International Immunology, Vol. 15, No. 4, pp. 515–524
doi: 10.1093/intimm/dxg050

Developmental stages of myeloid dendritic cells in mouse bone marrow

Tatjana Nikolic[1], Marella F. T. R. de Bruijn[1,3], Manfred B. Lutz[2] and Pieter J. M. Leenen[1]

[1]Department of Immunology, Erasmus MC, University Medical Center, PO Box 1738, 3000 DR Rotterdam, The Netherlands
[2]Department of Dermatology, University of Erlangen, D-91052 Erlangen, Germany

[3]Present address: Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DC, UK

Keywords: bone marrow, dendritic cells, myeloid development

Abstract

The lineage relationship of dendritic cells (DC) with other hematopoietic cell types has been studied extensively, resulting in the identification of different bone marrow (BM) progenitors that give rise to distinct DC types. However, the identity of the different maturation stages of DC precursors in the BM remains unclear. In this study we define the *in vivo* developmental steps of the myeloid DC lineage in mouse BM. To this end, BM cells were separated according to their expression of CD31 (ER-MP12), Ly-6C (ER-MP20) and ER-MP58 antigens, and stimulated to develop into myeloid DC, using granulocyte macrophage colony stimulating factor as a specific growth factor. DC developed from three BM subpopulations: ER-MP12^{hi}/20^{-} (early blast cells), ER-MP12^{+}/20^{+} (myeloid blasts) and ER-MP12^{-}/20^{hi} (monocytes). The kinetic and phenotypic features of DC developing in vitro indicate that the three populations represent successive maturation stages of myeloid DC precursors. Within the earliest ER-MP12^{hi}/20^{-} population, DC precursors exclusively occurred in the myeloid-restricted ER-MP58^{hi} subset. By using switch cultures, we show that these BM precursor subpopulations, when stimulated to develop into macrophages using macrophage colony stimulating factor, retain the ability to develop into myeloid DC until advanced stages of maturation. Together, these findings support a common ER-MP12/20-defined differentiation pathway for both macrophages and myeloid DC throughout their BM development.

Introduction

Dendritic cells (DC) are bone marrow (BM)-derived cells that have the unique capacity to initiate a primary immune response by efficient antigen presentation to naive T cells. The DC system is widely distributed throughout the body, and comprises Langerhans cells in epithelial locations, veiled cells in lymph, interdigitating cells in lymphoid organs and interstitial DC in connective tissue of non-lymphoid organs (1). Many studies in the past few years have addressed the lineage relationships of DC with other hematopoietic lineages. Mainly, DC of two possible origins, lymphoid and myeloid, have been identified in human and mouse. Both types have been shown to originate from the BM from which they can be derived *in vitro* by stimulation with different growth factors (2–4). Myeloid DC have been generated *in vitro* from early common myeloid progenitors in BM and from monocytes in peripheral blood using granulocyte macrophage colony stimulating factor (GM-CSF) as a growth stimulus. In contrast, using Flt-3 ligand (Flt-3L), both myeloid and lymphoid DC can be generated *in vitro* from BM (5–7). A progenitor population for human lymphoid DC, which also can give rise to lymphoid cells, has been identified in BM and thymus (3). However, data about this DC lineage in the mouse are inconclusive since the identity of the mature lymphoid DC has been unclear. An earlier study on mouse thymic DC precursors has suggested CD8α as a marker of the lymphoid-related DC lineage (8). More recent reports, however, show that CD8α is not a reliable marker for lymphoid DC since it can be expressed also on myeloid DC (9) and that CD8α^{+} DC can be derived from myeloid progenitors (10). A new candidate for the lymphoid-related DC in the mouse has been proposed (11–13) which

The first two authors contributed equally to this work

Correspondence to: T. Nikolic; E-mail: t.nikolic@erasmusmc

Transmitting editor: P. W. Kincade

Received 1 August 2002, *accepted* 20 January 2003

shows characteristics equivalent to those of human plasmacytoid DC (PDC).

The developmental plasticity inherent to the DC system has been demonstrated in different studies, showing that both common lymphoid and common myeloid progenitors are able to differentiate into different types of DC (14–16). The studies by Manz *et al.* and Mebius *et al.* indicated that both types of progenitors give rise to both CD8α^+ and CD8α^- DC *in vitro* and *in vivo* (14,15), while Izon *et al.* showed a previously unrecognized link between early B cell and DC ontogeny (16). Furthermore, recent experimental evidence suggests the existence of a common DC precursor in the BM (17) as well as in the peripheral blood (18) for CD8α^+, CD8α^- and presumed PDC.

The studies cited above were mainly directed to define the origins of the different DC types and to identify their precursors in the BM. However, the identification of the different developmental stages of DC precursors in the BM still remains unclear. In this study, we have investigated in mouse BM the *in vivo* differentiation pathway of myeloid DC progenitors identified by differential ER-MP12/CD31 and ER-MP20/Ly-6C expression. In addition, we addressed the developmental relationship of myeloid DC precursors with macrophage precursors.

Methods

Mice

Female C57BL6/J and C57BL6/Ly-5.1-Pep^{3b} mice between 11 and 13 weeks of age were used in this study. Female C3HeB/Fej mice were used as a source of allogeneic responder T cells. Animals were specific pathogen free, and kept with free access to food and water in the animal care facility at the Erasmus University Rotterdam under the institutional guidelines for usage of experimental animals.

mAb and conjugates

mAb used for cell sorting, flow cytometric analysis, immunocytochemistry and read out of T cell activation in the mixed leukocyte reaction (MLR) were either generated in our laboratory or obtained from BD PharMingen (Alphen aan de Rijn, The Netherlands). These were: biotinylated ER-MP12 (anti-CD31); FITC–ER-MP20 (anti-Ly6C); undiluted hybridoma culture supernatants ER-MP58 (19,20), ER-MP21 (anti-CD71/transferrin R) and M5/114 (anti-MHC class II), obtained in our laboratory; and 53-6.7–FITC (anti-CD8α), RM4-5–PE (anti-CD4) and HL3–phycoerythrin (PE) (anti-CD11c) purchased from BD PharMingen. Biotinylated ER-TR3 (anti-MHC class II) antibody was kindly provided by BMA Biomedicals (Augst, Switzerland). PE–streptavidin, TriColor–streptavidin and PE–goat anti-rat IgG (mouse absorbed) were used as conjugates (Caltag, San Francisco, CA).

Cell suspensions

Single-cell suspensions of BM, isolated from femora and tibiae, were prepared as described previously (20,21). Similarly, single-cell suspensions of spleen and lymph nodes were prepared and used for T cell isolation as described (22). Cultured BM-derived DC were isolated from Teflon culture bags or from culture plates. Cells were washed in PBS supplemented with 5% FCS (heat inactivated), 60 μg/ml penicillin and 100 μg/ml streptomycin when sterile suspensions were required. Prior to phenotypic analysis, cultured cells were washed with PBS supplemented with 0.5% (v/v) BSA (Organon Teknika, Boxtel, The Netherlands) and 20 mM sodium azide.

Cell sorting and flow cytometric analysis

For cell sorting, BM cells were labeled with two (ER-MP12 and ER-MP20) or three (ER-MP12, ER-MP20, and ER-MP58) mAb as described previously (20,21). Prior to sorting (FACS Vantage; Becton Dickinson, Amsterdam, The Netherlands), cell suspensions were filtered over a 30-μm sieve (Polymon PES; Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-running sorted samples. Purity was >95%, unless stated otherwise. Suspensions were kept at 4°C throughout the staining and sorting procedure. Sorted cells were counted in a Bürker hemocytometer.

For flow cytometric analysis, samples of cultured cells ($\geqslant 2 \times 10^4$ cells) were aliquoted into 96-microwell plates (round bottom; Nunc, Roskilde, Denmark) and labeled with antibodies as described before (21). Samples were analyzed on a FACSCalibur flow cytometer using CellQuest analysis software (Becton Dickinson).

Immunocytochemistry on poly-L-lysine-coated slides

For immunocytochemical analysis, aliquots of cultured cells were placed at 37°C, 7% CO_2 in complete medium for 1 h to adhere to poly-L-lysine-coated microwell slides (Nutacon, Schiphol-Oost, The Netherlands). Next, cell preparations were gently washed once with PBS (37°C), fixed in 1% paraformaldehyde in PBS for 10 min at room temperature, and washed with PBS and with PBS supplemented with 0.5% BSA. Cells were incubated with primary mAb, followed by rabbit anti-rat IgG conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch, West Grove, PA) (both incubations 30 min at room temperature). Diaminobenzidine (DAB; Sigma, St Louis, MO) was used as substrate. Between incubations, cell preparations were washed in PBS supplemented with 0.5% (v/v) Tween 20. After the DAB reaction was completed (3–5 min at room temperature), the cell preparations were dehydrated and coverslipped using Entallan (Merck, Darmstadt, Germany). Antibody reactivity was determined under a light microscope. Culture supernatant of the Y3 myeloma followed by the second stage antibody was used as negative control.

Growth factors

Conditioned medium of LADMAC cells was used as a source of mouse macrophage colony stimulating factor (M-CSF) (23). Conditioned medium was prepared as described elsewhere (19). Recombinant mouse GM-CSF (rGM-CSF) was purchased from Biosource International (Camarillo, CA).

rGM-CSF- and M-CSF-stimulated BM cultures

DC were generated by culturing total BM or isolated subsets in Teflon culture bags (24) or 24 well plates (Nunc) in RPMI-1640 (no HEPES; Biowhittaker, Verviers, Belgium) supplemented

with 10% FCS (heat inactivated; 0.2 μm filtered), 60 μg/ml penicillin, 100 μg/ml streptomycin and 20 ng/ml rGM-CSF. Cells were cultured at 37°C, 7% CO_2 for various periods of time, as indicated, for up to 14 days. Differentiation of isolated BM subsets, to macrophages, was induced by culturing in Teflon culture bags (24) in IMDM (with glutamax I; Gibco, Invitrogen BV, Breda, The Netherlands) supplemented with 20% FCS (heat inactivated; 0.2 μm filtered), 15% LADMAC-conditioned medium as a source of M-CSF, 100 μM 2-mercaptoethanol, 60 μg/ml penicillin and 100 μg/ml streptomycin. Cells were cultured for 4 or 5 days at 37°C, 7% CO_2 as indicated.

MLR

In vitro generated DC (stimulator cells) and T cells (responder cells) isolated from the spleen and lymph nodes were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% FCS (heat inactivated; 0.2 μm filtered), 60 μg/ml penicillin, 100 μg/ml streptomycin, 20 mg/l sodium pyruvate and 50 μM 2-mercaptoethanol (further referred to as MLR medium). T cells were resuspended at a concentration of 2×10^6/ml and the concentration of DC varied depending on the desired ratio of DC:T cells. The cells (100 μl of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C, 7% CO_2. DC and T cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by concanavalin A (final concentration 1.25 μg/ml) (Sigma) was used as a positive control.

After 4 days of co-culture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with anti-CD4, anti-CD8 and ER-MP21 antibodies was performed, and 15,000 events within the living gate was acquired. Analysis was performed using CellQuest analysis software. The percentage of ER-MP21 (CD71/transferrin R)$^+$ cells, as a measure of proliferating cells, was determined within the CD4$^+$ and CD8$^+$ population. This method of analysis provides comparable results to those obtained with the [^{3}H]thymidine incorporation method (unpublished results) and in addition allows us to analyze, in more detail, the characteristics of the responding T cell population

Results

Distinct DC precursors in the BM can be identified by ER-MP12/20 labeling

Based on ER-MP12/CD31 and ER-MP20/Ly-6C expression, we can fractionate BM into six separate, morphologically and phenotypically distinct subsets (Fig. 1) as we showed previously (25,26). We also demonstrated that in this way the different hematopoietic lineages (lymphoid, erythroid, granulocytic and monocytic) and three different stages of macrophage precursors could be distinguished (21,26). To examine in which of the cell populations DC precursors were present, BM subsets were sorted and cultured with GM-CSF. At day 10 of culture, the development of DC was evaluated by examining the cultures under an inverted light microscope (Table 1). DC were clearly visible in cultures derived from ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} subsets. No DC or other cell

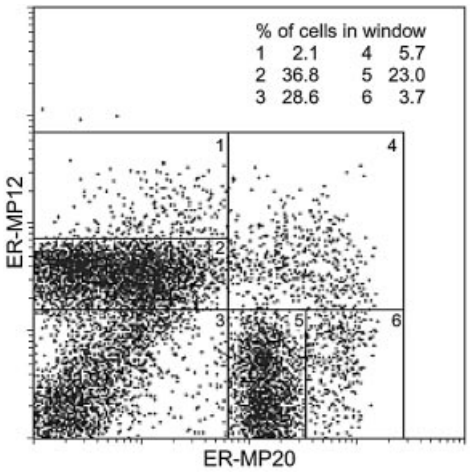

Fig. 1. Flow cytometric dot-plot of ER-MP12/20-labeled BM cells. Upon double labeling of BM cells with these mAb, six distinct subsets can be discerned. Gates used for cell sorting are shown. Percentages are the mean of five experiments.

Table 1. DC-like cells generated from ER-MP12/20 bone marrow subsets

BM subset	Overall cell growth[a]	DC-like cells visible in culture[b]		MHC class II^{++} cells with DC morphology (immunocytochemistry) (%)			
		Day 6	Day 10	Day 6		Day 10	
				Exp. I	Exp. II	Exp. I	Exp. II
ER-MP12^{hi}20$^-$	+	±	+	6	ND	15	9
ER-MP12^{med}20$^-$	–	–	–	–	–	–	–
ER-MP12$^-$20$^-$	–	–	–	–	–	–	–
ER-MP12$^+$20$^+$	+	+	+	20	43	16	ND
ER-MP12$^-$20^{med}	–	–	–	–	–	–	–
ER-MP12$^-$20^{hi}	+	+	+	23	26	40	33
TBM	+	+	+	19	ND	23	ND

[a]During entire culture.
[b]Results are for Experiments I and II as they were identical.
ND, not enough cells present on the poly-L-lysine-coated slides to determine percentage MHC class II$^+$ cells.

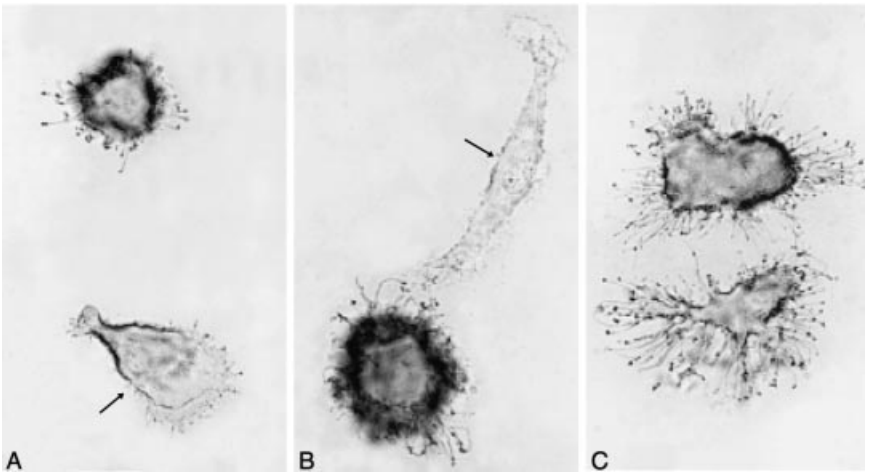

Fig. 2. Immunocytochemical MHC class II staining on cells from day 6 GM-CSF-stimulated cultures. DC (identified as MHC class II^{hi} and typical dendritic morphology) from cultures grown from (A) ER-MP12^{hi}20^{-}, (B) ER-MP12^{+}20^{+} and (C) ER-MP12^{-}20^{hi} BM cells are shown. Macrophages were also present in the cultures (arrow) and differed from DC in morphology and MHC class II expression (lower levels). Original magnification ×590.

types could be grown from the ER-MP12^{med}20^{-}, ER-MP12^{-}20^{-} and ER-MP12^{-}20^{med} subsets by GM-CSF stimulation. The latter BM subsets consisted of morphologically identifiable cells of the lymphoid, erythroid and granulocytic lineage respectively (21,26).

The presence of DC in cultures was also examined by assessing MHC class II expression in immunocytochemistry (Fig. 2). DC derived from different precursor populations showed the same typical DC morphology with MHC class II molecules present preferentially on the cell surface. However, the frequency of MHC class II^{hi} DC at day 6 (Table 1) in the ER-MP12^{hi}20^{-} culture was lower than that in the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} cultures (an average of 6 versus 33 and 25% respectively), suggesting that precursors from the ER-MP12^{hi}20^{-} population needed more time to develop into DC. This was in agreement with the results from visual examination of the cultures. By day 10, the frequency of DC had increased in the cultures derived from the ER-MP12^{hi}20^{-} subset (on average 12%), but was still lower than that in the ER-MP12^{+}20^{+} (16%) and ER-MP12^{-}20^{hi} (on average 37%) subsets. In conclusion, on the basis of differential ER-MP12 and ER-MP20 expression, we can distinguish three subsets in mouse BM from which DC can be derived with GM-CSF.

DC derived from different ER-MP12/20 populations develop into fully mature cells

Immature and mature DC differ in the level of MHC class II molecule expression on their surface. To determine the maturation state of DC derived with GM-CSF from different BM subpopulations, we double-stained cells at the end of culture with CD11c and anti-MHC class II antibodies. On the gated CD11c+ population, we measured the expression of MHC class II molecules (Fig. 3A): 30, 76, 76 and 91% CD11c^{+}MHC class II^{hi} mature DC were present in cultures derived from ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, ER-MP12^{-}20^{hi} and TBM respectively. In addition, all cultures contained a relatively low percentage of CD11c^{+}MHC class II^{lo} immature DC, except for the culture derived from the ER-MP12^{hi}20^{-} DC precursors in which a significant amount of DC was still immature (50%). These immature cells responded very well to overnight lipopolysaccharide (LPS) stimulation as this induced a significant increase of the percentage of mature CD11c^{+}MHC class II^{hi} cells (Fig. 3B). All cultures contained also some CD11c^{-}MHC class II^{-} cells (not shown), which are mainly granulocytes also developing *in vitro* with GM-CSF.

To investigate the functionality of DC derived from different precursor subpopulations, we assessed their antigen-presenting capacity by co-culturing GM-CSF derived cells with purified T cells (Fig. 3C). DC derived from all three BM subpopulations were good activators of T cells. The MLR stimulatory capacity was enhanced after LPS stimulation, particularly in the culture derived from the ER-MP12^{hi}20^{-} subpopulation when tested at lower DC:T cell ratios.

Three ER-MP12/20-defined subpopulations represent successive maturation stages of DC precursors in the BM

Morphological and functional analysis of DC cultures showed that different BM subpopulations develop with different kinetics into similar DC, which suggests that precursors in these populations might be in a different maturation stage at the time of isolation. To investigate this, we sorted the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} subpopulations from the BM, stimulated them with GM-CSF *in vitro*, and analyzed the expression of ER-MP12 and ER-MP20 by cultured cells at various time points (Fig. 4).

At day 2 of culture, cells from the ER-MP12^{hi}20^{-} subpopulation had up-regulated ER-MP20 on their surface and about a half of them were double positive for both markers. By day 5 all cells derived from this population were ER-MP20^{+} while most of them showed no ER-MP12 expression anymore. By day 8, the culture contained ER-MP12/20 double-negative cells and those that expressed medium levels of ER-MP20. The ER-MP12^{+}20^{+} population followed a similar pathway of development. At day 2, most of the cells had down-regulated ER-MP12

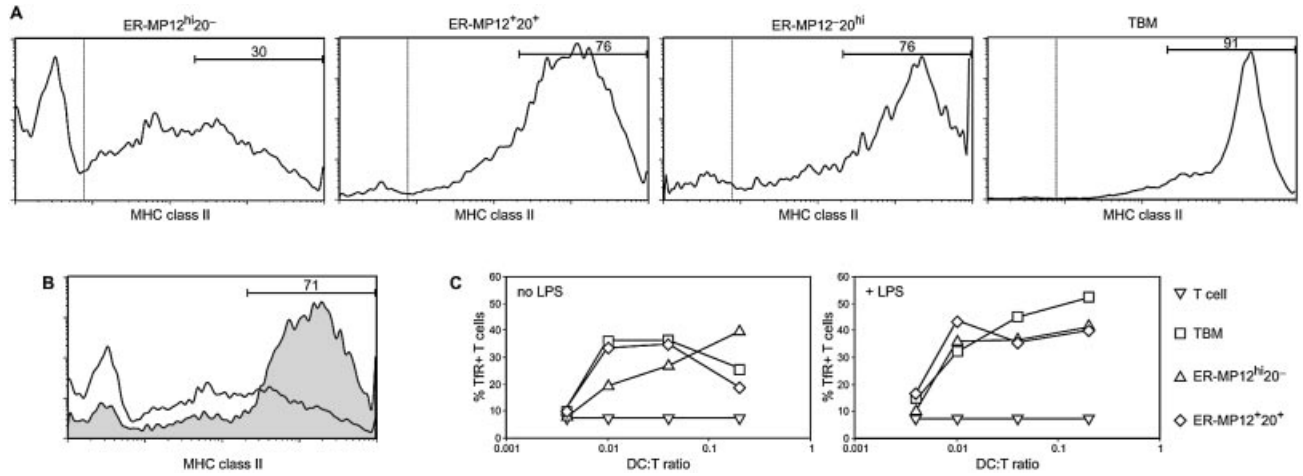

Fig. 3. Flow cytometric and functional analysis of DC generated from BM precursors. (A) Cells derived from the total BM and three different precursor populations are double stained with MHC class II and CD11c antibodies. Histograms show the MHC class II expression by CD11c$^+$-gated cells. (B) DC derived from the ER-MP12^{hi}20$^–$ population matured upon 24 h LPS stimulation as measured by enhancement of MHC class II expression. The profile indicated by a solid line only represents the expression before LPS stimulation; the filled surface is after 24 h LPS stimulation. The dotted line marks the isotype control and numbers represent the percentage of MHC class II^{hi} cells. (C) Potential of DC, either or not treated with LPS, derived from different BM precursors to stimulate T cells in the MLR. T cell activation is measured by expression of transferrin R (ER-MP21) on their surface.

expression and were ER-MP12$^–$20$^+$. At day 5 of culture, some cells also down-regulated ER-MP20 and were double negative. By day 8 almost all cells had lost both markers on their surface and were ER-MP12$^–$20$^–$. Finally, the ER-MP12$^–$20^{hi} -derived cells also down-regulated ER-MP20 on majority of the cells by day 5 of culture. By day 8 most of the cells in this culture had died (they probably were fully differentiated already before day 8) so no phenotypical analysis could be performed at this time point. Importantly, in all investigated cultures cells successively pass through the ER-MP12$^+$20$^+$ and ER-MP12$^–$20^{hi} stages and finally become ER-MP12$^–$20$^–$.

In the same cultures we followed the development of DC by using the DC marker CD11c. A common pattern of *in vitro* DC development was observed in cultures from all three fractions: first CD11c expression was induced followed by a prompt down-regulation of ER-MP20 (Fig. 5). CD11c$^+$ cells were present already at day 2 in the culture derived from the ER-MP12$^–$20^{hi} subpopulation. CD11c$^+$ cells were also present at day 2 in cultures derived from the ER-MP12$^+$20$^+$ subpopulation, but at much lower frequency. The percentage of CD11c$^+$ cells was increased in both cultures at day 5 and continued to rise in cultures derived from the ER-MP12$^+$20$^+$ subpopulation to reach 91% at day 8 of culture. In contrast, in cultures derived from the ER-MP12^{hi}20$^–$ precursors, CD11c$^+$ cells were present only from day 5 (10%) and reached 45% at day 8 of culture, implying that these precursors are indeed the earliest precursors able to differentiate into DC when stimulated with GM-CSF.

The maturation sequence of BM DC precursors, indicated by these phenotypic studies, is consistent with the decreasing proliferative capacity of the cells with increasing maturity. GM-CSF-stimulated culture of the distinct subsets in semi-solid medium demonstrated that the ER-MP12^{hi}20$^–$ subset generated predominantly large colonies (>50 cells), the ER-MP12$^+$20$^+$ subset predominantly clusters (<50 cells) and some smaller colonies, while the ER-MP12$^–$20^{hi} subset gave rise primarily to small clusters. Liquid culture recoveries were in line with these findings. In a representative experiment, 1×10^5 cells from the ER-MP12^{hi}20$^–$ population produced 4×10^6 cells after 7 days of culture, thus multiplying their starting number 40 times. From 4×10^5 ER-MP12$^+$20$^+$ cells, 3.6×10^6 progeny cells (9-fold) were generated, while 4×10^5 ER-MP12$^–$20^{hi} cells gave rise to only 2.8×10^5 cells after culture (0.7-fold).

The earliest ER-MP12^{hi}20$^–$ DC precursors have the phenotype of myeloid-committed cells

Previously, we showed that the same ER-MP12/20-defined subpopulations in the BM contain macrophage precursors as we found now for DC precursors (21). In addition, we also showed that within the ER-MP12^{hi}20$^–$ subpopulation, early myeloid-committed cells could be purified from the other hematopoietic differentiation capacities on the basis of differential expression of a third marker, ER-MP58 (20). Since we found that DC could also be generated from the ER-MP12^{hi}20$^–$ BM subset, we asked whether the earliest DC precursors also have a myeloid-committed phenotype by examining their ER-MP58 expression. For this purpose, ER-MP12^{hi}20$^–$ BM cells were fractionated according to their level of ER-MP58 expression (Fig. 6) and cultured in the presence of GM-CSF. Cultures were examined microscopically for the presence of DC at different time points. Starting from day 7 (Table 2), morphologically mature DC could only be detected in cultures of the ER-MP58^{hi} subset within the ER-MP12^{hi}20$^–$ population. This onset of DC appearing in culture was similar to what was observed when the total ER-MP12^{hi}20$^–$ subset was cultured (see above). No DC-like cells could be grown from the ER-MP58^{med} and ER-MP58$^{–/lo}$ subsets under these culture conditions. Together, these data show that, like macrophage precursors, GM-CSF-responsive DC precursors present in the ER-MP12^{hi}20$^–$ BM subset are characterized by a high level

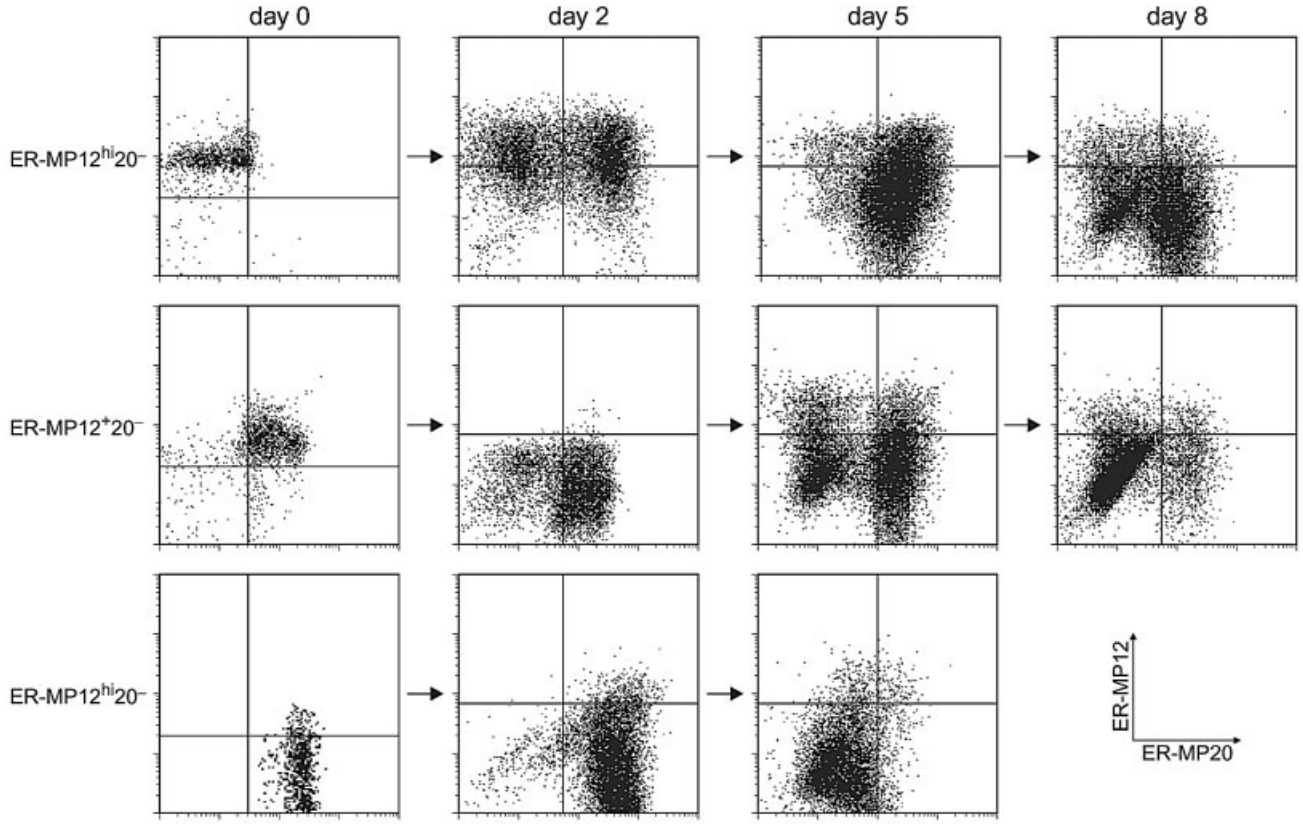

Fig. 4. Successive expression of ER-MP12 and ER-MP20 during the *in vitro* culture of BM precursor populations. Sorted populations were cultured with GM-CSF, and at various time points double-labeled with ER-MP12 and ER-MP20. Starting from the earliest precursor population, ER-MP12^{hi}20$^{–}$, all cells show the same pattern of ER-MP12/20 expression, indicating that they follow the same ER-MP12^{hi}20$^{–}$ → ER-MP12^{+}20^{+} → ER-MP12$^{–}$20^{hi} developmental pathway.

of ER-MP58 expression and thus have a myeloid-committed phenotype.

BM progenitors maturing along the macrophage lineage in vitro *maintain the capacity to generate DC*

To approach the question whether macrophages and DC share progenitors throughout their BM development, we cultured BM subsets sequentially with M-CSF ('primary culture') and GM-CSF ('secondary culture'). ER-MP12^{hi}20$^{–}$58^{hi} (early myeloid precursors) and ER-MP12^{+}20^{+} (myeloid blasts) were induced first to develop along the macrophage lineage *in vitro* (21). M-CSF was then replaced by GM-CSF and the potential of macrophage precursors to form DC was measured.

After 4 days in M-CSF-stimulated culture of the ER-MP12^{hi}20$^{–}$58^{hi} subset, ~90% of the cultured cells expressed the ER-MP20 antigen at a high level (Fig. 7A), representing BM monocytes as previously shown (21). Changing the growth stimulus to GM-CSF at this point resulted in the appearance of DC only 1 day later as determined by morphological inspection. The more mature ER-MP12^{+}20^{+} subset developed further along the macrophage pathway *in vitro*, since after 5 days of M-CSF-stimulated culture approximately half of the cells still expressed the ER-MP20 antigen, whereas the other cells in the culture had lost their ER-MP20 expression (Fig. 7B). When these cells were further stimulated by GM-CSF, similarly to ER-MP12^{hi}20$^{–}$58^{hi}-derived cultures, DC were visible after only 1 day of secondary culture and in increased numbers after 3 days of culture. Apart from DC, macrophages (ER-MP20$^{–}$) also developed in cultures from both precursor fractions as indicated by the presence of cells with (i) high forward and perpendicular light scatter (data not shown), indicative of a complex cell type like the mature macrophage with its numerous vacuoles, etc., and (ii) a high autofluorescence level, also characteristic of mature macrophages. After 9 days of GM-CSF-stimulated secondary culture, 28 and 71% DC developed from ER-MP12^{hi}20$^{–}$58^{hi} and ER-MP12^{+}20^{+} derived cultures respectively. These cells expressed MHC class II molecules at a high level and had typical DC morphology, as determined in immunocytochemistry (Fig. 7C and D). Thus, myeloid precursors from the BM developing for 4 or 5 days along the macrophage lineage in M-CSF-stimulated culture still have the potential to generate DC.

Discussion

In the past few years a significant amount of data has been generated about different DC types and their relationship to other hematopoietic lineages. Recently, it has been shown that both common myeloid and common lymphoid precursors can give rise to different DC types both *in vitro* and *in vivo* (14–16). Mature myeloid and lymphoid DC have been identified

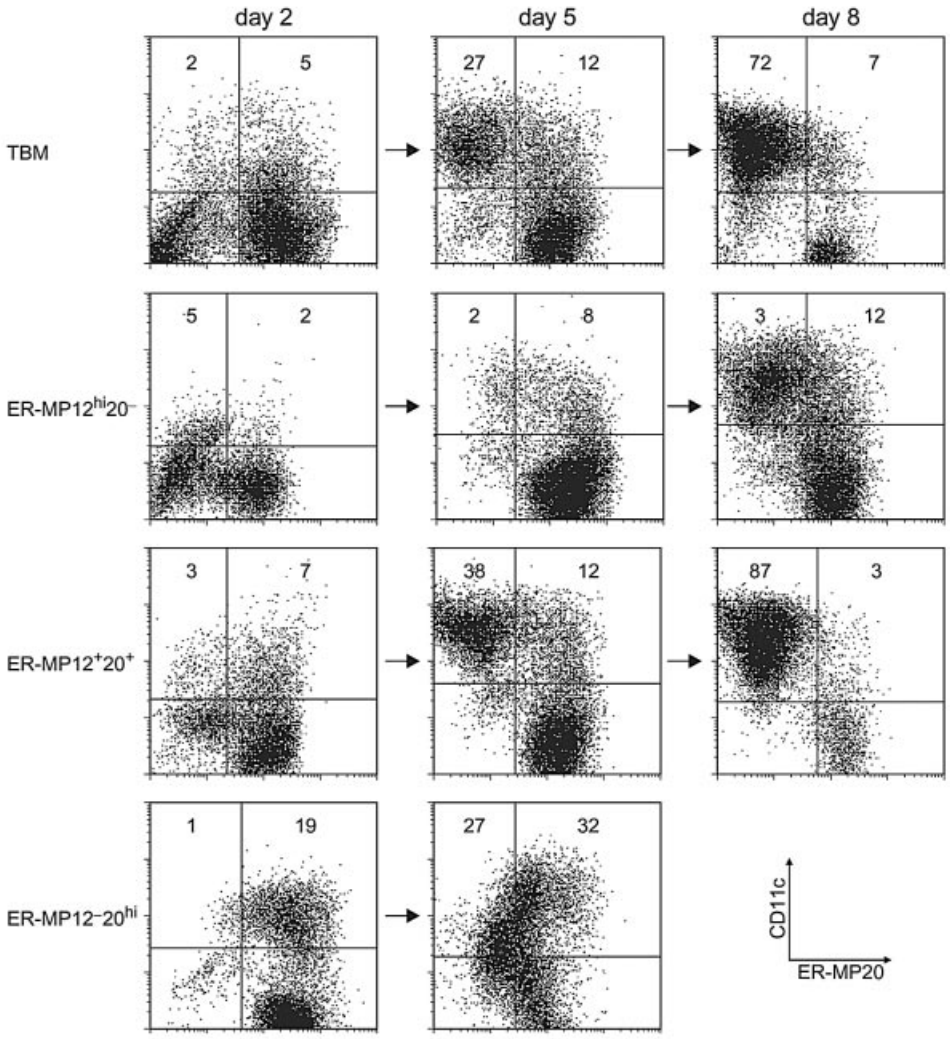

Fig. 5. Kinetics of the *in vitro* DC development from different BM precursors. Subpopulations were sorted from the BM and cultured for 8 days in the presence of GM-CSF. At different time points cells were labeled with ER-MP20 and CD11c. The DC development is marked by up-regulation of CD11c on the cell surface.

phenotypically in human and also in mouse (3,4). However, the exact maturation pathways of either of the types have remained unclear.

In this study we have examined the different stages of BM DC precursor development *in vivo*, as defined by the expression of ER-MP12/CD31, ER-MP20/Ly-6C and ER-MP58 antigens. DC are derived *in vitro* with GM-CSF only from the ER-MP12^{hi}20$^{–}$ (early blasts), ER-MP12^{+}20^{+} (myeloid blasts) and ER-MP12$^{–}$20^{hi} (BM monocytes) (21) subpopulations, while no cell growth could be detected from the other three sorted populations, ER-MP12^{med}20$^{–}$, ER-MP12$^{–}$20$^{–}$ and ER-MP12$^{–}$20^{med}. These populations represent subsets containing morphologically identifiable precursors and mature cells of the other hematopoietic lineages (lymphoid, erythroid and granulocytic respectively). Our results show that GM-CSF-responsive DC precursors reside in the same ER-MP12/20-defined BM subsets as M-CSF-responsive macrophage precursors (19–21).

Table 2. DC generated from ER-MP12^{hi}20$^{–}$ bone marrow subsets

BM subset	Overall cell growth[a]		DC visible in culture	
		Day 5	Day 7	Day 14
ER-MP12^{hi}20$^{–}$58^{hi}	+	–	+	+
ER-MP12^{hi}20$^{–}$58$^{med/lo}$	±	–	–	–
ER-MP12^{hi}20$^{–}$58$^{–/lo}$	–	–	–	–

[a]During entire culture.

Earlier reports show that mature DC develop from the BM within 8 days in GM-CSF-stimulated culture (27). In agreement with this we observe that the majority of DC derived from any of the BM subpopulations express significant amounts of MHC class II molecules, which, together with their good potential to stimulate allogeneic naive T cells *in vitro*, indicates that mature

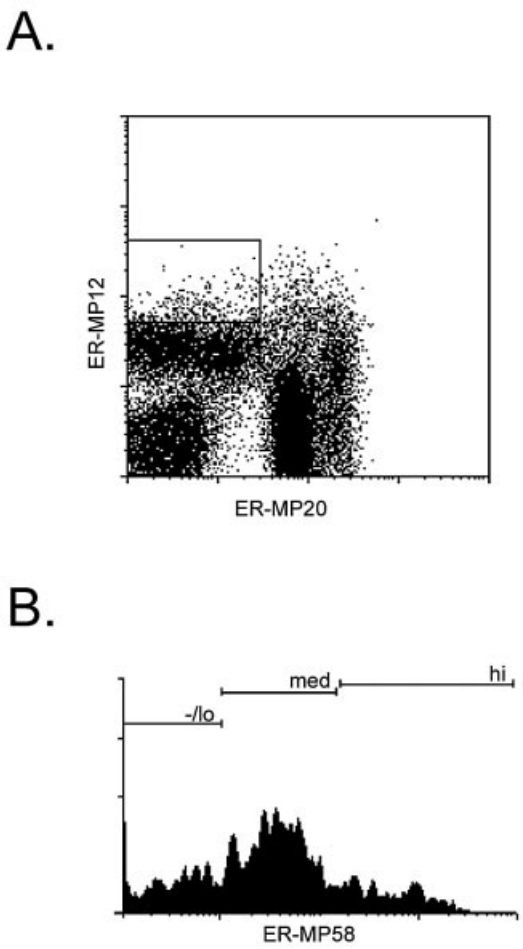

Fig. 6. ER-MP58 expression within the ER-MP12^{hi}20^{-} gate. Cells were triple labeled with ER-MP12, ER-MP20 and ER-MP58. ER-MP12^{hi}20^{-} cells were gated (A) and sorted on the basis of their ER-MP58 expression as indicated (B).

DC develop in these cultures. However, in cultures derived from the ER-MP12^{hi}20^{-} cells, immature DC, expressing medium levels of MHC class II, were also present in significant numbers after 7 days of culture. These cells mature upon LPS stimulation, suggesting that they need an extended period of time to develop into mature DC since they develop from early precursors.

This delay in reaching a mature state is in agreement with the observation that during the *in vitro* development, a difference in time of appearance of CD11c^{+} cells exists in cultures grown from the ER-MP12^{hi}20^{-} versus the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} BM subsets (day 5 versus 2 respectively). This further implies that ER-MP12^{hi}20^{-} DC precursors are less mature than the other two subsets, and need more time to develop into DC than ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} cells. In addition, the percentage of CD11c^{+} DC derived from the GM-CSF-stimulated ER-MP12^{+}20^{+} cells is 2-fold higher than that found with the ER-MP12^{hi}20^{-} BM subset as a starting population (90 versus 45%). Probably the higher proliferative capacity of the ER-MP12^{hi}20^{-} population contributes to the observed difference in maturation kinetics. Both the size and extent of colony/cluster formation and culture recoveries indicate that ER-MP12^{hi}20^{-} cells have the highest potential to proliferate, which then decreases to be the lowest among ER-MP12^{-}20^{hi} cells. The high recoveries obtained from the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{+} fractions make it highly unlikely that contaminating populations (<5%) in the sorted fractions account for the observed results. Together, these findings reinforce the notion that ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} populations represent different stages of DC development, since they show a reciprocal relationship between the ability to proliferate and the ability to develop into mature DC in a short period of time.

The successive expression of ER-MP12 and ER-MP20 antigens during the *in vitro* culture with GM-CSF strongly suggests that the three DC precursor populations, which we defined in the BM, differentiate along the pathway with the order ER-MP12^{hi}20^{-} $\rightarrow$ ER-MP12^{+}20^{+} $\rightarrow$ ER-MP12^{-}20^{hi}. Furthermore, this same sequence was previously shown for the macrophage precursors (21) suggesting that GM-CSF-responsive myeloid DC and macrophage precursors follow a similar ER-MP12/20-defined developmental pathway.

The population that contains the earliest DC precursors in the BM, ER-MP12^{hi}20^{-}, can be further divided into three subpopulations based on the expression of the early myeloid marker ER-MP58 (20). Of these three subsets, only ER-MP12^{hi}20^{-}58^{hi} BM cells are able to differentiate into DC in rGM-CSF-stimulated cultures. Since high level expression of ER-MP58 within the ER-MP12^{hi}20^{-} BM population has been shown to mark the earliest myeloid committed precursors, able to give rise to macrophages with M-CSF (20), we conclude that macrophages and myeloid DC most probably originate from the same precursors in the BM. Recently, Akashi *et al.* have described the existence of a common myeloid progenitor in mouse BM that gives rise to all myeloid lineages (10). The comparison of its colony forming ability and further characteristics with those of the ER-MP12^{hi}20^{-}58^{hi} population strongly suggests that the latter represents a similar, if not identical, multipotent myeloid precursor population.

In a recently published paper (7), Gilliet *et al.* show that the mouse PDC (CD11c^{+}CD11b^{-}B220^{+}) can be generated *in vitro* from the total BM, when Flt-3L is used as the growth factor. They also show that addition of GM-CSF to the culture medium completely blocks generation of PDC and increases myeloid DC development. Since we used GM-CSF alone, this is in agreement with the observation that in our cultures, no plasmacytoid, but only myeloid DC, were generated as all cells in the culture expressed high levels of CD11b (data not shown). An interesting question arises regarding the potential of our three populations to generate PDC with Flt-3L. For the BM monocyte population (ER-MP12^{-}20^{hi}) we regard it unlikely that they may give rise to lymphoid-related PDC, as we have shown previously that BM monocytes are all CD11b^{+} and thus myeloid cells (21), Further, it has been reported that the CD31^{+}/Ly-6C^{+} (i.e. our ER-MP12^{+}20^{+}) population contains precursor cells capable of differentiating into different cell types: CD8α^{+} DC, CD8α^{-} DC, PDC and macrophages, depending on the experimental conditions (17). Our current results support the concept that developing myeloid DC, which might eventually express CD8α, and macrophages progress through the same precursor stages in the BM

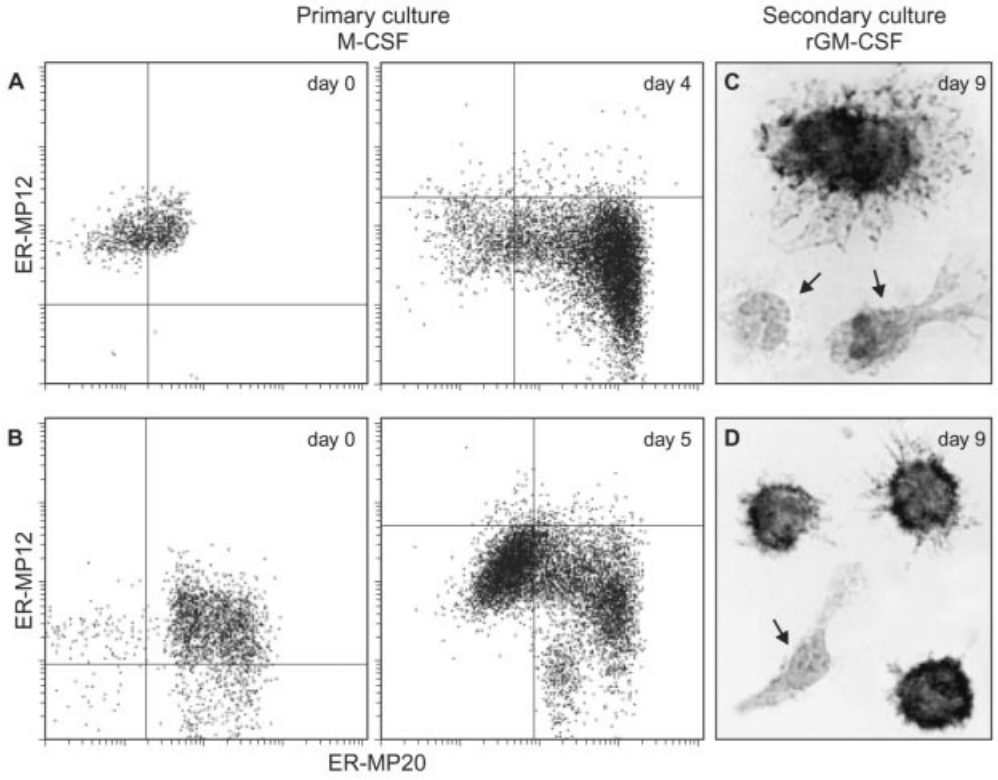

Fig. 7. DC development from precursor populations stimulated previously along the macrophage pathway. Bone marrow subsets ER-MP12^{hi}20^{-}58^{hi} (A) and ER-MP12^{+}20^{+} (B) were sorted and cultured for 4 and 5 days respectively in the presence of M-CSF to stimulate macrophage development. At the end of the primary culture, ER-MP12 and ER-MP20 expression was determined in two-color flow cytometry to assess the maturity of the cultured cells. Next, cells were grown for 9 days in secondary rGM-CSF-stimulated cultures. After this period cultures were analyzed for the presence of DC by immunocytochemical staining for MHC class II (C and D). Macrophages were also present in these secondary cultures (arrow). Original magnification (C and D) ×670.

including the CD31^{+}/Ly-6C^{+} stage. However, as we have shown earlier, the CD31^{+}Ly-6C^{+} population in BM is phenotypically heterogeneous (21) and might contain cells with different developmental capabilities; clonal assays are essential to prove whether PDC and myeloid DC derive from single precursors. Finally, we define the DC precursor, at the stage prior to the one previously reported, which is capable of differentiating in both myeloid DC and macrophages (CD31^{hi}/Ly-6C^{-}). As mentioned above, since GM-CSF directs cell development specifically to myeloid lineage, it is unclear from our data whether PDC might originate from the same population.

Taken together, in this report, we define different stages of GM-CSF-responsive DC precursors present in the BM and show their *in vivo* developmental pathway. Furthermore, we show that, throughout their BM development, myeloid DC precursors progress through the same stages as macrophages. Even when cells are induced *in vitro* with M-CSF to follow the macrophage developmental pathway, they can still be redirected to develop into DC when M-CSF is replaced with GM-CSF. This is in line with our hypothesis that over a prolonged stretch of development, progenitors of the mononuclear phagocyte system may either develop into macrophages or DC, depending on local conditions. However, additional clonal experiments are required to confirm this hypothesis. Finally, we feel that the ER-MP12/20/58 identification of distinct BM subsets provides a powerful means to study the developmental pathway of lymphoid-related PDC as opposed to the presently studied myeloid DC.

Acknowledgements

We thank Begona Grana Suares for her enthusiastic input in the initial phase of this study, and Willem van Ewijk and Paola Ricciardi-Castagnoli for stimulating discussions and support. This study was made possible by research grants from the Netherlands Organization for Scientific Research and the Dutch Diabetes Research Foundation.

Abbreviations

BM	bone marrow
DC	dendritic cell
Flt-3L	Flt-3 ligand
GM-CSF	granulocyte macrophage colony stimulating factor
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
MLR	mixed lymphocyte reaction
PDC	plasmacytoid dendritic cell
PE	phycoerythrin

References

1 Thomas, R. and Lipsky, P. E. 1996. Dendritic cells: origin and differentiation. *Stem Cells* 14:196.

2 Grabbe, S., Kampgen, E. and Schuler, G. 2000. Dendritic cells: multi-lineal and multi-functional. *Immunol. Today* 21:431.
3 Liu, Y. J., Kanzler, H., Soumelis, V. and Gilliet, M. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol.* 2:585.
4 Ardavin, C., Martinez del Hoyo, G., Martin, P., Anjuere, F., Arias, C. F., Marin, A. R., Ruiz, S., Parrillas, V. and Hernandez, H. 2001. Origin and differentiation of dendritic cells. *Trends Immunol.* 22:691.
5 Brasel, K., De Smedt, T., Smith, J. L. and Maliszewski, C. R. 2000. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 96:3029.
6 Blom, B., Ho, S., Antonenko, S. and Liu, Y. J. 2000. Generation of interferon alpha-producing predendritic cell (Pre-DC)2 from human CD34$^+$ hematopoietic stem cells. *J. Exp. Med.* 192:1785.
7 Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X. L., Trinchieri, G., O'Garra, A. and Liu, Y. J. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 195:953.
8 Wu, L., Li, C. L. and Shortman, K. 1996. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med.* 184:903.
9 Merad, M., Fong, L., Bogenberger, J. and Engleman, E. G. 2000. Differentiation of myeloid dendritic cells into CD8alpha-positive dendritic cells *in vivo*. *Blood* 96:1865.
10 Akashi, K., Traver, D., Miyamoto, T. and Weissman, I. L. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404:193.
11 Nakano, H., Yanagita, M. and Gunn, M. D. 2001. Cd11c$^+$b220$^+$gr-1$^+$ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.* 194:1171.
12 Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F. and Trinchieri, G. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2:1144.
13 Nikolic, T., Dingjan, G. M., Leenen, P. J. M. and Hendriks, R. W. 2002. A subfraction of B220$^+$ cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. *Eur. J. Immunol.* 32:686.
14 Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L. and Akashi, K. 2001. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 97:3333.
15 Mebius, R. E., Miyamoto, T., Christensen, J., Domen, J., Cupedo, T., Weissman, I. L. and Akashi, K. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45$^+$CD4$^+$CD3$^-$ cells, as well as macrophages. *J. Immunol.* 166:6593.
16 Izon, D., Rudd, K., DeMuth, W., Pear, W. S., Clendenin, C., Lindsley, R. C. and Allman, D. 2001. A common pathway for dendritic cell and early B cell development. *J. Immunol.* 167:1387.
17 Bruno, L., Seidl, T. and Lanzavecchia, A. 2001. Mouse pre-immunocytes as non-proliferating multipotent precursors of macrophages, interferon-producing cells, CD8alpha$^+$ and CD8alpha$^-$ dendritic cells. *Eur. J. Immunol.* 31:3403.
18 del Hoyo, G. M., Martin, P., Vargas, H. H., Ruiz, S., Arias, C. F. and Ardavin, C. 2002. Characterization of a common precursor population for dendritic cells. *Nature* 415:1043.
19 Leenen, P. J., Melis, M., Slieker, W. A. and Van Ewijk, W. 1990. Murine macrophage precursor characterization. II. Monoclonal antibodies against macrophage precursor antigens. *Eur. J. Immunol.* 20:27.
20 de Bruijn, M. F., Ploemacher, R. E., Mayen, A. E., Voerman, J. S., Slieker, W. A., van Ewijk, W. and Leenen, P. J. 1996. High-level expression of the ER-MP58 antigen on mouse bone marrow hematopoietic progenitor cells marks commitment to the myeloid lineage. *Eur. J. Immunol.* 26:2850.
21 de Bruijn, M. F., Slieker, W. A., van der Loo, J. C., Voerman, J. S., van Ewijk, W. and Leenen, P. J. 1994. Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. *Eur. J. Immunol.* 24:2279.
22 Radosevic, K., Casteels, K. M., Mathieu, C., Van Ewijk, W., Drexhage, H. A. and Leenen, P. J. 1999. Splenic dendritic cells from the non-obese diabetic mouse induce a prolonged proliferation of syngeneic T cells. A role for an impaired apoptosis of NOD T cells? *J. Autoimmun.* 13:373.
23 Sklar, M. D., Tereba, A., Chen, B. D. and Walker, W. S. 1985. Transformation of mouse bone marrow cells by transfection with a human oncogene related to c-*myc* is associated with the endogenous production of macrophage colony stimulating factor 1. *J. Cell Physiol.* 125:403.
24 van der Meer, J. W., van de Gevel, J. S., Elzenga-Claassen, I. and van Furth, R. 1979. Suspension cultures of mononuclear phagocytes in the Teflon culture bag. *Cell. Immunol.* 42:208.
25 Slieker, W. A., de Rijk-de Bruijn, M. F., Leenen, P. J. and van Ewijk, W. 1993. ER-MP12 antigen, a new cell surface marker on mouse bone marrow cells with thymus-repopulating ability: I. Intrathymic repopulating ability of ER-MP12-positive bone marrow cells. *Int. Immunol.* 5:1093.
26 van der Loo, J. C., Slieker, W. A., Kieboom, D. and Ploemacher, R. E. 1995. Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12. *Blood* 85:952.
27 Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N. and Schuler, G. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223:77.