Progressive and Controlled Development of Mouse Dendritic Cells from Flt3+CD11b+ Progenitors In Vitro

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Dendritic cells (DC) are the most professional APC of the immune system and represent key components for induction of primary immune responses and maintenance of immunological tolerance (1–3). DC originate from hematopoietic stem cells in bone marrow through consecutive steps of differentiation, which can be distinguished by phenotype, localization, and function. Four steps of DC development can be delineated: bone marrow progenitor cells develop into circulating precursor DC in blood, which enter tissues where they reside as sentinel immature DC and encounter pathogens. Following Ag uptake and activation by inflammatory signals, immature DC undergo a process of maturation, which is tightly linked with their migration to secondary lymphoid organs, where they initiate primary immune responses (1, 2).

Different DC subsets have been identified both in lymphoid and nonlymphoid organs, but their relationship and developmental origins remain unclear or highly controversial (4–7). It has been proposed that mature DC originate from both myeloid and lymphoid-committed precursors. In mouse, CD11c+CD11b+ DC represent the classical myeloid tissue DC that are found in almost all tissues. CD8α expression was used to subdivide DC subsets (4–6, 8), but does not delineate DC origin (9–11). Plasmacytoid DC (pDC) represent yet another DC subclass, which was initially characterized by production of large amounts of type 1 IFN in response to virus and bacteria (12, 13).

Recent studies on gene knockout mice revealed novel insights into how DC develop. ReλB−/− mice lack CD11c+CD11b+ DC, whereas other DC subsets are apparently normal (14–16). Mice deficient for IFN consensus sequence binding protein lack pDC (17). Id2−/− mice lack Langerhans cells, the cutaneous contingent of DC, and also the CD8α+ splenic DC subset (18, 19). B cells and pDC were increased in these mice (18, 20). These studies suggest that specific DC subsets develop through independent pathways. Similarly, a bone marrow Flt3+ precursor population contains early DC precursors for all DC subsets and comprises Flt3+ common lymphoid precursors (CLP) and Flt3+ common myeloid precursors (CMP; Ref. 7).

A large number of protocols have been developed for generating DC in vitro. Frequently, mouse DC are obtained in vitro from bone marrow based on the protocol initially described by Inaba et al. (21, 22) using GM-CSF. Flt3 ligand (FL) was observed to increase DC numbers in vivo and in vitro, and this relates well to Flt3 expression on early DC precursor in mouse bone marrow (Ref. 7 and references therein, 23–26). Furthermore, FL and GM-CSF were demonstrated to differentially regulate development of CD11c+CD11b+ DC and pDC, respectively (25).

A variety of cytokines and growth factors have been reported to efficiently expand hematopoietic progenitors in vitro, including stem cell factor (SCF), FL, insulin-like growth factor 1 (IGF-1), IL-3,
IL-6 and soluble IL-6R fusion protein (hyper-IL-6), and different combinations thereof (Refs. 27–32, and references therein). DC precursors were first amplified by SCF, FL, Tpo, and hyper-IL-6, and then induced to differentiate into DC by GM-CSF and IL-4 (18, 32). Using this culture system, fully functional DC were obtained and used to monitor the changes in gene expression during DC development by DNA microarrays (18, 32).

In this study, we have extended these studies to mouse DC and developed a two-step amplification/differentiation system to study a Flt3+CD11b+ progenitor from mouse bone marrow. This progenitor was first amplified in vitro under the aegis of a specific stem cell cytokine/growth factor mixture and then differentiated into DC with GM-CSF. By transcriptional profiling, we identified several stem cell marker genes including Flt3, IL-6R, c-kit/SCF receptor, and the stem cell Ags CD93/AA4.1 and CD133/AC133, which are expressed on Flt3+CD11b+ progenitors, and their expression declines during DC differentiation.

Materials and Methods

Cell lines and culture

For generation of Flt3+CD11b+ progenitor cells and DC, bone marrow suspensions were prepared from C57BL/6 and BALB/c mice (Charles River). Cells were seeded at $2 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (all from Invitrogen Life Technologies), and 50 μM 2-ME (Sigma-Aldrich) containing recombinant murine SCF (100 ng/ml), 25 ng/ml FL (PeproTech), 40 ng/ml recombinant long-range IGF-1 (Sigma-Aldrich), 5 ng/ml rIL-6/soluble IL-6R fusion protein (hyper-IL-6; Ref. 33), 20 U/ml recombinant mouse GM-CSF, and 10–30 M dexamethasone. After 3 days in culture, cells were subjected to Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/ml; Eurobio) to remove residual erythrocytes, dead cells, and debris. Medium with growth factors was replenished every 2 days, and cells were maintained at $2 \times 10^6$ cells/ml cell density. After 7 days, differentiation into DC was induced in culture medium supplemented with 200 U/ml recombinant mouse GM-CSF. Every 2 days, medium was replenished, and cells were maintained at $2 \times 10^6$ cells/ml cell density. Alternatively, DC were generated from mouse bone marrow using the protocol described by Inaba et al. (21, 34, 35).

In some experiments, 100 ng/ml TNF-α or 1 μg/ml LPS was added to induce DC maturation. Cell numbers were determined with an electronic cell counter device (CASY1; Schärfe Systems).

Hemopoietic stem cells from bone marrow of 8- to 12-wk-old NMRi mice were depleted of lineage-positive cells using immunomagnetic beads (Miltenyi Biotech) and negative cells were then sorted with a FACSVantage device (BD Biosciences) for c-kit (ACK45) and Sca-1 (E13-161.7) into lin –c-kit+Sca-1+ and lin –c-kit+Sca-1– populations, and lin –c-kit+Sca-1+ cells were used for further analysis.

Cell proliferation assay

A total of $5 \times 10^4$ cells were incubated in 200 μl of medium in a 96-well flat-bottom microtiter plate for 72 h at 37°C with the indicated growth factors and combinations thereof. Samples were then pulsed for 2 h with 0.75 μCi/well [3H]thymidine (29 Ci/mmol; Amersham Biosciences) and harvested onto glass fiber filters. Radioactivity was measured by liquid scintillation counting in a Microbeta counter (Wallac).

Flow cytometry

Flow cytometry analysis for surface Ag expression was performed as previously described (35) using the following Abs for staining and sorting: FITC-conjugated anti-MHC class II (I-A/I-E; clone 2C9), CD93-FTTC (AA4.1), CD11c-PE (HL3), CD11b-bio (M1/70), CD49f (GoH3), CD54 (3E2), CD80 (1G10), CD135/Flt3-PE (A2F10.1), and re- spective isotype controls (all purchased from BD Biosciences). CD8α-TRI-COLOR (CT-CD8α), CD40-bio (2/23), and CD86 (RMMP2), isotype- matched FITC- or PE-labeled secondary Abs, streptavidin-FITC and streptavidin-TRI-COLOR were from Caltag Laboratories. Anti-mouse CD83 (clone Michel 17) was obtained from Biocarta. Anti-mouse CXCXR4/CD11b+ progenitors were provided by R. Forster (Hannover Medical School, Hannover, Germany). Anti-prominin/AC133 Ab (36, 37) was kindly provided by W. Huttner and D. Corbeil (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Cells were analyzed by FACScalibur flow cytometer using CellQuest software (BD Biosciences).

Cytokine production in response to LPS, CpG oligodeoxynucleotide (ODN), and viruses

For analyzing IL-12 and IFN-α production in response to LPS, CpG ODN, or viruses, 1–10$^5$ DC/ml (day 10 of differentiation) were stimulated with either LPS (1 μg/ml), CpG ODN 1668 (100 ng/ml), influenza virus (multiplicity of infection (MOI), 1 and 10), and HSV (MOI, 1 and 3), and supernatants were collected after 24-h incubation. ELISA for detection of IFN-α (PBL Biomedical Laboratories) and IL-12 p70 (eBiosciences) was performed according to the manufacturer’s instructions.

Chemotaxis assay

Chemotaxis assay was performed as described by Kellermann et al. (38) with minor modifications (35). Briefly, Transwell inserts (5-μm pore size; Costar) were preincubated in medium to block unspecific binding, and 2 × 10$^5$ cells were seeded in the upper compartment. ELC chemokine (100 ng/ml) was added to the lower compartment to analyze migration toward gradient. After 90 min, 1 × 10$^5$ Dynabeads (15-μm diameter; Dynal Meters) were added to the lower chamber to allow normalization for variations in the experimental procedure. Cells and beads were recovered, and samples were reacted with fluorescein-di-ace to stain for live cells and analyzed by flow cytometry. By gating on beads, the ratio of beads to fluorescein-di-ace-positive cells was determined and allowed a precise calculation of the number of transmigrated cells.

In vitro stimulation of TCR transgenic T cells

CD8+ T cells from OT-I mice express a transgenic TCR that recognizes OVA257–264 peptide on H-2K$^b$ (39, 40). Splenocytes of OT-I mice were prepared and CD8+ T cells were obtained by immunomagnetic bead purification using MACS anti-CD8 microbeads (Miltenyi Biotech) according to manufacturer’s instructions. CD4+ T cells from DO11.10 mice expressing a transgenic TCR that recognizes OVA257–264 peptide on I-A$^b$ (41) were prepared accordingly using anti-CD4 microbeads (Miltenyi Biotech) for purification. Cells were pulsed with 0.1 μM OVA257–264 peptide (SINNFEKL) for 16 h. After washing, cells were then reseeded in RPMI 1640 medium with 10% FCS, and labeling was performed with 10 μM CFSE for 10 min at 37°C. Labeling was stopped by adding 1 vol of cell culture medium, and cells were washed an additional two times in medium.

CFSE labeling of OT-I splenocytes

Fluorescence labeling of OT-I splenocytes with CFSE (Molecular Probes) was performed essentially as described (42). In brief, single-cell suspensions of splenocytes were isolated from OT-I mice and erythrocytes were lysed in hypotonic buffer. Cells were resuspended in PBS at 1 × 10$^7$ cells/ml, and labeling was performed with 10 μM CFSE for 10 min at 37°C. Labeling was stopped by adding 1 vol of cell culture medium, and cells were washed an additional two times in medium.

Adaptive transfer and in vivo activation of OT-I T cells

A total of 5 × 10$^4$ CFSE-labeled OT-I spleen cells in 500 μl of PBS was injected into the tail vein of sex-matched C57BL/6 recipients. One day later, 5 × 10$^4$ untreated DC, DC pulsed with 0.1 μM OVA257–264 peptide (SINNFEKL) for 30 min, or PBS only were applied to recipient mice by i.v. injection. As a positive control for effective activation, OVA protein in INF-γ was injected s.c. Three days later, single-cell suspensions were prepared from spleen of recipients and stained with PE-labeled anti-CD8, anti-CD4, and anti-CD11b mAbs. A population of double-negative OT-I cells was detected and analyzed by flow cytometry. By gating on beads, the ratio of beads to fluorescein-di-ace-positive cells was determined and allowed a precise calculation of the number of transmigrated cells.

Adaptive transfer and in vivo differentiation of Flt3+CD11b+ progenitors

Flt3+CD11b+ progenitors were generated from C57BL/6-Ly5.1 as described above. Amplified progenitors were i.v. injected at a ratio of 1:1 or 1:4 into lethally irradiated (5 Gy twice with a 3-h interval) C57BL/6 Ly5.2 recipients, along with 1 × 10$^7$ unfractionated Ly5.2 bone marrow cells to ensure mouse survival (43). Alternatively, Flt3+CD11b+ progenitors were cultured without FL for 16 h and sorted for Flt3 and CD11b into Flt3+CD11b+ and Flt3+CD11b− cells using a FACSVantage device. Sorted cell populations were then i.v. injected at a ratio of 1:1 along with
fl. 1 × 10^6 unfraccionated Ly5.2 bone marrow cells. At various times after cell transfer, single-cell suspensions were prepared from spleen of recipients, and three-color staining with FITC-labeled anti-Ly5.1 (A20; BD Biosciences) and CD11c-PE in combination with CD8α or CD11b was performed to examine the generation of donor-derived DC. Donor-derived hematopoietic lineage cells were distinguished by FITC-labeled anti-Ly5.1 in combination with CD11b-1b (M1/70, Gr-1-bio (RB6-8C5), CD19 (1D3), and CD3e-bio (145-2C11; all from BD Biosciences). Three recipient mice were used for each setting in individual experiments.

RNA isolation and DNA microarray analysis

RNA isolation, cDNA and cRNA synthesis, DNA microarray hybridization and analysis for DC and Flt3^CD11b^ progenitor cells were conducted essentially as described before (18, 32). In brief, total RNA was isolated with RNeasy kits including DNase digestion (Qiagen). Five micrograms of total RNA was used to generate cDNA according to the Expression Analysis Technical Manual (Affymetrix). cRNA was generated with the Bioarray High-Yield Transcript Labeling kit (ENZO). Total cellular RNA from a total of 75,000 lin^c-kit^-Sca-1^- stem cells per experiment was isolated with RNeasy Mini kit (Qiagen). In vitro transcription-based RNA amplification was performed essentially as described (44), using the Ambion Megascript T7 kit for two rounds of in vitro transcription reaction. Finally, 10 μg of cRNA per sample was hybridized to Affymetrix mouse U74A arrays at 45°C for 16 h. DNA chips were stained, washed, and scanned according to the manufacturer’s protocol.

Bioinformatics

Scanned GeneChip.DAT files were analyzed by the Affymetrix Microarray Suite analysis software. Normalization and conversion of relative expression intensities into log ratios was performed with GeneSpring software (Silicon Genetics) as described (18). The normalization procedure in GeneSpring converts negative values to 0. For further analysis, genes were filtered for the criteria to appear as abundant transcripts (expression call "present" by the Affymetrix Microarray Suite analysis software) in at least one sample per experiment. Classification of genes encoding for mouse CD molecules was done using information from various databases including Entrez PubMed (www.ncbi.nlm.nih.gov/entrez/query.fcgi), the mouse genome informatics database (www.informatics.jax.org/), and the PROW database for CD molecules (www.ncbi.nlm.nih.gov/prow/). Hierarchical clustering (45) for those filtered CD molecules was done in GeneSpring using the Pearson correlation with a separation ratio of 0.5 and a minimum distance of 0.001. Microarray data were submitted to GEO database (www.ncbi.nlm.nih.gov/geo/; series accession nos. GSE575, GSE692, and GSE693).

Results

In vitro growth of Flt3^CD11b^ progenitors from bone marrow

Mouse bone marrow cells were cultured for up to 3 wk in the presence of FL, SCF, IGF-1, hyper-IL-6, dexamethasone, and low concentrations of GM-CSF (Fig. 1, A and B). These factors were chosen based on previous studies demonstrating expression of the respective receptors on hematopoietic lin^-c-kit^-Sca-1^- stem cells and DC precursor cells (Refs. 46 and 47; R. D. Kirsch, T. Hieronymus, and M. Zenke, unpublished data; see also Fig. 5A). Next, we quantitatively assessed the activity of FL, SCF, IGF-1, hyper-IL-6, and GM-CSF on progenitor growth by using unfraccionated bone marrow and established progenitor cells (day 7 of culture) and by measuring DNA synthesis in response to factors. When individual factors were applied, SCF, FL, and GM-CSF were found to be most effective (Fig. 1A). The activities of hyper-IL-6 and IGF-1 were less pronounced individually, yet these factors exhibited synergistic effects when applied in various combinations. FL together with either SCF or hyper-IL-6 plus IGF-1 clearly enhanced proliferation rates. A maximal response on bone marrow cells was observed when all factors were applied (Fig. 1A). This was different for established cultures where addition of GM-CSF decreased proliferation rates, presumably because GM-CSF down-regulates Flt3 and IGF-1 receptors (see Fig. 5, cluster IV, and data not shown). In addition, we noticed that an efficient outgrowth of progenitor cells from bone marrow was critically dependent on dexamethasone, which significantly reduced the rate of spontaneous differentiation (T. Jorgas, T. Hieronymus, and M. Zenke, unpublished data) as observed in other cell systems (29, 48, 49).

Total number of cells had increased by ~100-fold within 3 wk of culture (Fig. 1B). Cells retained their capacity to respond to growth factors (either individually or in combination) with time in culture (data not shown). Amplified progenitor cells were spherical (Fig. 1C) and expressed Flt3/CD135 and CD11b (Fig. 1D and see Fig. 4A). Surface Flt3 expression on cultured cells was not detected in FL-supplemented medium, presumably due to receptor internalization in response to ligand, but was readily observed in the absence of FL. Cells were negative for lineage markers such as CD3, CD4, CD8, CD11c, CD14, CD34, B220, and Ter119 (see Fig. 6, A and D, and data not shown). Therefore, we will in the following refer to these precursor cells as Flt3^-CD11b^ progenitors.

To address the question of the frequency of the Flt3^-CD11b^ progenitors in vivo, we analyzed isolated bone marrow cells for expression of Flt3/CD135 and CD11b (Fig. 1E). Flt3 expression was found on 3.8 ± 0.9% (mean value ± SEM; n = 5) of total bone marrow in accordance with previous results (11). Importantly, 5.4% of the CD11b^- cells (ranging from 2.8 to 8.6%; n = 5) coexpressed Flt3, demonstrating that the Flt3^-CD11b^ progenitors are present in bone marrow with a frequency of ~2–2.5%. Thus, the amplification rates achieved during in vitro culture are ~4000- to 5000-fold corresponding to ~12 cell division cycles.

Phenotypic and functional characterization of DC derived from amplified Flt3^-CD11b^ progenitors

Flt3^-CD11b^ progenitors were then induced to differentiate into DC by replacing growth-promoting factors (day 7) with a high dose of GM-CSF only (Fig. 1B), thereby using conditions that are frequently used for bone marrow-derived DC (21, 34, 35). Following induction of differentiation, cells underwent two to three additional cell division cycles (days 7–11), which led to a ~34-fold increase in total cell numbers (n = 8; ranging from 13.5- to 37-fold). At days 11–12, cells ceased proliferation and concomitantly acquired prominent dendritic processes and cytoplasmic projections that represent hallmarks of DC phenotype (Fig. 1C). Using this two-step culture system, we were able to obtain 0.7-2.1 × 10^5 DC from one bone marrow preparation.

DC development was monitored by assessing the expression profile of specific cell surface proteins by flow cytometry. Expression of MHC class II and CD11c, two commonly used cell surface Ags of mouse DC, was measured at various time points throughout differentiation. As shown in Fig. 2, Flt3^-CD11b^ progenitor cells expressed neither MHC class II nor CD11c. Importantly, expression of these cell surface proteins increased during DC development and, within 8–9 days of differentiation, resulted in a homogenous population of CD11c^- and MHC class II^- cells. To further characterize the progenitor population and the DC obtained, cells were examined for expression of a variety of cell surface molecules known to be involved in DC function. We found the Flt3^-CD11b^ progenitor cells to be negative for CD40, CD54, CD80, and the DC activation marker CD25. Interestingly, these molecules were also absent or expressed at low levels on differentiated DC, indicating that the DC obtained are immature (Figs. 1–3 and Table I). To induce DC maturation, cells were treated for 16 h with either 100 ng/ml TNF-α or 1 μg/ml LPS, and expression of surface Ags was assessed. As expected, both stimuli effectively up-regulated the expression of MHC class II, CD25, CD40, CD54, CD80, CD83, and CD86 on DC (Figs. 2 and 7, Table I, and data not shown). Thus, with the differentiation conditions applied, we consecutively generated homogenous populations of immature and mature DC.
Cytokine and chemotactic responses of in vitro-differentiated DC

In vitro-differentiated DC responded to various pathogen-associated molecules (LPS and CpG ODN) or influenza virus (Flu) and HSV (Fig. 3A). DC effectively produced IL-12p70 in response to LPS, whereas the response to CpG ODN was less pronounced.

FIGURE 1. Growth of bone marrow-derived Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells and their differentiation into DC. A, Growth factor response of bone marrow cells (□) and established progenitor cells at day 7 of culture (■). Factors were applied to cells as indicated, and [<sup>3</sup>H]thymidine incorporation was determined 72 h later. Results (mean triplicate values ± SD) from one representative of three individual experiments are shown. B, Growth kinetics of Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells (⊙) and differentiated DC (■) from bone marrow. Cells were cultivated as described in Materials and Methods. Differentiation of DC from Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells was induced at day 7 of cell culture. Cumulative cell numbers were determined in regular time intervals after Ficoll density gradient purification of progenitor cells at day 3 of culture. Results are mean values ± SD of five and eight different experiments for growth of progenitor cells and differentiation of DC, respectively. The total number of cells increased ~2-fold within the first 3 days under growing conditions, indicating effective response to the factor combination used (mean value ± SEM, 2.0 ± 0.1; n = 20). C, Morphology of Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells at day 7 (inset) and DC at day 17 of culture (day 10 differentiation condition) by phase contrast microscopy on fibronectin-coated coverslips. Bars represent 10 µm. D, Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells (day 7) were cultured with FL (+FL) or without FL (−FL) for 6 and 16 h as indicated, and cells were analyzed for Flt3/CD135 and CD11b surface expression by flow cytometry. Open areas represent staining with isotype control Ab.

FIGURE 2. Time course of MHC class II and CD11c surface expression on differentiating DC from Flt3<sup>+</sup>CD11b<sup>-</sup> progenitor cells. After 7 days of culture, Flt3<sup>+</sup>CD11b<sup>-</sup> progenitors were induced to differentiate into DC by applying GM-CSF (day 0). The kinetics of MHC class II and CD11c surface expression by flow cytometry is shown (days 0–11). Cells treated with TNF-α at day 10 for an additional 24 h are also shown (day 11+TNF-α). Open areas represent staining with isotype control Ab.

Cytokine and chemotactic responses of in vitro-differentiated DC

In vitro-differentiated DC responded to various pathogen-associated molecules (LPS and CpG ODN) or influenza virus (Flu) and HSV (Fig. 3A). DC effectively produced IL-12p70 in response to LPS, whereas the response to CpG ODN was less pronounced. There was
no IL-12p70 production in response to Flu and HSV viruses. However, these viruses induced production of IFN-α (Fig. 3A).

DC express chemokine receptors and respond to various chemokines (1, 2). CCR7 represents a chemokine receptor with a pivotal role in DC migration toward secondary lymphoid organs. Therefore, Transwell experiments were performed to evaluate the migratory capacity of DC in response to CCR7 ligand ELC at different stages of differentiation. DC at day 10 of differentiation were highly active in migration toward ELC, whereas Flt3⁺CD11b⁺ progenitor cells were as expected inactive (Fig. 3B). Stimulation of DC with TNF-α resulted in an increased number of transmigrated cells both in the presence and absence of ELC (data not shown). DC at day 7 of differentiation showed only a limited capacity to migrate in response to ELC, indicating the immature phenotype at this stage of DC differentiation (Fig. 3B).

In vitro-generated DC are fully active in Ag-specific T cell activation in vitro and in vivo

One of the major hallmarks of DC is their potent capacity to activate naive T cells in an Ag-dependent manner. We therefore determined the Ag-specific MHC class I- and II-restricted T cell stimulation capacity of the Flt3⁺CD11b⁺ progenitor cell-derived DC in vitro and in vivo. Chicken OVA and Ag-specific CD8⁺ and CD4⁺ T cells of TCR transgenic OT-I and DO11.10 mice, respectively, were used. OT-I T cells recognize OVA257–264 peptide in context of MHC class I H-2-Kb, whereas DO11.10 T cells detect MHC class II I-Ad-presented OVA323–339 peptide. First, syngeneic DC were activated with TNF-α for 16 h, or left untreated. Cells were then pulsed with the respective peptides and analyzed in co-cultures with CD8⁺ or CD4⁺ T cells of TCR transgenic mice for
ag-specific T cell proliferation. DC effectively stimulated proliferation of naive T cells in vitro, which was further enhanced by TNF-α treatment of DC, whereas progenitor cells and unpulsed DC were inactive (Fig. 3, C and D). DC obtained by the frequently used one-step culture system with GM-CSF by Inaba et al. (21) were analyzed in parallel and found to be 2- to 4-fold less efficient in T cell activation than the DC generated by the two-step culture system described in this paper (Fig. 3, C and D, lower panels). This might be because with the two-step culture system DC differentiation is highly synchronous and yields homogenous DC populations of the same maturation state. Second, the capability of DC to prime naive T cells in vivo was analyzed. CFSE-labeled OT-I T cells were transferred into syngeneic wild-type recipient mice and immunized 1 day later with unpulsed or OVA peptide-pulsed DC. Three days later, splenocytes were examined for proliferative response of transferred OT-I T cells by flow cytometry. Immunization with OVA peptide-pulsed DC effectively stimulated T cell proliferation as demonstrated by a shift of CFSE-positive transgenic T cells (Fig. 3E) and a 1.5 ± 0.2-fold increase in total cell number compared with control (mean value ± SEM of four independent experiments). This result is similar to the effective stimulation of T cell proliferation using OVA-protein with Freund’s adjuvant (mean value ± SEM, 1.9 ± 0.2; n = 4). In addition, the potency for in vivo T cell stimulation of DC generated from Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors was similar to that of DC obtained with GM-CSF from bone marrow (Fig. 3E) using the frequently used protocol by Inaba et al. (21, 34, 35). This result clearly demonstrate the potency of the Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cell-derived DC to prime naive T cells in vivo.

Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells differentiate into DC in vivo

Finally, we addressed the question of whether the in vitro-amplified Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors have the capacity to differentiate into DC in vivo. Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells from Ly5.1 mice were generated and transferred into lethally irradiated Ly5.2 recipient mice either as bulk cultures or as sorted populations of Flt3<sup>+</sup>CD11b<sup>+</sup> or Flt3<sup>+</sup>CD11b<sup>+</sup> cells (Fig. 4, A and B). After 1 and

### Table I. Mean fluorescence intensities (MFI) of selected surface Ags in development of DC from Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells<sup>a</sup>

<table>
<thead>
<tr>
<th>Surface Ags</th>
<th>Progenitor</th>
<th>DC</th>
<th>DC+TNF-α</th>
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</thead>
<tbody>
<tr>
<td>CD11c (n = 9)</td>
<td>4.8 ± 1.8</td>
<td>473 ± 190</td>
<td>470 ± 164</td>
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<tr>
<td>MHC II (n = 9)</td>
<td>4.7 ± 1.4</td>
<td>284 ± 51</td>
<td>1380 ± 361</td>
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<tr>
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<td>31.0 ± 17</td>
<td>53 ± 9</td>
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<tr>
<td>Iso control II (n = 6)</td>
<td>4.8 ± 1.4</td>
<td>31.0 ± 17</td>
<td>53 ± 9</td>
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</table>

<sup>a</sup> Progenitors were grown for 7 days and differentiated into DC and stimulated with TNF-α at day 10 of differentiation for 16 h (DC + TNF-α) or left untreated (DC). Cells were stained for the indicated surface Ags using either directly labeled mAbs or indirect staining as described in Materials and Methods. Values represent mean MFI ± SD from (n) independent experiments.

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**FIGURE 4.** Generation of DC from Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells in vivo. A. In vitro-amplified Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors from Ly5.1 mice were sorted for Flt3 and CD11b expression into Flt3<sup>+</sup>CD11b<sup>+</sup> and Flt3<sup>-</sup>CD11b<sup>+</sup> cells and used for adoptive transfer experiments (B). B. Unfractionated Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells and sorted cell populations (A) were transferred i.v. into lethally irradiated Ly5.2 recipient mice. Single-cell suspensions from spleen of recipient mice were analyzed for the presence of donor-derived CD11c<sup>+</sup> DC 1 wk after transfer. Donor-derived DC were further characterized for expression of CD11b and CD80. The data shown are one representative of six individual experiments. C. In vitro-amplified Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors from Ly5.1 mice were examined for the generation of hematopoietic lineage cells. One week after transfer, single-cell suspensions from spleen of recipient mice were analyzed for the presence of monocytes/macrophages (CD11b), granulocytes (Gr-1), B cells (CD19), and T cells (CD3e). The data shown are one representative of six individual experiments.
2 wk, spleens of recipients were analyzed for donor-derived cells. CD11c<sup>+</sup> cells were already detectable 1 wk after precursor transfer. For unfraccionated cell populations, 8.1–16.0% of total Ly5.1 donor cells were CD11c<sup>+</sup> (mean = 12.3; n = 6) and were found to be both CD11b positive and negative as well as CD8α positive and negative (Fig. 4B). Similarly, adoptive transfer of highly purified Flt3<sup>+</sup>CD11b<sup>+</sup> cells gave CD11c<sup>+</sup> cells that were CD11b<sup>+</sup> or CD11b<sup>−</sup> and CD8α<sup>+</sup> or CD8α<sup>−</sup> (Fig. 4B, right panel). These results indicate that Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells can give rise to the main conventional DC subtypes in vivo. CD11c<sup>+</sup> cells were also found comprising CD11b<sup>+</sup> macrophages and granulocytes, and no cells were found to express lymphoid-related lineage surface markers such as CD19 and CD3e (Fig. 4, B, right panel, and C). Adoptive transfer of highly purified Flt3<sup>+</sup>CD11b<sup>+</sup> cells gave the same result, which might be due inefficient staining of these cells because of the absence of surface Flt3.

In summary, our data clearly show that the two-step in vitro amplification/differentiation system described in this paper generates large numbers of homogeneous Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells with sustained proliferative capacity. Additionally, these progenitor cells can be induced to undergo synchronous differentiation and develop into fully functional DC in vitro and in vivo. Thus, the system provides an excellent means of generating DC at successive stages of development that is most suitable for further cellular, biochemical, and molecular studies.

**Gene expression profiling of cell surface Ags during DC differentiation**

Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors and DC at different stages of development were subjected to transcriptional profiling by DNA microarrays to gain new insights into differentially regulated genes involved in the DC differentiation program, thereby applying a strategy that was successfully applied before for human DC (18, 32). RNA from Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells and DC was prepared and subjected to DNA microarray analysis using Affymetrix GeneChip arrays that contain probe sets for ~6000 known genes and ~6500 expressed sequence tags. DC at days 7 and 10 of differentiation, and DC treated with TNF-α at day 9 for an additional 24 h (DC day 10 plus TNF-α) were analyzed. Samples from three independent experiments were generated, and RNA probes were hybridized to the microarrays.

Surface Ags are frequently used to characterize stem and early progenitor cells, and changes in surface Ag expression represent one of the hallmarks of DC differentiation. Approximately 200 genes encoding CD molecules are represented on the DNA microarray, and 122 surface Ags were found to be expressed in progenitor cells and/or differentiated DC. Hierarchical clustering (45) was used to unveil specific patterns inherent to the DC differentiation program. This method revealed distinct clusters of differentially regulated genes and also genes that were abundantly expressed in Flt3<sup>+</sup>CD11b<sup>+</sup> progenitors and DC and that did not significantly change during differentiation. Four major clusters of regulated genes were identified: clusters I–III contain genes that were highly expressed in developing DC but not (or at low levels) in Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells, and cluster IV comprises genes that were highly expressed in progenitors and down-regulated during DC differentiation (Fig. 5; a comprehensive list of the clustered genes is provided as supplementary material).4

Cluster I contains genes with prominent expression in TNF-α-treated DC, including molecules that specify activated and mature DC such as CD40, CD54, and CD83, the costimulatory molecules CD80 and CD86, and the chemokine receptor CCR7 (CD197; Fig. 5) (1, 2). Cluster II comprises genes expressed in DC that were not further enhanced by TNF-α, such as IL-4 and IL-7 receptors, FcR receptor, and chemokine receptor CCR5 (CD195). Cluster III contains genes with highest expression in DC at day 7 of differentiation and that were down-regulated when cells further progressed in differentiation. Interestingly, this cluster contains a larger number of cell adhesion molecules, some of which have been implicated in DC migration and/or homing like CD31 or CD44. Moreover, novel molecules, such as CD164/multi-glycosylated core protein 24 (MGC-24) and CD166/activated leukocyte cell adhesion molecule, which have so far not been related to DC phenotype or function, were found in this cluster. CD164/MGC-24 and CD166/activated leukocyte cell adhesion molecule were described originally to be expressed in hemopoitetic progenitor cells and are implicated as playing a role in homeostatic control of growth and/or migration, including neural and organ development, hematopoiesis, immune response, and tumor progression (50, 51). As expected, mannose receptor (CD206) is also found in cluster III.

Finally, cluster IV comprises genes that were highly expressed in Flt3<sup>+</sup>CD11b<sup>+</sup> progenitor cells and down-regulated during DC differentiation. The receptors for FL and IGF-1, CD135/Flt3, and

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4 The online version of this article contains supplemental material.
CD221/IGF1R, respectively, are found in this cluster, which is in line with the prominent effect of these cytokines on Flt3+CD11b+ progenitor cell growth and FACS analysis (Fig. 1, A, B, and D). Transcripts for the IL-6 signal transducer gp130 (CD130) and the GM-CSF receptor α- and β-chains (CD116 and CD131) were up-regulated during differentiation and are therefore contained in cluster I. Interestingly, cluster IV comprises also known stem cell Ags like CD49f/integrin α6, CD93/Ly68/AA4.1, and CD133/AC133/prominin (Refs. 36, 37, 46, and 47; R. D. Kirsch, T. Hieronymus, and M. Zenke, unpublished data).

**Flt3+CD11b+ progenitor and stem cells exhibit an overlapping surface Ag repertoire**

To further extend this analysis, we compared the surface Ag repertoire of Flt3+CD11b+ progenitor cells to that of lin−c-kit+Sca-1+ hemopoietic stem cells (46, 47; R. D. Kirsch, T. Hieronymus, and M. Zenke, unpublished data). Flt3+CD11b+ progenitor cells expressed 73 of the 206 CD molecules analyzed, whereas hemopoietic stem cells expressed 59 (Fig. 6). Surprisingly, the majority of surface Ags (49) were found in both hemopoietic stem cells and Flt3+CD11b+ progenitor cells, whereas only 10 and 24 were solely found in stem cells and progenitor cells, respectively (Fig. 6B). CD molecules that were expressed by Flt3+CD11b+ progenitor cells only include, e.g., the integrins CD11b and CD18 (see Fig. 1D and also Fig. 7).

**Flt3+CD11b+ progenitors express several cytokine receptors that are related to the stem cell phenotype**

To further extend these observations, surfact Ag expression of Flt3+CD11b+ progenitors was analyzed by flow cytometry. Progenitors express surface CD49f, CD93/A4.1, and CD133/Prominin, and their expression was lost when cells differentiate into DC (Fig. 7). CXCR4 chemokine receptor (CD184) was expressed on Flt3+CD11b+ progenitor cells and further up-regulated during DC differentiation.

**Stem cell Ag expression on Flt3+CD11b+ progenitor cells**

To further extend these observations, surfact Ag expression of Flt3+CD11b+ progenitors was analyzed by flow cytometry. Progenitors express surface CD49f, CD93/A4.1, and CD133/Prominin, and their expression was lost when cells differentiate into DC (Fig. 7). CXCR4 chemokine receptor (CD184) was expressed on Flt3+CD11b+ progenitor cells and further up-regulated during DC differentiation.

**FIGURE 6.** Comparative gene expression analysis for CD molecules of Flt3+CD11b+ progenitor cells and lin−c-kit+Sca-1+ hemopoietic stem cells. Gene expression in Flt3+CD11b+ progenitor cells and lin−c-kit+Sca-1+ stem cells was assessed by DNA microarrays. Results shown are mean intensity values ± SD of normalized data from two independent replicate sets of stem cell hybridization data and five independent experiments for Flt3+CD11b+ progenitor cells, respectively. Only those genes are shown that were recognized as abundant transcripts by the Microarray Suite analysis software. A, Genes found to be expressed both in lin−c-kit+Sca-1− stem cells (□) and Flt3+CD11b+ progenitor cells (■). B, Summary of expressed genes encoding mouse CD molecules in Flt3+CD11b+ progenitor cells and stem cells. C and D, Genes exclusively found to be expressed in stem cells (□) or progenitor cells (■), respectively. A comprehensive list of the analyzed genes is provided as supplementary material. All microarray data are available in GEO database (series accession nos. GSE 692 and GSE 693).
Development, which is in line with previously published reports on human DC (52, 53). Flt3⁻CD11b⁺ progenitors abundantly expressed the myeloid Ags CD11b and CD16/32, but were negative for B220 (Fig. 7 and data not shown). As expected, several well-characterized DC markers such as MHC class II, CD11c, CD25, CD40, CD80, CD83, and CD86 were highly induced during DC differentiation (Figs. 2 and 7, Table I). Differentiated DC were CD4⁻ and CD8α⁻ (data not shown) and thus represent classical myeloid CD11c⁺CD11b⁺CD8α⁻ DC.

Taken together, the Flt3⁻CD11b⁺ progenitors obtained express several stem cell Ags and effectively differentiate into myeloid DC in vitro.

Discussion

In this paper, we used a two-step culture system for amplification of Flt3⁻CD11b⁺ progenitor cells and their differentiation into fully functional DC. Transcriptional profiling with DNA microarrays was used to study the gene expression repertoire of the Flt3⁻CD11b⁺ progenitor and the ongoing changes during DC differentiation. One of the major findings is the broad overlap of surface marker expression of Flt3⁻CD11b⁺ progenitors and hemopoietic stem cells. Flt3⁻CD11b⁺ progenitors express, e.g., Flt3, IL-6R and c-kit/SCF receptor, the CD34-like molecule CD164/MGC-24 and the ABC transporter CD243/MDR1, and also the stem cell Ags CD93/AA4.1, CD133/AC133, and CD49f/integrin α6, and their expression declines during DC differentiation. Additionally, following administration of GM-CSF, Flt3⁻CD11b⁺ progenitors synchronously differentiate into fully functional DC, and this allows studying the consecutive steps that determine DC differentiation.

DC constitute a complex system of heterogeneous cells varying in their tissue distribution in the organism and differing in surface phenotype and/or specific functions (1, 2). It has been reasoned that different DC subsets are derived from different hemopoietic lineages (4, 6). However, their lineage origin and the underlying molecular mechanisms that determine DC development have remained controversial. In recent years, several in vitro systems that recapitulate DC differentiation in cell culture have been established (54–56). Although most in vitro culture systems focus on human DC, similar systems for mouse DC are just being developed (24, 57, 58). In this study, we describe a novel two-step culture system for mouse DC where a Flt3⁻CD11b⁺ progenitor from mouse bone marrow is first amplified with a specific cytokine combination and then differentiated into fully functional DC with GM-CSF. This approach follows a strategy that we successfully used before for human DC (18, 32, 56).

Our two-step culture system for mouse Flt3⁻CD11b⁺ progenitor cells employs SCF, FL, IGF-1, GM-CSF, hyper-IL-6, and the glucocorticoid dexamethasone. This factor combination was chosen based on microarray studies of lin⁻c-kit⁺Sca-1⁺ hemopoietic stem cells that demonstrated expression of their cognate receptors. These cytokines yield an effective outgrowth and amplification of Flt3⁻CD11b⁺ progenitors and overcome former limitations in number, purity, and homogeneity for such progenitors. However, whether other cytokines contribute to the generation and maintenance of hemopoietic precursors with the same or even broader differentiation potential is currently being investigated.

Addition of glucocorticoid receptor antagonist dexamethasone to Flt3⁻CD11b⁺ progenitor cultures significantly increased cell numbers and ensured homogeneity of the progenitor population. Dexamethasone appears to impose a differentiation block (T. Jorgas, T. Hieronymus, and M. Zenke, unpublished data) similar to that observed in other in vitro culture systems (29, 48, 49).

During the amplification phase, up to 10⁶ Flt3⁻CD11b⁺ progenitor cells are readily obtained from one bone marrow preparation. Further cultivation with GM-CSF results in an ordered and highly synchronized differentiation of precursor cells into DC, which thus enables the discrimination of consecutive steps of maturation. DC are fully functional as APCs both in vitro and in vivo, which qualifies this two-step culture system for various applications including vaccination studies (T. Hieronymus, T. C. Gust, and M. Zenke, unpublished data).

An interesting question is how the Flt3⁻CD11b⁺ progenitor cells described in this paper relate to hemopoietic stem cells and to various hemopoietic precursors, such as CLP and CMP (9, 10, 27, 59) and Flt3⁻ DC precursors (7).

Gene expression profiling by high density oligonucleotide microarrays provides a powerful means of exploring transcriptional patterns on a genome-wide scale. By using microarray analysis, we recently identified the basic helix-loop-helix transcription factor Id2 as one major determinant for DC development (18). Hemopoietic cells are commonly characterized by their surface Ag expression. Therefore, microarray data of Flt3⁻CD11b⁺ progenitors were evaluated for their surface Ag expression pattern, and this was compared with that of lin⁻c-kit⁻Sca-1⁺ hemopoietic stem cells (46, 47); R. D. Kirsch, T. Hieronymus, and M. Zenke, unpublished data). It was found that Flt3⁻CD11b⁺ progenitors exhibit a considerable overlap of the surface markers with hemopoietic stem cells including Flt3, IL-6R, c-kit/SCF receptor, and the stem cell Ags CD93/AA4.1, CD133/AC133, and CD49f/integrin α6.

The integrin α6 (CD49f) was also detectable on Flt3⁻CD11b⁺ progenitor cells as expected from the microarray data, and its expression declined with differentiation. Interestingly, in a recent paper, Melton and coworkers (47) propose the interaction with extracellular matrix via integrin α6/β1 as an essential stem cell property.

Flt3⁻CD11b⁺ progenitors do not express IL-7R, which represents a hallmark of CLP (59). Initial evidence suggests that
Flt3+CD11b+ progenitors are also different from the Flt3+CD11b+ progenitors identified by D’Amico and Wu (7). Whether Flt3+CD11b+ progenitors give rise to hemopoietic lineages other than DC is currently being investigated. An interesting question relates to the in vivo frequency of the Flt3+CD11b+ progenitor, and we demonstrate that its frequency in bone marrow is ~2–2.5%.

An Flt3+ macrophage precursor has been described that is CD11b+ and can be sequentially differentiated into osteoclasts, DC, and microglia in vitro (26). The expansion of this precursor population by FL in vitro is 4- to 5-fold within 11 days and thus rather limited compared with the amplification rates achieved in our two-step culture system. We attribute this to the unique combination of cytokines in addition to FL that are present in our amplified culture.

Enrichment of specific hemopoietic cell populations is frequently based on protocols that sort cells according to specific surface Ag composition, e.g., by FACS or immunomagnetic bead selection. Specific changes in the expression of cell surface molecules represent one of the hallmarks of DC differentiation including molecules involved in Ag uptake and presentation, migration, cell adhesion, and cell-cell interaction. The surface Ag repertoire represents one of the hallmarks of DC differentiation included in this paper provides valuable information for sorting strategies.

Finally, following addition of GM-CSF, Flt3+CD11b+ progenitors differentiate into fully functional DC. Thus, the two-step culture system described in this paper provides an excellent means of studying the successive stages of DC differentiation under well-defined and controlled conditions. Monitoring of surface Ag expression by transcriptional profiling perfectly confirms our data on the phenotypic and functional properties of the DC and is consistent with previous published data (1, 2). In this respect, the application of an in vitro DC differentiation system followed by transcriptional profiling appears to be particularly well suited for the identification of novel genes, which so far have not been implicated in DC development and/or function.

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