Interleukin-3Rα$^+$ Myeloid Dendritic Cells and Mast Cells Develop Simultaneously from Different Bone Marrow Precursors in Cultures with Interleukin-3

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The distinct developmental routes of dendritic cells and mast cells from murine bone marrow cultures with interleukin-3 are unclear. We found that short-term bone marrow cultures with interleukin-3 after 8–10 d consist of about 10%–30% dendritic cells and 70%–90% mast cell precursors, and only after 4–6 wk do homogeneous populations of mast cells emerge. Phenotypical and functional analysis of interleukin-3/dendritic cells revealed a high similarity with myeloid dendritic cells generated with granulocyte-macrophage colony stimulating factor in the expression of myeloid dendritic cell markers (CD11c$^+$ B220$^-$ CD8α$^-$ CD11b$^+$), major histocompatibility complex II and costimulatory molecules, endocytosis, maturation potential, interleukin-12 production, and T cell priming. Interleukin-3/dendritic cells expressed higher levels of interleukin-3 receptor, however. To dissect the interleukin-3/dendritic cell and mast cell development, we sorted fresh bone marrow cells into six subsets by the antibodies ER-MP12 (CD31) and ER-MP20 (Ly-6C). Both interelukin-3/dendritic cells and granulocyte-macrophage colony stimulating factor/dendritic cells develop from the same bone marrow populations, including the ER-MP12^{neg}, ER-MP20^{high} bone marrow monocytes. In contrast, mast cells only developed from ER-MP12$^{int+high}$, ER-MP20^{neg} bone marrow cell subsets, indicating that different precursors exist for interleukin-3/dendritic cells and mast cells. Established mast cell cultures could not be converted to dendritic cells or stimulated to express major histocompatibility complex II molecules *in vitro* or home to lymph node T cell areas *in vivo*. In summary, we show that dendritic cells generated from bone marrow precursors with interleukin-3 are clearly myeloid and develop via a different pathway compared to bone marrow mast cells. ***Key words: bone marrow/dendritic cells/development/IL-3/mast cells J Invest Dermatol 121:280–288, 2003***

Dendritic cells (DC) and mast cells (MC) represent sentinel cells of the immune system, equipped to defend infectious microbes. Therefore they are predominantly located in surface tissues such as the skin or mucosa. Both MC and DC are of bone marrow (BM) origin. Whereas MC seem to form their own distinct hematopoietic lineage (Rodewald *et al*, 1996; Austen and Boyce, 2001), several DC subsets derived from myeloid, lymphoid, and plasmacytoid precursor cells have been described (Shortman and Liu, 2002).

Interleukin-3 (IL-3) was originally described as a growth factor stimulating progenitor cells of the myeloid lineage (Ihle, 1992); only recently has it been reported that it also contributes to lymphoid lineage development (Brown *et al*, 1999). IL-3 has additionally been described as a growth factor for MC. Two major subsets of MC can be distinguished according to their tissue distribution and growth requirements. Whereas connective-tissue-type MC can be generated from murine BM cells in cultures with IL-3 and IL-4 (Tsuji *et al*, 1990), the mucosal type of MC can be generated from murine BM cells with IL-3 alone (Nakahata *et al*, 1986).

The growth requirements for DC are more complex due to their higher heterogeneity and plasticity during DC development. For human DC it has been found that IL-3 promotes the development of human myeloid DC *in vitro* (Caux *et al*, 1996; Ebner *et al*, 2002). *Ex vivo*, however, the expression of the IL-3Rα chain has been attributed as representing a distinctive marker for human plasmacytoid DC (Grouard *et al*, 1997; Cella *et al*, 1999; Rissoan *et al*, 1999).

In the mouse, lymphoid-related CD11c$^+$ CD11b$^-$ B220$^-$ CD8α$^+$ DC subsets can be generated with a cocktail containing IL-3, but not granulocyte-macrophage colony stimulating factor (GM-CSF), from T cell precursors in thymus (Saunders *et al*, 1996; Kelly *et al*, 2001) or B cell precursors in the liver (Lu *et al*, 2001), which are strictly dependent on IL-3 but not GM-CSF. The generation of murine CD11c$^+$ CD11b$^-$ B220$^+$ CD8α$^-$ plasmacytoid DC from BM cells has been shown with Flt3L (Gilliet *et al*, 2002), whereas isolated plasmacytoid DC from spleens and lymph nodes show an increased survival on IL-3 (Nakano *et al*, 2001). Murine plasmacytoid DC also express low amounts of

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Abbreviations: BM, bone marrow; DC, dendritic cell; MC, mast cell; PE, phycoerythrin; SAC, *Staphylococcus aureus* Cowan I.

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IL-3Rα (Asselin-Paturel *et al*, 2001; O'Keeffe *et al*, 2002). The generation of $CD11c^+$ $CD11b^+$ $B220^-$ $CD8\alpha^-$ myeloid DC with IL-3 and a differential analysis of the generation of MC with IL-3 within the same cultures has not been reported so far.

Standard methods using IL-3 as a growth factor for BM liquid cultures exclusively described the generation of MC, which remain the only cell type after 4–6 wk of culture (Huff and Lantz, 1997). Therefore we investigated the lineage origin of IL-3/DC cultures and compared them with GM-CSF/DC and MC cultures from BM.

Here we show, using the same liquid culture protocol as used for BM-DC, that DC constitute 10%–30% of the cells that are generated after 8–10 d of culture with IL-3. Such DC are highly similar in phenotype and function to myeloid DC generated with GM-CSF but express the IL-3Rα chain at high levels. IL-3/DC and MC appear to follow distinct pathways during BM development. Together, our data indicate that IL-3 promotes myeloid DC and MC development but from distinct BM precursors and that differentiated MC do not convert into DC.

MATERIALS AND METHODS

Cell culture reagents and cytokines For standard cell culture we used R10 medium, which consisted of RPMI 1640 medium (Boehringer Ingelheim Bioproducts, Verviers, Belgium) supplemented with 10% filtered fetal bovine serum (PAA, Cölbe, Germany), 50 μM β-mercaptoethanol, 100 U per ml penicillin, 100 μg per ml streptomycin, and 2 mM L-glutamine (all Sigma, Deisenhofen, Germany). In some experiments serum-free HL-1 medium was used (BioWhittaker Europe, Verviers, Belgium) supplemented in the same way as R10 without fetal bovine serum.

Cytokines purchased for use were as follows: IL-3 (100 U per ml), GM-CSF (200 U per ml), tumor necrosis factor α (TNF-α, 500 U per ml) (all Peprotech/TEBU, Frankfurt, Germany); lipopolysaccharide (LPS, 1 μg per ml, *Escherichia coli*, 0127:B8, Sigma); *Staphylococcus aureus* Cowan I strain (SAC, 10 μg per ml, Merck, Darmstadt, Germany). All Animal experiments have been approved by the German authorities (Regierung spräsidium Mittelfranken, approval number 621-2631-32-6102).

Mice BALB/c and C57BL/6 mice were either purchased from Charles River/Wiga (Sulzfeld, Germany) or bred within our own facilities. TNFR $1 + 2^{-/-}$ mice (on C57BL/6 background, kindly provided by H. Blüthmann, Roche, Basel, Switzerland) were used to compare allo-mixed leucocyte reaction activity of GM-CSF/DC with IL-3/DC.

Isolation of BM cells and generation of BM-DC The isolation of BM cells and the generation of murine BM-DC with GM-CSF has been described extensively before (Lutz *et al*, 1999). IL-3/DC were prepared similarly but using IL-3 instead of GM-CSF. Cells were fed at days 3 and 6, and analyzed at day 8, as described.

Flow cytometry Cells (2×10^5) were stained with 50 μL hybridoma culture supernatants containing 0.1% sodium azide or directly conjugated first and second step antibodies at 5–20 μg per ml for 30 min on ice. Primary and secondary antibodies were diluted in phosphate-buffered saline containing 5% fetal bovine serum and 0.1% sodium azide, which also served as washing medium. For cytoplasmic staining, cells were fixed with 1% formaldehyde and the staining was performed in the presence of 0.5% saponin (Sigma). Fluorescence analysis was performed with a FACScan (Becton Dickinson). The following antibodies were used as hybridoma cell culture supernatants (sup) or purified antibodies (pure) for surface and cytoplasmic staining: IL-3Rα (pure, CD123, 5B11, Pharmingen), CD86 (pure, anti-B7-2, GL1, Pharmingen), CD117 (pure, c-kit, 2B8, Pharmingen), CD14 (pure, Pharmingen), 2A1 (intracellular, sup), B220 (pure, Pharmingen), T cell receptor αβ (TCR-αβ, H57-597, sup), CD8α (pure, 53-6.7, Pharmingen), CD11b (M1/70, sup), CD11c (N418, sup), CD13 (ER-BMDM-1, sup), CD205 (NLDC145, sup), followed by antihamster-fluorescein isothiocyanate (FITC) or antirat-FITC antibodies (both Pharmingen) and phycoerythrin (PE) conjugated anti-major histocompatibility complex (MHC) class II (M5/114), or CD11c-PE or CD11c-FITC (Pharmingen). Isotype controls were rat IgG2a (MAC3AFRC, sup, or PE-conjugated R35-95, Pharmingen), rat IgG2b (Lo-DNP11, sup), hamster IgG (pure, G235-2356, anti-TNP, Pharmingen). Staining for the high affinity FcεR I was performed by incubating cells for 1 h at 4°C with 10 μg per ml mouse IgE-anti-DNP (Sigma, clone SPE-7). During the last 30 min 10 μg per ml FITC-conjugated rat-antimouse IgE (Pharmingen) was added. Detection of apoptotic cells was performed with an Annexin V FITC kit (BenderMed Systems, Vienna, Austria) according to the manufacturer's descriptions.

Endocytosis GM/DC and IL-3/DC were cultured until day 10. Then, 2×10^5 DC were incubated with 1 mg per ml FITC-conjugated dextran, FITC-conjugated ovalbumin, or Lucifer Yellow (all Molecular Probes, Leiden, The Netherlands) for 0, 15, 30, 60 min on ice or at 37°C. Then all samples were double stained with PE-conjugated 2G9 (anti-MHC class II) monoclonal antibodies before fluorescence-activated cell sorter (FACS) analysis. Mean fluorescence values within the gate for endocytically active immature DC were plotted as described previously (Lutz *et al*, 1997).

***In vitro* and *in vivo* priming** For *in vitro* primary allogeneic mixed leucocyte reaction, BM cells from female TNFR $1 + 2^{-/-}$ mice were cultured until day 9 under standard conditions with either GM-CSF or IL-3 and treated with LPS for 24 h. GM-CSF/DC generated from wild-type mice produced higher percentages of spontaneously mature DC in culture than IL-3/DC, due to endogenous TNF-α production in the cultures with GM-CSF (Yamaguchi *et al*, 1997). Therefore TNF-α-unresponsive BM-DC from TNFR $1 + 2^{-/-}$ mice were used to generate exclusively immature DC from both GM-CSF and IL-3 cultures, which still have the capacity to mature upon LPS stimulation. For the allo-mixed leucocyte reaction the cells were harvested at day 8, washed, and transferred into a 96-well flat-bottomed plate (Becton Dickinson) at titrated numbers together with $CD4^+$ T cells (BALB/c, 3×10^5 cells per well) derived from pooled lymph nodes and purified using Cellect mouse CD4 cell-enrichment columns (TEBU, Frankfurt, Germany). After 3 d, triplicate cultures were pulsed with 1 μCi [^{3}H]-thymidine (Amersham) for 16 h and harvested onto filtermats with an ICH-110 harvester (Inotech, Dottikon, Switzerland); filters were counted in a 1450 Microplate Counter (Wallac, Turku, Finland).

For *in vivo* priming with allogeneic BM-DC, BM cells from BALB/c mice were cultured until day 9 with either IL-3 or GM-CSF. Nonadherent cells were injected subcutaneously into each hind footpad of female C57BL/6 mice (10^5 per 50 μL phosphate-buffered saline per footpad). After 8 d the popliteal and inguinal lymph nodes were removed. From the single cell suspension of the lymph node cells, 4×10^5 cells per well were seeded into a 96-well flat-bottomed plate (Becton Dickinson) and restimulated with mitomycin-C-treated (Sigma) BALB/c spleen cells at titrated numbers. After 3 d the triplicate cultures were pulsed with 1μCi [^{3}H]-thymidine (Amersham) and harvested as described above.

***In vivo* migration of fluorescent MC** BM-MC were cultured for 4 wk and then labeled with 5 μM carboxyfluorescein (Molecular Probes) for 15 min at room temperature. MC were washed and injected subcutaneously (2×10^6 cells per footpad) or intravenously (1×10^7 cells). After 24 h popliteal lymph nodes or the spleen, respectively, were removed. Cryosections were stained with 3D6 antibody recognizing sialoadhesion CD169 (kindly provided by L. Martinez-Pomares and S. Gordon, Oxford, UK), followed by an antirat PE-conjugated secondary antibody (Pharmingen) to mark the subcapsular sinus of the lymph nodes or the marginal zone of the spleen.

IL-12 production BM cells from female BALB/c mice were cultured until day 8 either with IL-3 or GM-CSF, and then harvested and washed; 1×10^6 cells per well were transferred into a bacteria-quality 24-well plate (Becton Dickinson), and stimulated with SAC ± interferon-γ (IFN-γ, 100 U per ml, Pharmingen) ± LPS. After 48 h supernatants were analyzed for IL-12 p70 by ELISA by using an Opti EIA Mouse IL-12 p70 kit (Pharmingen). Analysis of intracellular IL-12 p40 production was performed with a Cytofix/Cytoperm Plus Kit (Pharmingen) according to the manufacturer's instructions. Cells were double stained with PE-conjugated CD11c (N418) or MHC II (M5/114) monoclonal antibody to gate on DC and non-DC before analysis by FACS.

Cell sorting Fresh BM cells were stained for CD31 (ER-MP12) and Ly-6C (ER-MP20) expression as described previously (de Bruijn *et al*, 1994) and cells within the indicated gates were sorted with a MoFlo high speed cell sorter (Cytomation Bioinstruments, Freiburg, Germany). Sorted BM subpopulations were cultured with IL-3 or GM-CSF for 8 d or 3 or 5 wk before the nonadherent cells were analyzed by FACS.

RESULTS

Simultaneous generation of DC and MC in short-term BM cultures supplemented with IL-3 BM cells are supposed to develop into MC after culture in IL-3 within a time period of about 4 wk. Here we cultured BM cells in IL-3 for 8 d and analyzed them for their morphology and by flow cytometry. A large proportion of cells at day 8 were already positive for CD117 (**Fig 1*a***) and the high affinity FcεR I (not shown), indicating the development of MC. Cytospin preparations of such cells showed the typical granular staining for MC with May–Grünwald–Giemsa reagent (**Fig 1*b***).

It has been described that MC can acquire MHC II expression under certain culture conditions using GM-CSF and IL-4 (Frandji *et al*, 1993; 1995). At this early time of day 8 BM culture in IL-3, a substantial proportion of cells was positive for MHC II, and only some of them coexpressed low amounts of CD117 or FcεR I (**Fig 1*a***). When we double stained these MHC II$^+$ cells for DC markers we found them positive for CD11c (**Fig 1*a***), CD14, and for intracellular 2A1 (not shown). The CD11c$^+$ DC did not express B220, CD8α, or CD205, but CD11b, partially Gr-1, and CD13, indicating that they are myeloid DC and not lymphoid DC (Henri *et al*, 2001) or plasmacytoid DC (Hochrein *et al*, 2002) (**Fig 1*a***). This expression pattern is nearly identical to myeloid DC that are generated from BM with GM-CSF (Lutz *et al*, 1999). A clear difference between IL-3/DC and myeloid GM-CSF/DC was observed regarding their expression of the IL-3Rα chain, which was almost absent on GM-CSF-derived BM-DC but clearly expressed on DC generated with IL-3. IL-3Rα chain expression by IL-3-derived DC is comparable to that on mature MC from a 4-wk-old culture generated with IL-3 (**Fig 1*c***).

In the following experiments we wanted to substantiate the presumed DC identity of the cells obtained from IL-3-stimulated cultures by assessing major DC functions such as endocytosis, maturation capacity, IL-12 production, and T cell priming and homing.

The endocytosis capability of immature IL-3/DC is comparable to that of GM-CSF/DC Immature myeloid DC *in vivo* reside in peripheral tissues as sentinels awaiting the penetration of microorganisms. DC at this developmental stage bear multiple receptor-dependent and receptor-independent mechanisms to take up foreign antigens. These antigens are processed intracellularly and are subsequently transported to the T cell areas of the draining lymph nodes for antigen presentation (Banchereau and Steinman, 1998). Two of the best studied mechanisms of DC endocytosis are macropinocytosis and mannose-receptor-mediated uptake of fluorescence-labeled tracers, such as Lucifer Yellow and FITC-ovalbumin or FITC-dextran, respectively (Sallusto *et al*, 1995; Lutz *et al*, 1996; 1997). When we compared the level of antigen uptake of these tracers by GM-CSF/DC and IL-3/DC no differences were found (**Fig 2**). Also the phagocytic activity of FITC-conjugated latex beads was comparable (not shown).

Phenotypical and morphologic maturation of IL-3/DC DC mature upon contact with proinflammatory stimuli, microbial products from viruses or bacteria, or CD40 signals from T cells, which results in upregulation of MHC and costimulatory molecules (Banchereau and Steinman, 1998). When IL-3/DC cultures were kept in IL-3 alone, only very few CD11c$^+$ MHC II^{high} expressing cells, representing mature DC, were detectable. The CD11c$^+$ cells also did not express CD40 or CD86, and very little CD80 (**Fig 3*a***, ctrl). A mild maturation of IL-3/DC could be induced, however, by stimulation with TNF-α and all markers were clearly upregulated by LPS (**Fig 3*a***). The maturation of DC was confirmed by the induction of dendritic morphology, which was again mild with TNF-α but very strong with a SAC preparation (**Fig 3*b***).

IL-3/DC and GM-CSF/DC are similarly potent to produce IL-12 p40 and p70 after stimulation DC can be stimulated to secrete cytokines such as IL-12, which promotes polarization of T helper cells towards the Th1 direction (Cella *et al*, 1996; Koch *et al*, 1996; Vieira *et al*, 2000). There has been ample discussion about the superiority of human plasmacytoid IL-3Rα$^+$ DC *versus* myeloid monocyte-derived DC or murine lymphoid-related CD8α$^+$ *versus* myeloid CD8a$^-$ DC in their capability to produce IL-12 (summarized in Moser and Rock, 2000). When we tested our myeloid GM-CSF/DC and IL-3/DC for IL-12 p40 production by intracellular FACS staining we found that only a fraction of the CD11c$^+$ GM-CSF-cultured cells but none of the CD11c$^-$ cells produced IL-12 p40 (**Fig 4*a***). Further analysis revealed that only the mature MHC II^{high}, CD11c$^+$ GM-CSF/DC were responsible for the IL-12 p40 production (**Fig 4*b***). Interestingly, in the IL-3-stimulated cultures both the CD11c$^+$ and CD11c$^-$ subpopulations produced IL-12 p40 (**Fig 4*a***). Both IL-3/DC and GM-CSF/DC were also stimulated with SAC alone or in combination with IFN-γ or IFN-γ + LPS to induce bioactive IL-12 p70. Whereas ELISA supernatants from SAC or SAC + IFN-γ stimulated cells contained comparable amounts of IL-12 p70 for both IL-3/DC and GM-CSF/DC, IL-3/DC were superior to GM-CSF/DC when the triple cocktail SAC + IFN-γ + LPS was used (**Fig 4*c***).

IL-3/DC but not MC facilitate homing to the lymph node T cell areas and priming of allogeneic T cells IL-3/DC and GM-CSF/DC were tested for their potential to stimulate allogeneic CD4$^+$ T cells *in vitro*. To eliminate the effects of endogenously produced TNF on immature and LPS-matured stages of GM-CSF/DC and IL-3/DC, BM-DC were generated from TNFR 1 + 2$^{-/-}$ mice (see also *Materials and Methods*). Unstimulated IL-3/DC were slightly weaker stimulators of T cell proliferation than GM-CSF/DC, but when both DC types were pretreated with LPS the T cell proliferation was comparable (**Fig 5*a***).

A prerequisite for T cell priming *in vivo* is the ability of the antigen presenting cell to migrate into the T cell areas of lymphoid organs. When IL-3/DC or GM-CSF/DC (C57Bl/6) were injected subcutaneously into allogeneic mice (BALB/c) and the draining lymph node cells were restimulated after 10 d with the same allotype of spleen cells as used for the priming (C57Bl/6), an increased proliferation of draining lymph node cells was observed in mice that were preinjected with IL-3/DC or GM-CSF/DC (**Fig 5*b***). Although GM-CSF/DC were superior in T cell stimulation *in vivo*, a clear priming effect was also observed with IL-3/DC.

Although MC have been shown to migrate into lymph nodes *in vivo* (Wang *et al*, 1998), we found that fluorescence-labeled MC do indeed enter the lymph nodes but cannot reach the T cell areas. MC remain in the subcapsular sinus of the lymph nodes after subcutaneous injection and the red pulp of the spleen after intravenous injection (**Fig 5*c***), indicating that they are not involved in antigen presentation to T cells *in vivo*.

Taken together, we generated DC from BM precursor cells with IL-3 that show phenotypical and functional characteristics highly similar to those described for BM-DC generated with GM-CSF (Inaba *et al*, 1992; Scheicher *et al*, 1992; Lutz *et al*, 1999).

IL-3/DC and MC develop from different progenitor cells in BM Using IL-3 in BM cultures DC and MC were generated simultaneously. DC development stimulated by IL-3 reaches the highest proportion of DC around day 9 or 10 of culture (not shown) equivalent to GM-CSF/DC (Lutz *et al*, 1999). MC development continues for more than 8 wk and ends with a highly pure MC population after 4–6 wk because other primary BM cell types do not survive for such an extended period of culture (Lantz *et al*, 1997, and our own observations).

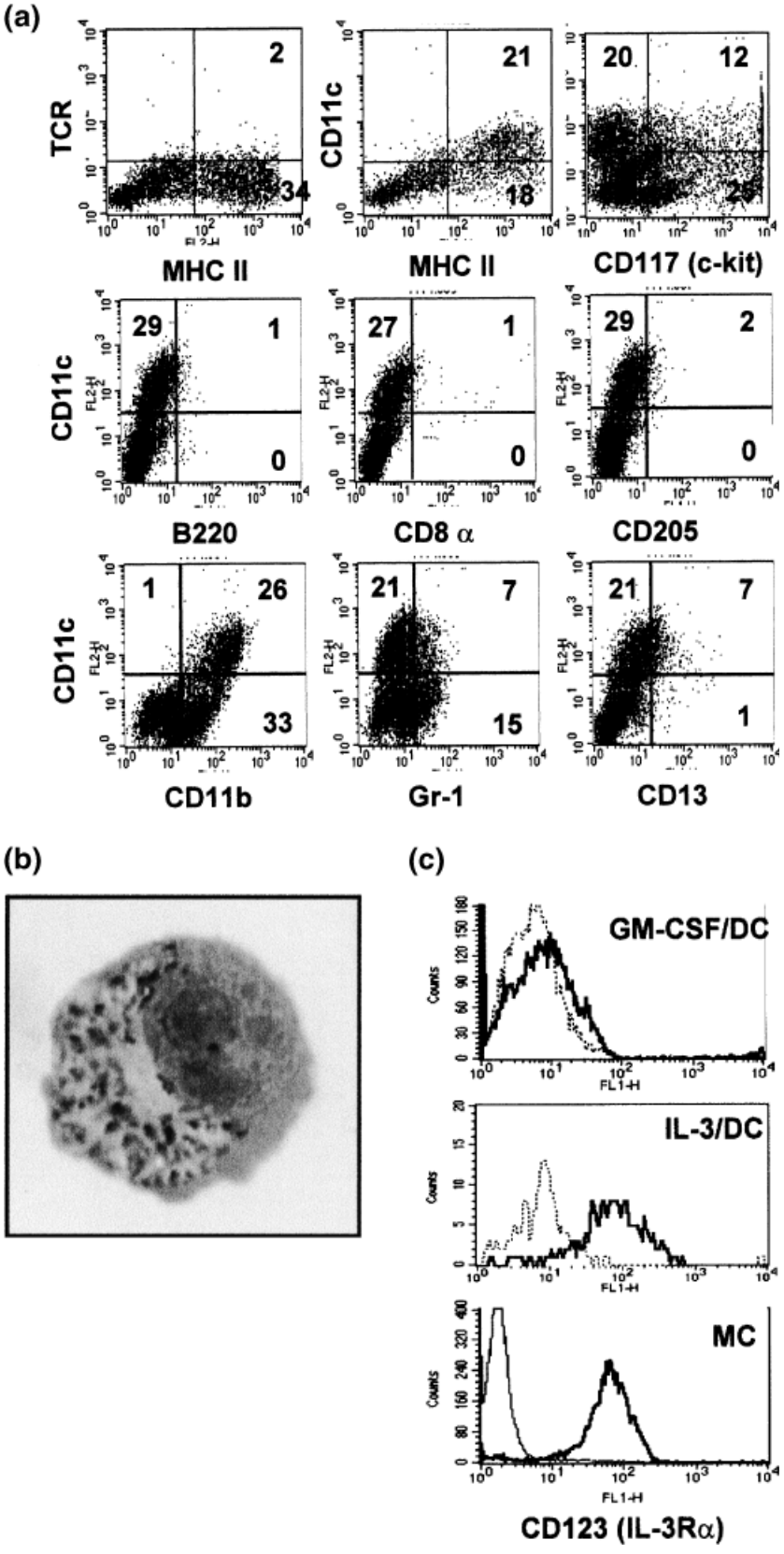


Figure 1. Simultaneous generation of MC and IL-3Rα$^+$ DC in BM cultures supplemented with IL-3. BM cells were cultured for 8 d with IL-3. (*a*) Double stainings were performed for MHC II *versus* TCR-αβ (negative control) and CD11c, or for CD11c *versus* the indicated markers. (*b*) MC and their precursors within the cultures could be identified by their cytoplasmic granularity visible after May–Grünwald–Giemsa staining of a cytospin preparation (original magnification 1000×). (*c*) DC were generated with IL-3 and GM-CSF until day 8 and pure MC were generated by culture with IL-3 for 4 wk and then analyzed for CD123 (IL-3Rα) expression. Day 8 cultures were gated on MHC II$^+$ cells.

Therefore, we addressed the question whether there is a common progenitor for IL-3/DC and MC. Two markers have been described to follow the development of BM cells from the earliest stem cells to enriched subsets for committed erythroid, lymphoid, granulocytic, and macrophage/DC development (de Bruijn *et al*, 1994; Nikolic *et al*, 2003). When fresh BM cells were double stained with the antibodies ER-MP12 (CD31, PECAM-1) and ER-MP20 (Ly-6C) six distinct populations could be detected (**Fig 6*a***). The ER-MP12^{neg}/20^{high} region (**Fig 6*a***, R6) contains BM monocytes, which show restricted development into

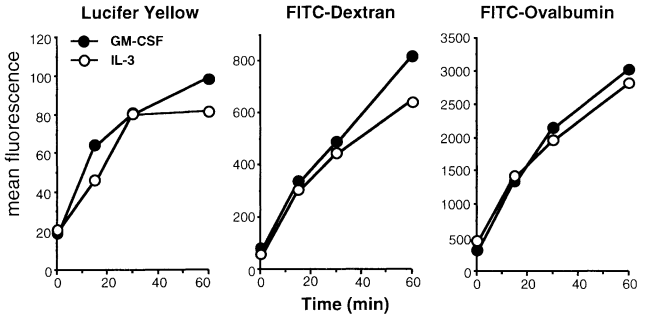


Figure 2. Endocytosis capacities of IL-3/DC and GM-CSF/DC. BM cells were cultured with GM-CSF or IL-3 for 8 d. Macropinocytosis was analyzed with Lucifer Yellow and FITC-ovalbumin, and mannose-receptor-mediated uptake with FITC-dextran, after 0, 15, 30, and 60 min; cells were double stained with MHC II. The mean fluorescence values of MHC II^{low} expressing immature DC are shown.

macrophages and DC when cultured in GM-CSF, whereas ER-$MP12^{neg}/20^{int}$ cells (**Fig 6*a***, R5) represent exclusively neutrophilic polymorphonuclear granulocytes (de Bruijn *et al*, 1994, and our own unpublished observations). Here we sorted fresh BM cells within the same gates (ER-$MP12^{neg}/20^{high}$ and ER-$MP12^{neg}/20^{int}$) and cultured them with IL-3, or as a control with GM-CSF. As described, the cells cultured with GM-CSF for 8 d developed into DC and macrophages (**Fig 6*b***, from R6) and MHC II^{-} $CD11c^{-}$ cells, most probably granulocytes (**Fig 6*b***, from R5), respectively. In contrast, with IL-3 no cells grew within the granulocyte gate (**Fig 6*b***, from R5, ER-$MP12^{neg}/20^{int}$), but equal amounts of DC could be generated from the ER-$MP12^{neg}/20^{high}$ population (**Fig 6*b***, from R6).

To address whether BM monocytes might represent common precursors for IL-3/DC and MC, the same BM population that gives rise to IL-3/DC (cells within R6 of **Fig 6*a***) was sorted for ER-$MP12^{neg}/20^{high}$ (R6) and cultured for 3 wk. All DC had died in cultures with IL-3 and, as known, with GM-CSF. Only adherent macrophages remained, but no nonadherent MC could be detected. This shows that DC develop from different precursors within the BM than MC, although both respond to IL-3.

It remained to be established which region R1–R4 would give rise to MC after culture for more than 4 wk in IL-3. Therefore fresh BM cells were stained for ER-MP12/20 and the cells within the regions R1–R4 were sorted and cultured in IL-3 for 38 d. Only the cells within the regions R1 and R2 developed into MC (**Fig 7*a***, ***b***), whereas cells sorted from the regions R3 and R4 had all died after this time. DC could be generated with GM-CSF or IL-3 after 8 d from the regions R1, R2, and R4 (**Fig 7*c***, ***d***). As shown recently for GM-CSF-stimulated DC cultures (Nikolic *et al*, 2003), the regions R1 and R4 may be considered

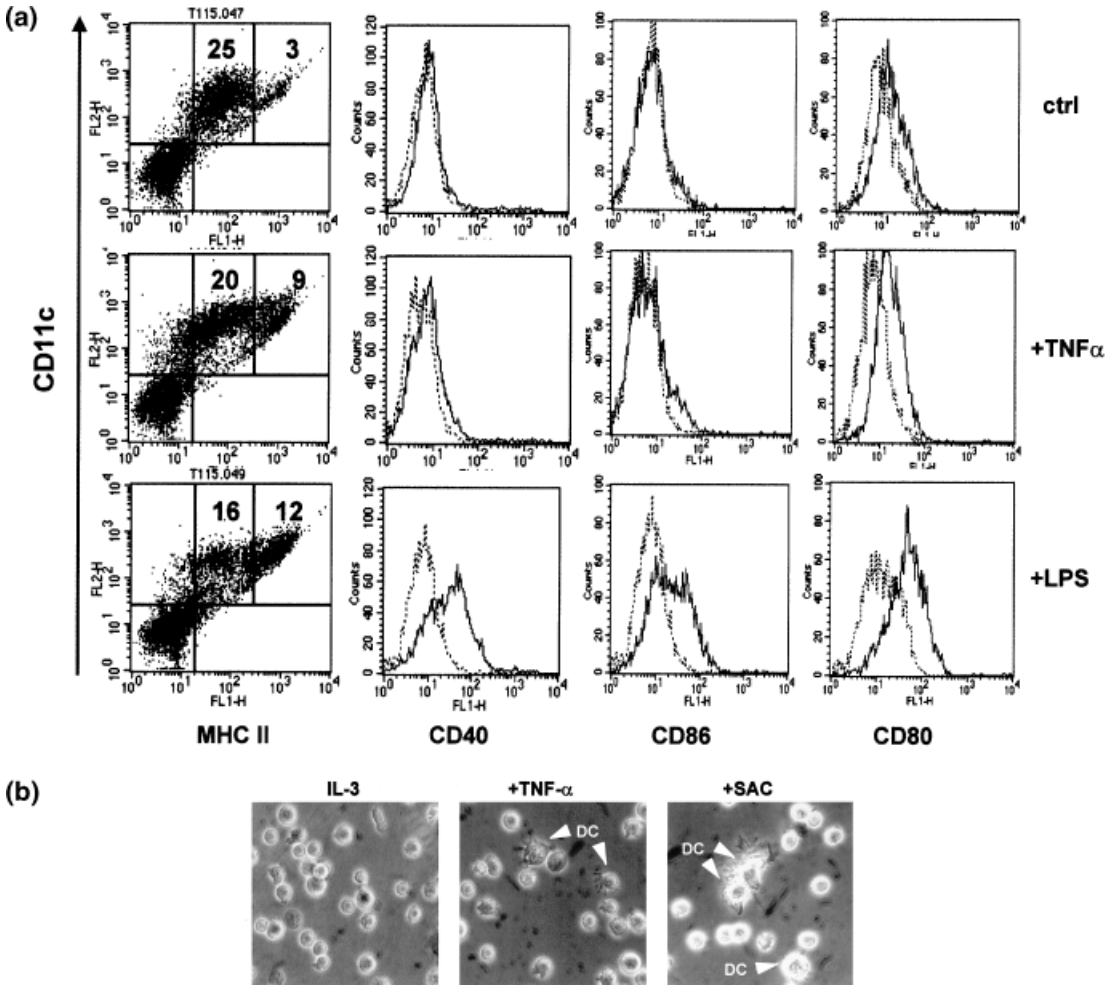


Figure 3. Maturation of IL-3/DC measured by MHC II and B7-2 expression and acquisition of veiled morphology. BM cells were cultured with IL-3 until day 8 and then stimulated overnight with the indicated reagents. (*a*) FACS analysis for CD11c *versus* MHC II and histograms gated on $CD11c^{+}$ cells of the markers CD40, CD80, and CD86 of unstimulated cells (ctrl) or TNF- or LPS-stimulated cells are shown (*straight lines*), with isotype stainings as overlays (*dotted line*). Numbers within the dot plots represent percentages of mature DC within the quadrants. (*b*) Phase contrast photographs were taken from untreated cultures with IL-3 alone (IL-3) or with the indicated stimuli. Original magnification 400 ×.

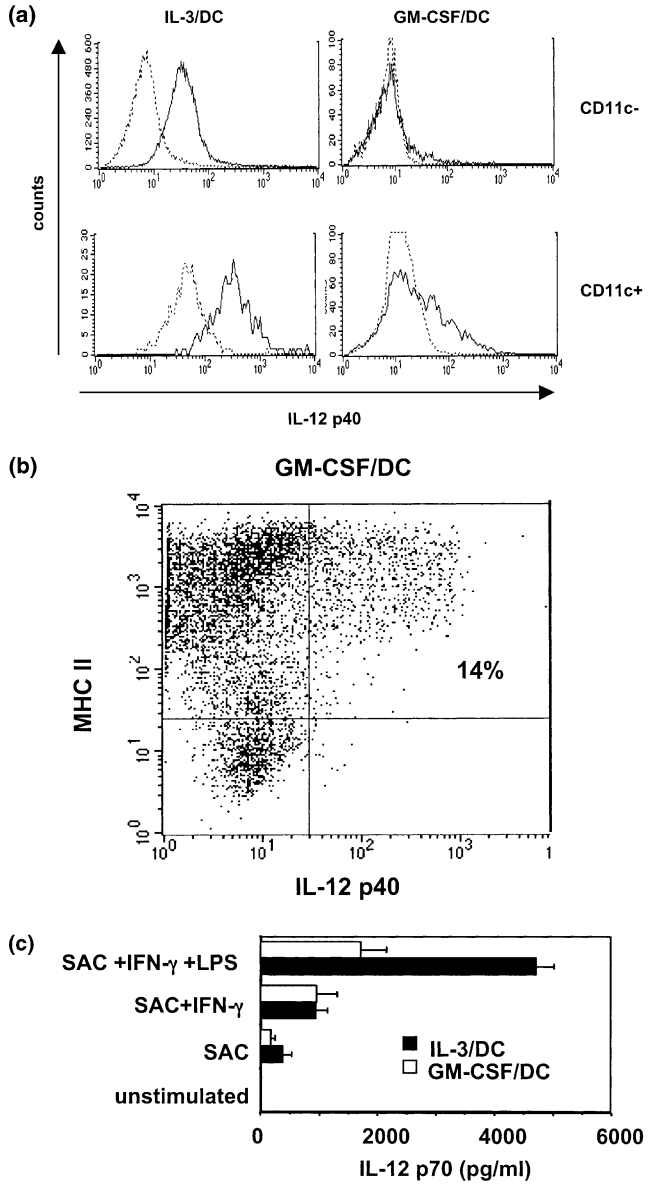


Figure 4. IL-3/DC and GM-CSF/DC produce similar amounts of IL-12. (*a*), (*b*) BM cells were cultured with IL-3 or GM-CSF until day 8 and then incubated overnight with cytoperm solution and LPS for cytokine production. Then, the cells were fixed and stained intracellularly for IL-12 p40 (*continuous line*) or an isotype control monoclonal antibody (*dotted line*) and on the surface for CD11c. (*a*) Histograms for IL-12 p40 staining of gated $CD11c^+$ and $CD11c^-$ cells. Both subsets produce IL-12 p40. (*b*) GM-CSF/DC were double stained for MHC II, indicating that only the most mature DC (MHC II^{high}) produce IL-12 p40. (*c*) IL-3/DC and GM-CSF/DC were cultured until day 8 and then stimulated with SAC alone or in combination with LPS and IFN-γ to detect IL-12 p70 after 48 h by ELISA.

as the major sources for DC development also for IL-3-stimulated cultures. In our experiments, R2 cells also generated some DC in cultures with GM-CSF or IL-3. Given the low yields from this fraction, however, we cannot completely rule out R1 contamination. For the development of MC the cells from region R1 also show a higher expansion potential than R2 cells. The region R1 represents the source of the earliest myeloid progenitor cells responsive to GM-CSF before they develop into R4 and finally R6 (de Bruijn *et al*, 1994; Nikolic *et al*, 2003). R2 is known to contain the earliest hematopoietic stem cells with long-term repopulating activity *in vivo* (van der Loo *et al*, 1995).

Together, DC development with IL-3 from BM cells could be observed mainly from the populations R1, R4, and R6, and possibly to a limited extent from R2. MC can predominantly be derived from cells within the region R1 and less effectively from R2. This might indicate that DC and MC derive from the same pool of early GM-CSF- or IL-3-responsive cells, but DC follow different routes of differentiation within the BM as followed with the markers ER-MP12 (CD31) and ER-MP20 (Ly-6C). DC development from the different regions seems to follow the same routes, however, whether the subsets are cultured with GM-CSF or IL-3.

MC do not convert into DC To test whether established MC cultures can develop into DC, an 8-wk-old BM-MC culture was washed and recultured in IL-3, GM-CSF, or GM-CSF plus IL-4 for 2 and 11 d. No MHC II or CD11c DC marker expression could be detected in such MC cultures, however, and the MC showed increased signs of apoptosis (annexin V^+) in cultures without IL-3 (**Fig 8**).

DISCUSSION

Only the development of MC has been reported so far when murine BM cells are cultured with IL-3 (Nakahata *et al*, 1986; Huff and Lantz, 1997). Therefore we investigated whether murine BM cultures supplemented with IL-3 can also give rise to DC, and what their lineage origin and relation to MC might be.

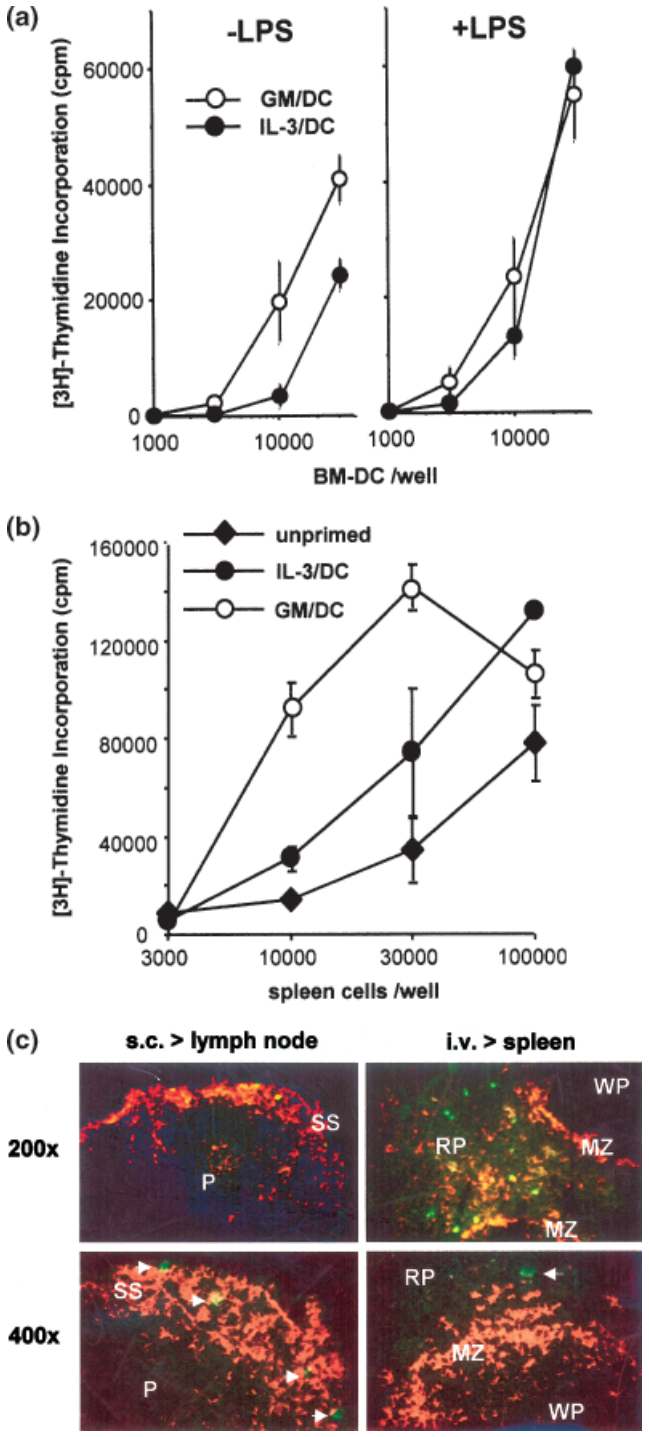
Early day 8–10 BM cultures with IL-3 contain around 70% MC or their precursor stages but also about 30% MHC II^+ $CD11c^+$ cells that coexpress typical myeloid BM-DC markers such as CD11b, CD14, and low amounts of CD13, Gr-1, and intracellular 2A1 (not shown), similarly to GM-CSF/DC (Lutz *et al*, 1999). In contrast, the lymphoid DC markers CD8α and CD205 as well as plasmacytoid DC marker B220 are not expressed. Other typical features of myeloid DC, such as endocytosis, maturation potential, IL-12 production, and T cell priming and homing *in vivo* were also comparable to myeloid DC that have been generated with GM-CSF, as described previously (Lutz *et al*, 1999). The only clear difference to GM-CSF/DC is that IL-3/DC express higher levels of CD123, the IL-3Rα chain. Therefore our data strongly argue for the generation of $CD11c^+$ $CD11b^+$ $B220^-$ $CD8\alpha^-$ myeloid DC in early day 8–10 cultures with IL-3.

Previously, it has been reported that MC from IL-3-stimulated BM cultures could be induced by GM-CSF or GM-CSF plus IL-4 to express MHC II and costimulatory molecules and therefore these cells could be used as professional antigen presenting cells *in vitro* (Frandji *et al*, 1993; 1995; Tkaczyk *et al*, 2000) and *in vivo* (Villa *et al*, 2001). In contrast, we found that pure cultures of BM-MC, which were only obtained after 6–8 wk of culture, could not be induced to express MHC II molecules or develop into $CD11c^+$ cells upon culture in GM-CSF or GM-CSF plus IL-4. *In vivo* migration of MC during immune responses can occur from peripheral organs into the lymph nodes of mice (Wang *et al*, 1998). We also observed MC entering the lymph nodes or spleen, but they did not reach the T cell areas, but rather remained in the subcapsular sinus of lymph nodes or red pulp of the spleen. Consequently, MC also could not induce allogeneic T cell priming *in vivo* (not shown). Short-term day 8 BM cultures with IL-3, however, which contain IL-3/DC, could readily induce allogeneic T cell priming *in vivo*. Based on these data, we argue that MC cannot act as professional, migrating antigen presenting cells, but that BM-MC cultures might be contaminated by IL-3/DC, which then exert these functions.

A remarkable difference between the IL-3- and GM-CSF cultures is that IL-3/DC developed together with MC, whereas GM-CSF/DC grow in concert with neutrophils (Lutz *et al*, 1999). In our cultures IL-3 seems to promote the development of DC and MC with different kinetics, as DC are detectable at maximal

levels around days 8–10 and MC around 4–8 wk. To address the question of a lineage relation between IL-3/DC and MC, we sorted BM cells into six populations by the markers ER-MP12 and ER-MP20. MC development was more restricted than IL-3/DC development, as MC grew only from the sorted ER-MP12/20 populations R1 and R2, whereas IL-3/DC could be generated additionally from R4 and R6 cells, similar to GM-CSF/DC. This argues against a close lineage relationship between later myeloid stages of IL-3/DC and MC, but does not exclude a common early progenitor within BM. For myeloid DC development one might speculate whether an early myeloid progenitor is equally responsive to IL-3 or GM-CSF.

A physiologic role for the overlapping functions of IL-3 on the generation of DC and MC even from separate lineages remains unclear so far. Neutrophil generation is promoted by GM-CSF, as is the development of DC and macrophages. Functionally, neutrophil mobilization is associated with Th1 immune responses, whereas MC activation is observed during allergic responses and Th2 immunity. Studies investigating IL-3$^{-/-}$ mice revealed that IL-3 is not essential for MC and basophil generation, but certain types of immune responses were impaired, such as delayed-type



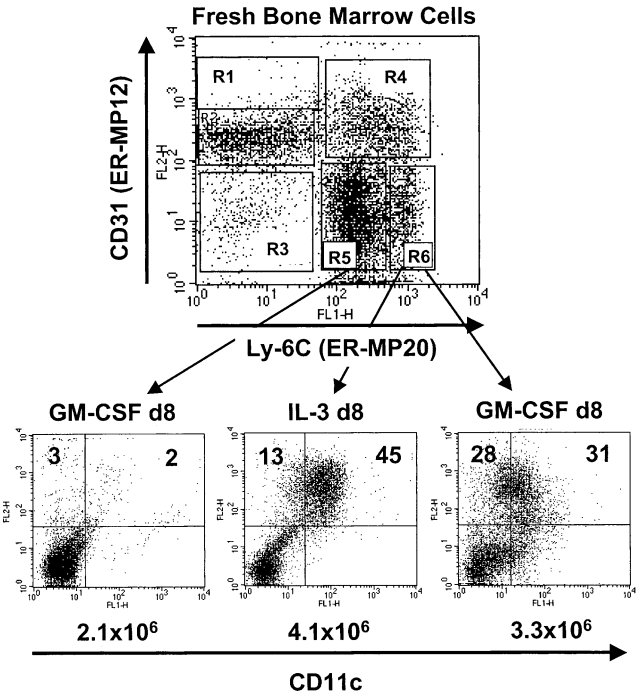
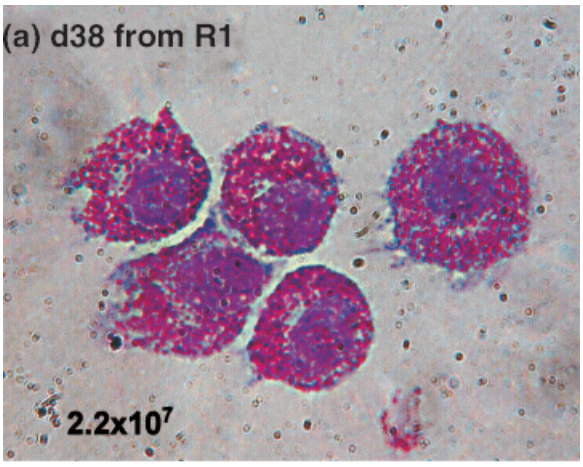
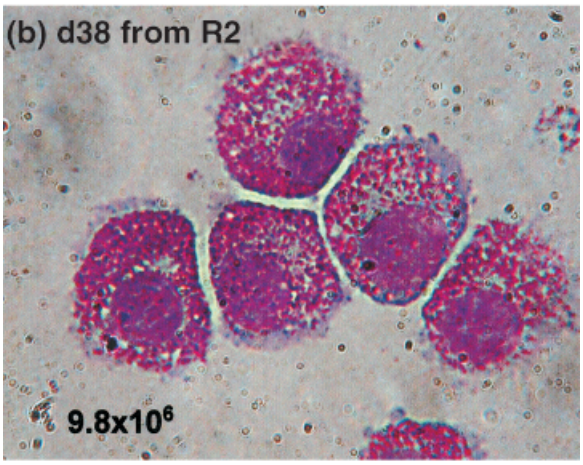
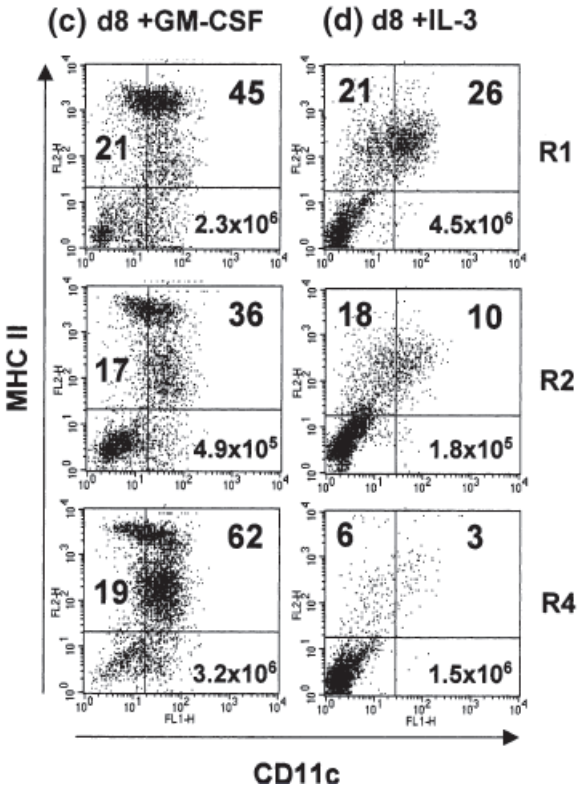


Figure 6. IL-3/DC and GM-CSF/DC but not MC derive from the same late BM precursor cell population. (*a*) Fresh BM cells were sorted for CD31^{neg} Ly-6C intermediate and high expressing cells indicative for restricted granulocyte (R5) and macrophage/DC development (R6), and 5×10^5 cells per well were cultured with GM-CSF. (*b*) FACS analysis of cells cultured with IL-3 and GM-CSF to detect DC (after 8 d). Double staining was performed for MHC II and CD11c to detect DC. Numbers within the quadrants represent percentages of cells. Numbers below the dot plots represent the total cell yields at day 8. No nonadherent cells could be detected from cells sorted from R5 and cultured with IL-3 (after 8 d or 3 wk) and from R6 with GM-CSF after 3 wk.

Figure 5. Capacities of T cell priming by DC generated in IL-3 or GM-CSF and MC homing. (*a*) IL-3/DC and GM-CSF/DC were generated from TNFR 1 + 2 double deficient mice (see *Materials and Methods*). Unstimulated and LPS-stimulated DC were cocultured with allogeneic T cells for 3 d before proliferation was assessed. Error bars represent standard deviations. The figure is representative for four independent experiments. (*b*) IL-3/DC and GM-CSF/DC were generated from C57/BL6 mice until day 8, and then 2×10^5 DC were injected subcutaneously into BALB/c mice. Draining lymph node cells of not injected and DC-injected mice were restimulated with allogeneic spleen cells (C57/BL6) *in vitro* and proliferation tested after 3 d. Error bars represent standard deviations. The figure is representative for three independent experiments. (*c*) MC were cultured for 6 wk in IL-3, labeled with the fluorescent tracer carboxyfluorescein, and then injected subcutaneously into the hind footpads or intravenously into syngeneic mice. After 24 h draining lymph nodes or spleen were removed and cryosections were stained with 3D6 antibody, marking the subcapsular sinus (SS) but not paracortical T cell areas (p), or the marginal zone (MZ) but not the red pulp (RP) or white pulp (WP), respectively. Green cells indicate migrated MC (also by *arrows*). Original magnifications were 200 × or 400 ×. The figure is representative for two independent experiments.







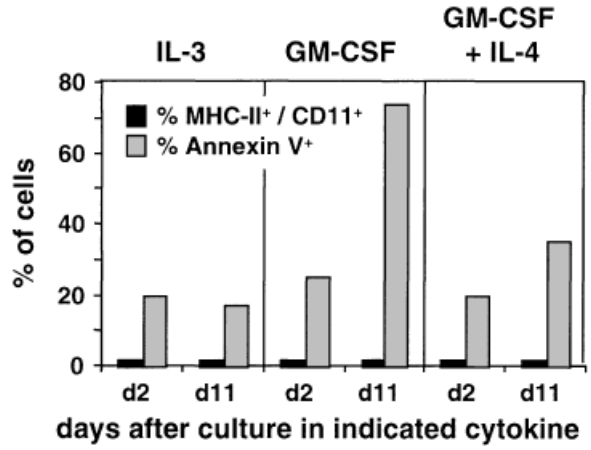


Figure 8. Terminally differentiated MC cannot be induced to express MHC II or convert to CD11c$^+$ DC with GM-CSF ± IL-4. Cultures of pure MC after 8 wk were left in IL-3 or washed and cultured in GM-CSF or GM-CSF ± IL-4 for 2 or 11 d. Then cells were analyzed by FACS for MHC II and CD11c expression to detect DC and annexin V binding to detect apoptosis.

hypersensitivity reactions (Mach *et al*, 1998) and immune responses to parasites (Lantz *et al*, 1998). Given the fact that DC colocalize with MC in surface organs such as the skin where infections take place, IL-3 might exert additional functions also on DC. This fact needs now further evaluation in delayed-type hypersensitivity reactions or infection models.

Although both GM-CSF/DC and IL-3/DC can be induced to secrete Th1 polarizing IL-12 *in vitro*, a cellular cooperation between DC and neutrophils or DC and MC might polarize towards Th1 or Th2, respectively. For human DC cultured with IL-3 plus IL-4 it has been reported that such cells rather polarize for Th2 responses (Ebner *et al*, 2002). This topic of DC instruction for Th polarization (Kalinski *et al*, 1999) and the role of MC in influencing this process are currently under investigation.

Taken together, we described the simultaneous generation of DC and MC from murine BM cultures by IL-3. Given the fact that these two cell types colocalize preferentially in surface organs such as the skin, IL-3 might play a dual role on DC and MC for the induction of Th2 responses such as allergies.

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Figure 7. MC development from BM is more restricted than IL-3/DC development. Fresh BM cells were stained with ER-MP12 and ER-MP20 antibodies as in Fig 6, and the sorted populations within regions R1, R2, R3, and R4 were cultured for 8 or 38 d with a starting cell number of 1.5×10^5 per culture. (*a*), (*b*) MC could only be generated from sorted cells within the R1 and R2 regions as detected by May–Grünwald–Giemsa staining of cytospins. (*b*) DC development was analyzed after 8 d and detected by staining for MHC II and CD11c, analyzed by FACS. From R3 no DC could be generated. Large numbers within the dot plots represent percentages of cells within the respective quadrants; small numbers indicate the total cell number yielded from the cultures at day 8 or day 38.

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