3-L reporter mouse line, termed FL-GL, that expresses eGFP and luciferase as individual proteins under the control of the flt3l promoter and cis regulatory elements present in the BAC (Fig. 1A). Thus, expression analysis of eGFP or luciferase activity reports on flt3l transcription. Noninvasive bioluminescence imaging of FL-GL mice revealed strong light emission throughout the body, suggesting ubiquitous activity of the flt3l promoter (Fig. 1B), which was confirmed by ex vivo analysis of individual organs (Fig. 1C). In agreement with ubiquitous promoter activity, Flt3-L protein was detected in all organs albeit with different amounts. The lung, kidney, and liver contained the highest amount of Flt3-L (>700 pg/organ), whereas all other organs investigated contained lower amounts (~200 pg/organ; Fig. 1D, 1E). Although soluble Flt3-L was present in serum (Fig. 1F), we observed similar organ Flt3-L content following intracardial perfusion of mice, indicating that the amount of blood-borne Flt3-L had no influence (data not shown). Although the spleen contains relatively high numbers of DCs and their precursors, we observed only an intermediate to low amount of Flt3-L in that organ (Fig. 1D, 1E). Surprisingly, Flt3-L expression in BM, the ultimate source of Flt3-L-dependent hematopoietic precursors, was even lower than in spleen (Fig. 1G).

In order to investigate Flt3-L expression at the single-cell level, we quantified eGFP expression in FL-GL mice by flow cytometry (referred to in this article as FL-eGFP). Since DC differentiation is initiated in BM and finalised in peripheral organs such as spleen (2, 16, 44), we focused our analysis on these two organs. In addition we also investigated expression in the lung because of its high Flt3-L protein content (Fig. 1D, 1E). Independently of the organ investigated, all immune and non-immune cells analyzed in FL-GL mice expressed FL-eGFP, indicating ubiquitous flt3l transcription across different cell types (Fig. 2 and Supplemental Figs. 1 and 2). Similar results were obtained by RT-PCR (data not shown). There were nevertheless quantitative differences between cell types. NK cells showed the highest FL-eGFP level, followed by mast cells, basophils, NKT cells, T cells, B cells, and myeloid cells (Fig. 2A–D, Supplemental Fig. 2). Because the frequency of mast cells in the spleen is extremely low, we analyzed this population in the peritoneal exudates of naive mice. FL-eGFP expression within each cell population was homogenous, indicating that there were no differences depending on the subpopulation analyzed or their activation status (Fig. 2A, Supplemental Fig. 1C, and data not shown). Nonimmune cells such as endothelial cells, epithelial cells, and fibroblasts showed intermediate FL-eGFP levels comparable to those of B cells and myeloid cells (Fig. 2A–D and Supplemental Fig. 1).

The difference in Flt3-L mRNA expression between NK cells and B cells was similar to the difference in FL-eGFP mean fluorescence intensity (2.2-fold; Fig. 2E), indicating that flow cytometric quantification of FL-eGFP in FL-GL mice indeed parallels flt3l mRNA expression. Next, the hierarchy of flt3l promoter activity in lymphoid cells was confirmed at the protein level using NK cells, B cells, and T cells sorted from wild type (wt) spleen. NK cells expressed the highest amount of Flt3-L (~1.5 pg/10⁶ cells), whereas B cells expressed the lowest (~0.05 pg/10⁶ cells; Fig. 2F). Thus, FL-eGFP intensity highly correlated with Flt3-L protein content among NK, B, and T cells ($r^2 = 0.98219$). Because of the low yield of stromal and rare immune cells obtained, we were not able to quantify Flt3-L protein in these cell populations.

These results show that the flt3l promoter is ubiquitously active in different organs and cells. Unexpectedly, expression of Flt3-L in NK cells was the highest, whereas expression in nonimmune cells was in general intermediate.

**DC development in lymphoid organs is differentially regulated by the source of Flt3-L**

It is known that hematopoietic stem cells and DC precursors are in close association with both the immune and nonimmune cell compartments (21, 45). Therefore we next investigated whether DC development relies on a specific source of Flt3-L expression. For this investigation, we produced BM chimeric mice restricting Flt3-L production either to the hematopoietic compartment (wt...
BM into lethally irradiated \( f_{lt3}^{-/-} \) recipients; \( wt \rightarrow f_{lt3}^{-/-} \) or nonhematopoietic compartment (\( f_{lt3}^{-/-} \rightarrow wt \)), and then cDCs and pDCs were enumerated following reconstitution. All sets of chimeras contained similar numbers of leukocytes except for \( f_{lt3}^{-/-} \rightarrow f_{lt3}^{-/-} \) mice in some organs (Supplemental Fig. 3A), resembling the reduced cellularity in \( f_{lt3}^{-/-} \) mice (27). The \( wt \rightarrow wt \) chimeras contained similar numbers of cDCs and pDCs as \( wt \) B6 mice in spleen and BM, whereas \( f_{lt3}^{-/-} \rightarrow f_{lt3}^{-/-} \) mice had strongly reduced numbers of DCs, validating our BM chimera approach to investigate DC homeostasis. Interestingly, the Flt3-L source required for full DC development differed depending on the organ or DC population investigated.

In the spleen, \( \sim -2\% \) of leukocytes are cDCs and \( \sim -0.5\% \) are pDCs (Fig. 3A). The hematopoietic source of Flt3-L (\( wt \rightarrow f_{lt3}^{-/-} \)) was sufficient and mandatory to reach normal cDC and pDC numbers in the spleen (Fig. 3A–C). In contrast, restricted expression of Flt3-L to the stromal, nonhematopoietic compartment (\( f_{lt3}^{-/-} \rightarrow wt \)) was only able to induce partial generation of splenic cDCs and pDCs reaching \( \sim -50\% \) of normal numbers (Fig. 3A–C). The chimerism in the NK cell, B cell, and myeloid compartments was \( \sim 97\% \) of cells derived from the donor BM, whereas that of NKT and T cells was \( \sim 75\% \). Nevertheless, similar results in DC reconstitution were obtained in BM chimeras using rag1\(^{-/-}\) recipient mice, in which all NKT and T cells were of donor origin (Supplemental Fig. 3B). The phenotype of cDCs in the BM chimeras was similar in terms of CD11c, MHC-II, CD8, and CD11b expression (Supplemental Fig. 3C), indicating that there was no bias in the development of certain cDC subpopulations. Thus, Flt3-L production by hematopoietic cells is sufficient and necessary to generate a normal DC compartment in the spleen, whereas the contribution of Flt3-L from stromal origin is limited.

In BM, most DCs are pDCs (\( \sim -0.8\% \)), whereas cDCs represent only \( \sim -0.1\% \) of leukocytes (Fig. 3D). Using the same set of BM chimeras, we also observed that Flt3-L production by immune cells was mandatory and sufficient for pDC development in BM (Fig. 3D, 3F). However, both the hematopoietic and nonhematopoietic sources of Flt3-L were each sufficient and thus redundant for development of the less abundant cDCs (Fig. 3D, 3E).

These results show that although both the immune and nonimmune compartments in BM and spleen are capable of Flt3-L production, there are important differences regarding the requirement for normal DC development. Flt3-L produced by immune cells was mandatory and sufficient for cDC and pDC generation in the spleen (Fig. 3B, 3C) and for BM pDCs (Fig. 3F). In contrast, there was a high degree of redundancy in Flt3-L production for cDC generation in the BM (Fig. 3E).

Flt3-L provided by nonhematopoietic cells is required for CD103\(^+\)CD11b\(^+\) DC homeostasis in nonlymphoid organs

CD11c\(^+\)MHC-II\(^+\) DCs in nonlymphoid organs are heterogeneous in terms of function and development (10, 11). Approximately 70% of lung DCs are CD103\(^+\)CD11b\(^−\) and are largely dependent on Flt3-L for their development, whereas CD103\(^−\)CD11b\(^+\) DCs represent \( \sim -20\% \) of total lung DCs and do not require Flt3-L for their development (10). We thus investigated the source of Flt3-L required for generating lung CD103\(^+\) DCs using the BM chimera approach described in this study. Total leukocyte numbers in the lung were not significantly altered in the different BM chimeras (Supplemental Fig. 3A). However, there was a tendency toward

\[ \text{mean } \pm \text{ SEM (} n = 3 \text{). One representative of at least three independent experiments is shown. EC, endothelial cells with phenotype as in } A; \text{ EpC, epithelial cells (CD19} \text{CD3} \text{NK1.1} \text{Ter119} \text{CD45.2} \text{CD31} \text{EpCAM}). \]
FIGURE 3. Influence of the cellular source of Flt3-L on DC generation in the spleen and BM. A, Representative FACS dot plot analysis showing the percentages of CD11c<sup>+</sup>MHCII<sup>+</sup> cDCs (left panels) and CD11c<sup>+</sup>PDLCA-1<sup>+</sup> pDCs (right panels) among live splenocytes. B, Number of splenic CD11c<sup>+</sup>MHCII<sup>+</sup> cDCs in the different BM chimera mice calculated based on their percentages as shown in A. C, Number of CD11c<sup>+</sup>PDLCA-1<sup>+</sup> pDCs per spleen of BM chimera mice based on their percentage as shown in A. D–F, Similar to A–C for dendritic cells in the BM. Data are expressed as mean value ± SEM (n = 3–6). One representative of three independent experiments is shown. n.s., nonstatistically significant. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA).

Reduced DC numbers by restricting Flt3-L production to hematopoietic cells (wt→flt3<sup/li>−/−</sup>), whereas exclusive Flt3-L production by the non-hematopoietic compartment (flt3<sup/li>−/−</sup>→wt) resulted in normal numbers of DCs (Fig. 4A, 4B). When focusing on different DC populations, we observed that this tendency of decreased DC numbers in wt→flt3<sup/li>−/−</sup> mice was caused by a strong and significant reduction in Flt3-L–dependent CD103<sup>+</sup> DC populations (Fig. 4A, 4C). As expected, the number of lung CD103<sup>+</sup> DCs, which do not require Flt3-L, for development, was not decreased in the different chimeras (Fig. 4A, 4C). Thus, Flt3-L produced by the nonhematopoietic compartment is sufficient and dominant for generating normal numbers of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the lung. Similar results were obtained for this subset of DCs in other organs such as the kidney and pancreas (Fig. 4). In the liver, however, both the hematopoietic and nonhematopoietic sources of Flt3-L were required for normal CD103<sup>+</sup> DC development (Fig. 4), highlighting organ-specific differences in Flt3-L requirements.

The requirement of Flt3-L for CD103<sup>+</sup> DC development in nonlymphoid organs was more complex. Development of this subpopulation in the kidney, pancreas, and liver was dependent on Flt3-L expression, but not in the lung (Fig. 4). Interestingly, the requirement for a hematopoietic or nonhematopoietic origin of Flt3-L differed among the former organs (Fig. 4F, 4I, 4L).

Contribution of the hematopoietic and nonhematopoietic compartments to total Flt3-L expression

After having shown that the Flt3-L source required for generating normal numbers of DCs varied among the spleen, BM, kidney, pancreas, and liver, we next investigated whether this was due to a differential contribution of the immune and nonimmune compartments to the total Flt3-L protein in those organs. Limiting Flt3-L production to the immune (wt→flt3<sup/li>−/−</sup>) or nonimmune (flt3<sup/li>−/−</sup>→wt) compartments resulted in intermediate levels of Flt3-L protein in the spleen compared with control chimeras (wt→wt; Fig. 5A). No differences were found between wt→flt3<sup/li>−/−</sup> and flt3<sup/li>−/−</sup>→wt chimeras, indicating that both sources equally contributed to the total Flt3-L amount in the spleen. This finding was in clear contrast with the requirements for DC generation because the hematopoietic source of Flt3-L was sufficient and necessary, but the nonhematopoietic source was not (Fig. 3B, 3C). In BM, however, most of the Flt3-L was derived from immune cells, with nonhematopoietic cells providing only ∼5% of the amount (Fig. 5B). This finding again did not correlate with cDC generation in BM, because both sources were sufficient and redundant to obtain a normal cDC compartment (Fig. 3E). Finally, most of the Flt3-L in serum and nonlymphoid organs originated from nonhematopoietic cells (Fig. 5C–G), the latter reflecting the requirement of this source for normal CD103<sup>+</sup> DC homeostasis in the lung, kidney, and pancreas.

These results show that the contribution of the hematopoietic and nonhematopoietic compartments to total organ amount of Flt3-L greatly differs in spleen, BM, and nonlymphoid organs. Unexpectedly, Flt3-L content in the spleen and BM did not correlate with the number of splenic cDCs and pDCs, and BM cDCs, respectively. Thus the source rather than the amount of Flt3-L in the spleen and BM was dominant for generating normal numbers of DCs in those organs, strongly suggesting that DC precursors come into close interaction with certain Flt3-L–producing cells during their differentiation.

The number of pre-cDCs in spleen is independent of the source of Flt3-L

Generation and differentiation of early myeloid progenitors in BM is promoted by Flt3/Flt3-L expression (27, 46). Therefore, we in-
vestigated whether the number of downstream DC precursors in the spleen was modulated depending on the source of Flt3-L. Although the number of splenic pre-cDCs in mice lacking Flt3-L was halved, we found a normal pre-cDC compartment independently of whether Flt3-L was of hematopoietic or nonhematopoietic origin (Fig. 6A). These data indicate that a hematopoietic source of Flt3-L, which is required for normal cDC generation in the spleen, is not critical for maintenance of splenic pre-cDCs but promotes their final differentiation.

Pre-cDCs are initially generated in the BM from MDPs and CDPs (21). Like in the spleen, the number of pre-cDCs in BM did not depend on the source of Flt3-L (Fig. 6A). Similar findings were obtained for MDPs and CDPs in BM (Fig. 6B, 6C). These findings are in agreement with a redundancy in the source of Flt3-L for normal BM cDC generation (Fig. 3E) and underline the fact that although nonhematopoietic cells produce only a minor fraction of Flt3-L present in the BM, they are able to support normal numbers of DC precursors and their progeny in that organ.

**FIGURE 4.** Influence of the cellular source of Flt3-L on DC generation in non-lymphoid organs. Flt3-L produced by nonhematopoietic cells is required for DC generation in the lung. A, Representative phenotypic analysis of lung CD11c^hi^MHC-II^+^ DCs in BM chimera mice. Numbers indicate the percentage of events in the respective gate. B, The numbers of total CD11c^hi^MHC-II^+^ DCs per one lung in BM chimera mice was calculated based on their percentages among CD45^+^ cells (as shown in A, left panel). C, Numbers of DC subpopulations in the lung in BM chimera mice (gated as shown in A, right panel). D–F, Similar to A–C for the kidney. G–I, for pancreas. J–L, For the liver. Data are expressed as mean value ± SEM (n = 3–6). Shown is one representative of three experiments. n.s., nonstatistically significant. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA).
whether there was a specific leukocyte population required to provide Flt3-L for DC development in that organ. To investigate this question, we performed cell depletion and DC repopulation experiments.

NK and NKT cells produced the highest Flt3-L amount on a per-cell basis in the spleen (Fig. 2A, 2B). However, deficiency of NK cells in IL15−/− mice (47) did not result in a reduced pool size of cDCs in the spleen in the steady state (Supplemental Fig. 4A). In addition, we followed an independent approach by depleting NK and NKT cells with anti-NK.1.1 Ab in CD11c.DOG mice whose DC compartment was being repopulated following a single dose of DC-depleting DT (Fig. 7A, left panel). Because of de novo DC generation in the spleen, the splenic DC compartment 4–5 d after DT administration in CD11c.DOG mice is mostly repopulated, comprising ∼75% of the normal number of DCs (Supplemental Fig. 4B) (16). Therefore, we hypothesized that if NK or NKT cells are essential at providing Flt3-L for DC development, the number of cDCs at day 5 after DT treatment would be reduced in CD11c.DOG mice treated with anti-NK.1.1 Ab compared with CD11c.DOG mice not treated with anti-NK.1.1 Ab. Injection of Ab against NK.1 resulted in more than 95% depletion of NK and NKT cells in the spleen (Supplemental Fig. 4C). As expected, the cDC pool in CD11c.DOG mice was in expansion 5 d after DT administration, reaching ∼75% of that in B6 mice (Fig. 7A, right panel, and Supplemental Fig. 4D). Similar results were observed in NK cell-depleted CD11c.DOG mice (Fig. 7A, right panel, and Supplemental Fig. 4D). Comparable findings were obtained for splenic pDCs (data not shown).

We next followed a similar approach to elucidate the role of monocytes and granulocytes for splenic cDC development (Fig. 7B, left panel). In this case, we used anti–Gr-1 Ab to deplete granulocytes (Gr-1hu) and monocytes (Gr-1−; Supplemental Fig. 4E). Again, we did not observe a role of these cells for cDC (Fig. 7B, right panel, and Supplemental Fig. 4F) or pDC (data not shown) differentiation in spleen.

We then investigated whether Flt3-L production by T and B lymphocytes was necessary to support splenic DC generation. T and B cell-deficient rag1−/− mice contain significantly fewer cDCs in the spleen than do wt mice (Fig. 7C, 7D). However, comparing splenic cDC number in B6 and rag1−/− mice lacking lymphocytes may be misleading, because the spleen anatomy and cellular composition in rag1−/− mice are completely altered, resulting in a drastically reduced size. We thus compared rag1−/− and flt3l−/−/rag1−/− mice, which have similar spleen cellularity (Supplemental Fig. 4G) and can thus be directly compared for cDC numbers. flt3l−/− mice contain more than 5-fold fewer splenic cDCs than B6 wt mice, whereas splenic cDC numbers are reduced only by half in flt3l−/−/rag1−/− mice compared with rag1−/− mice (Fig. 7D). The number of cDCs in flt3l−/− reflects the general role of Flt3-L expression on cDC generation in comparison with B6 mice, whereas the number of cDCs in flt3l−/−/rag1−/− mice shows the role of Flt3-L production by cells other than T and B lymphocytes in comparison with rag1−/− mice. Thus, expression of Flt3-L in mice lacking T and B cells leads to only a minimal increase in splenic cDCs, suggesting that lymphocyte-expressed Flt3-L contributes to DC generation. We attempted to discriminate between the role of B and T cells in normalizing DC numbers by transferring the corresponding cells into rag1−/− mice. Although we observed avid lymphopenia-induced proliferation, there was only a residual reconstitution of the T and B cell compartments (∼1–2 × 10^6 of each cell type per spleen from day 7 after transfer); consequently, we did not observe a significant increase in the number of splenic cDCs (data not shown). In addition, we quantified DC numbers in the spleen of B cell-deficient jht−/− mice or T cell-deficient lck−/− mice. However, both jht−/− and lck−/− mice showed a strongly disturbed spleen homeostasis with a generalized alteration in the number of specific immune cells, thus making a conclusion on DC homeostasis not possible (data not shown).

These experiments show that Flt3-L production by NK, NKT, granulocytes, and monocytes is redundant and not critical for DC
homeostasis in the spleen, and they suggest that Flt3-L production by T or B cells influences cDC generation in the spleen.

Discussion

BM-derived DC precursors constitutively seed peripheral organs, where they finally differentiate into DCs (2–4, 21, 44). Generation of DCs in lymphoid organs and CD103 + DCs in nonlymphoid organs depends on Flt3-L for differentiation of immediate pre-cDC precursors expressing the Flt3-L receptor Flt3 (10, 16, 20, 29). In this study, we investigated the source of Flt3-L that is required for generating a normal DC pool focusing on the spleen, BM, lung, pancreas, kidney, and liver. Our results show that Flt3-L expression is ubiquitous regarding organs and cell types but, interestingly, specific cellular sources are required for DC development in different organs. In accordance with this, we find that the cell type producing Flt3-L and not the total organ amount governs the extent of DC generation in the lymphoid organs we investigated.

Using FL-GL mice, a novel Flt3-L reporter strain expressing eGFP and luciferase under the endogenous flt3l promoter, we show that Flt3-L is constitutively expressed in all organs (Fig. 1B, 1C). This finding is in agreement with previous work investigating flt3l mRNA expression (34, 48). Ubiquitous promoter activity resulted in Flt3-L protein expression in all organs in a hierarchical manner. Lungs contained the highest Flt3-L amount, whereas lymphoid organs with extensive DC generation such as the spleen and BM expressed rather low amounts (Fig. 1D, 1E, 1G). In terms of DC homeostasis, we believe that ubiquitous Flt3-L expression outside the BM is necessary to maintain constitutive DC generation in different organs because Flt3 + pre-cDC precursors, which seed peripheral organs, require Flt3-L for their final differentiation (10, 16, 29).

Although comprehensive studies on primary cells are lacking, T cells, fibroblasts and several stromal cell lines are believed to be major producers of Flt3-L on a per-cell basis (34, 35, 38, 39, 48). Contrary to this, we found that all cells investigated in the BM, thymus, spleen, and lung produced Flt3-L, but NK cells were the highest producers, whereas T cells, stromal cells, and other non-immune cells expressed intermediate levels comparable to other immune cells (Fig. 2 and data not shown). It is uncommon that hematopoietic factors are produced by most cells throughout the body. For example IL-7, a cytokine driving T cell differentiation, is mostly produced by BM stromal cells and the thymus (49, 50), whereas T cell progenitors differentiate. Flt3-L–dependent DC development is, however, not confined to a specific organ but proceeds in most locations of the body, which is in agreement with ubiquitous Flt3-L expression.

Because the cellular composition varies considerably in different organs, DC precursors may obtain the necessary Flt3-L from different sources.
specific cell populations in the various organs. Thus, it may be advantageous that most cell types express Flt3-L, with some cells being more important for DC development than others depending on the organ in question. We investigated this question by experimentally restricting Flt3-L production to either the hematopoietic or nonhematopoietic compartments using BM chimera mice. Indeed, although all cells expressed Flt3-L in the lung, the nonhematopoietic compartment was required to generate a normal CD103+ DC compartment (Fig. 4C). Similar results were obtained for other lymphoid organs. This result was in accordance with a major contribution of nonimmune cells to total Flt3-L protein in nonlymphoid organs (Fig. 5). In contrast, normal splenic cDC and pDC development required Flt3-L production by immune cells (Fig. 3B, 3C), although both immune and nonimmune cells equally contributed to total splenic Flt3-L (Fig. 5A). The opposite was observed for cDC development in BM: although both sources were sufficient to establish the cDC compartment (Fig. 3E), almost all Flt3-L was derived from hematopoietic cells (Fig. 5B). Thus, the cell type producing Flt3-L rather than the total organ content dictated splenic DC development. The differences observed in lymphoid and nonlymphoid organs regarding the Flt3-L source for DC development may be a result of the different progenitor cells involved and the cellular milieu with which they interact. cDCs and CD103+ DCs derive from pre-DCs, whereas CD103–CD11b+ DCs in nonlymphoid organs are thought to originate from Ly6C+ monocytes (20, 22, 23). In addition, it is highly likely that these precursors interact with different cell types in the various organs.

The size of the pre-cDC compartment in the spleen was independent of the origin of Flt3-L (Fig. 6A), strongly suggesting that the Flt3-L produced by hematopoietic cells in the spleen is not required for recruitment of circulating pre-cDCs but rather for their final differentiation. This is in agreement with previous reports showing that Flt3 receptor is critical for pre-cDC differentiation (29). Our findings are strongly suggestive of DC development in the spleen being promoted either within confined cellular niches or by dynamic interactions with specific cell populations. Recent data using two-photon imaging showed that pre-DCs are highly motile during their conversion into DCs (21), arguing for dynamic interactions during DC differentiation. Further investigation is needed to clarify this issue.

Although Flt3-L production by immune cells was required to establish a normal cDC compartment in the spleen (Fig. 3B), we hypothesized that production by certain immune cell types was redundant because of the ubiquitous Flt3-L expression (Fig. 2B). Indeed, absence or depletion of NK cells, NKT cells, granulocytes, or monocytes did not interfere with the number of splenic cDCs or with reconstitution of the DC compartment following punctual elimination of DCs in CD11c.DOG mice (Fig. 7A, 7B, and Supplemental Fig. 4). However, we found that T and B cells contributed to splenic cDC homeostasis. We obtained this evidence by comparing the effect of Flt3-L expression in mice containing or not containing T and B cells. The spleen of flt3l−/− mice contained more than 5-fold fewer cDCs than its wild type counterpart, whereas lymphocyte-deficient rag1−/−/flt3l−/− spleens contained half the number of cDCs than lymphocyte-deficient spleens with otherwise normal Flt3-L expression (rag1−/−/flt3l−/−; Fig. 7C, 7D). These findings demonstrate that there exists a high degree of redundancy within innate cell-derived Flt3-L for DC generation, and they suggest that T and B cells specifically contribute to DC homeostasis in spleen.

DC development is unique, being a continuum initiated in the BM and finalized in peripheral organs where DC precursors terminally differentiate into subpopulations such as pDCs, cDCs, and CD103+ DCs in a Flt3-L–dependent fashion. In this study, we

**FIGURE 7.** Contribution of different immune cell populations to cDC homeostasis in spleen. A, The left panel shows the experimental design for anti-NK1.1-mediated NKT cell depletion and quantification of cDCs in the spleen of CD11c.DOG mice. NKT cell depletion was maintained by multiple injections of anti–NK1.1-depleting Ab starting at day −4. DCs were depleted by a single DT application at day 0. The resulting NKT cell depletion is shown in Supplemental Fig. 4C. The right panel shows quantification of the number of splenic cDCs in different experimental groups as indicated. B, The left panel shows the experimental plan for anti–Gr-1-mediated depletion of monocytes and granulocytes and quantification of cDCs in the spleen of CD11c.DOG mice. Multiple anti–Gr-1 injections were given from day −1 in CD11c.DOG mice and DCs were depleted by a single DT application at day 0. Gr-1+ cell depletion is shown in Supplemental Fig. 4E. The right panel shows quantification of the number of splenic cDCs in different experimental groups as indicated. C, representative dot plots showing the percentage of cDCs in B6, flt3l−/−, rag1−/−, and flt3l−/−/rag1−/− mice. D, The number of cDCs in the spleen of the indicated mice based on their percentages as shown in C. For all experiments, similar results were obtained in three independent experiments. Data are expressed as mean value ± SEM (n = 3–6). n.s., nonstatistically significant. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA).
provide a basis for understanding how DC generation occurs in such a diverse range of organs throughout the body. Although Flt3-L is widely expressed by immune and nonimmune cells, a high degree of flexibility in the cellular source is required for DC generation depending on the organ in question. This study highlights the adaptation of DC development to specific cellular environments encountered in different organs. Future investigations on the regulation of Flt3-L expression and DC precursors during inflammation will help to understand DC homeostasis in pathologies such as cancer and autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

References

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Supplemental Figure Legends

SUPPLEMENTAL FIGURE 1. Flt3-L-eGFP expression on stromal cells and T cell subpopulations. A, Representative flow cytometric analysis showing the successive gating strategy to identify non-immune, stromal cells in spleen and BM as well as quantification of flt3l-eGFP expression on the indicated stromal populations. Lin: Ter119, Gr-1, CD19, NK1.1. Endothelial cells are identified as Lin’CD3’CD45.2’CD35’CD31+. PI, propidium iodide. B, Top panels, representative flow cytometry dot plots showing the gating strategy to identify stromal and parenchymal cells in the lung (top panels). Bottom panels, histogram overlays showing the flt3l-eGFP expression in the indicated cell non-immune populations. Lin: Ter119, Gr-1, CD19, CD3, NK1.1. Epithelial cells are Lin’CD45.2’CD31’Ep-CAM+. Endothelial cells are Lin’CD45.2’Ep-CAM’CD31+. Alveolar macrophages and other highly autofluorescence cells have been excluded using a blank fluorescence channel. C, Representative flow cytometric analysis showing flt3l-eGFP expression by different splenic T cell populations. Live T cells were identified as PI’CD3’NK1.1’. Full histogram, B6; black and dark grey lines, indicated T cell populations from FL-GL mice.
SUPPLEMENTAL FIGURE 2. Gating strategy to identify basophils (A, B) and mast cells (C) by flow cytometry. Right panels show the intensity of Flt3L-eGFP in the indicated cell populations. A representative of three experiments is shown.

SUPPLEMENTAL FIGURE 3. A, Leukocyte numbers in different organs of the indicated BM chimeras. Leukocytes were identified by flow cytometry by means of CD45 expression. Results were obtained 11 weeks after reconstitution and are expressed as mean ± S.E.M (n=3 mice). Similar results were obtained in three independent experiments. B, Number of splenic CD11c^{hi}MHC-II^{+} cDCs in different BM chimeras as indicated. Lethally-irradiated rag1^{-/-} mice were used as BM recipients. Shown is a representative of two independent experiments (n=4). Data is expressed as mean ±S.E.M. C, Representative flow cytometric analysis of splenic cDCs in the indicated BMx. Numbers indicate the percentage of cells. *, P<0.05; **, P<0.01; ***, P<0.001; n.s., non-statistically significant (ANOVA).

SUPPLEMENTAL FIGURE 4. Depletion of different immune cell types in the spleen and its impact on cDC numbers. A, Numbers of CD11c^{hi}MHC-II^{+} cDCs in the spleen of B6 and IL-15^{−/−} mice that lack NK cells. B, Number of splenic CD11c^{hi}MHC-II^{+} cDCs in CD11c.DOG mice after a single DT administration. cDCs fully recover at day 6 after DT. C, Representative FACS dot plots of spleen cells in mice treated or not with αNK1.1 and summary of data showing depletion of NK (CD3^{−}NK1.1^{+}) and NKT (CD3^{+}NK1.1^{−}) cells in the spleen of the indicated mice (right panel). Numbers on the dot plots indicate the percentage of the respective
population. The experimental protocol for administration of DT and \( \alpha \)NK1.1 is shown in Figure 7A. D, Representative dot plots showing the percentage of CD11c\(^{hi}\)MHC-II\(^{+}\) cDCs in the spleen of the indicated mice. The experimental protocol for administration of DT and \( \alpha \)NK1.1 is shown in Figure 7A. E, Representative FACS dot plots of spleen cells in mice treated or not with \( \alpha \)Gr-1 and summary of data showing depletion of monocytes (SSC\(^{lo}\)CD11b\(^{+}\)Gr-1\(^{+}\)) and granulocytes (SSC\(^{hi}\)CD11b\(^{+}\)Gr-1\(^{++}\)) cells in the spleen of the indicated mice (right panel). Numbers on the dot plots indicate the percentage of the respective population. The experimental protocol for administration of DT and \( \alpha \)Gr-1.1 is shown in Figure 7B. Depletion of Gr-1\(^{+/+}\) cells was confirmed by lack of anti-ratIgG 2b staining on CD11b\(^{+}\) splenocytes (data not shown). G, Leukocyte numbers in the spleen of the indicated mice. Similar results were obtained in three independent experiments. Data are expressed as mean value + SEM (n=3-8). n.s.: non-statistically significant; *, P<0.05; (ANOVA).
SUPPLEMENTAL FIGURE 2  
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A  Basophils - Spleen

B  Basophils - Bone marrow

C  Mast cells- Peritoneal lavage
SUPPLEMENTAL FIGURE 3  Miloud et al

A

Spleen

Bone marrow

Lung

Kidney

Pancreas

Liver

B

Dendritic cells / spleen

Gated on:

Live splenocytes

CD11c\(^{hi}\)MHC-II\(^{+}\)

CD8

CD11b

C

MHC-II

**

n.s.

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