Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon–producing and dendritic cell development

Nobuyuki Onai, Aya Obata-Onai, Roxane Tussiwand, Antonio Lanzavecchia, and Markus G. Manz

Institute for Research in Biomedicine, CH-6500 Bellinzona, Switzerland

Flt3 ligand (Flt3L) is a nonredundant cytokine in type I interferon–producing cell (IPC) and dendritic cell (DC) development, and IPC and DC differentiation potential is confined to Flt3+ hematopoietic progenitor cells. Here, we show that overexpression of human Flt3 in Flt3− (Flt3−Lin−IL-7Rα−Thy1.1−c-Kit+) and Flt3+ (Flt3+Lin−IL-7Rα−Thy1.1−c-Kit+) hematopoietic progenitors rescues and enhances their IPC and DC differentiation potential, respectively. In defined hematopoietic cell populations, such as Flt3− megakaryocyte/erythrocyte-restricted progenitors (MEPs), enforced Flt3 signaling induces transcription of IPC, DC, and granulocyte/macrophage (GM) development–affiliated genes, including STAT3, PU.1, and G-/M-/GM-CSFR, and activates differentiation capacities to these lineages. Moreover, ectopic expression of Flt3 downstream transcription factors STAT3 or PU.1 in Flt3− MEPs evokes Flt3 receptor expression and instructs differentiation into IPCs, DCs, and myelomonocytic cells, whereas GATA-1 expression and consecutive megakaryocyte/erythrocyte development is suppressed. Based on these data, we propose a demand-regulated, cytokine-driven DC and IPC regeneration model, in which high Flt3L levels initiate a self-sustaining, Flt3-STAT3– and Flt3-PU.1–mediated IPC and DC differentiation program in Flt3+ hematopoietic progenitor cells.

Differentiation of hematopoietic stem cells (HSCs) to mature hematopoietic cells is characterized by progressive loss of developmental options and restriction to one lineage (1). In both mice and men, HSCs as well as multiple developmental intermediates with limited cellular expansion and restriction to specific mature cell types have been identified. These include myeloid progenitors, as clonal common myeloid progenitors (CMPs) that give rise to either granulocyte/macrophage progenitors (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs; references 2 and 3), and clonal common lymphoid progenitors (CLPs; references 4 and 5), which robustly produce the respective mature cell types. Hematopoietic differentiation is regarded as a multi-linear, unidirectional process, and regeneration and expansion of specific lineages are largely regulated extrinsically by different hematopoietic cytokines. However, it is unclear whether under physiologic conditions cytokines are capable to instruct HSCs and subsequent progenitors to differentiate to lineage-restricted progenitors (extrinsic lineage determination). Alternatively, HSCs and subsequent progenitors commit to lineage-restricted progenitors by intrinsic differentiation programs (intrinsic lineage determination), and restricted progenitors are consecutively stimulated by hematopoietic cytokines produced upon demand (6, 7).

On the molecular level, access to lineage developmental options and readiness to receive lineage-permissive and -instructive signals might be determined by graded, relative expression levels of diverse transcription factors and cytokine receptors (8, 9). Indeed, it has been demonstrated that genetic deletion or overexpression of different single transcription factors is sufficient to reprogram committed progenitors or mature cells to alternative hematopoietic lineages.
(10): Pax5-deficient pre-B cells lose B cell differentiation potential and mature into T and myelomonocytic cells, but re-expression of Pax5 restores B cell commitment (11, 12); ectopic expression of GATA-1 instructs HSCs and CMPs and converts CLPs and GMPs to the megakaryocyte/erythrocyte lineage, respectively (13); and enforced expression of C/EBPα and C/EBPβ in B cells leads to macrophage differentiation (14). Furthermore, it has been shown that artificial expression of GM-CSF receptor and stimulation with the cognate ligand redirect CLPs and early T cell progenitors to myeloid lineage outcomes (15–17). This proves that hematopoietic lineage instruction can be mediated extrinsically by cytokines, at least in these experimental settings.

DCs are important regulators of innate and adaptive immune responses and are involved in initiation of immunity as well as in maintenance of self-tolerance (18–20). DCs are cells of the hematopoietic system and are continuously replenished from hematopoietic stem and progenitor cells (1). In mice, multiple DC subsets that differ in maturation state, phenotype, location, and in some functions were identified (21). Here, for simplicity, these will grossly be divided into CD11c+ “B220−” natural type I interferon–producing cells (IPC; also called plasmacytoid cells or plasmacytoid pre-DCs) and CD11c+ “B220−” “conventional” DCs, consisting of CD11c+CD8α+CD4+CD11b+, CD11c+CD8α−CD4+CD11b+, and CD11c+CD8α+CD4−CD11b+ subpopulations (21). Although it was suggested that IPCs as well as conventional CD11c+CD8α+ DCs are derived from lymphoid-committed progenitors (21), it was demonstrated recently that any of the IPCs and conventional DCs can be generated via lymphoid and myeloid progenitors (22–27). Specifically, all IPCs and conventional DCs are generated by mouse CMPs, GMPs, CLPs, and pro–T1 cells, whereas IPC and DC differentiation potential is lost once definitive MEP or B cell commitment occurs (22–27). Thus, in contrast to other hematopoietic lineages, IPC and DC potentials are conserved along lymphoid and myeloid developmental pathways.

Flt3 is a receptor tyrosine kinase with homology to c-Kit (the receptor for stem cell factor) and c-fms (the receptor for M-CSF; reference 28) that has a nonredundant role in steady-state differentiation of IPCs and DCs in vivo: Flt3 ligand (Flt3L)–deficient mice and mice with hematopoietic system–confined deletions of STAT3, a transcription factor activated in the Flt3 signaling cascade, as well as mice that are treated with flt3 tyrosine kinase inhibitors, show massively reduced IPCs and DCs (29–31). Conversely, injection or conditional expression of Flt3L in mice increases IPCs and DCs, with up to 30% of mouse spleen cells expressing CD11c (32–34). Furthermore, Flt3L as a single cytokine is capable of inducing differentiation of IPCs and DCs in mouse bone marrow cell cultures (35).

Flt3 is expressed in mouse short-term HSCs and multipotent progenitors (36, 37) in most CLPs and CMPs, at lower levels on fractions of GMPS and pro–T1 cells, as well as on mature steady-state IPCs and DCs. It is down-regulated on pro–B cells, further downstream T cell progenitors, and absent on MEPs (33, 38). To determine what might define IPC and DC developmental potential in lymphoid- and myeloid-committed cells, we and others showed that in vitro and in vivo IPC, DC, and Langerhans cell differentiation potential is confined to Flt3-expressing hematopoietic progenitors (33, 38, 39). Furthermore, we demonstrated that injection of Flt3L expands Flt3+ but not downstream Flt3− progenitors and drives IPC and DC development along both lymphoid and myeloid differentiation pathways (33).

Based on these data, we postulate that high environmental Flt3L levels and consecutive Flt3 signaling might be both the earliest event and a continuous regulator that determines IPC and DC developmental outcomes in bone marrow hematopoietic progenitor cells. Thus, we were interested to test whether enforced Flt3 expression and signaling would be sufficient to instruct IPC and DC development from Flt3− progenitor cells and enhance IPC and DC development from Flt3+ progenitor cells, respectively.

RESULTS

Enforced expression of human Flt3 in Flt3− and Flt3+ progenitors rescues and enhances IPC and DC developmental potential, respectively

We used a bicistronic retroviral transduction system to transduce human Flt3 into progenitor cells. The constructs carrying either GFP or huFlt3-GFP are shown in Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051645/DC1. Lin−IL-7Rα−Thy1.1−c-Kit+ bone marrow cells that contain a heterogeneous fraction of progenitors, including multipotent progenitors and myeloid-committed cells, and are devoid of Thy1.1+ HSCs, IL-7Rα+ lymphoid–committed cells, and mature lineage cells, were sorted into Flt3− (Flt3− progenitors also containing Flt3− MEPs) and Flt3+ (Flt3+ progenitors containing multipotent progenitors and Flt3+ CMPs) cell fractions (Fig. 1 A; references 33, 36–38) and consecutively retrovirally transfected as described in Materials and methods. Fig. S1 B shows typical 18-h coculture transduction efficiencies (18–26%) in Flt3− and Flt3+ progenitors as determined by GFP expression.

To study the effects of enforced huFlt3 expression on IPC and DC development, Flt3− and Flt3+ progenitors were transduced with control–GFP or huFlt3–GFP and cultured in human Flt3L-Ig fusion protein (huFlt3L-Ig)− and stem cell factor (SCF)–supplemented media. Cultures were analyzed for cell numbers and the presence of IPCs and DCs at days 4, 8, and 12. Freshly isolated Flt3− progenitors did not express CD11c or MHC class II (not depicted). As expected, unmanipulated Flt3− progenitors (not depicted) as well as GFP-transduced Flt3− progenitors gave rise to no or very few CD11c+ MHC class II+ cells (Fig. 1 B, top). In contrast, huFlt3–GFP–transduced Flt3− progenitors differentiated into CD11c+ MHC class II+ cells that increased in relative numbers from day 4 to 8 of culture (Fig. 1 B, bottom). Similarly, GFP-transduced as well as huFlt3-transduced Flt3+ progenitor cells gave
rise to CD11c+ MHC class II+ and CD11c+B220+ cells, with huFlt3-transduced Flt3+ progenitor cells producing slightly higher relative numbers of both cell types (Fig. 1 C). To quantify absolute CD11c+ cell production, cell numbers were determined at days 4, 8, and 12 of culture. Numbers peaked at day 8 of culture, with huFlt3-transduced Flt3+ progenitors producing slightly higher relative numbers of both cell types (Fig. 1 C). To quantify absolute CD11c+ cell production, cell numbers were determined at days 4, 8, and 12 of culture. Numbers peaked at day 8 of culture, with huFlt3-transduced Flt3+ progenitors producing slightly higher relative numbers of both cell types (Fig. 1 C). To quantify absolute CD11c+ cell production, cell numbers were determined at days 4, 8, and 12 of culture. Numbers peaked at day 8 of culture, with huFlt3-transduced Flt3+ progenitors producing slightly higher relative numbers of both cell types (Fig. 1 C). 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producing the highest cell numbers (~14-fold expansion of input cells), followed by intermediate expansion (6–9-fold) of both GFP-transduced Flt3+ and huFlt3-transduced Flt3+ progenitors, and low expansion (3–4-fold) of GFP-transduced Flt3+ progenitors (Fig. 1 D). The total cellular expansion was paralleled by a peak expansion of CD11c+ MHC class II+ and CD11c+B220+ cells at day 8 of culture (Fig. 1, B–E). Interestingly, huFlt3-transduced Flt3+ progenitors produced significantly higher total numbers of both CD11c+ MHC class II+ and CD11c+B220+ cells compared with GFP-transduced Flt3+ progenitors (Fig. 1 E), with somewhat higher relative CD11c+B220+ cell numbers (Fig. 1, C and E).

Next, we evaluated IPC- and DC-associated surface antigen expression and function of CD11c+B220+ and CD11c+B220+ cells derived from different progenitor cell populations at day 8 of culture. Consistent with typical mouse IPC and DC phenotypes, CD11c+B220+ cells expressed Gr-1, Ly6C, and CD45RA, whereas CD11c+B220− cells expressed CD11b and intermediate levels of CD80 and CD86, respectively (Fig. 2 A). Furthermore, CD11c+B220+ cells produced substantial amounts of IFN-α upon stimulation with either influenza virus or CpG, whereas CD11c+B220− cells did not (Fig. 2 B). CD11c+B220− cells, but not CD11c+B220+ cells, displayed typical DC morphology (Fig. 2 C) and were efficient stimulators in allogeneic MLR cultures, as evaluated by thymidine incorporation (Fig. 2 D). Therefore, CD11c+B220+ cells phenotypically and functionally were typical IPCs, whereas CD11c+B220− cells were typical conventional DCs. Collectively, these results indicate that huFlt3 signaling rescues and enhances the development of functional IPCs and DCs from Flt3− and Flt3+ hematopoietic progenitor cell populations, respectively. In addition, it suggests that strong Flt3 signaling slightly skews development toward an IPC phenotype.

Enforced expression of huFlt3 is sufficient to rescue IPC and DC development from MEPs and enhances IPC and DC development from GMPs

In normal mouse and human hematopoiesis, IPC and DC developmental potentials are maintained from Flt3+ CMPs to downstream Flt3+ GMPs, but are lost in Flt3− MEPs (23–27, 33, 38). Thus, we tested whether enforced huFlt3 expression in MEPs would be sufficient to rescue IPC and DC development. As comparator cell population, we used huFlt3-transduced GMPs and GFP-transduced CMPs (GFP−CMPs). As reported previously for unmanipulated progenitors, GFP-transduced MEPs (GFP−MEPs) gave rise to no or very few CD11c+ cells (Fig. 3 A, top). In contrast, huFlt3+MEPs gave rise to CD11c+B220+ cells and CD11c+B220− cells at day 8 in huFlt3L-Ig− and SCF-supplemented cultures, at least as efficient as GFP+GMPs (Fig. 3, A–C). Similarly as from huFlt3-transduced Flt3+ progenitor cells, CD11c+B220+ and CD11c+B220− cell development was significantly enhanced from huFlt3-transduced GMPs (Fig. 3, B and C). Upon influenza virus or CpG stimulation, huFlt3+MEP− and huFlt3+GMP− derived CD11c+B220+ cells produced IFN-α (Fig. 3 D), suggesting that these cells are functional.

As reported previously for untransduced CMPs (22–27, 38), GFP−CMPs generated both IPCs and DCs. The efficacy of GFP−CMPs to produce these offspring cells was about threefold higher than that observed from huFlt3+GMPs (Fig. 3 C). Thus, enforced huFlt3 expression is sufficient to rescue IPC and DC developmental potential in MEPs to levels comparable to their developmental counterparts, GFP−GMPs. Furthermore, huFlt3 expression enhances IPC and DC development from GMPs, but not to levels observed in the upstream CMP population.

Enforced expression of huFlt3 permits myelomonocytic development from huFlt3+MEPs but not megakaryocyte/erythrocyte development from huFlt3+GMPs

Because huFlt3 signaling in MEPs activated IPC and DC development, a differentiation option normally confined to Flt3+ progenitors as CMPs and GMPs, we were interested to test whether huFlt3 signaling in MEPs would also reestablish myeloid CFU activity. GFP−transduced CMPs, GMPs, and MEPs gave rise to their respective colony types, but with somewhat lower plating efficacy as compared with freshly isolated CMPs, GMPs, and MEPs (Fig. 4 A; reference 2). huFlt3+MEPs gave rise to not only erythroid-affiliated colonies but also granulocyte/macrophage (GM)-affiliated colonies, including CFU-G, CFU-M, and CFU-GM (Fig. 4 A). Compared with CMPs and GMPs, the myelomonocytic colony-forming efficiency of huFlt3+MEPs was lower; however, the diversity of colony formation resembled that of CMPs with the exception that no CFU-Mix colonies developed. No substantial difference in CFU activity was observed in huFlt3+GMPs compared with GFP−GMPs (Fig. 4 A). Thus, huFlt3 signaling in MEPs reestablishes myelomonocytic CFU activity, whereas huFlt3 signaling in GMPs does not affect their overall CFU activity and particularly does not reestablish megakaryocyte/erythrocyte readout.

Enforced expression of huFlt3 in MEPs is sufficient to rescue IPC, DC, and myelomonocytic cell development in vivo

To test the robustness of Flt3 transduction–mediated effects observed in vitro, we compared in vivo reconstitution activity of GFP−MEPs, huFlt3+MEPs, and GFP−CMPs. 2 × 104 cells of each progenitor population combined with 2 × 105 cells of host bone marrow cells were transplanted into lethally irradiated mice, and spleen progeny cells were analyzed on day 7. As reported previously for MEPs, GFP−MEPs produced ~0.7% of nucleated GFP+ spleen cells that consisted mostly of Ter119+ erythroid cells, but no DCs, IPCs, or Gr-1− myeloid cells (Fig. 4 B, top; references 2, 23, and 25). In contrast, huFlt3+MEPs gave rise to ~2.7% of nucleated GFP+ spleen cells containing CD11c+ MHC class II+ conventional DCs, low numbers of CD11c+B220+ IPCs, as well as Ter119+ erythroid and Gr-1− myeloid cells (Fig. 4 B, middle). As expected, GFP−CMPs gave rise to ~6.0% of nucleated GFP+ spleen cells that contained DCs, IPCs, as well as...
erythroid and myeloid cells (Fig. 4 B, bottom; references 2, 23, and 25). This formally demonstrates that enforced expression of huFlt3 is sufficient to rescue in vivo IPC, DC, and myelomonocytic cell development from MEPs.

HuFlt3 signaling in MEPs induces activation of DC and myeloid development–associated genes

To evaluate the immediate consequences of huFlt3 signaling on gene transcription profiles that might be involved in the consecutive activation of IPC, DC, and myeloid development–associated genes, we analyzed the transcription of a panel of IPC, DC, and myeloid development–associated genes by RT-PCR in huFlt3+–MEPs. STAT3, an indispensable transcription factor for Flt3L-mediated DC development (30), was hardly detectable in GFP+–MEPs but was clearly upregulated in huFlt3+–MEPs. Furthermore, huFlt3+–MEPs but not GFP+–MEPs expressed myelomonocytic development–associated genes, such as the cytokine receptors for G-CSF, M-CSF, and GM-CSF, as well as the transcription factors C/EBPα and PU.1, similar to that found in GFP+–GMPs, huFlt3+–GMPs, and normal CMPs (Fig. 5 A; references 2 and 40). However, transcription factors RelB, ICSBP, and Id2 could not be detected in any of the retrovirus-transduced MEPs and GMPs, whereas they were detectable in unmanipulated CMPs (Fig. 5 A). Real-time RT-PCR revealed low-level STAT3 expression in GFP+–MEPs and similar high levels of STAT3 expression in huFlt3+–MEPs, GFP+–GMPs, huFlt3+–GMPs, and CMPs (Fig. 5 B). Furthermore, PU.1 expression levels in huFlt3+–MEPs increased to levels found in CMPs, whereas expression levels of this gene were somewhat higher in GM-committed GFP+–GMPs and huFlt3+–GMPs (Fig. 5 B).
Consistent with their maintained Meg/E-developmental potential, Meg/E-related genes, such as \(\text{EpoR}\), \(\beta\)-globin, GATA-1, and GATA-2, were still transcribed in huFlt3\(^+\)-MEPs, although the expression of NF-E2 decreased (Fig. 5 A). Interestingly, huFlt3\(^+\)-GMP showed some transcription of Meg/E development–associated genes as \(\beta\)-globin and GATA-1. However, GATA-2 and NF-E2 transcripts were not detectable, and huFlt3\(^+\)-GMPs were not able to give rise to Meg/E lineage colonies (Figs. 4 A and 5 A). Collectively, these results demonstrate on a molecular level that MEPs have a latent IPC, DC, and myelomonocytic lineage potential that is inducible by enforced Flt3 signaling.

**HuFlt3 signaling in MEPs leads to downstream STAT3 phosphorylation**

To test activation of STAT3 in huFlt3\(^+\)-MEPs, we performed intracellular phospho–STAT3 staining in retrovirus-transduced MEPs that were Flt3L deprived and then stimulated with huFlt3L-Ig. Indeed, phosphorylation of STAT3 was detected in huFlt3\(^+\)-MEPs but not in GFP\(^+\)-MEPs, indicating that as expected (30) STAT3 is a downstream activated transcription factor of enforced huFlt3 signaling (Fig. 6 A).

**Enforced STAT3 or PU.1 expression instructs MEPs to differentiate into IPCs, DCs, and GM lineage cells**

Given the importance of STAT3 for IPC and DC development, and the finding that STAT3 and PU.1 were up-regulated in huFlt3\(^+\)-MEPs, we tested whether STAT3 and PU.1 could directly activate IPC, DC, and myelomonocytic development from MEPs. Mouse STAT3 and PU.1 were transduced into MEPs using retrovirus expression vectors, respectively (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20051645/DC1). However, survival of cells was low when cultured in SCF alone or SCF and huFlt3L-Ig (not depicted). To possibly support the survival of MEPs, we first added thrombopoietin (TPO), followed by TPO and huFlt3L-Ig to SCF in consecutive cultures. STAT3\(^+\)-MEPs...
and PU.1<sup>+</sup>-MEPs differentiated into CD11c<sup>+</sup>B220<sup>+</sup> and CD11c<sup>+</sup>B220<sup>−</sup> cells at day 8 in both SCF as well as TPO (not depicted) and, with even higher efficacy in SCF, TPO, and huFlt3L-Ig, supplemented cultures (Fig. 6 B). Interestingly, enforced expression of STAT3 or PU.1 in MEPs in turn led to the up-regulation of mouse Flt3 mRNA levels (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20051645/DC1).

To test myeloid CFU activity of these cells, STAT3<sup>+</sup>-MEPs and PU.1<sup>+</sup>-MEPs were plated in methylcellulose assays. Both STAT3<sup>+</sup>-MEPs and PU.1<sup>+</sup>-MEPs gave rise to CFU-G, CFU-M, and CFU-GM colonies, but not to Meg/E-affiliated colonies (Fig. 6 C).

Finally, we evaluated the expression of GATA-1, a non-redundant transcription factor, for megakaryocyte/erythrocyte development by real-time RT-PCR. GATA-1 expression was down-regulated in STAT3<sup>+</sup>-MEPs and PU.1<sup>+</sup>-MEPs compared with that in GFP<sup>+</sup>-MEPs, huFlt3<sup>+</sup>-MEPs, and CMPs (Fig. 6 D). Thus, enforced expression of STAT3 and PU.1 in MEPs reprogrammed them to differentiate into IPCs, DCs, and myelomonocytic cell lineages and inhibited Meg/E lineage potentials, indicating that strong Flt3 downstream signals were capable of inducing complete lineage conversion.

**DISCUSSION**
A standing question in early hematopoiesis is whether cytokine signaling is sufficient to induce cell fate decisions. Here, we showed that enforced expression of huFlt3 in Flt3<sup>−</sup> progenitors rescued their potential to differentiate into functional IPCs and DCs with comparable in vitro differentiation efficiency as Flt3<sup>−</sup> progenitors (Figs. 1 and 2). We also demonstrated that enforced expression of huFlt3 in MEPs, which under normal conditions cannot give rise to IPCs and DCs (23–27) and are contained in Flt3<sup>−</sup> progenitor cells, induced
in vitro IPC and DC differentiation comparable to that observed from GFP+ GMPs (Fig. 3). Furthermore, huFlt3+ MEPs differentiated into IPCs and DCs upon in vivo transfer, the most informative assay available to prove the robustness of in vitro observations (Fig. 4 B). Thus, these data demonstrate that enforced expression and signaling of huFlt3 in Flt3− progenitors deliver an instructive signal to activate latent IPC and DC differentiation programs.

Overexpression of huFlt3 in total Flt3+ progenitors, in Flt3-expressing GMPs, and in CLPs (of which ~70% are Flt3+) led to a gain of higher relative and absolute (two- to threefold) numbers of IPCs and DCs in vitro (Figs. 1 D and E, and 3 C, and not depicted). Thus, beyond activation, increased Flt3 signaling also enhanced IPC and DC development. The gain in offspring cells was consistently higher for IPCs than for DCs (Figs. 1 E and 3 C), in line with our previous findings that after 10 d of in vivo Flt3L injection, spleen IPCs and DCs were expanded on average 28- and 21-fold, respectively (33). This suggests that a continuous strong Flt3 signal might induce a shift toward relatively higher IPC levels.

We previously found that Flt3 is expressed in lymphoid- and myeloid-committed progenitor cells, and in vivo Flt3L application mediates the expansion of both cell types without changing their biology (33). The enforced expression of huFlt3 in MEPs not only led to a gain of IPC and DC developmental capacity, but, with the exception of mixed colony formation, also to a gain of CFU activity of upstream myeloid progenitors as well as to differentiation of erythroid and myelomonocytic cells in vivo (Fig. 4 A). In contrast, huFlt3 signaling in GMPs did not activate megakaryocyte/erythrocyte potential (Fig. 4 A). This implies that beyond activation and enhancement of IPC and DC development,
Flt3 signaling is not immediately deterministic but primarily opens access to an IPC, DC, and myelomonocytic differentiation program. Thus, we propose that final IPC and DC lineage outcome might be a gradual process, depending on continuous strong Flt3 signaling.

What are the Flt3 signaling–initiated downstream molecular events? It was shown that hematopoietic system–confined deletion of STAT3 transcription factor leads to the inhibition of Flt3-driven IPC and DC development (30). Furthermore, human Flt3 transfection and stimulation with Flt3L in mouse myeloid 32Dcl3 cells leads to the induction of PU.1 and C/EBPα expression (41). These transcription factors are indispensable for granulocyte and monocyte development (42), and it was shown that PU.1 cooperatively with C/EBPα activates myeloid development–associated cytokine receptor genes, including G-CSFR, M-CSFR, and GM-CSFR (42). Interestingly, PU.1-deficient mice, in addition to other hematopoietic defects, lack either CD8α− or both CD8α− and CD8α+ DCs, depending on the type of PU.1 deletion (43, 44).

Here, we showed that enforced huFlt3 signaling in MEPs results in enhanced expression of IPC, DC, and GM lineage development related transcription factors STAT3, PU.1, and C/EBPα, as well as expression of G-, M-, and GM-CSF cytokine receptors (Fig. 5). Thus, at least in terms of these RNA transcripts, huFlt3+–MEPs but not GFP+–MEPs resembled the gene expression profiles of CMPs (2, 40).

Importantly, enforced expression of STAT3 or PU.1 in Flt3− MEPs was again sufficient to permit the development of both IPCs and DCs (Fig. 6 B). This, however, was only possible once TPO was added to SCF or SCF and Flt3L in cultures. Thus, TPO possibly substitutes for a survival signal otherwise delivered by Flt3. In addition, or alternatively, TPO might be involved in the phosphorylation of overexpressed STAT3 (45). Interestingly, enforced expression of STAT3 or PU.1 in MEPs led to the up-regulation of mouse Flt3 mRNA levels (Fig. S3). This in turn likely allowed culture–supplemented human Flt3L to cross-reactively stimulate STAT3- or PU.1-transduced cells via mouse Flt3. These results suggest a self-sustaining effect of Flt3 signaling–induced Flt3 transcription via downstream STAT3 and PU.1.

As enforced expression of huFlt3 in MEPs did not terminate megakaryocyte/erythrocyte differentiation potential,
whereas huFlt3 expression in GMPs did not lead to a gain of these differentiation potentials (Fig. 4A), how can Flt3 signaling be integrated in megakaryocyte/erythrocyte versus IPC, DC, and GM lineage commitment? By using PU.1
g reporter mice, PU.1 expression was recently mapped in early hematopoietic progenitor cells (46). It was shown that PU.1+Flt3+ CMPs contain high myelomonocytic developmental potential, whereas PU.1−Flt3− CMPs and PU.1− MEPS have high megakaryocyte/erythrocyte potential (46). The data presented here suggests that Flt3 might be critical in PU.1 regulation, although this likely will not be an exclusive event. GATA-1 is a nonredundant transcription factor for megakaryocyte and erythrocyte development (47). DNA binding activity of GATA-1 can be suppressed by enforced PU.1 expression, resulting in a differentiation block and apoptotic cell death of an erythroid cell line (48). Conversely, GATA-1 inhibits the binding of PU.1 to c-Jun, a critical co-activator of myeloid gene transactivation by PU.1 (49). Furthermore, GATA-1 interferes with DNA binding activity of STAT3 and inhibits TPO-dependent growth of the Ba/F3 cell line (50). Thus, as suggested previously for PU.1 and GATA-1 (51, 52), relative dosage of gene transcription and protein levels will likely determine lineage outcomes. Indeed, STAT3 and PU.1 expression levels in huFlt3+−MEPs were increased to levels of normal CMPs and were somewhat lower than those observed in GFP+−GMPs or huFlt3+− GMPs (Fig. 5). Thus, it is possible that Meps with relatively lower huFlt3 and consecutive STAT3 and PU.1 expression do not fully inhibit GATA-1, whereas high Flt3 expressing and signaling cells develop to IPC, DC, or GM lineages. Of note, enforced expression of STAT3 and PU.1 in Meps suppressed GATA-1 expression and inhibited megakaryocyte/erythrocyte development (Fig. 6, C and D). HuFlt3 overexpression in GMPs in turn induced some EpoR, B-gllobin, and GATA-1 mRNA expression; however, this was not sufficient to reactivate megalakaryocyte/erythrocyte development as demonstrated for enforced high-level GATA-1 expression in GMPs (Figs. 4A and 5A; reference 13).

Because overexpression of huFlt3 in Flt3− progenitors does not occur under physiologic conditions, what do these findings imply for normal hematopoiesis? Flt3 is expressed on short-term HSCs, multipotent progenitors, CLPs, CMPs, and GMPs, and in vivo injection of Flt3L resulted in increased numbers of these cells as well as IPCs and DCs, whereas Meps and their progeny remained unchanged (32, 33). The data presented here demonstrate that enforced Flt3 cytokine receptor signaling is sufficient to activate as well as enhance IPC and DC differentiation programs, suggesting that instructive cytokine signaling might indeed occur in hematopoiesis. Thus, we speculate that once Flt3+ short-term HSCs and their offspring Flt3+ cells are exposed to Flt3L-rich environments, these cells will be instructed to differentiate into IPCs and DCs. This might be enhanced by a self-sustaining process in which Flt3 downstream transcription factors STAT3 and PU.1 in turn maintain Flt3 receptor expression. However, Flt3 signaling does not immediately silence other developmental options. It is likely that most Flt3-expressing progenitors will not continuously be stimulated via Flt3L but will receive and activate alternative signals, and thus consecutively acquire myeloid or lymphoid, but not IPC or DC, cell fates. Beyond previous studies, our data further emphasize that IPC and DC development does not fit into a deterministic “lymphoid” nor “myeloid” lineage, but rather a “Flt3-permissive” developmental model, whereas Flt3-expressing progenitors maintain IPC and DC differentiation options in response to Flt3L as long as no competing signal shuts these down. It will be of interest to test whether downstream dividing Flt3+ common IPC and DC progenitors with silenced alternative developmental programs exist and which critical factors are involved in final IPC or DC lineage termination.

MATERIALS AND METHODS

Mice. C57BL/6 (CD45.2), C57BL/Ka-Thy1.1 (CD45.1), and BALB/c mice (Charles River Laboratories) were maintained at the Institute for Research in Biomedicine animal facility in accordance with the Swiss Federal Veterinary Office guidelines.

Flow cytometry and cell sorting. Hematopoietic progenitors were isolated as described previously with minor modifications (2, 37). Bone marrow cells were immuno-magnetically preenriched for c-Kit+cells using APC-conjugated c-Kit antibodies (2B8; ebioscience) and APC microbeads (Miltenyi Biotech). Cells were then stained with monoclonal antibodies as indicated below. Flt3+ and Flt3− hematopoietic progenitors were sorted as lineage− (CD3ε, 145-2C11; CD4, GK1.5; CD8, 53-67; B220, RA3-6B2; CD19, MB19-1; CD11b, M1/70; Gr-1, RB6-8C5; and TER119, TER119), IL-7Rα− (ATR34), Thy1.1− (19X5E), c-KIt+, and Flt3+− (A2F10.1) cells. Thus, Flt3− and Flt3+ hematopoietic progenitors did not contain Thy1.1, HSCs or IL-7Rα− lymphoid progenitors. Myeloid progenitors were sorted as Lin−Sca-1− (E13-161-7) c-KIt−CD34+ (RAM34) FcyR− (2.5G2; CMPs), Lin−Sca-1−c-KIt−CD34+FcyRhigh (GMPs), and Lin−Sca-1−c-KIt−CD34+FcyR− (MEPs) cells. For IPC and DC analysis and sorting, additional monoclonal antibodies against the following antigens were used: CD11c (N418), MHC class II (I-A/I-E; M15/114.15.2), Ly6C (AL-21), CD45Rα (A7R34), CD11c (N418), MHC class II (I-A/I-E; M15/114.15.2), Ly6C (AL-21), CD45Rα (A7R34), CD80 (16-10A1), and CD86 (GL-1). Cells were sorted and analyzed using a FACS Calibur and FACS Aria (Becton Dickinson).

Retroviral transduction of hematopoietic progenitors. The full length of human Flt3, mouse STAT3, and PU.1 cDNA was inserted into a retroviral expression vector, pMYs-ires-GFP, respectively (53). These constructs were transiently transfected into Phoenix-Ampho cells by Lipofectamine (Invitrogen). The amphotropic retrovirus supernatants were used to transduce GP+E-E86 cells. After 2 d, the brightest GFP-expressing GP+E-E86 cells were FACS sorted and expanded. For transduction of hematopoietic progenitor cells, GP+E-E86 cells were 20-Gy irradiated and plated in 24-well plates at 1.5 × 10^6 cells per well for 24 h. Progenitor cells were transduced by coculture with GP+E+E86 for 18 h in IMDM (Invitrogen) containing 2% FCS, 4 μg/ml polybrene (Sigma-Aldrich), 100 ng/ml human Flt3-Lg fusion protein (huFlt3-Lg), 10 ng/ml mSCF (R&D Systems), and 10 ng/ml mIL-11 (R&D Systems). Transduced cells were removed by gentle pipetting and then subjected to further assays.

In vitro IPC and DC differentiation assays. Retrovirus-transduced Flt3− and Flt3+ progenitors as well as CMPs, GMPs, and Meps were cultured in IMDM, supplemented with 10% FCS, 10−4 M 2-ME, sodium pyruvate, and antibiotics, 100 ng/ml huFlt3-Lg, and 10 ng/ml mSCF as indicated. Half of the media was replaced every 3 d and new cytokines were added. Human Flt3-Lg fusion protein was produced in Drosophila cells as described previously (54).
In vitro myeloid colony formation assays. For myeloid colony-forming assays, GPP+–CMPs, GPP+–GMPs, and GPP+–MEPs were sorted after viral transduction and were cultured in IMDM-based methylcellulose media (Methodcult H4100; StemCell Technologies Inc.), containing 30% FCS, 1% bovine serum albumin, 2 mM l-glutamine, and 50 μM 2-ME, 10 ng/ml mSCF, 10 ng/ml mIL-3 (R&D Systems), 10 ng/ml mIL-6, 10 ng/ml mGM-CSF (R&D Systems), 10 ng/ml mTpo (R&D Systems), 1 U/ml hEpo (Roche), and 100 ng/ml huFlt3L-Ig. Colonies were determined and enumerated under an inverted microscope consecutively from day 3 to 8. In some cases, to confirm colony types, colonies were picked using fine-drawn Pasteur pipettes, spun on slides, Giemsa stained, and evaluated by light microscopy.

In vivo reconstitution assays. 2 × 10^6 CD45.2 GPP+–MEPs, huFlt3+–MEPs, or GPP+–CMPs each were injected intravenously into lethally irradiated (2 × 6 Gy in a 4-h interval from a Cesium 137 source; Biobeam 8000; STS GmbH) congenic mice (CD45.1) in U-bottom 96-well plates with 2 × 10^5 recipient-type CD45.1 whole bone marrow cells. Mice were killed on day 7. The progeny of donor-derived cells were isolated as described previously (33) and evaluated using FACS analysis.

MLR. Graded numbers of sorted, irradiated (25 Gy) IPCs or DCs were plated in U-bottom 96-well plates with 2 × 10^5 immunomagnetically selected (CD4 microbeads; Miltenyi Biotec) BALB/c spleen CD4+ T cells in a final volume of 200 μl RPMI 1640 supplemented with 10% FCS. Cells were cultured for 5 d and pulsed with 1 μCi [3H]thymidine (Amersham Biosciences) per well during the last 16 h of culture. [3H]thymidine incorporation was measured on a β-plate counter (Tracker Beta TriLux; EG&G WALLAC).

IFN-α production. To evaluate IFN-α production, sorted CD11c+ B220+ IPCs or conventional CD11c+ B220+ DCs derived from retrovirus-transduced progenitors were cultured for 24 h at 10^5 cells/200 μl in U-bottom 96-well plates in RPMI 1640 supplemented with 10% FCS, 2-ME, penicillin G, and streptomycin. Either 40 HAU/ml influenza virus (strain A/Beijing/353/89; National Public Health Institute, Helsinki, Finland) or 1 μM CpG-A-ODN (ggTGCATCGATGCggggggG; lowercase letters indicate base with phosphorothioate-modified backbones) was added at start of culture and again at 12 h. Culture supernatants were assayed using an IFN-α ELISA kit (Performance Biomedical Laboratories).

RT-PCR analysis. Total RNA was extracted from sorted progenitors as indicated using TRIzol reagent (Invitrogen) followed by DNAse I (Invitrogen) treatment. The cDNA was synthesized using random hexamers as well as SuperScript II reverse transcriptase (Invitrogen) and amplified using specific primers as described previously (13). For real-time PCR, cDNA products equivalent to RNAs from 200 progenitors were amplified using an Applied Biosystems 7900HT Fast Real-Time PCR System. The data were normalized by the level of 18s rRNA, PU.1, and STAT3, and 18s rRNA were purchased from Applied Biosystems.

Intracellular phospho-STAT3 staining. Retroviral-transduced MEPs were cytotoxic starved for 24 h in 1% FCS-IMDM. Cells were then incubated with or without 100 ng/ml huFlt3L-Ig and analyzed at indicated times for phospho-STAT3 by FACS according to the manufacturer's instructions (Cell Signaling).

Statistical analysis. Results of experiments are reported as mean ± SD. Differences were analyzed using Student’s t test.

Online supplemental material. Fig. S1 shows the diagrams of pMY-IRES-GFP and pMY-huFlt3-IRES-GFP bicistronic retroviral expression vector constructs and virus transduction efficiency in progenitor cells. Fig. S2 shows the diagrams of pMY-mSTAT3-IRES-GFP and pMY-mPU.1-IRES-GFP bicistronic retroviral expression vector constructs. Fig. S3 shows the analysis of mouse Flt3 mRNA expression in GPP+, huFlt3–, STAT3+, and PU.1-transduced M Ep s, as well as CMPs. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20051645/DC1.

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