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Tight Control of STAT5 Activity Determines Human CD34-Derived Interstitial Dendritic Cell and Langerhans Cell Development

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Despite the crucial function of dendritic cells (DC) in immunity, the molecular mechanisms regulating human DC development remain poorly defined. STAT5 regulates various hematopoietic lineages and is activated by GM-CSF, a critical cytokine in DC development. In this study, we investigated the role of STAT5 during differentiation of human CD34+ hematopoietic progenitors into precursor DC (pre-DC) and their subsequent differentiation toward interstitial DC and Langerhans cells. Inhibiting STAT5 activity by dominant-negative STAT5 promoted Langerhans cell commitment of hematopoietic progenitors but resulted in loss of pre-interstitial DC development, showing subset-specific regulation. Increasing the low endogenous STAT5 activity by ectopic STAT5 activation downregulated expression of the critical DC transcription factor PU.1 and abrogated commitment to either DC lineage. In contrast, high STAT5 activity was beneficial in already committed pre-DC: terminal DC differentiation was associated with increased endogenous STAT5 phosphorylation levels, JAK2–STAT5 inhibition reduced terminal DC differentiation, and conditional STAT5 activation in pre-DC improved development of BDCA-1+, DC-SIGN+, and Langerin+ DC with normal maturation and T cell stimulation. These data show that STAT5 critically regulates human DC development, with specific requirements for the level of STAT5 activation at distinct differentiation stages. By regulating STAT5 activity, cytokines present at specific locations and under different pathophysiological conditions can determine the fate of DC precursors. The Journal of Immunology, 2011, 186: 000–000.

Although dendritic cells (DC) represent only a very small fraction of the hematopoietic system, their specialized functions are crucial for both the induction of immunity and the maintenance of tolerance (1). Multiple DC subtypes can be recognized based on surface markers, localization, functional abilities, and ontogeny, but the identified subsets do not completely overlap between the different species (2–5). The human myeloid DC subsets, including interstitial DC (intDC) and Langerhans cells (LC), are related to conventional DC identified in mice (2, 6).

Being relatively short-lived, DC are continuously replenished from organ-resident or blood-derived precursors (7). Direct precursor DC (pre-DC), as well as immune cells with their own functionality but able to generate DC, such as monocytes, constantly develop from early hematopoietic progenitors in a complex cytokine-driven process including several developmental stages at different anatomic locations (2, 7–11). Because the cytokine milieu varies between anatomic locations, the intracellular signaling cascades required for DC development may differ between the separate developmental stages. Furthermore, different cytokines and probably also signaling pathways are induced under various pathophysiological conditions. Despite their important role in the regulation of immunity, the molecular mechanisms responsible for the development of human DC are largely unknown.

Signal transducer and activator of transcription 5 (STAT5) plays an important role in hematopoiesis, regulating self-renewal, proliferation, and lineage decisions of hematopoietic stem cells and hematopoietic progenitor cells (HPC) (12–16). Most myeloid cells express cytokine receptors that are able to induce STAT5 activation, and STAT5 has been shown to regulate the development of various myeloid lineages (13, 17). GM-CSF is one of the cytokines triggering STAT5 phosphorylation via activation of JAK2 (18). Initially, the role of GM-CSF for DC development in the steady state was thought to be limited (19, 20), but it was recently shown that next to Flt3 ligand, GM-CSF is also required for steady-state DC development (21). In addition, GM-CSF is thought to play an important role in DC differentiation during inflammation (5, 7).

In mice, GM-CSF–induced STAT5 activation has been shown to inhibit plasmacytoid DC development (22). However, despite the importance of STAT5 in hematopoiesis and myeloid lineage development, the role of STAT5 in human myeloid DC development remains unclear. The current study demonstrates that development...
of human myeloid DC subsets is critically regulated by STAT5 activity. Rather than acting in an on–off binary manner, STAT5 activity needs to be tightly regulated for optimal differentiation of intDC and LC from human CD34+ hematopoietic progenitors. In addition to the level, the timing of STAT5 activation appeared essential. Depending on the developmental stage, manipulation of STAT5 activity resulted in lineage skewing, or inhibition or enhancement of DC differentiation. These data indicate that regulation of STAT5 activation represents a mechanism by which cytokines determine the fate of DC precursors. Insight in the molecular mechanisms regulating human DC development will improve the understanding of how DC replenishment from their hematopoietic progenitors is regulated and homeostasis of these important immune cells is maintained.

Materials and Methods
Generation of CD34-derived myeloid DC
Umbilical cord blood samples were obtained ex utero according to legal guidelines. CD34+ hematopoietic progenitor cells were isolated and differentiated toward intDC and LC as described previously (23, 24). Briefly, CD34+ cells, isolated through positive selection using anti-CD34–coated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany), were cultured with CD34+ hematopoietic progenitors is regulated and homeostasis of these important immune cells is maintained.

FIGURE 1. Endogenous STAT5 activity levels increase with CD34-derived myeloid DC differentiation. A, CD34-derived myeloid DC were differentiated under control conditions. Cells were harvested at days 3, 7, and 10, total cell lysates were prepared, and equal amounts of protein were separated on SDS-PAGE. Expression of p-STAT5 and total STAT5 was determined, and the ratio was calculated. Tubulin expression was analyzed to confirm equal loading. A representative experiment and mean ± SEM p-STAT5/STAT5 ratios of four to seven independent experiments with different donors are shown. B, Control cultures were harvested at days 3, 7, and 10. Cells were fixed, and intracellular FACS staining for p-STAT5 and total STAT5 was performed. For day 10, some experimental variation was apparent. FACS plots representing the majority of 10 independent experiments with different donors are shown. Specific p-STAT5 staining, calculated by subtracting the mean fluorescence intensity of the isotype control from the mean fluorescence intensity measured for p-STAT5, is indicated by the numbers in the FACS plots. C, CD34+ HPC were retrovirally transduced with STAT5a-wt, STAT5a1*6, or a control vector. Transduced cells were isolated by FACS sort at day 7. Total cell lysates were prepared, and equal amounts of protein were separated on SDS-PAGE. Expression of p-STAT5 and total STAT5, as well as tubulin, was determined. A representative experiment of three independent experiments with different donors is shown. *p < 0.05 (paired Student t test).
Intracellular: For analysis of intracellular proteins, cells were fixed (30 min) and subsequently permeabilized (15 min) by incubation in 2% formaldehyde (Merck) and Perm Buffer III (BD Biosciences). Labeling of phosphorylated or total STAT5 was performed on ice by 30 min incubation with Abs against Y694-phosphorylated STAT5 (p-STAT5; rabbit polyclonal; Cell Signaling Technology, Danvers, MA) or total STAT5 (3H7, rabbit monoclonal; Cell Signaling Technology), followed by 30 min incubation with biotin-labeled goat anti-rabbit Abs (Dako). Labeling was visualized by a third incubation with fluorochrome-conjugated streptavidin (BD Biosciences).

Apoptosis. Phosphatidylserine exposure and membrane permeability were analyzed in annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Cells were incubated with fluorochrome-conjugated annexin V (BD Biosciences) for 30 min on ice and subsequently taken up in 7-aminomycin D (BD Biosciences) or 1 μg/ml propidium iodide (PI; Sigma-Aldrich).

Assessment. Assessment was performed using a FACSCalibur or FACS-Cantoll (BD Biosciences), and data were analyzed using FlowJo software (http://www.flowjo.com). Surface markers and intracellular proteins were analyzed on viable cells, gated on forward scatter/side scatter (FSC/SSC).

Cell isolation by FACS sort

Cells were sorted using a FACSaria SORP (BD Biosciences). Cells transduced with a construct expressing eGFP were isolated based on eGFP expression. STAT5a1*6-ER–transduced cells were incubated with a biotin-conjugated Abs against NGFR/CD271 (C40-1457; BD Biosciences), followed by a second incubation with fluorochrome-conjugated streptavidin and fluorochrome-conjugated Abs against CD1a and CD14. Labeled cells were life gated on FSC/SSC and sorted into iNGFR–CD14–CD1a– and iNGFR+CD14+CD1a+ fractions.

Histological staining

Cytospins were prepared from 5 × 10⁶ cells. After fixation in methanol for 5 min, cytospins were stained in a 50% eosin methylene blue solution according to May–Grunwald (Sigma-Aldrich) for 20 min. After rinsing in water for 5 s, the nuclei were counterstained with 10% Giemsa solution (Merck) for 15 min. Micrographs were acquired at room temperature with an Axioskop 20 microscope (Carl Zeiss, Sliedrecht, The Netherlands) fitted with a ×40/1.3 Plan Neofluor objective using Immersol 518N oil (Carl Zeiss), a Nikon Digital Sight DS-U1 camera (Nikon, Lijnden, The Netherlands), and NIS Elements D3.0 image acquisition software (Nikon).

Allogeneic MLR

Responder T cells were isolated from auffy coat through negative selection using PE-labeled Abs against BDCA-1, CD14, CD19 (J4.119; Beckman Coulter), CD56, and CD123 (SSDCLY107D2; Beckman Coulter), and anti-PE–, anti-CD15–, and anti-CD235–coated microbeads (Miltenyi Biotec) (24, 26). Irradiated DC (30 Gy) were added in graded doses to 2 × 10⁶ allogeneic T cells in 96-well round-bottom plates in RPMI 1640 containing 8% heat-inactivated FBS and penicillin/streptomycin. Proliferation was quantified by incubation with 1 μCi (37 kBq) [methyl-³H] thymidine (NEN Life Science Products, Boston, MA) during the last 18 h of 6-d cultures. To determine IFN-γ concentrations in day 5 supernatants, the commercially available ELISA kit for human IFN-γ (eBioscience, San Diego, CA) was used according to the manufacturer’s instructions. The detection limit of this assay was 4 pg/ml.

Western blot

Total cell lysates were produced using Laemmli sample buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μl bromophenol blue, and 35 mM 2-mercaptoethanol) and boiled for 5 min. Equal amounts of total protein, determined by Lowry method, were separated by 8% SDS-PAGE, transferred to an Immobilon-FL membrane transfer membrane (Millipore, Bedford, MA), and incubated with Odyssey blocking buffer (Westburg, Leusden, The Netherlands) for 30 min before probing with Abs against human PU.1 (rabbit polyclonal; Cell Signaling Technology), p-STAT5 (rabbit polyclonal; Cell Signaling Technology), total STAT5 (3H7, rabbit monoclonal; Cell Signaling Technology), β-tubulin (mouse monoclonal; Sigma-Aldrich), or β-actin (C4, mouse monoclonal; Santa Cruz Biotechnology, Heidelberg, Germany). Subsequently, blots were incubated with goat anti-

FIGURE 2. STAT5 activity is required for intDC differentiation but redundant for LC commitment. CD34+ HPC were retrovirally transduced with STAT5aΔ750 or a control vector. A. Cells were harvested at day 7 and analyzed for the expression of CD1a and CD14. Representative FACS plots of eGFP+ cells are shown (n = 7). B. Numbers of eGFP+ cells 2 d after transduction (day 4) were calculated from the trypan blue negative cell count and the percentage of eGFP+ cells as determined by FACS. At day 7, the cells were counted and analyzed for the expression of eGFP, CD14, and CD1a. The absolute numbers of eGFP+CD14+CD1a– pre-intDC and eGFP+CD14+CD1a+ pre-LC were calculated and normalized for the difference in transduction efficiency using the day 4 eGFP+ cell count. Shown are mean ± SEM pre-intDC and pre-LC absolute cell numbers standardized to control (n = 3). C and D, eGFP+ cells were isolated by FACS sort at day 7 and further cultured under control conditions. Day 10 cells were counted with trypan blue exclusion and analyzed for the expression of Langerin and BDCA-1 by flow cytometry. Numbers of Langerin+ and BDCA-1+ cells were calculated and standardized to control. Representative FACS plots (C) and mean ± SEM absolute cell numbers (D) are shown (n = 3). E and F, Day 13 cells were stimulated with LPS. After 18 h, cells were harvested, thoroughly washed, and used in allogeneic MLR. T cell proliferation was quantified by incubating the cells with [methyl-³H] thymidine during the last 18 h of 6-d cultures (E). ELISA determined IFN-γ concentrations in supernatants harvested at day 5 (DC/T cell ratio 1:2) (F). Data represent mean ± SD of triplicate cultures of a representative experiment (n = 2). *p < 0.05 (paired Student t test).
Results

**STAT5 activity is required for intDC differentiation but redundant for LC commitment**

To determine the role of STAT5 in CD34-derived myeloid DC development, we first analyzed the phosphorylation status of STAT5, a measure of STAT5 activation, at different time points during culture. Although STAT5 activation was observed at all time points, increased STAT5 phosphorylation was found at later differentiation stages (Fig. 1A, 1B). To investigate the importance of this transcription factor, STAT5 activity was inhibited by retroviral introduction of STAT5αΔ750, a dominant-negative mutant that contains the Y694 phosphorylation site and can bind to DNA but is unable to activate transcription (25). Ectopic expression of this truncated mutant was confirmed by Western blot (Fig. 1C).

CD34⁺ HPC differentiate to CD1a⁺ intDC and CD1a⁺ LC through subset-specific pre-DC that can be distinguished based on their CD14⁺CD1a⁻ and CD14⁺ CD1a⁺ phenotypes, respectively (23, 24). In contrast to control cultures in which both CD14⁺CD1a⁻ pre-Interstitial DC (pre-intDC) and CD14⁺ CD1a⁺ pre-LC were present at day 7, no pre-intDC developed from STAT5αΔ750-transduced HPC (Fig. 2A, 2B). Conversely, the proportion (p < 0.01) as well as the absolute numbers of pre-LC were increased (Fig. 2A, 2B), indicating that STAT5 activity is redundant or may impede LC commitment. Under control conditions, both terminally differentiated intDC and LC express the myeloid DC marker BDCA-1, whereas only LC acquire Langerin upon terminal differentiation (23, 24). Classification of the Langerin⁺ cells as LC was further supported by their specific expression of E-cadherin and cutaneous leucocyte Ag (data not shown). As demonstrated by the increase in Langerin⁺ cells at day 10, STAT5 inhibition induced skewing toward LC (Fig. 2C, 2D). Whereas in control cultures 80% of cells differentiated into BDCA-1⁻ myeloid DC, BDCA-1 was only expressed by 40% of cells derived from STAT5αΔ750-transduced HPC (Fig. 2C, 2D), demonstrating the loss of intDC. Cells derived from STAT5-inhibited cultures had an impaired ability to induce proliferation and IFN-γ production by allogeneic T cells (Fig. 2E, 2F), which could result from reduced DC numbers as well as impaired DC function. The expression of costimulatory molecules by DC from STAT5-inhibited and control cultures was comparable (Supplemental Fig. 1), suggesting intact Ag-presenting ability of DC expressing STAT5αΔ750. Thus, whereas CD34-derived intDC development requires STAT5 activity, this transcription factor does not appear to be necessary for commitment to the LC lineage.

**Increased STAT5 activity in CD34⁺ HPC inhibits myeloid DC commitment**

To investigate further how STAT5 activity influences myeloid DC commitment, CD34⁺ HPC were retrovirally transduced with STAT5α-wt or the constitutively active STAT5 mutant STAT5α1*6. Both introduction of STAT5α-wt and STAT5α1*6 increased STAT5 phosphorylation, but STAT5α1*6 increased STAT5 phosphorylation to a larger extent (Fig. 1C). Compared with control cultures, a 44 ± 9% and 51 ± 12% reduction in expansion was observed for STAT5α-wt-transduced and STAT5α1*6-transduced cultures, respectively. More importantly, however, myeloid DC development was dramatically reduced in these cultures. Almost no pre-intDC could be observed (Fig. 3A, 3B). Although pre-LC were less affected, the development of these cells was also significantly reduced by STAT5α-wt (Fig. 3A, 3B).

**FIGURE 3.** Increased STAT5 activity in CD34⁺ HPC inhibits myeloid DC commitment. CD34⁺ HPC were retrovirally transduced with STAT5α-wt, STAT5α1*6, or a control vector. A and B, Cells were harvested at day 7 and analyzed for the expression of CD1a and CD14 by flow cytometry. The percentages CD14⁺CD1a⁻ pre-intDC and CD14⁺ CD1a⁺ pre-LC within the eGFP⁺ population were determined. Representative FACS plots of eGFP⁺ cells (A) and mean ± SEM percentage of pre-DC within the eGFP⁺ cells (B) are shown (n = 6). C and D, Day 13 cells were analyzed for the expression of BDCA-1 and Langerin by flow cytometry. Representative FACS plots of eGFP⁺ cells (C) and mean ± SEM percentage of BDCA-1⁺ and Langerin⁺ cells within the eGFP⁺ population (D) are shown (n = 3). E, eGFP⁺ cells were isolated by FACS sort at day 7. At day 13, cells were harvested. Cytospins were prepared, stained with May–Gruenwald Giemsa solution, and analyzed by light microscopy (original magnification ×400). The results are representative of three experiments. *p < 0.05 (paired Student t test).
STAT5a1*6 further reduced pre-LC development (Fig. 3A, 3B), demonstrating an inverse correlation between the increase in STAT5 activity and pre-LC differentiation capacity. In accordance with the loss of pre-DC, no differentiated DC were present at day 13, as demonstrated by the absence of BDCA-1 and Langerin expression (Fig. 3C, 3D). In addition, the cells generated hardly expressed costimulatory molecules and induced only low T cell proliferation (data not shown). STAT5a-wt and STAT5a1*6 cultures contained a heterogeneous cell population (Fig. 3E). Analysis of lineage-specific surface markers revealed expression of CD71, an early maker of erythrocyte development, but not glycophorin A, which is expressed by fully differentiated erythrocytes (Supplemental Fig. 2). Although STAT5a-wt cultures contained cells whose morphology resembled that of granulocytes (Fig. 3E), CD15 expression could not be detected (Supplemental Fig. 2). The leukocyte marker CD45 was expressed by all cells (Supplemental Fig. 2). Whereas control cultures demonstrated almost no proliferation between days 10 and 13, a 3-fold expansion was observed in the cultures ectopically expressing STAT5 (data not shown). Together, these data demonstrate that increased STAT5 expression abolishes the myeloid DC differentiation capacity of CD34+ HPC.

**FIGURE 4.** STAT5 promotes intDC differentiation from purified pre-intDC. CD34+ HPC were retrovirally transduced with 4-OHT-inducible STAT5a1*6-ER. At day 7, transduced pre-intDC (iNGFR+CD14+CD1a⁻) were isolated by FACS sort. Cells were further cultured in the presence of either 4-OHT or its solvent ethanol. A and B, Cells were harvested at days 8, 9, and 10 and analyzed for the expression of CD14 and CD1a. The percentages of CD1a⁻ and CD1a⁺ cells were determined, and the ratio was calculated. Representative FACS plots of day 9 cells (A) and mean ± SEM CD1a⁻/CD1a⁺ ratios (B) of at least four independent experiments with different donors are shown. C, Day 8 cells were analyzed for Annexin V/PI staining. Representative FACS plots are shown (n = 4). D, Day 9 cells were harvested, counted with trypan blue exclusion, and analyzed for the expression of CD1a by flow cytometry. Absolute numbers of CD1a⁺ cells were calculated. E, Three independent experiments with different donors are shown. F, Representative FACS plots of at least three independent experiments showing DC-SIGN and BDCA-1 expression at day 10. F and G, Day 10 cells were stimulated with LPS. At day 12, cells were harvested, thoroughly washed, and used in allogeneic MLR. T cell proliferation was quantified by incubating the cells with [methyl-3H]thymidine during the last 18 h of 6-d cultures (F). ELISA determined IFN-γ concentrations in supernatants harvested at day 5 (DC/T cell ratio 1:2) (G). Data represent mean ± SD of triplicate cultures of a representative experiment (n = 3). H, CD34⁺ HPC were cultured under control conditions until day 7. Then, CD14⁺CD1a⁻ pre-intDC were isolated by FACS sort and further cultured in the presence or absence of JAK2 inhibitor AG490. At day 9, cells were harvested and analyzed for the expression of CD1a and CD14 by flow cytometry. Representative FACS plots of three independent experiments with different donors are shown. *p < 0.05 (paired Student t test).

**STAT5 promotes intDC differentiation from pre-intDC)** Because GM-CSF is an important inducer of myeloid DC differentiation as well as STAT5 activation (18, 21), the negative effects of increased STAT5 activity on DC development were surprising. We therefore retrovirally transduced HPC with 4-OHT-inducible STAT5a1*6-ER, which allows specific modulation of STAT5 activity levels at different time points during differentiation. Similar to the effects of STAT5a1*6 expression (Fig. 3), immediately increasing STAT5 activity by addition of 4-OHT to STAT5a1*6-ER-transduced HPC from the moment of transduction blocked pre-DC differentiation (data not shown). To determine the effect of increased STAT5 activity in purified committed pre-intDC, STAT5a1*6-ER-transduced cells were cultured without 4-OHT until day 7. CD14⁺CD1a⁻ pre-intDC were then isolated and differentiated toward intDC in the presence or absence of 4-OHT. As previously reported (24), terminal differentiation of pre-intDC to intDC involves the acquisition of CD1a (Fig. 4A). Conditional STAT5 activation by addition of 4-OHT resulted in increased CD1a expression (Fig. 4A, 4B), suggesting that STAT5 increased differentiation. After 1 d, the ratio of CD1a⁺ and CD1a⁻ cells was similar for control and 4-OHT cultures, but 2- and 3-d incubation with 4-OHT resulted in a relative increase in CD1a⁺ cells.
STAT5 regulates human myeloid DC development

compared with control cultures (Fig. 4B). No difference in viability was found (Fig. 4C), resulting in similar cell yields in control and 4-OHT cultures (Supplemental Fig. 3A). The similar yields and increased percentage of CD1a+ cells resulted in a variable but consistent increase of absolute CD1a+ cell numbers in three independent experiments (Fig. 4D). These data support the view that conditional STAT5 activation improved differentiation rather than induced apoptosis of the less differentiated CD1a− cells. Enhanced differentiation was further demonstrated by the augmented DC-SIGN expression, whereas most cells expressed BDCA-1 irrespective of 4-OHT addition (Fig. 4E). None of these effects were observed when 4-OHT was added to control cells, either untransduced or transduced with an empty control vector (Supplemental Fig. 4A–D and data not shown), confirming that the observed effects resulted specifically from increased STAT5 activation. Furthermore, DC generated in the presence of increased STAT5 activation were functional, as demonstrated by the normal LPS-induced CD86 and HLA-DR expression (Supplemental Fig. 3B). T cell stimulatory capacity was also unaffected, and 4-OHT–treated DC even modestly increased IFN-γ secretion by allogeneic T cells (Fig. 4F, 4G).

To confirm the importance of STAT5 in terminal intDC differentiation and to investigate the function of endogenous STAT5 activity, JAK2−STAT5 signaling was inhibited in pre-intDC by treatment with the JAK2 inhibitor AG490. JAK2 inhibition impaired differentiation, as demonstrated by a modest but reproducible reduction in CD1a expression (67 μM: 16 ± 2%; p < 0.05) (Fig. 4H). Taken together, these data show that whereas increased STAT5 activation negatively affects DC development from CD34+ HPC, it has a beneficial effect on the differentiation of cells that have already committed to the DC lineage, leading to improved differentiation of pre-intDC to intDC.

STAT5 promotes LC differentiation from pre-LC

We next investigated the consequences of increasing STAT5 activity in purified pre-LC. Similar to pre-intDC, a time-dependent relative increase in differentiated cells was observed in 4-OHT–treated cultures compared with control, as demonstrated by the increased percentage of Langerin+ cells (Fig. 5A, 5B). Although already evident after 1 d, this became increasingly clear at later time points (Fig. 5B). Viability and cell yields were relatively unaffected 1 and 2 d after 4-OHT addition (Fig. 5C, Supplemental Fig. 3C), resulting in increased Langerin+ cell yields at day 9 (Fig. 5D). We have previously shown that LC generated in this culture system are more susceptible to apoptotic death after terminal differentiation than intDC (24). In accordance, the enhanced LC differentiation was accompanied by increased apoptosis and reduced cell yields at day 10 (Supplemental Fig. 3C, 3D). In addition to Langerin, BDCA-1 expression was also increased in 4-OHT–treated cultures (Fig. 5E). In control or untransduced cultures, 4-OHT did not affect LC development (Supplemental Fig. 4E–I and data not shown). Similar to intDC, 4-OHT–treated LC exhibited normal LPS-induced maturation (Supplemental Fig. 3E) and T cell stimulatory capacity (Fig. 5F, 5G). The importance of STAT5 in terminal LC differentiation was further confirmed by the reduced Langerin expression in JAK2–inhibited cultures (Fig. 5H).

Taken together, these data demonstrate that STAT5 activation promotes terminal differentiation of pre-LC.

STAT5 modulates DC lineage commitment by regulation of PU.1 expression levels

Although STAT5 activity promoted terminal LC differentiation from pre-LC (Fig. 5), STAT5 was redundant for and even impeded LC commitment of HPC, as demonstrated by the LC skewing induced by introduction of dominant-negative STAT5 (Fig. 2). The latter was remarkably similar to the reported LC skewing induced by TGF-β (27), a finding that was confirmed by the increased
independent experiments with different donors are shown. Control conditions, or under control conditions in the presence of TGF-β, were harvested at day 0, 6 h after the start of culture. Day 10 cells had been cultured in the presence or absence of TGF-β and inhibition of STAT5 transcription factor activity were found upon TGF-β addition to our cultures (Fig. 6A and data not shown). To investigate this further, we analyzed the effects of TGF-β addition on STAT5 activity. As determined by both FACS and Western blot, TGF-β reduced STAT5 phosphorylation in CD34-derived myeloid DC cultures at day 0 and day 10 (Fig. 6B, 6C). This was associated with a significant increase in PU.1 (Fig. 6C), a protein whose expression is known to be upregulated by TGF-β (28). In addition, PU.1 expression in STAT5aΔ750 cultures was somewhat increased compared with control, whereas PU.1 levels were dramatically reduced in cells with increased STAT5 activity (Fig. 6D). Furthermore, the expression of CD11b, which is transcriptionally regulated by PU.1 (29, 30), was also inversely correlated with STAT5 activity (Fig. 6E). Because PU.1 is a critical transcription factor for DC development (31), these data suggest that the regulation of CD34-derived myeloid DC commitment by STAT5 is at least partly mediated by regulation of PU.1 expression.

**Discussion**

DC, the most important APCs, are relatively short lived and therefore need to be continuously replenished. However, our knowledge on how this is achieved remains limited. In this study, we show that STAT5 is active at all stages of differentiation during human CD34-derived intDC and LC development, but that higher activation levels are present in further differentiated cells. Tight regulation of STAT5 transcription factor activity is required for optimal development of these myeloid DC. Depending on the DC developmental stage, manipulation of STAT5 activity levels results in a) skewing toward a specific myeloid DC subset, b) a complete blockade in myeloid DC development, or c) enhanced myeloid DC differentiation.

Whereas intDC development required STAT5 activity, this transcription factor was redundant for and even impeded the differentiation of CD34+ HPC to pre-LC. The relatively low endogenous STAT5 activity during development of pre-DC from HPC, together with our finding that ectopic activation of STAT5 in early progenitors abolished commitment to the DC lineage, indicates that STAT5 activity needs to be carefully regulated. It was speculated very recently that STAT5 activation promotes development of mouse conventional DC (22, 32), a DC lineage related to the human myeloid DC subsets (6). However, our data not only show myeloid subset-specific requirements for STAT5 activity but also clearly demonstrate that this process is not regulated in a simple on–off binary manner. Instead, careful regulation of the level of STAT5 activity appears crucial.

The skewing toward the LC lineage upon inhibition of STAT5 activity showed similarities with TGF-β–induced LC commitment as previously reported (27, 28). In line with previous studies demonstrating TGF-β–induced inhibition of STAT5 transcription factor activity (33–35), we observed reduced STAT5 phosphorylation upon addition of TGF-β. Furthermore, both exposure to TGF-β and inhibition of STAT5 transcription factor activity were associated with an increased expression of PU.1, a critical transcription factor for DC development (28, 31, 36, 37). These data suggest that TGF-β–induced LC skewing may be mediated by inhibition of STAT5 activity, resulting in elevated PU.1 expression.
levels favorable for LC commitment (28, 38) (Fig. 7). Because PU.1 has an instructive role in cytokine-driven DC development (28, 31), but cannot replace the cytokines inducing DC differentiation (28), PU.1 can only favor DC development within the proper microenvironment. Therefore, the seeming contradiction between the increased PU.1 expression but inhibited intDC development upon STAT5 inhibition could be explained by other transcriptional targets of STAT5 involved in intDC development (Fig. 7).

The inverse correlation between STAT5 activity and PU.1 expression could also be responsible for the block in myeloid DC development upon ectopic STAT5 activation in HPC. PU.1 is indispensable for both myelopoiesis (39) and DC development (31, 36, 40). Thus, the loss of PU.1 upon increased STAT5 activity could account for the reported block in myelopoiesis (12, 14) and the abolished DC development shown in this study. Although we cannot fully exclude that PU.1 loss is the consequence rather than the cause of reduced DC development, STAT5a1*6-ER–transduced HPC stimulated for 24 h with 4-OHT demonstrated 40% reduction in PU.1 mRNA levels (A.T.J. Wierenga, unpublished observations), supporting the view that PU.1 expression is regulated by STAT5. Moreover, a recent study showed that increased STAT5 activity inhibits myelopoiesis and promotes erythrocyte differentiation through GATA-1, a functional antagonist of PU.1 (41). Although no mature erythrocytes could be observed in the current study, the induction of CD71 expression by increased STAT5 activity suggests partial differentiation toward the erythrocyte lineage. Erythrocyte differentiation has been shown to benefit from ectopic STAT5 expression (16), but their full differentiation probably requires more than STAT5 activation alone.

Although STAT5 activation should be low in CD34+ HPC to allow DC commitment, increased STAT5 phosphorylation was observed during pre-DC to DC development, and ectopic activation of STAT5 in pre-DC improved their terminal differentiation. Although seemingly contradictory, altered roles of STAT5 at distinct developmental stages appear relevant in vivo. Serum concentrations of GM-CSF and other STAT5-activating cytokines are normally low (42), inducing low levels of STAT5 activation optimal for the commitment of early hematopoietic progenitors to DC lineages. However, pre-DC not only continuously replenish DC during steady state but are also attracted to sites of infection or inflammation to increase the DC pool locally (43). The high GM-CSF concentrations at these sites will result in increased STAT5 activation in the pre-DC, aiding in their prompt differentiation to fully functional DC (Fig. 7). Unfortunately, currently available in vivo models to investigate human DC development do not provide the opportunity of differential manipulation during distinct phases of DC differentiation. It will therefore remain a major challenge to confirm the important role of STAT5 as shown here in an in vivo model that allows careful regulation of STAT5 activity at different stages of DC development. In addition, replication of the current data in an in vitro system that includes Flt3 ligand to mimic better the cytokine situation in the steady state would be interesting, as the combination of GM-CSF, SCF, and TNF-α used in the current study may contribute to a more proinflammatory environment than generally present in vivo.

Enhanced differentiation induced by increased STAT5 activation in pre-DC did not reduce the functionality of the resulting DC. Furthermore, these intDC even modestly increased IFN-γ secretion by allogeneic T cells. This could indicate a direct role for STAT5 in DC function, which would be in accordance with previous studies suggesting regulation of DC maturation or function by JAK2–STAT5 signaling (44–46). Alternatively, the increased proportion of BDCA-3+ DC in these cultures (L. van de Laar, unpublished observations) could also contribute to Th1 skewing, as the recently characterized BDCA-3+ DC were shown to promote Th1 differentiation (47).

In conclusion, we show a critical function for STAT5 in controlling development of human intDC and LC. STAT5 is redundant for LC commitment but required to induce pre-intDC differentiation of HPC. However, STAT5 activation levels should be low to allow commitment to either DC lineage. In contrast, high STAT5 activity promotes terminal differentiation of already committed pre-DC. Thus, specific requirements for the level of STAT5 activation exist at distinct stages of intDC and LC differentiation. Regulation of the STAT5 activation status may therefore be one of the mechanisms by which cytokines determine the fate of possible DC precursors at various locations under different pathophysiologic conditions. Fundamental insight into the intracellular signal transduction pathways and transcription factors involved in human DC development will improve the understanding of how continuous DC replenishment from their hematopoietic progenitors is regulated and homeostasis of these important immune cells is maintained.

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References
Supplementary Legends

Figure S1. Similar expression of costimulatory molecules by DC in control and STAT5aΔ750 cultures

CD34⁺ HPC were transduced, cultured and FACSorted as for Figure 2E,F. LPS was added from day 13. At day 15, cells were analyzed for the expression of CD1a, BDCA-1, CD83, CD86 and HLA-DR. Representative FACS plots show CD83, CD86 and HLA-DR expression by CD1a⁺BDCA1⁺-gated DC (n=3).

Figure S2. Expression of lineage markers

CD34⁺ HPC were transduced and FACSorted as for Figure 3E. The expression of CD3, CD14, CD15, CD20, CD33, CD42b, CD45, CD56, CD61, CD71 and GPA was analyzed at day 13. Representative FACS plots are shown (n=2).

Figure S3. Effects of conditional STAT5 activation on yields, survival and costimulatory molecule expression

CD34⁺ HPC were transduced, FACSorted and cultured as described in Figure 4 (AB) and 5 (C-E). AC) At day 8, 9 and 10, cells were harvested and counted with trypan blue exclusion. Cell yields were standardized to the control cultures. Data represent mean±SEM standardized yields of at least 3 independent experiments with different donors. BE) LPS was added from day 10. Representative FACS plots show CD86 and HLA-DR expression at day 12 (n=3). D) Day 10 cells were analyzed for Annexin V/7-AAD staining. Representative FACS plots are shown (n=3). * p<0.05, paired students t-test
**Figure S4. Treatment of control pre-DC with 4-OHT**

Control CD14<sup>-</sup>CD1a<sup>-</sup> pre-intDC (A-D) or CD14<sup>-</sup>CD1a<sup>+</sup> pre-LC (E-I) were FACSorted at day 7 and further cultured with 4-OHT or EtOH. Data are representative of at least 2 independent experiments with different donors. **AB)** Cells were analyzed for CD1a and CD14 expression at day 8, 9 and 10. The percentages CD1a<sup>-</sup> and CD1a<sup>+</sup> cells were determined, and the ratios were calculated and standardized to the control cultures. Representative FACS plots of day 9 cells (A) and mean±SEM CD1a<sup>-</sup>/CD1a<sup>+</sup> ratios (B) are shown. **CD)** Representative FACS plots showing DC-SIGN and BDCA-1 expression at day 10. Data are representative of at least 2 independent experiments with different donors. **EF)** Cells were analyzed for CD1a and Langerin expression at day 8, 9 and 10. The percentages of Langerin<sup>-</sup> and Langerin<sup>+</sup> cells were determined, and the ratios were calculated and standardized to the control cultures. Representative FACS plots of day 9 cells (E) and mean±SEM Langerin<sup>-</sup>/Langerin<sup>+</sup> ratios (F) are shown. **G)** Cells were analyzed for Annexin V/7-AAD staining at day 10. Representative FACS plots are shown. **H)** Cells were harvested at day 8, 9 and 10 and counted with trypan blue exclusion. Data represent mean±SEM cell yields, standardized to control. **I)** Representative FACS plots showing CD1a and BDCA-1 expression at day 10.
CD83

CD86

HLA-DR

% of max

log10 fluorescence

- isotype
- Control
- STAT5aΔ750