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*J Immunol* 2005; 175:1399-1405; ;
http://www.jimmunol.org/content/175/3/1399
The Colony-Stimulating Factor 1 Receptor Is Expressed on Dendritic Cells during Differentiation and Regulates Their Expansion

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The lineage of dendritic cells (DC), and in particular their relationship to monocytes and macrophages, remains obscure. Furthermore, the requirement for the macrophage growth factor CSF-1 during DC homeostasis is unclear. Using a transgenic mouse in which the promoter for the CSF-1R (c-fms) directs the expression of enhanced GFP in cells of the myeloid lineage, we determined that although the c-fms promoter is inactive in DC precursors, it is up-regulated in all DC subsets during differentiation. Furthermore, plasmacytoid DC and all CD11chigh DC subsets are reduced by 50–70% in CSF-1-deficient osteopetrotic mice, confirming that CSF-1 signaling is required for the optimal differentiation of DC in vivo. These data provide additional evidence that the majority of tissue DC is of myeloid origin during steady state and supports a close relationship between DC and macrophage biology in vivo. The Journal of Immunology, 2005, 175: 1399–1405.

Materials and Methods

Mice

MacGreen mice were produced on a B6 × CBAF1 background as described previously (5). Homozygous op/op and heterozygous op/wt mice were supplied by the Herston Medical Research Centre (Brisbane, Australia). C57BL/6 mice were supplied by the Animal Research Centre (Western Australia, Australia). All mice used were 5–12 wk of age.

Cytokine treatment

Recombinant progenipoietin-1 (ProGP-1) (Pfizer) was diluted in 1 µg/ml murine serum albumin in PBS before injection. Mice were injected s.c. with diluent or 20 µg/animal/day ProGP-1 once daily from days −7 to −1. Complete blood counts were performed on EDTA peripheral blood (PB) samples using a Sysmex SE-9000 (Sysmex) automated analyser.

Monoclonal Abs

The following mAbs were purchased from BD Pharmingen: FITC-conjugated CD11c (HL3) and IgG2a isotype control; PE-conjugated CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), CD40 (3/23), CD45R/B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL1), I-AJ-E (2G9), and IgG2b isotype control; and PE-Cy5-conjugated CD8 CD4 and IgG2b isotype control. PE-conjugated CD115 was purchased from Serotec. Purified mAb against CD3 (KT3), CD19 (HB935), Gr1 (RB6-8C5), Thy1.2 (HO-13-4), Ter119, FcγR II/III (2.4G2), and biotinylated F4/80 were produced in house.

Cell preparation

DC purification was undertaken as described previously (6). Briefly, low-density cells were enriched from digested lymphoid tissues, bone marrow, or lysis buffer-treated heparinized blood by Nycodenz density gradient centrifugation. In some experiments, non-DC lineage cells were depleted by coating with rat IgG Abs to B cells (CD19), T cells (CD3, Thy1), granulocytes (Gr-1), and erythroid cells (Ter-119). The coated cells were then removed by magnetic beads coupled to anti-rat IgG (Dynal ASA, Oslo, Norway). For mixed lymphocyte cultures (MLC), splenic T cells were purified by depleting B cells (B220 and CD19), monocytes (CD11b), granulocytes (Gr-1), and erythroid cells (Ter-119) using magnetic bead depletion. For culture experiments, highly purified DC populations (>98%) were obtained by FACS (MoFlo; DakoCytomation). Peritoneal cells were lavaged from the peritoneal cavity with HBSS containing EDTA (Sigma-Aldrich).

Real-time RT-PCR for CSF-1R

For real-time RT-PCR analysis, equivalent numbers of sort-purified cells were resuspended in TRIzol (Invitrogen Life Technologies), snap frozen on
dry ice, and RNA extracted, according to the manufacturers protocol. cDNA was immediately reverse transcribed using avian myeloblastosis reverse transcriptase (Promega), according to the manufacturers protocol, and cDNA stored at −20°C. Real-time PCR was undertaken using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen Life Technologies) conducted on a Rotor-Gene3000 (Corbett Research) and data analyzed using Rotor-Gene v5.0 (Corbett Research). Primers used for CSF-1R reactions were 5'-CCACCATCACCAGATGCTCAAGAT-3' (forward) and 5'-CTCAGACATGTGACCG3' (reverse). Primers used for 2-microglobulin reactions were 5'-TTTCTGGTGCTCTCACTGACCG-3' (forward) and 5'-GCAGTTCAGTATGTTCGGCTTCCCA-3' (reverse). The thermal cycler conditions were as follows: 12 min 50°C, 10 min 95°C, 40–50 cycles denaturation (15 s, 95°C), and combined annealing/extension (1 min, 60°C). CSF-1R cDNA copy numbers were then normalized for variations in the efficiency of RNA extraction and cDNA transcription against the 2-microglobulin housekeeping gene.

Cell culture
Sort-purified CD11c+ c-fms/EGFP+ PB cells were cultured at 10⁵/ml for 7–9 days in 10% FCS/IMDM (JRH Biosciences) supplemented with GM-CSF, IL-3, and IL-4 (all at 100 ng/ml). Sort-purified CD11c+ and CD11c−/B220+ DC were cultured for 18 h at 10⁵/ml in 10% FCS/IMDM supplemented with GM-CSF, IL-3 (each at 100 ng/ml), LPS (1 μg/ml), and phosphorothioated oligo CpG (1668; 0.5 μM) (7). For MLC, 10⁶ magnetic bead-purified C57BL/6 T cells were cultured for 5 days with varying numbers of sort-purified CD11c+ DC. [3H]Thymidine (1 Ci/well) was added on day 4, and proliferation was determined 18 h later using a Betaplate Reader (Wallac).

Results
CD11c+bhigh and CD11c+dim/B220+ DC express c-fms
We first compared c-fms expression by peritoneal macrophages and splenic DC subsets using three-color flow cytometry. For these experiments, DC were highly enriched from MacGreen and C57BL/6 spleens by gradient centrifugation and magnetic bead depletion of lineage-positive cells. CD11c+bhigh (myeloid) and CD11c+dim/B220+ (plasmacytoid) cells were gated (Fig. 1a) and examined for c-fms/EGFP expression. Normalized CSF-1R mRNA levels were determined by SYBR green real-time PCR analysis of total RNA isolated from sort-purified cell populations as described in Materials and Methods. Data are presented as mean values with SDs from triplicate samples and are representative of one of three independent experiments with similar results (c). The capacity of sort-purified wild-type or MacGreen CD11c+bhigh DC to stimulate allogeneic (H-2b) T cell proliferation was determined in an MLC using [3H]thymidine incorporation as a readout (d).

FIGURE 1. Splenic CD11c+bhigh and CD11c+dim/B220+ DC express c-fms. Splenic DC were enriched from MacGreen or C57BL/6 mice by gradient centrifugation and lineage depletion and CD11c+bhigh and CD11c+dim/B220+ populations gated (a) and examined for c-fms/EGFP or CSF-1R (CD115) expression (thick line) (b). Negative controls (fine line) are from c-fms/EGFP-negative littermates or isotype control mAb, respectively. Peritoneal F4/80+ macrophages served as a positive control for c-fms/EGFP and CSF-1R expression. Normalized CSF-1R mRNA levels were determined by SYBR green real-time PCR analysis of total RNA isolated from sort-purified cell populations as described in Materials and Methods. Data are presented as mean values with SDs from triplicate samples and are representative of one of three independent experiments with similar results (c). The capacity of sort-purified wild-type or MacGreen CD11c+bhigh DC to stimulate allogeneic (H-2b) T cell proliferation was determined in an MLC using [3H]thymidine incorporation as a readout (d).
of CSF-1R mRNA in F4/80+ macrophages was 5-fold that of CD11c+DC, which was 2-fold higher than the CD11cdimB220+ DC. As expected, CSF-1R mRNA was not detected in CD4+ T cells. Finally, the presence of the EGFP reporter gene in the MacGreen mice did not alter the stimulatory capacity of CD11c+ DC (Fig. 1d).

Examination of EGFP expression within the skin, thymus, and lymph nodes from MacGreen mice revealed EGFP bright cells included cells with DC morphology. In this regard, within epidermal sheets, Langerhans cells were clearly demarcated by EGFP expression, whereas cells with pronounced interdigitating processes were evident within the thymic cortex. In addition to the strong expression of EGFP in the macrophages of the subcapsular sinus, note the extensive dendritic network of EGFP-positive cells through the remainder of the lymph node (Fig. 2).

Because MacGreen mice permitted enhanced detection of c-fms promoter activity, we used these mice to extend our analysis of CD11c+ DC CSF-1R expression within various lymphoid organs. For these experiments, DC were enriched from lymphoid tissues by density gradient centrifugation. Greater than 90% of CD11c+ cells within DC preparations from bone marrow, thymus, spleen, and lymph nodes expressed c-fms/EGFP. In contrast, within the PB DC preparations, 20–30% of the CD11c+ cells were EGFP negative (Fig. 3), corresponding to ~6% of blood mononuclear cells.

PB contains a CD11c+c-fms/FPpos DC precursor population

Because a CD11c+MHC-II+ DC precursor population of similar frequency to that of the CD11c+c-fms/FPpos PB population was recently identified in murine PB (8), we investigated whether the CD11c+c-fms/FPpos PB population related to this DC precursor. To limit the number of animals required, we treated the MacGreen mice with ProGP-1, a chimeric protein with receptor agonist activity for both fetal liver tyrosine kinase-3 (Flt3) and G-CSF receptor that is known to expand both CD11c+ and CD11cdimB220+ DC in blood and lymphoid organs (9, 10). ProGP-1 pretreatment of the MacGreen mice resulted in a 100-fold increase in the blood white cell count and a 40-fold expansion of blood CD11c+ DC, and this expansion was associated with both the CD11c+c-fms/FPpos and CD11c+c-fms/FPneg populations (Fig. 4a). Phenotypic analysis of the CD11c+c-fms/FPpos population from ProGP-1-treated animals revealed the cells to be MHC class II+, CD8+, CD4+, F4/80+, CD11b+, CD62Ldim, and B220+ (Fig. 4b). Notably, this phenotype is identical to that of the DC precursor described by del Hoyo (8).

Because the PB DC precursors have been shown to exhibit the capacity to reconstitute all splenic DC populations, we examined whether the differentiation of CD11c+c-fms/FPneg population would induce the expression of c-fms/EGFP. Following 7–9 days in culture in the presence of GM-CSF, IL-4, and IL-3, sort-purified CD11c+c-fms/FPneg DC precursors differentiated into large DC-like cells (based on forward and side scatter characteristics) and expressed c-fms/EGFP (Fig. 5).

All DC subsets are reduced in CSF-1-deficient op/op mice

Because CSF-1R was found to be associated with DC maturation, we asked whether CSF-1 signaling was required for the generation
and/or differentiation of DC. For these studies, we used op/op mice, which have a null mutation in the CSF-1 gene that results in congenital osteopetrosis (11) due to a deficiency of osteoclasts and macrophages (12). As expected, F4/80-positive peritoneal macrophages were largely absent in op/op mice (Table I). Examination of DC within the op/op spleen revealed a 43 and 70% reduction in the CD11c<sup>high</sup> DC and CD11c<sup>dimm</sup>B220<sup>+</sup> compartments, respectively, compared with the normal heterozygous op/wt littermates. Interestingly, following culture, the op/op CD11cdimmB220<sup>+</sup> sets (CD8<sup>+</sup>) exhibited enhanced expression of CD40 and CD86 as compared with the normal heterozygote op/wt DC (Fig. 6).

**DC cell function and maturation is not altered in op/op mice**

Functional testing of sort-purified CD11c<sup>high</sup> DC in MLC revealed that op/op DC were as potent at stimulating allogeneic T cell (C57/B6) proliferation as normal heterozygote op/wt DC (Fig. 6). Although the data suggest that these c-fms/EGFP<sup>−/−</sup>CD11c<sup>+</sup> cells are DC precursors, we cannot exclude the possibility that these are DC<sup>+</sup> cells of a separate lineage (e.g., lymphoid) or stage of trafficking. However, these putative DC precursors resemble common myeloid progenitors purified from bone marrow in which control elements of the c-fms locus are assembled into active chromatin, and c-fms mRNA expression is very low (13). The expression of c-fms is induced rapidly during macrophage lineage commitment. In contrast to the intense c-fms/EGFP transgene expression seen in mature DC populations, surface c-fms (CD115) was expressed on DC at low levels only (3-log less than on F4/80<sup>+</sup> cells). CSF-1 drives the expansion and differentiation of macrophages, which are well established to be of myeloid lineage. MacGreen mice express EGFP driven by the c-fms promoter, thus permitting identification of myeloid-derived cells. In this study, we demonstrate that the MacGreen mouse represents a highly sensitive means of amplifying CD115. This allowed assessment of the potential for expression of the CSF-1R and confirmed that the majority of all tissue DC subsets expressed c-fms/EGFP. Conversely, up to 30% of CD11c<sup>+</sup> DC within the PB lacked c-fms/EGFP expression. The c-fms/EGFP<sup>−/−</sup>CD11c<sup>+</sup> PB population exhibited a phenotype (class II<sup>−</sup>, CD11b<sup>+</sup>, B220<sup>+</sup>, and CD62L<sup>+</sup>) identical to that of a previously described blood DC precursor (8) and could be induced to express c-fms/EGFP upon differentiation in vitro. Furthermore, following culture, the op/op CD11c<sup>dimm</sup>B220<sup>+</sup> DC exhibited enhanced expression of CD40 and CD86 as compared with the op/wt CD11c<sup>dimm</sup>B220<sup>+</sup> DC.

**Discussion**

DC represent a heterogeneous population whose lineage relationship with other hemopoietic cell populations has long been questioned. Although DC were initially thought to be of myeloid origin, several recent studies using DC reconstitution experiments have established that both common myeloid and lymphoid precursors exhibit the capacity to differentiate into DC in vivo. However, the relative contribution of either of these precursor populations during normal DC development is not known. DC can be subdivided into multiple subsets based on phenotype and tissue distribution. Thus, within mouse spleen four unique CD11c<sup>+</sup> DC subsets (CD8<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>−</sup> CD4<sup>+</sup>, and B220<sup>+</sup>) can be identified. Furthermore, DC populations include Langerhans cells and dermal DC within the skin and CD8<sup>−</sup> DC within lymph nodes. Although the various DC subpopulations appear to be developmentally and functionally distinct, it is not clear whether this divergence can be attributed to distinct lineages, different stages of development/activation, or the influence of unique tissue specific niches.

**FIGURE 4.** PB contains a CD11c<sup>c</sup>c-fms/EGFP<sup>−/−</sup> DC precursor population. MacGreen mice were treated with control diluent or ProGP-1, and white counts were determined as described in Materials and Methods. c-fms/EGFP expression was then determined in CD11c<sup>c</sup>c-fms/EGFP<sup>−/−</sup> population (R2 in a above) by three-color flow cytometry using a panel of Ag-specific mAb (thick line). Isotype control mAbs are shown as a fine line (b).

**FIGURE 5.** CD11c<sup>c</sup>c-fms/EGFP<sup>−/−</sup> DC precursor up-regulates c-fms upon differentiation. Analysis of forward and side scatter characteristics and c-fms/EGFP expression by the CD11c<sup>c</sup>c-fms/EGFP<sup>−/−</sup> population before (day 0, thin line) and after culture in vitro (day 7, shaded peak). Thick line depicts level of c-fms/EGFP expression by freshly purified PB CD11c<sup>c</sup>c-fms/EGFP<sup>−/−</sup> DC.
80-positive macrophages), suggesting that the majority of c-fms expression in DC is not surface bound. This finding does not imply that the CSF-1R is inactive. For example, Langerhans cells have been shown to express functional CSF-1R and respond to CSF-1 (14). c-fms mRNA is barely detectable in bone marrow myeloid progenitors, yet the cells are clearly CSF-1 responsive (13). Tissue

<table>
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<tr>
<th>Peritoneal Macrophages</th>
<th>CD11c&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>CD11c&lt;sup&gt;dim&lt;/sup&gt;B220&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD11c&lt;sup&gt;hi&lt;/sup&gt; Subsets (% of CD11c&lt;sup&gt;hi&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>op/wt</td>
<td>1.1 ± 0.2</td>
<td>4.7 ± 0.5</td>
<td>55.7 ± 2.8</td>
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<tr>
<td>op/op</td>
<td>0.04 ± 0.02&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.7 ± 0.3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>21.6 ± 2.3</td>
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Peritoneal macrophages (×10<sup>5</sup>/lavage) and DC (×10<sup>5</sup>/spleen) from individual osteopetrotic CSF-1-deficient op/op (n = 5) or normal heterozygote littermate op/wt (n = 6) mice were characterized as described in Materials and Methods. Results expressed as mean ± SE. *p < 0.01; †p < 0.05.

**FIGURE 6.** T cell stimulatory capacity of freshly purified op/op and op/wt CD11c<sup>hi</sup> DC. Freshly purified CD11c<sup>hi</sup> DC from op/wt and op/op (H-2<sup>b</sup>/k) spleens were cultured in the presence of allogeneic (H-2<sup>k</sup>) T cells and proliferation determined by [³H]thymidine incorporation (a). Costimulatory molecule expression by in vitro-matured op/wt and op/op DC subsets. Sort-purified DC subsets from op/wt and op/op spleens were cultured in the presence of GM-CSF, IL-3, LPS, and phosphorothioated oligo CpG. Cells were harvested after 18 h and stained with PE-conjugated mAb as indicated (b). Data are from one experiment and represents one of three sets of cultures of DC subsets derived from individual op/wt and op/op spleens.
macrophages express surface CSF-1R at much higher levels than proliferating progenitors, and in these cells, the receptor mediates endocytic clearance of the growth factor (15). The level of c-fms mRNA is also down-modulated posttranscriptionally by GM-CSF, which promotes DC differentiation (16, 17), and surface CSF-1R is down-modulated acutely by TLR agonists (18). In contrast, EGFP remains intracellular and therefore is not subject to variables that influence both initial expression at the surface and subsequent degradation. Therefore, the expression of the CSF-1R reporter gene is not expected to be perfectly correlated with the presence of the receptor on the cell surface. Taken together, these data suggest that expression of c-fms promoter activity is a marker of maturation within all DC subsets, and its absence identifies immature DC precursors within the PB.

DC numbers are reported to be normal in op/op mice, and previous studies have noted GM-CSF to be the primary cytokine determining DC development rather than CSF-1 (19, 20). In elegant studies of macrophage and DC differentiation in vitro, IL-6 from stromal cells has been shown to enhance CSF-1R expression on monocytes and promote CSF-1-CSF-1R internalization. This results in the subsequent differentiation of monocytes to macrophages (21). In contrast, TNF-α blocks CSF-1R expression and internalization, leading to DC differentiation (22). These data suggest that the absence of the CSF-1R expression would preferentially block macrophage differentiation, consistent with the phenotype of the CSF-1 (op/op) and CSF-1R-deficient mice (23). Because previous studies used poorly quantitative immunohistochemical techniques and predate the description of DC subsets, we revisited the influence of CSF-1 on DC development in these mice using a quantitative approach encompassing the wider understanding of DC subset heterogeneity as published recently (24). These data confirm that the absence of CSF-1 results in a 50–70% reduction in DC numbers, supporting the view that the low levels of CSF-1R on DC are, indeed, functional. CSF-1 is also required for optimal production of osteoclasts, hence, the osteopetrotic phenotype of the op/op mice. However, the op/op mice recover relatively normal osteoclastogenesis with age, which is attributed to the ability of vascular endothelial growth factor-A and/or flt3 ligand, to provide partial compensation (25, 26). Both these ligands act through type III tyrosine kinase receptors closely related to c-fms. Given the biology of flt3 ligand as a DC promoter, it is very likely that it also provides partial compensation for the absence of CSF-1, and of course, GM-CSF (which actually cross-modulates CSF-1 that it also provides partial compensation for the absence of CSF-1, and promotes CSF-1-CSF-1R internalization, leading to DC differentiation (22). Our data confirm and highlight the differential expression of CSF-1R on macrophages and DC, and we propose that the differentiation of each lineage is at least in part determined at the cellular level by c-fms expression. Furthermore, the expression of c-fms by both myeloid and plasmacytoid DC and their dependence on CSF-1 as seen by the current studies in op/op mice also favors a common lineage for these two DC subsets. Recent studies confirming the ability of plasmacytoid DC to differentiate into myeloid DC following stimulation by dsRNA and type I IFNs further supports this concept of plasticity within DC subset lineages and their differentiation (30). Thus, although both common myeloid and lymphoid progenitors can give rise to both plasmacytoid and myeloid DC in vivo, these subsets can no longer be unambiguously defined on arbitrary assumptions of lineage (reviewed in Ref. 31). Our data provides further support of this concept, suggesting that the majority of tissue DC are of myeloid origin during steady state and that a close relationship between DC and macrophage biology exists in vivo.

Disclosures
The authors have no financial conflict of interest.

References


