Macrophage progenitor cells in mouse bone marrow

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Macrophage progenitor cells in mouse bone marrow

Macrofaag voorlopercellen in
het beenmerg van de muis

Proefschrift

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1 General introduction

Introduction

Macrophages were described by Elie Metchnikoff in 1884 as a population of phagocytic cells present in loose connective tissue[1]. Initially macrophages were classified as members of the reticuloendothelial system[2], which included reticular cells, endothelial cells, fibroblasts, histiocytes and monocytes. However, after recognition of the bone marrow origin of macrophages[3] the concept of the mononuclear phagocyte system has been developed which comprises mature macrophages as well as their precursors in peripheral blood and bone marrow[4,5]. The cells of the mononuclear phagocyte system form a heterogeneous population of cells which are widely distributed throughout the body (Table 1) and which have been shown to play essential roles in a broad variety of biological functions (Table 2) (reviewed in 6-9). To date, there is no single characteristic which marks all members of the mononuclear phagocyte system. Cells are instead assigned to the system on the basis of a combination of several characteristics. The lack of a definite mononuclear phagocyte marker and the extensive heterogeneity among members of the system has sometimes made it difficult to determine whether a particular cell type belongs to the mononuclear phagocyte system or not. For example, in the past there has been much debate whether dendritic cells (Langerhans cells, interdigitating cells, veiled cells, and *in vitro* dendritic cells[10]) should be included in the mononuclear phagocyte system (reviewed in 11). Recently, an increasing amount of data has accumulated supporting the view that at least part of the dendritic cells do indeed belong to the mononuclear phagocyte system (reviewed in 12). This will be discussed in more detail later in this chapter.

Considering the extensive functional and phenotypic heterogeneity of the mononuclear phagocyte system, the question arises how this system develops and how diversity within the system is generated. In the study described in this thesis we set out to examine if heterogeneity is generated already at the level of precursor cells in the bone marrow. Therefore, a search was conducted for cell surface markers allowing the identification and isolation of subsets of bone marrow macrophage progenitor cells, among which may be separate progenitor cells for different types of mature macrophages. In addition, markers allowing the discrimination of macrophage-committed progenitors will be useful tools for the determination of branch points between the various hematopoietic lineages.

This chapter considers the current understanding on the development of the mononuclear phagocyte system. In the first part of this chapter the development and maintenance of mononuclear phagocytes during adult life will be addressed, with a focus on the data available on the development from hematopoietic stem cells. The second part will cover the development of macrophages in ontogeny, which differs from macrophage development in the adult. Third, the currently available data regarding the multiple mechanisms by which mature macrophage heterogeneity arises are considered. The chapter concludes with an introduction to the experimental work described in this thesis.

Table 1 Distribution of mononuclear phagocytes

Bone marrow
Monoblasts
Promonocytes
Monocytes
Macrophages
Peripheral blood
Circulating and marginating monocytes
Tissues
Liver (Kupffer cells)
Lung (alveolar macrophages)
Connective tissue (histiocytes)
Spleen
Lymph nodes
Thymus
Bone marrow
Bone (osteoclasts)
Synovium (type A cells)
Mucosa-associated lymphoid tissue
Gastrointestinal tract
Genitourinary tract
Endocrine organs
Central nervous system (microglia)
Skin (histiocyte/Langerhans cells)
Serous cavities
Pleural macrophages
Peritoneal macrophages
Inflammatory tissues
Epithelioid cells
Exudate macrophages
Multinucleated giant cells

Adapted from van Furth et al.[5].

1 Development of mononuclear phagocytes in the adult mouse

In the concept of the mononuclear phagocyte system all macrophages are the progeny of monocytes which develop via exudate macrophages into resident macrophages. Monocytes are derived from hematopoietic progenitor cells in the bone marrow and enter the blood within 24 hours after their formation[13]. They have a half-time in circulation of approximately 18 hours[14] before entering the tissues to mature into the various types of macrophages. However, in the tissues a small proportion (less than 5%) of DNA-synthesizing macrophages have also been found, thought to be able to undergo one more round of cell division[15]. These cells were shown to be of bone marrow origin, but do not belong to the resident macrophage population as they were found to be recent immigrants in the tissues[15]. Thus, a small part of tissue macrophages

Table 2 Major mononuclear phagocyte functions

Host defence against microorganisms and tumor cells
- endocytosis of microorganisms, effete cells, and cell debris
- cytotoxic activity against microorganisms, virally infected cells, and tumor cells

Regulation of the inflammatory response
- production of inflammatory mediators, such as interleukin 1 and prostaglandin E_2
- secretion of molecules which attract other leukocytes, such as leukotriene B_4
- secretion of complement components

Regulation of the immune response
- antigen presentation and T cell activation
- accessory and regulatory function for T, B, and NK cells

Regulation of hematopoiesis
- production of hematopoiesis-stimulating factors
- regulation of iron metabolism
- removal of effete erythrocytes
- B lymphopoiesis (bone marrow)
- T lymphopoiesis (thymus)
- inhibition of hematopoiesis

Regulation of endocrine response
- ovary and testis
- bone
- anterior pituitary

Wound healing
- regulation of coagulation and fibrinolysis
- tissue debridement
- regulation of angiogenesis
- regulation of fibroblast proliferation and secretory activity

Adapted from Ginsel[7].

may be derived from local division of such cells[15]. In contrast to the view of bone marrow-derived macrophage populations, self-maintained mature macrophage subsets have also been reported, e.g. in the lung, peritoneal cavity and liver[16-19]. These subsets appeared to be independent of bone marrow monocyte production, and are either maintained by local macrophage precursors or by proliferation of resident macrophages. Thus, in the adult mouse mature macrophage subsets can be maintained by local precursors, influx of precursors (monocytes) from the circulation, or local proliferation of mature macrophages. In the following sections the development of macrophages from bone marrow and from local precursors will be discussed. Also lineage relationships with other hematopoietic cells will be considered. The role of mature macrophage proliferation in maintenance of the system will not be addressed, as this falls beyond the scope of this thesis (reviewed in 20).

Development of mononuclear phagocytes from bone marrow precursors

Development from bone marrow hematopoietic stem cells: The generation of mature blood cells from hematopoietic stem cells is a continuous process throughout life. In the adult mouse, bone marrow and spleen are the main sites of hematopoiesis[21]. All blood cells have a limited lifespan and need to be replaced by newly formed cells. The number of blood cells generated each day approximates the total number of cells present in primary hematopoietic tissues (in the mouse approximately 240 x 10^6 cells in bone marrow and 10 x 10^6 cells in spleen)[21]. All these cells are derived from a small pool of hematopoietic stem cells with a large proliferative and broad differentiation potential. The most primitive pluripotent hematopoietic stem cells (PHSC) have been defined as cells with the capacity for self-renewal and long-term repopulation of all blood cell lineages[22]. The frequency of these cells in bone marrow has been estimated to be 1 per 10^4-10^5 cells[23,24]. Under steady state conditions only a small fraction of these cells are in cycle, the others are quiescent.

When PHSC start to proliferate and differentiate, they gradually lose their self-renewal and proliferative capacity and become restricted to one of the hematopoietic lineages (Fig. 1). The main hematopoietic progenitor compartments recognized along the various differentiation pathways are multipotent stem cells, committed progenitor cells, and morphologically identifiable cells which finally become the mature cells entering the blood[21]. As stem and progenitor cells are defined on the basis of functional attributes, the different stem and progenitor cell subsets are named by the functional assays in which they are detected; *in vivo* e.g. multi-lineage long-term repopulating cells (LTRC)[25,26], cells with marrow-repopulating ability (MRA)[27,28], and cells forming colonies in the spleen (colony-forming unit in spleen, CFU-S)[29], *in vitro* e.g. cobblestone area-forming cell (CAFC) subsets, representing various classes of hematopoietic stem and progenitor cells[30,31], relatively immature high proliferative potential colony-forming cells (HPP-CFC)[32], and committed cells forming colonies in culture (colony-forming unit in culture, CFU-C)[33]. This way of defining cells may lead to difficulties in comparing results from different studies, as stem and progenitor cell populations isolated by phenotypic or physiological characteristics are heterogeneous in their self-renewal, proliferative and differentiation potential, and as yet no markers are available which unambiguously discriminate between the various classes of hematopoietic cells[34,35].

In myeloid development the multipotent CFU-GEMM (colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte) is the earliest cell that can be detected. This stem cell gives rise to the more committed bipotent progenitors of the granulocyte-macrophage lineages, the granulocyte-macrophage colony-forming cell (GM-CFC), and to committed progenitors of the erythroid and megakaryocytic lineages[25,33,36,37]. The GM-CFC is generally accepted to be the last common progenitor for macrophages and granulocytes[38,39]. Upon further commitment, the granulocyte and mononuclear phagocyte lineages diverge into the distinct granulocyte and macrophage colony-forming cells (G-CFC and M-CFC)[38,40]. Development beyond the M-CFC stage is paralleled by the acquisition of typical mononuclear phagocyte characteristics[41,42]. The most immature cell in the bone marrow recognized by these characteristics is the monoblast[43,44]. These

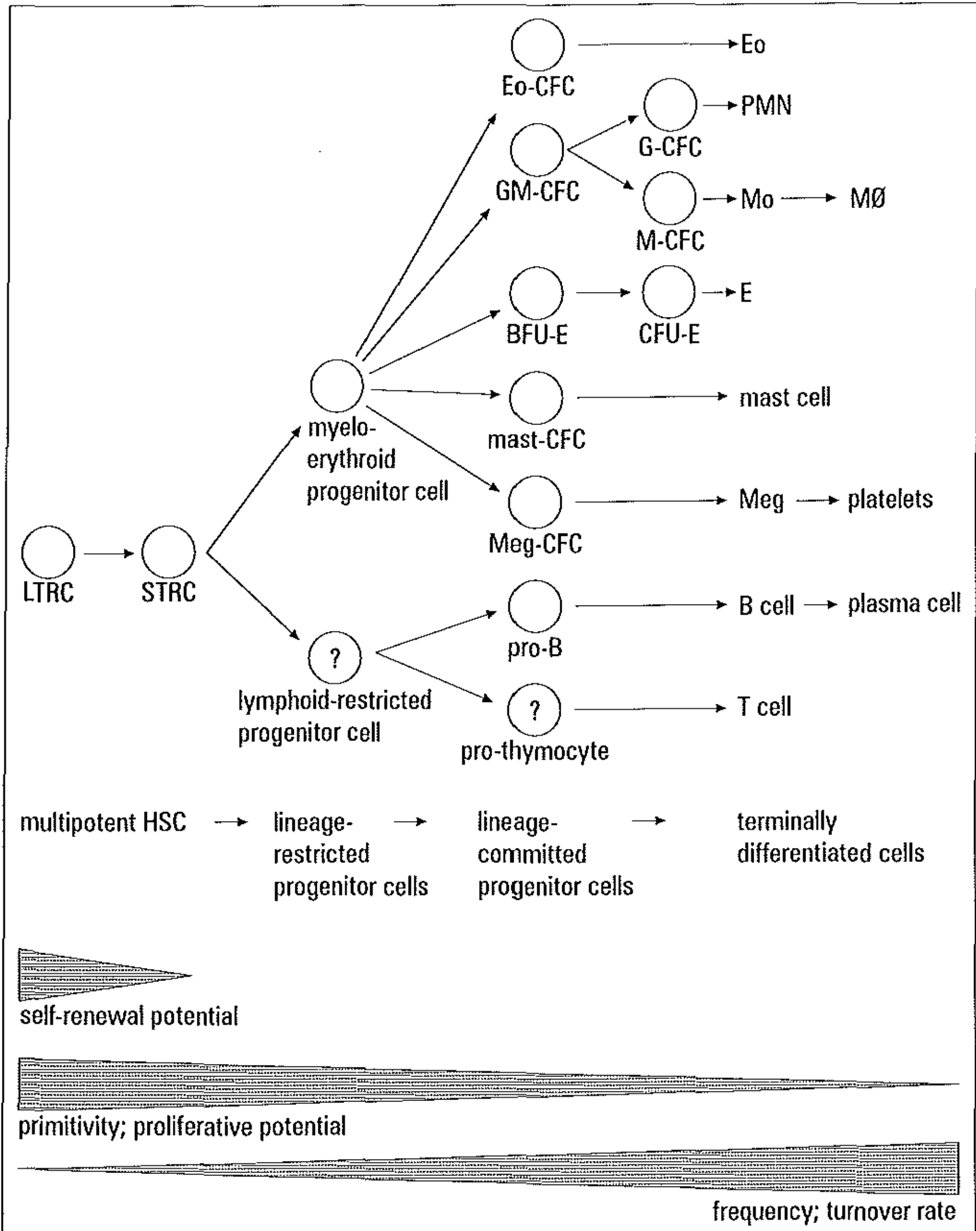

Figure 1. Schematic representation of hematopoiesis in adult bone marrow. LTRC: long-term repopulating cell; STRC: short-term repopulating cell; CFC: colony-forming cell; Eo: Eosinophil; GM: granulocyte/macrophage; G: granulocyte; PMN: polymorphonuclear phagocyte; M: macrophage; Mo: monocyte; Mϕ: macrophage; BFU-E: burst-forming unit-erythroid; CFU-E: colony-forming unit-erythroid; E: erythrocyte; Meg: megakaryocyte; pro-B: progenitor B cell; HSC: hematopoietic stem cell.

cells are thought to divide once and give rise to promonocytes[45]. Promonocytes also divide once and generate monocytes, the last stage of mononuclear phagocyte development in the bone marrow[46,47].

Isolation of early stages of mononuclear phagocyte development: The study of the mechanisms involved in myeloid lineage commitment requires the identification of lineage-specific markers which permit the isolation of different subsets of committed progenitor cells. The currently available cell separation protocols combine multiple cell characteristics in order to obtain bone marrow cell populations highly enriched in hematopoietic stem cell activity[48-53]. Although primitive hematopoietic stem cells can be separated from more committed progenitor cells in this way, the different classes of committed progenitor cells are still difficult to dissect[34]. Using density separation and counterflow centrifugal elutriation a highly enriched population of GM-CFC has been isolated from the bone marrow of cyclophosphamide-treated mice[54,55]. However, isolation of committed progenitors on the basis of cell surface antigen expression has the advantage that information on phenotypic changes associated with commitment may provide insight into molecules, genes, and ultimately mechanisms crucial in lineage commitment. Recently Trevisan and Iscove[34] reported on the phenotypic similarity of progenitor cells detected in a variety of short-term assays (CFU-multi, CFU-E/Meg, CFU-G/M, and short-term repopulation *in vivo*) for the expression of H-2K, CD45, AA4.1, HSA, CD71 and the lineage markers B220, Mac-1, Gr-1 and CD8. This indicates that none of these markers allow separation of myeloid-committed progenitor cells from other hematopoietic activities. In addition to the markers mentioned above, the macrophage marker F4/80 also cannot be used for identification of myeloid-committed progenitor cells, as bone marrow M-CFC do not express this antigen[56]. Recently, Antica et al.[57] reported that $Lin^{-}Thy\text{-}1^{lo}HSA^{+}Sca\text{-}2^{+}$ progenitor cells have the capacity to differentiate into the B and T and myeloid lineages, indicating that Sca-2 does not discriminate between early myeloid and lymphoid progenitors either. Thus, at present no markers are available to isolate specifically myeloid progenitors from the bone marrow.

Relationships with other hematopoietic lineages: Although the development of mononuclear phagocytes from hematopoietic stem cells is relatively well described, a number of questions remain concerning the lineage relationships of these cells with other hematopoietic cells. These include the lineage relationships between mononuclear phagocytes and granulocytes, B cells, dendritic cells, osteoclasts, and natural killer cells. Particularly the stage at which diversification from putative common precursors into the differentiated end cells occurs, remains a topic of investigation.

The granulocyte-macrophage colony-forming cell (GM-CFC) has generally been accepted as the most mature common progenitor of mononuclear phagocytes and granulocytes[21]. However, several data suggest that granulocytic cells as mature as promyelocytes can under certain *in vitro* conditions still differentiate into macrophages[6]. This has been shown for leukemic cell lines as well as for normal human bone marrow

derived promyelocytes[6,58]. Thus, the branch point of the granulocyte and macrophage lineages appears to be less clearly defined than initially thought.

In the generally accepted scheme of hematopoiesis (Fig. 1), myeloid and lymphoid lineages already diverge early. However, several data point to a close relationship between macrophages and B cells (reviewed in 59). Transformed $CD5^+$ early B cell lines which acquire macrophage characteristics upon *in vitro* maturation have been described[59,60]. These cells differ from conventional macrophages in that they coexpress B cell and macrophage characteristics. Recently, the normal counterpart of the transformed B/macrophage cell has been reported in cultures of bone marrow and spleen of mice[61,62], indicating that the double phenotype is not caused by inappropriate gene expression due to transformation. These data, together with the identification of a bipotent fetal liver progenitor for B cells and macrophages[63,64], have led to the proposal of a new model of macrophage relationships to B cells[59]. In this model conventional macrophages are derived from adult bone marrow monocytes, whereas the B/macrophage cells are derived from $CD5^+$ B cells generated in ontogeny from the bipotent fetal liver progenitor for B cells and macrophages. However, recently precursors for $CD5^+$ B cells of adult rather than fetal origin have also been detected in adult bone marrow[65]. Thus, the precise developmental origin of the precursor for B/macrophage cells still remains to be established.

The question whether dendritic cells (DC) belong to the mononuclear phagocyte system has been a topic of much debate. The dendritic cell system is widely distributed throughout the body and comprises Langerhans cells in the skin, veiled cells in the lymph, interdigitating cells in the lymphoid organs and interstitial DC in the connective tissue of non-lymphoid organs[10]. After the bone marrow origin of DC was established in 1979[66,67], many studies have addressed the lineage relationship of DC with other hematopoietic lineages. Three possible origins for DC have been suggested, which may exist next to each other. First, in the last decade a still increasing amount of data has been published in favor of a myelomonocytic origin of DC (reviewed in 12, 68, 69). These include (a) the expression of myeloid antigens by dendritic cells[70-73], (b) expression of GM-CSF receptors and dependence on GM-CSF for *in vitro* development[74-77], (c) phagocytic and macropinocytic activity in defined differentiation stages (reviewed in 78), (d) growth of mixed myeloid/DC colonies from bone marrow precursors[79], (e) development of dendritic cells from peripheral blood monocytes[80,81], and (f) the identification of a $CD1a^-CD14^+$ post-CFU intermediate cell type in cultures of $CD34^+$ bone marrow or cord blood cells which can differentiate into either macrophages or dendritic cells[82,83]. Thus, DC have been generated in GM-CSF-dependent cultures from different stages of myeloid development, ranging from early common progenitors for DC, granulocytes and macrophages in bone marrow to monocytes in peripheral blood. Second, several studies report on a lymphoid origin of DC. In human bone marrow, a common progenitor population for DC, T, NK and B cells has been identified, lacking erythroid and myeloid differentiation capacity[84]. Similarly, in the human thymus, a population capable of generating DC, T and NK cells has been reported[85,86]. The myeloid and/or B cell differentiation capacity of this thymic cell population remains to be tested. Also in mouse thymus an early precursor population

has been identified with the capacity to develop along the T, NK, DC and B cell lineages, but hardly or not along the myeloid lineage[87-89]. In none of these studies direct evidence was given for the existence of a common multipotent progenitor cell for T, DC, NK and B cells in either the thymic or bone marrow progenitor populations, due to a lack of suitable clonal assays. So far, only multipotent B/NK/DC progenitors could be demonstrated[84]. Yet, a recent study on mouse thymic DC precursors strongly supports the existence of a common precursor of DC and T cells, with CD8α as a marker of the lymphoid-related DC lineage[90]. Third, apart from the reported lineage relations with the myeloid and lymphoid lineages, evidence for a separate DC progenitor has also been reported[82,83,91,92]. In GM-CSF- and TNF-α-stimulated cultures of human $CD34^+$ bone marrow cells progenitor cells have been detected that give rise to pure DC colonies (CFU-DC)[91]. The cells generated in these colonies resemble epidermal DC[91]. Likewise, in liquid cultures of $CD34^+$ human bone marrow or cord blood cells a $CD1a^+CD14^-$ precursor has been detected which finally gives rise to Birbeck granule-positive DC[82,83]. These data suggest that epidermal Langerhans cells are derived from a separate lineage of DC which is independent of monocytes[83,92]. In conclusion, the precise lineage relationships of dendritic cells with other hematopoietic lineages are complex. On the basis of current data different pathways for DC development appear to exist, leading to different types of mature DC. One of these pathways is clearly linked to the monocyte/macrophage lineage.

The hematopoietic origin of osteoclasts has been well established[93-97]. Although generally accepted to be of myeloid origin[93], the exact divergence point of the osteoclast and myeloid lineages is still unclear. On the basis of experimental evidence several possible branch points have been suggested, ranging from early in myeloid differentiation (before the CFU-GM stage) till the stage of mature macrophages (reviewed in 98). However, the precise identification of one or more stages at which the osteoclast and myeloid lineages diverge, awaits the further identification and isolation of myeloid progenitor stages.

Despite their undisputed bone marrow origin[99-101] the precise relationship of natural killer (NK) cells with other hematopoietically derived cells has remained controversial. Detailed phenotypic characterization of NK cells did not lead to an unambiguous classification of NK cells to a defined lineage, since NK cells share surface markers with T lymphocytes, granulocytes and cells of the mononuclear phagocyte system[102]. It has been suggested that at least part of the NK cells are related to the mononuclear phagocyte system[103-106]. In addition, it has also been suggested that NK cells are derived from an early NK/T cell progenitor from which they branch off before rearrangement of the T cell receptor[107]. Still another possibility is that NK cells constitute a separate lineage and are thus derived from NK-committed progenitors. Neither such NK-committed nor NK/T progenitors have thus far been reported for normal adult bone marrow. Rather, the cells identified in bone marrow as NK progenitors seem to be part of a (multipotent) cell population that has the capacity to differentiate into both the myeloid and lymphoid lineages[108]. In summary, the lineage relationships of NK cells with other hematopoietic cells are still obscure. Similar to the situation for DC, different pathways for NK development may exist.

In conclusion, due to the limited identification of the precursor stages in the bone marrow and the great diversity of cells belonging to the mononuclear phagocyte system, the lineage relationships of the mononuclear phagocyte system with other cell types are still unclear.

Development of mononuclear phagocytes from local tissue precursors

Spleen: In rodents the spleen continues to be a hematopoietic organ throughout life. Under conditions of impaired bone marrow hematopoiesis, e.g. in leukemic mice, splenic hematopoiesis is augmented as a compensatory mechanism. In the mouse spleen erythroid cells are produced in the red pulp while myeloid progenitors are located under the capsule and scattered in the red pulp (ref. 21, and unpublished observations). Splenic macrophage progenitors differ from bone marrow macrophage progenitors in their expression of Ly-6C, suggesting that macrophages in the spleen and bone marrow arise from different progenitors[109]. Clonable splenic macrophage progenitor cells have been reported to give rise to mature macrophage colonies which differ in antigen presentation capacity[110-113]. Together these data are suggestive of the existence of different progenitors for functionally different mature macrophages. The spleen contains several mature mononuclear phagocyte populations, distinct both in phenotype and anatomical localization. Several studies have addressed the origin of these distinct populations. Following splenic macrophage elimination by administration of chlodronate liposomes, these subsets were shown to differ significantly in repopulation kinetics, varying from one week for total recovery of red pulp macrophages to more than one month for recovery of marginal zone macrophages[114]. Recently it was shown in macrophage-depleted radiation chimeras that all splenic macrophage populations can be bone marrow-derived[115]. In addition, it was found that under steady state conditions subpopulations of mature splenic macrophages retain the capacity to proliferate and thus may contribute substantially to the maintenance of the splenic macrophage populations[115,116]. These data correspond with previous findings[117,118]. For example, it has been shown that under steady state conditions 55% of all isolated splenic macrophages originate from blood-derived monocytes, while 45% is maintained by local proliferation[117]. Currently no data are available that indicate a clear contribution of local splenic (pre)monocyte precursors to the maintenance of mature splenic macrophages. Still, a role for such cells cannot be excluded[115].

Omentum: In the mouse precursors of the mononuclear phagocyte lineage have been reported to reside in the milky spots of the omentum[18,119,120]. These precursors are thought to contribute to the maintenance of the peritoneal macrophage population[119,121]. However, on the basis of the data available it is not clear whether these precursors are deposited in the milky spots early in ontogeny, or whether they are bone marrow (monocytic) derived[122].

Next to bone marrow, the spleen and omentum are the best studied for the presence of macrophage precursors. Little is known about these cells in other organs. In liver macrophage precursors have also been reported[123,124], but this was under experimental conditions that induced recruitment of these cells from the peripheral blood. Under

steady state conditions and after liver transplantation, DC could be grown from normal mouse liver[125,126]. However, although whole body perfusion was performed before harvesting the liver, it cannot formally be excluded that these cells arise from peripheral blood-derived cells that adhere to the liver vasculature.

2 Development of mononuclear phagocytes in ontogeny

Macrophage-like cells are among the first hematopoietic cells appearing in the mouse embryo. Using immunoelectron microscopy macrophages can be detected in the blood islands of the yolk sac at day 9 of gestation[127,128]. Because of their immature characteristics they are named "primitive macrophages"[127,128]. By day 10 of gestation these primitive macrophages have acquired a more mature phenotype and are named "fetal macrophages"[127,128]. It has been suggested that the primitive macrophages are not derived from monocytic cells but develop directly from local hematopoietic cells[127-130]. This suggestion is based on the absence of promonocytic and monocytic cells in the mouse yolk sac at day 9[127,128,131-133], while committed hematopoietic precursors with *in vitro* myeloid differentiation capacity have been detected in the yolk sac already at day 7/8 of gestation[131,134,135]. Cells of the monocytic lineage start to appear in the yolk sac at day 10 of gestation[127,132,133].

Around day 10 of gestation, when the yolk sac is connected to the developing embryonic vascular system, primitive/fetal macrophages enter the vitelline vessels, migrate through the blood to the fetal liver and colonize various embryonic tissues[127-129]. This is in contrast to adult macrophages which do not migrate through the blood. In addition, primitive/fetal macrophages have a high proliferative potential, reflected in the large proportion of cells in cycle[127,128,136], and therefore might be a self-sustaining population. Once in the tissues they gradually obtain features similar to those of resident macrophages in adult animals[137,138] and may persist there after birth[130,136]. Thus, it has been suggested that primitive/fetal macrophages constitute a separate line of macrophage development in ontogeny, independent of the monocytic lineage[130,136].

Recently several studies have addressed the origin of the adult hematopoietic system of the mouse. Definitive adult hematopoietic stem cells were shown to develop in the intraembryonic aorta-gonads-mesonephros (AGM) region[139,140], analogous to what has previously been found in nonmammalian vertebrates[141]. This is in contrast with the generally accepted idea that definitive hematopoiesis originates in the yolk sac[131]. Recently it was demonstrated by *in vitro* organ cultures of the yolk sac, liver and AGM that the AGM region autonomously and exclusively generates the definitive hematopoietic stem cells which form the adult hematopoietic system[140].

Monocytic cells, which are part of the adult hematopoietic system, appear in the yolk sac after the blood circulation is established[127,132,133]. This finding is suggestive of a seeding of the yolk sac by precursors from the AGM region. In accordance with this notion is the finding that in cultures of yolk sacs, isolated from the embryo before circulation, a reduced myeloid differentiation potential was found[142]. However, confirmation of the AGM origin of yolk sac monocytic cells requires further experimentation.

In Fig. 2 a scheme of macrophage development in ontogeny is presented, summarizing currently available data. Thus, macrophages are thought to develop along two distinct lines in ontogeny. The earliest detectable macrophages are the primitive macrophages in the yolk sac, which colonize embryonic tissues. Later in ontogeny the monocyte/macrophage lineage arises, presumably from the definitive hematopoietic stem cells generated in the AGM.

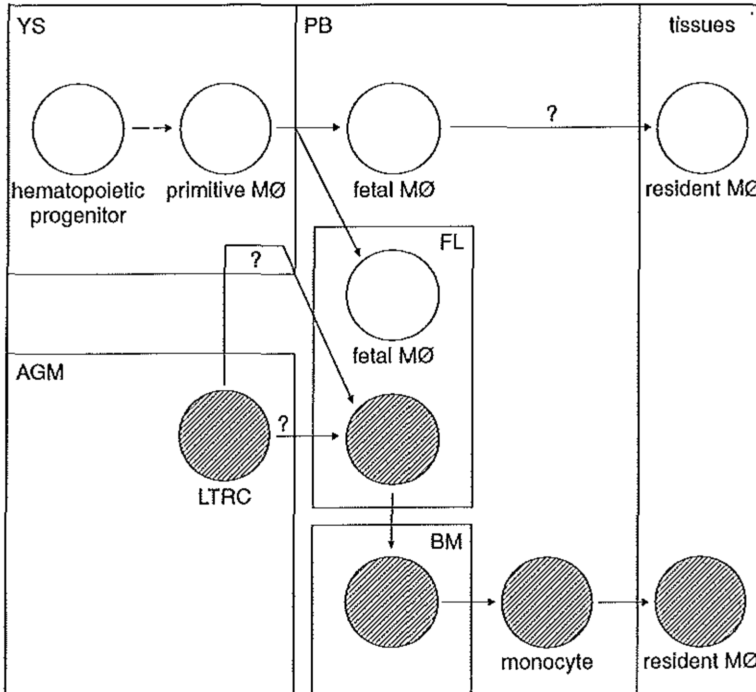

Figure 2. Development of mononuclear phagocytes in ontogeny. Macrophages are thought to develop along two separate lineages in ontogeny. First to appear are the primitive macrophages in the yolk sac, probably arising from a local hematopoietic precursor with *in vitro* myeloid differentiation capacity. Primitive macrophages mature into fetal macrophages without passing through the stage of promonocytes and monocytes, and colonize the embryonic tissues (including fetal liver). Once in the tissues they mature into cells resembling adult resident macrophages and are thought to persist after birth as a self-sustained population of cells. In fetal liver, these fetal macrophages may give rise to Kupffer cells (see section 3). Later in ontogeny, cells of the monocytic/macrophage lineage appear in the embryo. These cells are part of the final adult hematopoietic system and are presumably derived from the hematopoietic stem cells generated in the AGM. Once generated, cells of the definitive hematopoietic system spread through the embryo and finally colonize the bone marrow, the adult site of hematopoiesis. YS: yolk sac; AGM: aorta-gonads-mesonephros region; PB: peripheral blood; FL: fetal liver; BM: bone marrow; Mϕ: macrophage; LTRC: long-term repopulating cell. In the AGM, FL, and BM shaded cells represent cells of the definitive hematopoietic system, ranging from hematopoietic stem cells to mature blood cells.

3 Origin of mononuclear phagocyte heterogeneity

Based on differences in tissue localization, expression of surface molecules, and functional properties, macrophages can be divided into distinct subsets. As illustrated in Fig. 3, several mechanisms contributing to mature macrophage heterogeneity have been suggested. These are (i) the generation of self-maintained, bone marrow independent populations early in ontogeny; (ii) induction of heterogeneity in response to local conditions or stimuli encountered in peripheral tissues; and (iii) heterogeneity resulting from differences at the macrophage precursor level. In the following, experimental data supporting each of these mechanisms will be discussed shortly (for detailed reviews see ref. 6, 8, and 143).

Self-maintained populations generated in ontogeny

As described in section 2, a separate population of fetal macrophages originates from primitive yolk sac macrophages before the appearance of cells of the monocytic lineage. These macrophages migrate early in ontogeny into various fetal tissues and are thought to persist after birth as resident macrophages[130,136]. For several adult resident macrophage populations a fetal origin has indeed been described, including Kupffer cells[129,137], alveolar macrophages[144,145] and microglia[146]. Also for dendritic cells (Langerhans cells in the skin) a fetal macrophage origin has been suggested[147]. Evidence that certain peripheral macrophage populations under steady state conditions may be self-sustained instead of dependent on monocyte influx, was obtained from studies in which bone marrow and peripheral blood precursors were depleted by the use of the bone-seeking isotope ^{89}Sr[16,17,148-151] and from experiments using bone marrow chimeras or parabiotic animals[152,153]. These studies showed that the number of Kupffer cells, alveolar macrophages and resident peritoneal macrophages is independent of monocyte influx, indicative of maintenance by local proliferation. In addition, it has been shown that Langerhans cells are a long-lived and slow-cycling cell population, capable of self-reproduction[154,155]. However, several studies have reported also on a bone marrow origin of e.g. Kupffer cells and Langerhans cells[66,67,156,157]. Therefore, a dual origin of resident macrophages has been suggested, in which both local proliferation of resident cells (possibly generated during ontogeny), and monocyte influx contribute to the maintenance of resident macrophage populations[15,158,159].

Induction of heterogeneity by local factors in the peripheral tissues

According to the concept of the mononuclear phagocyte system, the differences observed between macrophages from different organs, or from different anatomical sites within one organ, are the result of the exposure of cells to different tissue micro-environments. Indeed it has been shown that tissues differ in their local concentrations of growth factors such as M-CSF[160,161] and GM-CSF[162,163]. Previously these growth factors were shown to contribute to phenotypic and functional heterogeneity of mature macrophages *in vitro*[21,164-166]. In addition, recent studies in mice deficient for these growth factors have confirmed and extended the *in vitro* data on the influence of M-CSF and GM-CSF on the functional activity of macrophages[167].

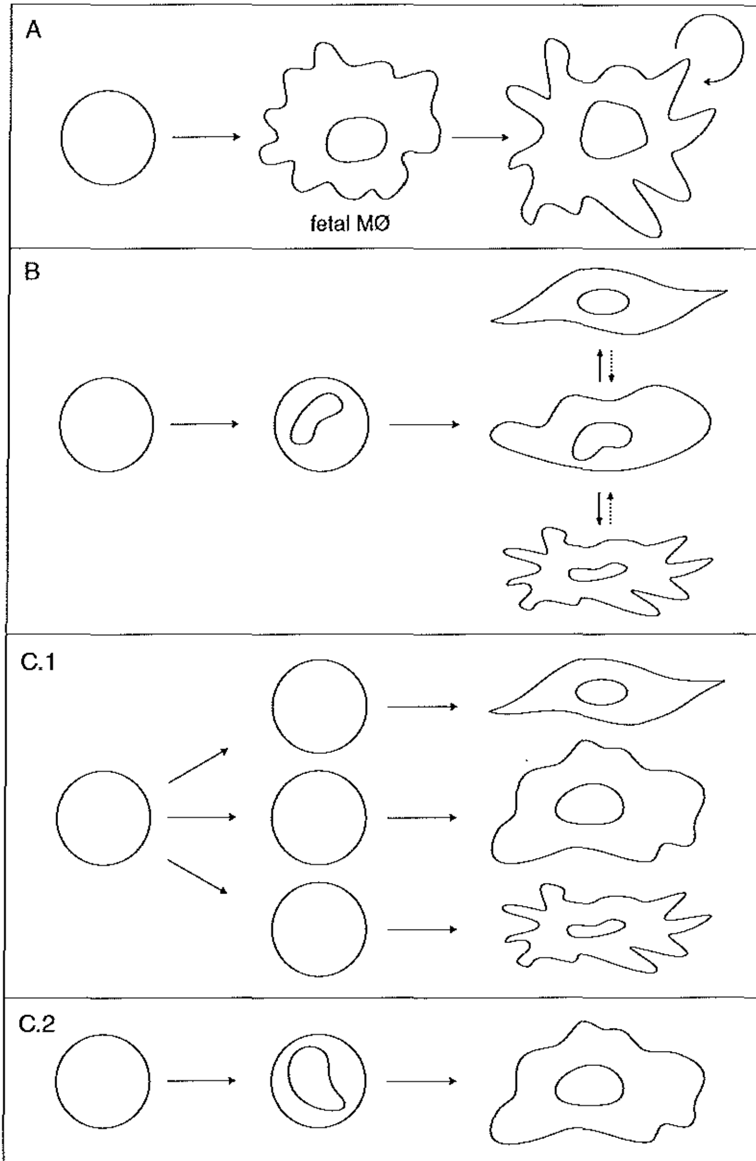

Figure 3. Hypothetical mechanisms contributing to mononuclear phagocyte heterogeneity. A. Self-maintained populations generated in ontogeny. In ontogeny resident macrophages may arise from fetal macrophages entering the tissues. In adult life such populations are self-maintained, independent of monocyte influx. B. Induction of heterogeneity by local factors in peripheral tissues. Mature macrophage heterogeneity may arise at the level of monocytes entering the various tissue microenvironments (differentiation), or at the level of macrophages responding to altering conditions in their microenvironment (activation). By definition, activation of macrophages is a reversible process. C. Induction of heterogeneity at the precursor level. C.1. Separate precursor populations that give rise to different types of mature macrophages may be generated during mononuclear phagocyte development. C.2. Differences in function and phenotype associated with different maturation stages may contribute to mononuclear phagocyte heterogeneity.

The osteopetrotic *op/op* mouse lacks the macrophage-specific growth factor M-CSF due to a spontaneous inactivating mutation in the M-CSF gene. As a result the number of macrophages and osteoclasts is severely reduced. However, not all macrophage populations were affected by the mutation. It appeared that populations of tissue macrophages clearly differ in their M-CSF dependency[168-171]. Three groups of macrophage populations could be discerned: (i) macrophage subsets that are totally M-CSF-dependent (less than 5% of normal cell numbers found in *op/op* mouse), (ii) partially M-CSF-dependent macrophage populations (5-80% of normal cells), and (iii) M-CSF-independent populations (>80% of normal numbers still present)[169]. For example, peritoneal macrophages, osteoclasts and spleen methallophils are almost completely dependent on M-CSF, whereas e.g. Langerhans cells and dendritic cells of lymphoid organs are M-CSF-independent[169,170]. It has been suggested that these differences in M-CSF dependency reflect functional differences, with M-CSF-dependent macrophages most likely playing a role in various tissue reactions, including responses to bacterial infection, tumor cell growth, and tissue remodeling[169,170]. M-CSF-independent macrophages, on the other hand, were hypothesized to be primarily involved in mounting an immune response[169,170].

In contrast to the *op/op* mouse, general analysis of the GM-CSF knockout mouse showed no reduction in the basal numbers of granulocytes or monocytes/macrophages[172,173]. However, the function of alveolar macrophages was severely impaired, indicating an important, irreplaceable, role for GM-CSF in the regulation of alveolar macrophage function[172,173].

In addition to these colony-stimulating factors, other factors like IFN-β, IFN-γ, TNF-α, LPS, IL-4, IL-13, IL-10 have been shown to contribute to macrophage diversity[143,174,175]. Obviously, cell-cell interactions, as well as interactions with the extracellular matrix may also play a role in generating macrophage heterogeneity. As yet, the precise mechanisms involved in the tissue-specific generation of macrophage diversity are not clear. It is to be expected that analysis of mutant mice will contribute significantly to our understanding of these processes.

Induction of heterogeneity at the precursor level

Heterogeneity can find its origin at the macrophage precursor level in two ways. First, precursors can differ in functional capacities as a result of differences in maturation stages and in this way contribute to the functional heterogeneity among mononuclear phagocytes. Second, subpopulations of precursor cells may exist, which develop along intrinsically different gene programs into distinct subtypes of mature macrophages. Maturation of bone marrow-derived macrophages has been shown to be accompanied by changes in phenotype such as increasing levels of β-galactosidase[176], decreasing levels of myeloperoxidase[177] and increasing or decreasing levels of cell surface markers (178 and chapter 2 of this thesis). In addition, maturation stage-related functional changes have also been reported. Phagocytic capacity, cytotoxicity, chemotactic responses, the production of various molecules associated with inflammation have all been shown to be expressed maximally at specific stages of maturation[179-185]. Phenotypic heterogeneity resulting from differences in maturation stage was also found

among freshly isolated human peripheral blood monocytes. Two monocyte subsets were identified on the basis of differential CD14 and CD16 expression, a $CD14^{+}16^{+}$ and a less mature $CD14^{++}16^{-}$ subset (reviewed in 186). On the basis of differences in the expression of several other cell surface markers and in cytokine production, it has been suggested that these monocyte subsets differ in their functional capacities, with the $CD14^{+}16^{+}$ cells being of a proinflammatory type[186].

The existence of separate precursor populations which give rise to different mature macrophage subsets has first been suggested by Bursuker and Goldman[187,188]. They found that bone marrow precursors could be divided into two populations: one giving rise to mononuclear phagocyte colonies expressing high levels of 5'-nucleotidase activity and one giving rise to colonies expressing low levels of this enzyme. As the level of enzyme expression in either of these populations could not be modulated, they suggested that the difference in enzyme expression reflected the existence of inherently different precursor subsets[188]. Since then, several studies have been published, supporting the existence of such macrophage precursor populations. These studies report on clonal differences of bone marrow and spleen derived macrophages in antigen presentation[110-113,189-191], cytotoxicity[192], and cytokine production[193]. In addition, immortalized bone marrow macrophage precursor cell lines have been shown to differ in their capacities to differentiate into either non-phagocytic, phagocytic, or class II expressing mature macrophages, suggestive of the existence of precursors with divergent differentiation pathways[194].

Blood monocytes form the link between macrophage precursors in the bone marrow and mature tissue macrophages. Thus, if heterogeneity originates already at the level of the bone marrow precursors, then such a heterogeneity should be reflected also at the level of blood monocytes. Indeed, monocyte subsets heterogeneous in phenotype and/or function have been detected in peripheral blood, e.g. a subset of monocytes expressing a proinflammatory phenotype, named P monocytes[195,196] and a $CD64^{-}$ subset with a high accessory and antiviral activity[197]. As no obvious maturational differences were found so far that could be underlying the observed heterogeneity, these data support the concept of different precursor subsets.

Previously it has been shown that macrophages derived *in vitro* from precursors using either M-CSF or GM-CSF as a growth factor differ morphologically and functionally[8,198-205]. Thus, the local concentrations of growth factors such as M-CSF and GM-CSF are likely to have an effect on the developmental pathway followed by each individual macrophage precursor. It remains to be established, however, to what extent microenvironmental stimuli early in macrophage development contribute to the generation of mature macrophage diversity. It is possible that predetermined developmental programs are followed once they are activated by local stimuli.

In summary, the currently available data indicate that heterogeneity of mature macrophages can originate also at the level of macrophage precursor cells in the bone marrow and peripheral blood. However, the precise developmental stage(s) at which heterogeneity is generated, the mechanisms involved in the generation of such heterogeneity, and the contribution of precursor heterogeneity to mature macrophage heterogeneity *in vivo*, remain to be established.

4 Introduction to the experimental work

As discussed in the previous sections, many questions still exist concerning the origin and development of the mononuclear phagocyte system. Further identification of the branch points of the mononuclear phagocyte system with other hematopoietic lineages and branch points between mononuclear phagocyte sublineages, may shed light on the processes involved in lineage commitment of progenitor cells. Such studies require the identification of mononuclear phagocytes at early stages of their development. To this end a panel of monoclonal antibodies has been generated in our laboratory against immortalized macrophage precursors from the bone marrow of the mouse[178,194]. Two of these antibodies, ER-MP12 and ER-MP20, each have been found to recognize cell surface molecules differentially expressed by bone marrow macrophage precursor cells. A third antibody, ER-MP58, appears to detect all M-CSF-responsive bone marrow progenitors[178]. It is not clear whether the observed heterogeneity in macrophage precursor phenotype reflects the existence of macrophage sublineages, or different stages of maturation along a linear differentiation pathway. The aim of the studies described in this thesis was therefore to (i) isolate bone marrow macrophage precursor subsets for analysis of their developmental potential, and (ii) to separate early myeloid-committed progenitor cells from hematopoietic progenitors with other or broader activities in order to facilitate studies on the processes involved in commitment to the myeloid/mononuclear phagocyte lineage.

In **chapter 2** the expression of the ER-MP12, ER-MP20, ER-MP58 markers and a selected panel of other currently available anti-mononuclear phagocyte markers was examined on macrophages developing in liquid bone marrow cultures stimulated with M-CSF. Such cultures give insight into which stage(s) of macrophage development are recognized by the antibodies. In this chapter data on the differential recognition of mononuclear phagocytes obtained from the bone marrow cultures and other models of macrophage development described in the literature are reviewed to come to a classification of anti-mononuclear phagocyte antibodies. In this classification, emphasis is on the interpretation of phenotypic characteristics in terms of macrophage developmental stages and functional capabilities.

The experiments described in **chapter 3** were undertaken to examine (i) whether the phenotypic heterogeneity detected among macrophage precursors by ER-MP12 and ER-MP20 monoclonal antibodies[178] could be extended in two-color flow cytometry, and (ii) whether this phenotypic heterogeneity reflected different maturation stages or the existence of macrophage sublineages.

In a parallel study on the ER-MP12/20 phenotype of hematopoietic stem and progenitor cells it was found that cells which have the capacity to confer hematopoietic reconstitution after intravenous transfer into irradiated recipient mice, reside in the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subsets[206]. To examine if progenitors of the T, B and myeloid lineages can be separated on the basis of differential ER-MP12/20 expression, the early *in vivo* differentiation capacity of the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subsets was determined after transfer into irradiated recipients (**chapter 4**).

The experiments described in **chapter 5** were designed to answer the question whether myeloid-committed cells present in the ER-MP12^{hi}20^{-} subset can be distinguished and purified from the other hematopoietic stem and progenitor cells in this subset (see chapter 4). For this purpose, bone marrow cells were triple labeled with ER-MP12/20 and ER-MP58, an antibody previously found to detect all M-CSF-responsive progenitors in mouse bone marrow[178]. Subsets differing in ER-MP58 expression were isolated from the ER-MP12^{hi}20^{-} bone marrow population by cell sorting and tested *in vitro* and *in vivo* for their capacity to differentiate along the myeloid, B, T, and erythroid lineages. In addition, the expression of the ER-MP58 antigen on various hematopoietic stem cell subsets was investigated.

As discussed in this introduction, dendritic cells (DC) are at least in part linked to the monocyte/macrophage lineage. In **chapter 6** we questioned in which of the ER-MP12/20/58-defined bone marrow subsets GM-CSF-responsive DC progenitors reside. For this purpose, ER-MP12/20/58 subsets were isolated by cell sorting and cultured in the presence of GM-CSF. After various periods of time, cultures were examined for the presence of DC-like cells by microscopic inspection of cellular morphology and immunocytochemical analysis of MHC class II expression. In addition, we asked till what ER-MP12/20-defined stage of development DC-like cells can be derived from macrophage precursors. To this end, ER-MP12/20/58 bone marrow subsets containing different stages of macrophage maturation were cultured in the presence of M-CSF to induce further development along the monocyte/macrophage lineage. After four to five days of culture the stimulant was changed to GM-CSF and the capacity of the developing macrophage precursors to give rise to DC-like cells was assessed as described above.

From the initial experiments described in chapter 3 we learned that upon double labeling of bone marrow with ER-MP12 and ER-MP20 monoclonal antibodies 6 bone marrow subsets can be discerned with a remarkable hematopoietic lineage homogeneity. These data suggested that ER-MP12/20 analysis of bone marrow could provide a new, relatively simple, and objective method for differential analysis of mouse bone marrow composition. In **chapter 7** we aimed at validating the use of this method in an experimental *in vivo* model. To this end, changes in bone marrow cellular composition were monitored in a sublethal infection with *Listeria monocytogenes*. Data obtained from ER-MP12/20 bone marrow subset analysis were compared with data obtained from analysis of lineage marker expression and from conventional morphological analysis. The correlation between the different methods was assessed by linear regression analysis.

In **chapter 8**, we have applied ER-MP12/20 bone marrow subset analysis (validated in chapter 7) in a study on the effects of dietary fish oil supplementation in mice. Fish oil consumption has been shown to enhance the resistance to infections in various experimental animal models[207]. Examination of the cellular composition of bone marrow and the frequency of M-CSF-responsive macrophage precursors contributed to shed more light on the mechanism(s) behind the enhanced resistance induced by dietary fish oil.

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2 Markers of mouse macrophage development detected by monoclonal antibodies

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Abstract

In this review, we present and discuss a selected panel of antibody-defined markers expressed during different stages of mouse macrophage development. We distinguish four categories of markers, which are characteristic of: (1) macrophage precursors and immature macrophages (ER-MP12, ER-MP20, ER-MP54, ER-MP58); (2) mature macrophages in general (F4/80, BM8, Mac-1, Mac-2, ER-BMDM1); (3) macrophage subsets (ER-HR3, ER-MP23, ER-TR9, Forssman antigen, MOMA-1, MOMA-2, Monts-4, SER-4), and (4) IFN-γ-stimulated macrophages (H-2Ia, LFA-1, ICAM-1, 158.2, MBR-2, TM-2, TM-4, and TM-5). It should be noted that many of the markers in this last category are inducible by other stimuli as well. The rigid classification of markers into four separate groups should be regarded as a digitalization of a continuum, thus inevitably implicating a simplification of the complex phenotypic changes that occur during mononuclear phagocyte development. Nevertheless, the current selection of antibodies against markers for different developmental stages of macrophages constitutes an important tool for characterization of mouse macrophages which participate in various biological processes.

Introduction

The cell surface of the mouse macrophage has been mapped extensively since the introduction of hybridoma technology in the late seventies. An array of monoclonal antibodies elicited against different macrophage populations has been produced and characterized (see ref. 1 for an overview). Yet, defining discrete, functional stages in mouse macrophage development using antibodies against discriminating determinants appeared to be much more complicated than describing functionally distinct lymphocyte subtypes.

The limited insight into the link between phenotype and function of different macrophage populations may be ascribed primarily to the complexity of the mononuclear phagocyte system (see recent reviews by us and others[1,2]). Different macrophages are involved in a wide variety of processes, such as (1) clearance of potentially harmful substances, (2) killing of microorganisms and tumor cells, (3) regulation of and participation in inflammatory and immune responses, and (4) regulation of hemato- and lymphopoiesis. This functional diversity is clearly reflected in the phenotypic diversity of the various mononuclear phagocyte populations, although it is often difficult to interpret observed phenotypic differences.

In this review, we will discuss a selected panel of markers, detected by monoclonal antibodies, useful for the phenotypic characterization of mouse macrophages. Emphasis will be on the interpretation of phenotypic characteristics – and on the limitations thereof – in terms of macrophage developmental stages and functional capabilities. To that end, we will first attempt to define the different processes in macrophage development, as many slightly different definitions are being used in the field.

We regard the phenotypic and functional changes that macrophages undergo during development as being caused by either maturation, differentiation, or activation of the cells (schematically depicted in Fig. 1). *Maturation* is the process by which cells acquire features typical of cells in later stages of development, and lose aspects of earlier stages. *Differentiation*, in our view, is a specific type of maturational process by which diversity is generated. Both maturation and differentiation are, in principle, irreversible. In contrast, *activation* of macrophages is a reversible process by which cells are stimulated to express particular functions or by which the accomplishment of certain existing functions is enhanced. These processes may occur concurrently, that is, a cell might both mature and become activated as a response to a specific stimulus.

In accordance with the different developmental processes, macrophage markers may be classified as (1) maturation markers, which are either positively or negatively correlated with the cell's maturation stage, (2) differentiation or subset markers, which are expressed differentially by cells of similar maturation stages, and (3) activation markers, whose expression can be induced or enhanced by particular external stimuli and is indicative of certain functional capabilities. Inevitably, the assignment of a particular marker to one of these categories depends significantly on the experimental models used to study maturation, differentiation and activation.

In our previous work, we have adopted and developed three different models for the identification of macrophage maturation markers, recognized by newly prepared and existing monoclonal antibodies. The first model consists of a panel of macrophage cell lines,

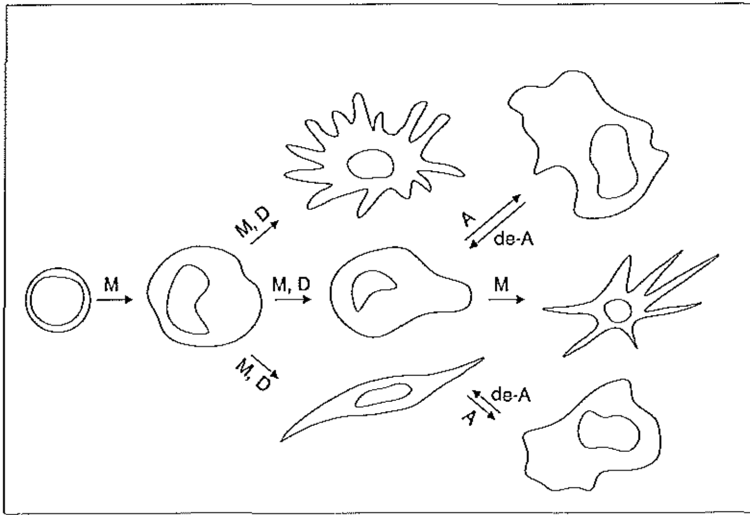

Figure 1. Schematic representation of different facets of macrophage development. M: maturation; D: differentiation; A: activation; de-A: de-activation.

which can be aligned in order of increasing maturity and represent stages ranging from CFU-GM to relatively mature exudate macrophages[3]. M-CSF-stimulated bone marrow cell cultures abrogated at different time points provides a second maturation model[4,5]. Thirdly, multiple macrophage precursor cell lines can be stimulated to develop into more mature stages by external stimuli, such as mouse post-endotoxin serum[6].

Models for the identification of macrophage differentiation or subset markers are not as clearly defined as the maturation models mentioned above. In the latter, homogenous populations of mature cells are generated from precursors, and diversification, which is by definition the hallmark of differentiation, is rarely observed. Yet, different macrophage subsets can be easily discerned *in situ* by immunohistochemical analysis of complex organs such as the spleen[7]. Here, phenotypically distinct macrophage subsets are found in different microanatomical locations. The developmental relationship between these subsets is unclear, but the cells do not show conclusive maturational differences that might explain their phenotypic heterogeneity. However, at some point in their development the distinct macrophage subsets share a common progenitor and thus represent the end-stages of a differentiation process. Therefore, to identify different subset markers we use immunohistochemistry of macrophages in lymphoid and non-lymphoid organs as the main technique.

Macrophage activation markers are, by definition, induced by specific external stimuli and the expression of these markers decreases again when the stimulus is removed. As a screening model, macrophage cell lines or primary bone marrow cultures are stimulated with IFN-γ, which is known to mediate several functional changes in a broad spectrum of macrophages. For instance, IFN-γ-stimulated macrophages show increased ability to present antigen to $CD4^+$ T cells, as a consequence of increased MHC class II expression, and are primed for further stimulation to become tumoricidal[8]. However, stimulating macrophages

with IFN-γ precludes the development of other functional stages. Thus, in general, activation markers are linked directly to the type of stimulus given, as well as to the type of responding macrophage.

In the following sections we will discuss a panel of macrophage markers, classified as either immature or mature markers, subset markers or activation markers according to the criteria mentioned above. Unfortunately, the discussion will show that very few of these markers, if any, are fully specific for a single category. Our experience is that the same holds true for other macrophage markers not included in the present selection. Yet, using combinations of multiple markers from the different categories for the phenotyping of mouse macrophages enables a plausible description of their developmental stage in terms of relative (im)maturity, belonging to a particular subset or being (un)stimulated.

Materials and Methods

Bone marrow cultures

Macrophage development was followed in liquid bone marrow cultures stimulated with M-CSF, as described before[6]. Briefly, bone marrow was harvested from BALB/c mice (10-20 weeks of age), which had been kept under clean, routine conditions in accordance with institutional guidelines for animal welfare. The cells obtained were cultured for varying periods of time in DMEM, supplemented with 10% fetal calf serum, 13% L-cell conditioned medium and antibiotics, and then phenotypically analyzed.

Monoclonal antibodies

The characteristics of and references for monoclonal antibodies described in this paper are summarized in Table 1. Hybridomas marked with "ER-" were established in our own department; M1/70, M3/38, M1/22.25, M5/114, YN1/1.7 and MA158.2 producing hybridomas were obtained from the American Type Culture Collection (Rockville, MD); monoclonal antibodies and/or hybridomas designated BM8, F4/80, H35.89, MOMA-1, MOMA-2, Monts-4, MIV 38, SER-4, TM-2, TM-4, and TM-5 were kindly provided by the respective producers and/or BMA Biomedicals AG (Augst, Switzerland). For cellular staining, either undiluted hybridoma supernatants or optimally diluted purified antibody preparations were used.

Immunofluorescence staining and flow cytometric analysis

Staining of cells was performed using routine procedures, essentially as described before[9]. Cellular fluorescence as a measure of antibody binding was determined using a FACScan (Becton Dickinson, Sunnyvale, CA) with logarithmic amplification of FITC fluorescence signals. Results are expressed either as percent positive cells compared to cells stained with a control monoclonal antibody, or as molecules equivalent to soluble FITC (MESF). The latter values were obtained by interpolation of cellular fluorescence values compared to a standard curve prepared using microspheres of known fluorescence intensity (Flow Cytometry Standards, Research Triangle Park, NC).

Table 1 Monoclonal antibodies against markers of mouse macrophage development

Antibody	Antigen	Mol.mass (kDa)	Antigen	Reference
Immature macrophage markers				
ER-MP12		140	single chain (glyco)protein	9
ER-MP58				9
ER-MP54		90, 80-85, 70-75		9
ER-MP20 [a]	Ly-6C	14	glycoprotein, usually PI-linked	9
Mature macrophage markers				
F4/80 [a]		160	single-chain glycoprotein	75
BM8		125		22
M1/70 [a]	Mac-1α, CD11b, Ly-40	170	CR3α, fibrinogen R[b], clotting factor X R, adhesion molecule	23
M3/38 [a]	Mac-2, CBP35	32-35	galactose-specific lectin; IgE- and laminin-binding protein	34
ER-BMDM1 [a]	CD13?	160	aminopeptidase N	38
Macrophage subset markers				
ER-HR3		76, 67 (non-red.) 69, 55 (red.)	two distinct single chain (glyco)proteins; high M_r form secreted	39, 40
ER-MP23		38	single chain (glyco-)protein	42
ER-TR9			involved in Mϕ neutral polysaccharide uptake	44
M1/22.25 [a]	Forssman Ag		glycosphingolipid hapten	76
MOMA-1				50
MOMA-2				53
Monts-4		80-100	(glyco-)protein	54
SER-4	SER; sialoadhesin	170-185	sialylated glycoconjugate R	55
Macrophage activation markers				
M5/114 [a]	H-2Ia	25-34	homodimer; Ag-peptide binding for presentation	77
H35.89 [a]	LFA-1α, CD11a	180	CD54 and ICAM-2 ligand	78
YN1/1.7 [a]	ICAM-1, MALA-2, CD54	95	CD11/CD18 ligand	79
MA158.2	158.2			69
MIV 38 [a]	MBR-2			71
TM-2		45		74
TM-4				D.M. Paulnock, personal comm.
TM-5				D.M. Paulnock, personal comm.

[a] Also other monoclonal antibodies with the same or similar reactivities have been reported; [b] R = receptor.

Results and Discussion

Table 1 shows a selection of monoclonal antibodies directed against markers of different mouse macrophage developmental stages, i.e. universal immature and mature macrophage markers, subset markers, and macrophage activation markers. As outlined above, the distinction between the different marker categories is not always absolute. In particular, the discrimination between universal mature macrophage markers and subset markers is more or less arbitrary, since no markers exist, to our knowledge, that are expressed by all macrophages, and are relatively specific for these cells. In other words, all mature macrophage markers are in fact subset markers. Yet, a distinction between the two categories is made on the basis of the widespread expression of universal mature markers by macrophages *in vitro* as well as *in vivo*, and the more restricted occurrence of subset markers.

Immature macrophage markers

The panel of immature macrophage markers, ER-MP12, -20, -54, and -58, has been generated against macrophage precursor hybrid cell lines and selected for recognition of markers expressed preferentially by immature stages of maturation using the *in vitro* models[9]. The decreasing expression of these markers upon maturation of macrophages is clearly illustrated by the decreasing percent of cells positive for these markers during M-CSF- stimulated bone marrow culture (Fig. 2). Additional studies by ourselves and others have confirmed and extended the initial characterization of these markers[10-12].

Using these markers for identification of macrophage precursor cells, it should be realized that, in the macrophage lineage, ER-MP12, -20, -54 and -58 antigens are restricted to precursor stages, but these markers are also expressed by other cell types. In recent experiments, about 40% of freshly isolated bone marrow cells was repeatedly found positive for ER-MP12, in contrast with 6-9% assessed earlier using less advanced technology (cf. ref. 13 and 9). This significant percent of bone marrow cells found positive for ER-MP12 already indicates that other hematopoietic cells are recognized as well. ER-MP12 also detects pluri- and multipotent stem cells and prothymocytes in bone marrow as well as particular lymphoid cells in bone marrow and peripheral lymphoid organs[12-15]. Although the identity of the ER-MP12 antigen has not yet been established, it bears similarity to CD34 with regard to high level expression by early hematopoietic cells.

The antigen recognized by ER-MP20 has been identified definitively as Ly-6C [11] and is expressed by monocytes and granulocytes, as well as by a subpopulation of $CD8^+$ T cells. Quantitative analysis of ER-MP20 antigen expression permits the identification of monocytes in bone marrow as these cells show the highest antigen levels[12]. Bone marrow macrophage colony-forming cells (M-CFC) are either ER-MP20 negative or show an intermediate level. The former are the most immature: they produce the largest colonies and pass through an ER-MP20 positive stage upon maturation *in vitro*[12]. Mature macrophages, however, are ER-MP20 negative. Therefore, ER-MP20 is a typical marker of intermediate stages of bone marrow-derived macrophage maturation. Surprisingly, in spleen all M-CFC are found in the $ER\text{-}MP20^{hi}$ fraction, whereas no M-CFC are present in the corresponding fraction in bone marrow[11] . Although the origin of this distinction is

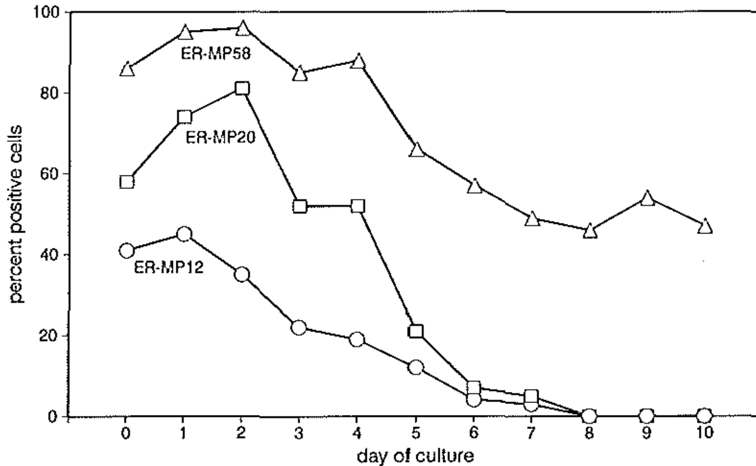

Figure 2. Expression of immature macrophage markers in M-CSF-stimulated bone marrow culture. Cells were harvested at different time points from liquid bone marrow cultures and analyzed phenotypically using flow cytometry. The number of morphologically identifiable mononuclear phagocytes (i.e. monoblasts, (pro)monocytes and macrophages) in these cultures increases from less than 10% at day 0 to 60-70% at day 4 and >98% at day 7. Data represent the mean net percent of marker-positive cells, compared to negative control values, as determined in at least two experiments; the standard deviations were less than 10%.

not known, it is possible that environmental differences determine expression of the ER-MP20 antigen by macrophage progenitors: the antigen is significantly induced or up-regulated after interferon-γ or interferon-α/β stimulation (unpublished data and ref. 16). The inducibility of ER-MP20 antigen expression is strikingly maturation-stage-dependent, as it can be induced in immature cells, but not in mature macrophages.

The ER-MP54 antigen was selected as a surface marker of macrophage precursor cell lines, but appeared to be absent from the cell surface of normal bone marrow cells[9]. Instead, bone marrow cells express this marker only cytoplasmically. Specific experimental conditions, such as corticosteroid treatment *in vitro*, may induce ER-MP54 surface expression on some non-transformed cells (unpublished). Immunohistological examination shows an ER-MP54 antigen distribution that is restricted to presumable myeloid cells: the majority of bone marrow cells and some clusters in the splenic red pulp (unpublished).

ER-MP58 demonstrates a similar, relatively specific, staining of myeloid cells in sections of bone marrow and spleen (unpublished). Yet, this marker is present on the cell surface of the majority of normal bone marrow cells, including virtually all M-CSF-responsive macrophage precursors[9]. Expression of ER-MP58 decreases significantly upon maturation of macrophages. However, reduced levels can still be demonstrated on bone marrow-derived macrophages *in vitro* (Fig. 2) as well as on some macrophage cell lines with mature features. Thus, high level ER-MP58 expression is characteristic of precursor cells, although the marker may be retained by some macrophages in more mature stages.

Taken together, the markers detected by ER-MP12, -20, -54, and -58 are expressed selectively by immature cells of the macrophage lineage. However, other cell types may be positive for these markers as well, implicating that expression of one or more of these

antigens does not necessarily identify the cells under study as macrophage precursors. If, on the other hand, the macrophage identity of such cells has been established, then expression of ER-MP12, -20, -54 and/or -58 antigens is highly suggestive of their immature nature.

Mature macrophage markers

The panel of universal mature macrophage markers that we regularly use to demonstrate macrophage maturity includes F4/80, BM8, Mac-1, Mac-2 and ER-BMDM1. Fig. 3 shows the expression of these markers during M-CSF-stimulated bone marrow culture. Despite the differences found in the percent of positive cells in freshly isolated bone marrow, all of these markers are expressed uniformly by the mature macrophages obtained from day 7 and beyond (Fig. 3a). Not only does the percentage of positive cells increase in these cultures as macrophages mature, the level of antibody binding per cell increases even more dramatically, indicating enhanced antigen expression by more mature cells (Fig. 3b). The differential staining of fresh bone marrow for the different markers clearly reflects the presence (e.g. Mac-1) or absence (e.g. ER-BMDM1) of these markers on other hematopoietic cell types, especially myeloid cells.

The F4/80 antigen is perhaps the most universal and widely used macrophage marker with relative specificity for this cell type. Expression of this marker commences early during macrophage development, but after the M-CFC stage[17]. *In vitro*, most if not all mature macrophages express the F4/80 antigen, but *in vivo* some macrophage populations do not show detectable F4/80 antigen levels, especially those in lymphoid microenvironments[18]. This lack of significant antigen expression may be due to high local concentrations of lymphocyte-derived cytokines, some of which have been shown to downregulate F4/80 antigen expression[19]. F4/80 is a selective, but not an exclusive marker for macrophages, since freshly isolated eosinophils as well as endothelial-like cells in bone marrow stromal cultures bind the F4/80 antibody[20,21].

BM8 is a macrophage marker that bears significant similarity to F4/80[22]. Although the apparent molecular masses of these antigens are different, 125 kDa for BM8 vs. 160 kDa for F4/80, the staining patterns obtained show a high degree of overlap. The original description of BM8 showed expression of the antigen only by cells beyond the monocytic stage. In our hands, however, monocytes and myeloid precursors also specifically bind small, but detectable amounts of the BM8 antibody as demonstrated by sensitive flow cytometry.

The Mac-1 (CD11b/CD18) antigen was one of the first macrophage markers defined by monoclonal antibody[23]. Expression of Mac-1 is detected on cells beyond the M-CFC stage in bone marrow and spleen[11,24] and increases significantly with maturation of macrophages *in vitro*[5]. Yet, Mac-1 is undetectable on many "tissue-fixed" macrophages, but it is expressed at different levels by virtually all "free" macrophage populations[25,26]. In addition, granulocytes and NK cells express Mac-1 as well as activated and memory $CD8^+$ cytotoxic T cells and some unusual B-1 lymphocytes[23,27-29]. Using blocking studies, it was found that anti-Mac-1 monoclonal antibody recognized the α-chain of type 3 complement receptor, a member of the integrin family[30]. This receptor is involved in adhesive processes with different ligands. First of all, CR3 mediates uptake of opsonized

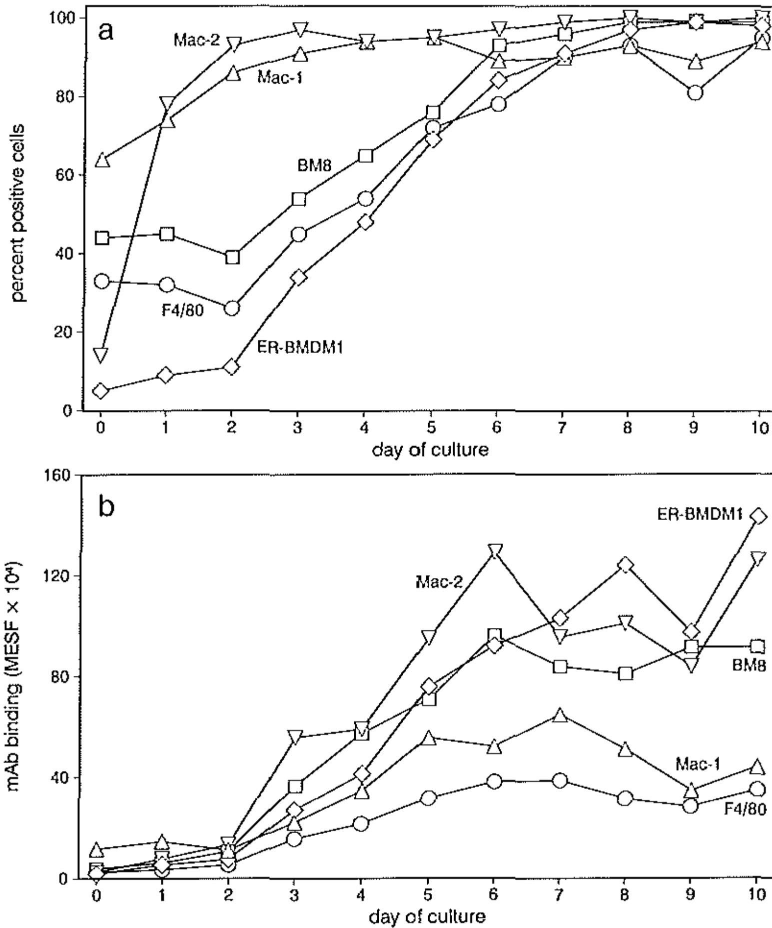

Figure 3. Expression of universal mature macrophage markers in M-CSF-stimulated bone marrow culture. (a) Mean net percent of marker-positive cells at different time points during *in vitro* macrophage development (cf. legends Fig. 2); (b) quantitative analysis of monoclonal antibody binding to developing macrophages. Data represent the mean net antibody binding to the marker-positive cells depicted in (a).

microorganisms and particles by binding of iC3b and C3d (reviewed in 31). In addition, it was shown for macrophage - *Listeria* interactions, that CR3 not only functions as a receptor for internalization but also seems to trigger processes essential for killing of the bacterium[32]. Second, CR3 binds other molecules, such as fibrinogen and clotting factor X, mostly through an Arg-Gly-Asp containing sequence in the ligand[31]. Third, CR3 is critically involved in the recruitment of myelomonocytic cells in response to various inflammatory stimuli, probably by binding to ICAM-1 (CD54) molecules on the inflamed endothelium (reviewed in 33).

Mac-2 was originally presented as a macrophage subpopulation-specific marker[34]. However, expression of this antigen closely correlates with macrophage maturation in all *in vitro* models (Fig. 3; ref. 3, 5, 6). It is induced in precursor cells at a relatively late stage, just preceding the ability of cells to become adherent. Cloning and sequencing of the Mac-2 gene showed that it does not contain a classic signal peptide or transmembrane domain, indicating that it is mainly an intracellular protein[35]. This is confirmed by the finding that the percent of Mac-2 positive spleen cells increases from about 20% to more than 70% upon permeabilization of the cell membrane[36]. The latter finding also implies that many cell types other than macrophages also express the Mac-2 antigen. Mac-2 has been identified as a galactose-specific lectin. Cell surface expression may thus be explained by binding of secreted molecules to surface-bound glycan moieties, since binding is abolished by incubation of cells in the presence of competing sugar[37].

The ER-BMDM1 antigen is, from the universal mature markers described here, induced last on developing macrophages (Fig. 3). Bone marrow cells as well as blood monocytes are essentially ER-BMDM1 negative, and expression is observed uniformly on all mature cells in the various models[38]. *In vivo*, however, only specific macrophage subpopulations are detected by this antibody, including about one third of the resident peritoneal and alveolar macrophages. Certain inflammatory macrophages, such as thioglycollate-elicited cells, express high levels of this marker. Furthermore, populations belonging to the dendritic leukocyte lineage are mostly ER-BMDM1 positive. The biochemical characteristics of the antigen - a molecular mass of 160 kDa and demonstrated aminopeptidase N activity - suggest that this marker is the mouse homologue of human CD13 [38].

Macrophage subset markers

As outlined before, the distinction between universal mature macrophage markers and subset markers is more or less arbitrary. Our criterion for classification is that subset markers are not universally expressed by all mature macrophages in the *in vitro* maturation models, in contrast to the universal mature markers. Also, subset markers show a restricted distribution *in vivo* (see below). The subset markers that we use (ER-HR3, ER-MP23, ER-TR9, Forssman antigen, MOMA-1, MOMA-2, Monts-4, and SER-4) are typical of different macrophage subsets in tissue sections of various organs, and all, except Forssman antigen, show a high degree of specificity for mononuclear phagocytes.

The macrophage subset marker ER-HR3 is expressed by a specific subpopulation of mouse macrophages, located especially in hematopoietic organs: bone marrow and splenic red pulp[39,40]. The number of ER-HR3 positive macrophages in the red pulp constitutes only a subset of all (F4/80 positive) red pulp macrophages, yet includes the so-called central macrophages of the erythropoietic islands. Induction of extramedullary hematopoiesis in the liver by phenylhydrazine treatment of mice is accompanied by the appearance of ER-HR3 positive central macrophages. This observation supports the notion that the ER-HR3 positive macrophage subset is functionally involved with erythropoietic activity[40]. Yet, other subsets which are not apparently involved with hematopoietic activity, such as those located in the lymph node paracortex and the ileal lamina propria, also express the ER-HR3 antigen[41].

ER-MP23 is a marker particularly expressed by connective tissue macrophages, or histiocytes[42]. Interestingly, macrophages in many, but not all, connective tissues located near epithelia express high levels of this antigen: e.g. dermal macrophages and macrophages in the reticular connective tissue of the submandibular gland are strongly ER-MP23 positive, but those in the intestinal lamina propria are generally ER-MP23 negative. In addition to a location in connective tissue, ER-MP23 positive cells may also occur sparsely in lymphoid tissues. Furthermore, ER-MP23 positive non-lymphoid cells, being either macrophages or dendritic leukocytes, are among the first cells to infiltrate the pancreatic islets of the pre-diabetic NOD mouse[43]. *In vitro* studies of ER-MP23 expression among different macrophages populations and derived cell lines have indicated that macrophages are either strongly positive for this marker, or negative/dull. This ER-MP23 phenotype of *in vitro* maintained cells is surprisingly stable; in our hands, an ER-MP23$^{-/dull}$ cell line could not be converted by a variety of stimulants into an ER-MP23^{hi} cell line and vice versa[42]. This led us to conclude that ER-MP23 might be a subset marker characteristic of a distinct lineage of macrophage development.

ER-TR9 antigen expression is characteristic of splenic marginal zone macrophages and macrophages located in the medullary and trabecular sinuses of lymph nodes[44,45]. These highly phagocytic populations are unique in their ability to retain and ingest neutral polysaccharides, such as Ficoll and dextran. Interestingly, *in vivo* administration of the ER-TR9 antibody abolished endocytosis of these polysaccharides, but did not influence the ability to ingest latex or carbon particles[46]. The ER-TR9 monoclonal antibody probably does not recognize the binding site of the neutral polysaccharide receptor, since injection of polysaccharides prior to administration of the antibody did not diminish binding of the latter.

The Forssman glycolipid antigen (Fo) is expressed by a subset of stromal macrophages mainly associated with early hemo-lymphopoietic development: macrophages in bone marrow stroma, splenic red pulp, and thymic cortex[47,48]. Furthermore, medullary macrophages in inguinal and axial lymph nodes, but not in mesenteric lymph nodes, express Fo. This marker is less specific for mononuclear phagocytes than the other subset markers from the present selection; in adult tissues also reticular cells in various organs and thymic epithelial cells are Fo^{+}, whereas developing mesenchymal cells express Fo transiently. Fo expression is inducible by IL-4 or IL-6 in a small percent of macrophages in a specific stage of development[49].

The antigen recognized by MOMA-1 is expressed by a macrophage subset typified by the metallophilic macrophages at the white pulp side of the marginal sinus in the spleen, and capsular and medullary sinus macrophages in peripheral lymph nodes[50]. These populations are characterized by a high level of nonspecific esterase activity, which, linked to their strategic location, is suggestive of a degrading and detoxifying function[51]. Injection of the MOMA-1 monoclonal antibody into neonatal mice led to a significant reduction of the presence of MOMA-1 positive cells, and concomitantly, to impaired humoral immune responses against thymus-dependent (TD) and thymus-independent type 2 (TI-2) antigens[52]. This suggests a role for the marginal metallophilic macrophages in the presentation of these antigens, although in the neonate a subpopulation of cells located in the red pulp also

shows MOMA-1 expression and may thus be involved in the immune responses against TD and TI-2 antigens.

The MOMA-2 monoclonal antibody is especially useful for the identification of macrophages in lymphoid tissues[53]. Most, if not all, macrophages in lymphoid microenvironments show high level expression of this marker. The antigen is located primarily in the cytoplasm, but it can also be demonstrated on the cell surface. The expression of the MOMA-2 antigen shows a high degree of similarity with the expression of acid phosphatase, including the localization of both markers to a cytoplasmic region of dendritic leukocytes. MOMA-2 is expressed early during the development of mononuclear phagocytes, since a significant fraction of bone marrow cells as well as all blood monocytes are positive.

Immunohistological examination of the expression of the Monts-4 antigen suggests that this marker is expressed by both MOMA-1 and MOMA-2 positive subsets[54]. Yet, many macrophage populations do not express Monts-4, including most splenic red pulp macrophages. Expression of the antigen decreases quickly upon culture of isolated Monts-4$^+$ macrophages, suggesting the necessity of continuous stimulation for Monts-4 expression. In agreement with this, we have never observed significant Monts-4 antigen expression by *in vitro* maintained macrophages, including transformed as well as growth factor-dependent cell lines and bone marrow-derived macrophages.

The macrophage subset marker SER, identified by monoclonal antibody SER-4, has been characterized most extensively in relation to the function of the antigen and the antigen-expressing macrophages in hematopoiesis[55]. Initially identified as a receptor for sheep erythrocytes, SER is now also known as sialoadhesin, a receptor for sialylated glycoconjugates occuring on developing hematopoietic cells. Antibodies against SER are able to block interaction between sialylated compounds and SER. Interestingly, ligand binding to SER does not induce phagocytosis, suggesting that SER's primary function is to maintain physical contact between cells and/or mediate signal transduction. The receptor is expressed by stromal macrophages at hematopoietic sites, such as resident bone marrow macrophages and a subset of splenic red pulp macrophages, but also by macrophages in lymphoid microenvironments, such as splenic marginal metallophils and lymph node subcapsular and medullary macrophages. Here, SER is thought to be involved in the interaction between stromal macrophages and activated T cells and plasmablasts[56].

Macrophage activation markers

A fourth category of macrophage markers comprises antigens induced by activating agents. Induced expression of such activation markers is accompanied by, and in the ideal situation indicative of, specific functional acquisitions. Generic activation markers, however, do not exist, as the phenotypic and functional response of a macrophage depends greatly on the nature of both the stimulus ánd the responding cell. Yet, IFN-γ is a major stimulator of multiple macrophage functions, and was therefore chosen as a "model activating agent" to identify macrophage activation markers. As the following discussion will show, many of the present activation markers are also induced by other agents.

Expression of MHC class II antigens, in the mouse H-2Ia, is induced in most macrophages by IFN-γ, as well as by a number of other stimuli including IL-4, GM-CSF

and IL-3 [57-60]. On the other hand, IFN-α/β and M-CSF have been shown to counteract the IFN-γ- or GM-CSF-mediated induction of Ia expression[59,61]. Colony-forming macrophage precursors do not express Ia antigens[24], but expression is induced during *in vitro* development in about 15-30% of the cells (unpublished observation; ref. 62). This apparent heterogeneity is not clonally determined, however[63]. Expression of Ia molecules is a prerequisite for macrophages to present antigen to $CD4^+$ T helper cells, but not a "proof" of their ability to do so; productive stimulation of Th cells requires appropriate co-stimulatory signals in addition to Ia-bound antigenic peptides[64].

LFA-1 (CD11a/CD18) belongs to the integrin family and shares its β-chain type with Mac-1 and p150/95 (CD11b and CD11c, resp.). Expression of LFA-1 is already found on immature cells, as part of bone marrow M-CFC is $LFA\text{-}1^+$ [24]. Yet, among more mature cells, LFA-1 can be considered a marker of primed and activated stages and its expression can be induced by several stimulants including IFN-γ, LPS, IL-4 and IL-3 [60,65]. Various macrophage populations, however, are refractory with respect to induction of LFA-1 [65]. As an integrin, LFA-1 is critically involved in many cellular interactions and is a ligand for ICAM-1, ICAM-2 and possibly others. However, to be functional, the LFA-1 molecule needs to be activated to reach a high-affinity state[66]. Therefore, expression of LFA-1 is a sign of potential, but not necessarily actual, involvement in LFA-1 – ICAM-interactions.

The major ligand of LFA-1, ICAM-1 (CD54), may also be considered a macrophage activation marker. Expression of this member of the immunoglobulin superfamily is induced by a variety of inflammatory cytokines, including IFN-γ, IL-1 and TNF-α[67]. ICAM-1 shows a widespread cellular and tissue distribution, including vascular endothelium, lymphocytes, monocytes, macrophages and dendritic leukocytes[68]. This suggests that the ICAM-1 – LFA-1 interaction is probably regulated at the level of activation of the LFA-1 molecule.

The antigen 158.2 is another IFN-γ-inducible marker for macrophages in both mature and immature stages of development[69,70]. In addition, expression of 158.2 can be induced by LPS, but not by IFN-α/β, muramyl dipeptide or bestatin. The initial characterization of this marker suggested a link between enhanced 158.2 expression and tumoricidal and/or bactericidal activity of macrophages. In our hands, however, IFN-γ also induces high level expression of this marker in macrophage precursor cells without inducing concomitant cytotoxic activities (unpublished results). 158.2 is weakly expressed by resident macrophages, but can be demonstrated at significant levels on exudate macrophages as well as exudate granulocytes.

MBR-2 is one of a cluster of antigenic determinants on the same or closely associated molecules[71]. Initially, this marker was defined on a subpopulation of B cells, but its presence on macrophages and erythrocytes was also recognized[72]. In contrast to the activation markers described above, MBR-2 is induced by IFN-γ only on macrophages in more mature stages of development (unpublished). Expression of MBR-2 is not fully dependent on induction by IFN-γ, however, since macrophages maturing *in vitro* show increasing expression of MBR-2 in the absence of IFN-γ stimulation[73]. Resident peritoneal macrophages also express significant levels of MBR-2. The immunohistochemical profile of anti-MBR monoclonal antibodies is characterized particularly by reactivity with capillary endothelia.

The TM-2, TM-4, and TM-5 antibodies were raised against IFN-γ-stimulated RAW264 macrophage tumor cells in order to obtain markers for intermediate stages in the tumoricidal activation pathway (ref. 74; D.M. Paulnock, personal communication). As such, these antigens are expressed by stimulated RAW264 cells, but not by IFN-γ-stimulated WEHI-3 cells that cannot be induced to express tumoricidal activity. Likewise, resident peritoneal macrophages, which cannot be induced to kill tumor cells, fail to express these antigens after stimulation. Induction of TM-2, -4 and -5 expression by responsive cells is relatively specific for IFN-γ exposure, since IFN-α/β, TNF-α, LPS, IL-4 and GM-CSF are ineffective in this respect. Analysis of TM-antigen profiles of different exudate macrophage populations revealed a more complex pattern, suggesting that TM-4 and -5 antigens are associated with an inflammatory phenotype, while induction of TM-2 occurs during the later stages of activation to tumoricidal activity.

Concluding remarks

The available monoclonal antibodies against mouse mononuclear phagocyte markers, of which the current panel is only a selection, permit us to identify different stages in the development of these cells. Using multiple markers from each category, we feel confident in determining from the cellular phenotype whether macrophages are immature, mature, belong to a specific subset, or are more or less stimulated. This classification is still rather crude, and definitely needs further refinement. This, however, can only be achieved when more insight is obtained into the developmental relationships between the various members of the mononuclear phagocyte system and the phenotypic hallmarks that identify the different stages. Considering the "rudimentary" classification, caution should be exercised in drawing conclusions from results obtained with single markers, since many experimental conditions unexpectedly induce or suppress the expression of particular antigens. Such an "aberrant" phenotype is likely to have functional significance. This, however, remains elusive until the function of the irregularly up- or down-modulated antigen has been clarified. Unfortunately, many markers described in this overview still lack a functional designation, thus leaving us with "good guesses" in relating the cellular phenotype to a specific cellular function. A major effort of the forthcoming years, therefore, will be to transform these "good guesses" into "solid evidence" by elucidating the functions of the various macrophage markers and translating these in terms of cellular capabilities.

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3 Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens

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Abstract

The characterization of early branch points in the differentiation of leukocytes requires identification of precursor cells in the bone marrow. Recently, we produced two monoclonal antibodies, ER-MP12 and ER-MP20, which in two-color flow cytometric analysis divide the murine bone marrow into six defined subsets. Here we show, using fluorescence-activated cell sorting followed by macrophage colony-stimulating factor-stimulated culture in soft agar, that precursors of the mononuclear phagocyte system reside only within the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} bone marrow subsets. Together these subsets comprise 15% of nucleated bone marrow cells. Furthermore, we provide evidence that the macrophage precursors present in these subsets represent successive stages in a maturation sequence where the most immature ER-MP12^{hi}20^{-} cells develop via the ER-MP12^{+}20^{+} stage into ER-MP12^{-}20^{hi} monocytes.

Introduction

Macrophages form a heterogeneous population of cells which play essential roles in a wide variety of biological processes (for reviews see 1 and 2). As yet, it is not clear whether their extensive diversity is generated solely at the level of the monocytes entering the tissue microenvironments, or also at the level of the macrophage precursors in the bone marrow. Studies on the early stages of macrophage development have been seriously hampered by the limited characterization and low frequency of macrophage precursors in the bone marrow. To approach this problem, we previously produced a panel of monoclonal antibodies using immortalized macrophage precursors as immunogens[3]. Two of these monoclonal antibodies, ER-MP12 and ER-MP20, were shown to detect phenotypic heterogeneity among bone marrow macrophage precursors[3]. In the present study we aimed at identifying distinct macrophage precursor subsets in mouse bone marrow using ER-MP12 and ER-MP20 monoclonal antibodies in two-color flowcytometric analysis and cell sorting. Bone marrow subsets sorted on the basis of their differential expression of ER-MP12 and ER-MP20 antigens were examined on cellular composition, macrophage colony/cluster forming capacity, cell surface expression of the macrophage maturation-related marker Mac-1, and expression of ER-MP12 and ER-MP20 antigens during macrophage maturation *in vitro*. In this report we show that three phenotypically distinct subsets of M-CSF-responsive bone marrow macrophage precursors can be identified. Moreover, our data indicate that these three macrophage precursor subsets reflect successive, phenotypically defined, stages of *in vivo* macrophage development.

Materials and Methods

Mice

Female C57BL/6-Ly-5.1-Pep^{3b} mice (breeding pairs kindly provided by Dr. I.L. Weissman, Stanford University, Stanford, CA) between 6 and 20 weeks old were used in all experiments. Animals were kept under clean routine laboratory conditions with free access to food and water.

Antibodies and conjugates

The monoclonal antibodies used in this study were M1/70 (anti-Mac-1)[4], ER-BMDM1 (anti-aminopeptidase N)[5], ER-MP12 [3], and ER-MP20 (anti-Ly-6C)[3,14]. Antibodies were applied as hybridoma culture supernatants or as purified monoclonal antibodies conjugated to FITC (fluorescein isothiocyanate, isomer I, Sigma Chemical Co., St. Louis, MO) or biotin (N-hydroxysuccinimidobiotin, Boehringer Mannheim GMBH, FRG) by standard procedures.

R-Phycoerythrin-conjugated streptavidin (SAV-PE; Caltag Laboratories, San Francisco, CA), Tri-Color-conjugated streptavidin (SAV-TC; Caltag Laboratories), FITC-conjugated rabbit-anti-rat IgG F(ab)'$_2$ fragments (RαRa-FITC; Cappel, Organon Teknika, Turnhout, Belgium), and R-phycoerythrin-conjugated goat-anti-rat IgG (mouse adsorbed; GαRa-PE; Caltag Laboratories), were used as second stage fluorescent reagents.

Preparation of cell suspensions

Bone marrow cell suspensions were prepared as described previously[6]. Briefly, femora and tibiae were ground, using a mortar, in Dutton's Balanced Salt Solution (Gibco, Breda, The Netherlands) supplemented with 5% fetal calf serum, 60 µg/ml penicillin and 100 µg/ml streptomycin (DBBS-FCS-PS). The cell suspension was aspirated through a 22-gauge needle and filtered over a nylon sieve (mesh size 100 µm; Polymon PES, Kabel, Amsterdam, The Netherlands) to remove connective tissue, bone fragments, and clumps of cells.

Cultured bone marrow cells were isolated from Teflon bags (see below) and washed with DBBS-FCS-PS prior to immunofluorescence labeling.

Immunofluorescence labeling, flow cytometric analysis and cell sorting

For phenotypic analyses, 1×10^6 - 5×10^6 freshly isolated bone marrow cells/well or 5×10^4 cultured cells/well were aliquotted into 96-microwell plates (round-bottom, Nunc, Denmark) and labeled with the appropriate antibodies in phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 20mM NaN_3 (PBS-BSA-NaN_3). All incubations were performed on ice for 30 min and were followed by three washes with PBS-BSA-NaN_3. For single-color analysis, cells were incubated first with hybridoma supernatant, washed and then incubated with RαRa-FITC, supplemented with 2% normal mouse serum (NMS) to avoid nonspecific binding. For two-color analysis, cells were incubated first with biotinylated monoclonal antibody, followed by FITC-labeled monoclonal antibody and SAV-PE simultaneously. For three-color analysis, cells were incubated first with hybridoma supernatant followed by GαRa-PE. After two washes, cells were washed in the presence of 3% normal rat serum to block free binding sites on GαRa-PE. Subsequently, cells were incubated with biotinylated monoclonal antibody followed by FITC-labeled monoclonal antibody and SAV-TC simultaneously. Percentages of positive cells obtained from three-color analysis were compared with those obtained from single stainings and were found to be identical (data not shown). Culture supernatant of the non-producing Y3 myeloma followed by RαRa-FITC (one-color analysis) or GαRa-PE (three-color analysis) was used as negative control, since, in our hands, control values obtained with Y3 supernatant are identical to those obtained with rat isotype control monoclonal antibodies (unpublished data). Phenotypic analyses were performed with a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA).

For cell sorting experiments, 2×10^8 bone marrow cells were incubated for 30 min with 2 ml DBSS-FCS-PS containing biotinylated ER-MP12, washed with a large volume of DBSS-FCS-PS and subsequently incubated (30 min) with 2 ml optimally diluted ER-MP20-FITC and SAV-PE simultaneously. After two washes, the cells were resuspended in PBS supplemented with BSA (0.5% w/v), D-glucose (0.45% w/v; Merck, Amsterdam, The Netherlands), penicillin (60 µg/ml) and streptomycin (100 µg/ml) to a final concentration of 3×10^6 cells/ml. Before sorting, the cell suspension was filtered over a 30 µm sieve (Polymon PES) to avoid clogging of the nozzle. All sorts were performed using a FACS Vantage cell sorter (Becton Dickinson). After sorting, viable cells were counted using a Bürker hemocytometer. The purity of the sorted cell populations was determined by FACScan analysis and normally exceeded 95%, unless stated otherwise.

M-CSF-stimulated bone marrow culture

The medium used for M-CSF-stimulated bone marrow culture (either in soft agar or in Teflon culture bags, see below) was α-modified DMEM (Gibco) supplemented with 20% L-cell conditioned medium (LCM) as a source of M-CSF, 20% FCS (heat inactivated; Hyclone Laboratories, UT), glutamine (2mM), penicillin (60 μg/ml), streptomycin (100 μg/ml), β-mercaptoethanol (10^{-4} M) and sodiumselenite (10^{-7} M). LCM was prepared as previously described[7]. The same LCM and FCS batches were used throughout the study.

To assess the frequency of macrophage precursors within the sorted subsets the macrophage colony- and cluster-forming capacity of the sorted cells was determined in M-CSF-stimulated culture in soft agar. Cloning was performed as described by Wijffels et al.[8] with minor modifications. Briefly, a 6% agar stock (Bacto-Agar, Difco Laboratories, Detroit, MI) was diluted with warm (42°C) culture medium. Cells were plated in 0.5 ml 0.3% agar-medium on top of a layer of 0.3 ml 0.5% agar-medium in 24 well plates (Costar, Cambridge, MA, USA). Per sorted bone marrow subset two different cell concentrations were plated in six wells/concentration: 1000 and 250 cells/well for the ER-MP12^{med}20$^-$, ER-MP12$^-$20$^-$, ER-MP12$^-$20^{med} subsets and unlabeled, unseparated bone marrow, and 250 and 100 cells/well for the ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} subsets. After 12 to 14 days of culture (37°C, 7% CO_2), the number of macrophage colonies ($\geq$ 50 cells) and clusters (< 50 cells) generated from each subset was assessed by examining the plates using an inverted light microscope at low magnification. LCM as a source of M-CSF specifically stimulates macrophage development, as only macrophage progeny could be detected using morphological and immunohistological analysis (data not shown).

For phenotypic analysis of *in vitro* matured mononuclear phagocytes, sorted cells were cultured in Teflon culture bags in M-CSF-containing medium[9]. Depending on the duration of culture, the initial cell number was adjusted to ensure optimal growth and viability. Thus, 2×10^4 cells were seeded in a volume of 2 ml medium for phenotypic analysis at day 2 of culture, and 10^4 cells, also in a volume of 2 ml, for analysis after 5 and 7 days of culture.

Morphological analysis

Differential morphological analysis of sorted bone marrow subsets was performed on May-Grünwald-Giemsa stained cytospin preparations. Per subset five hundred cells were analyzed.

Results

ER-MP12 and ER-MP20 monoclonal antibodies recognize morphologically distinct bone marrow subsets

Using the anti-macrophage precursor monoclonal antibodies ER-MP12 and ER-MP20, six phenotypically distinct subsets can be detected in the bone marrow of the mouse[6]. Two subsets express the ER-MP12 antigen but not the ER-MP20 antigen: an ER-MP12^{med}20$^-$

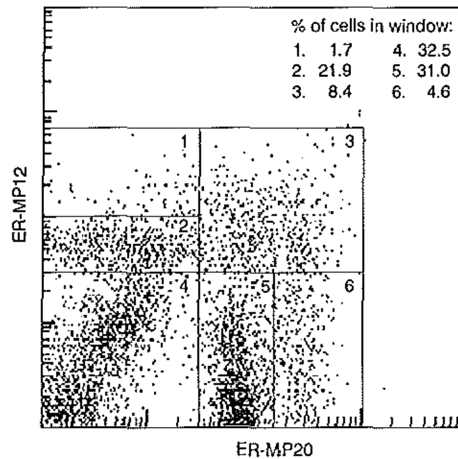

Figure 1. Two-color FACScan analysis of nucleated bone marrow cells labeled with ER-MP12 and ER-MP20 monoclonal antibodies. Percentages are the mean of eight experiments.

and an ER-MP12^{hi}20^{-} subset (Fig. 1). Similarly, two subsets exclusively express the ER-MP20 antigen: an ER-MP12^{-}20^{med} and an ER-MP12^{-}20^{hi} subset. Of the remaining two subsets, one expresses both ER-MP12 and ER-MP20 antigens, *i.e.* ER-MP12^{+}20^{+} (for this subset no distinction was made in levels of antigen expression), and one lacks both antigens, *i.e.* ER-MP12^{-}20^{-}. In this study we determined the cellular composition of the

Table 1 Morphological analysis of bone marrow subsets sorted on the basis of ER-MP58 expression

BM subset	Myeloid			Erythroid		Lymphoid	Meg.	Undiff. blasts
	Imm. prog.	Band + segm.	Monocytes	Erythro-blasts	Polychr. + norm.blasts			
ER-MP12^{hi}20^{-}	4[a)]	0	1	18	3	25	0	49
ER-MP12^{med}20^{-}	0	0	1	1	0	**87**	1	10
ER-MP12^{+}20^{+}	20	7	7	14	3	4	0	**45**
ER-MP12^{-}20^{-}	0	0	0	15	**76**	0	0	9
ER-MP12^{-}20^{med}	4	**91**	2	0	0	0	0	3
ER-MP12^{-}20^{hi}	19	0	**74**	1	0	0	0	6

The sorted bone marrow subsets were spun onto microscopic slides and stained with May-Grünwald-Giemsa. Per subset 500 nucleated cells were examined. [a)] Data represent the percentage of cells present in the ER-MP12/20 subsets. FACScan analysis of the sorted bone marrow subsets revealed that the purity of the sorted fractions varied between 87% for the ER-MP12^{+}20^{+} subset and >95% for the other subsets. In each subset the prevailing cell type is in bold. Imm. prog.: immature myeloid progenitors; band + segm.: band form and segmented granulocytes; polychr. + norm. blasts: polychromatophilic erythroblasts and normoblasts; meg.: megakaryocytic cells; undiff. blasts: morphologically undifferentiated blasts.

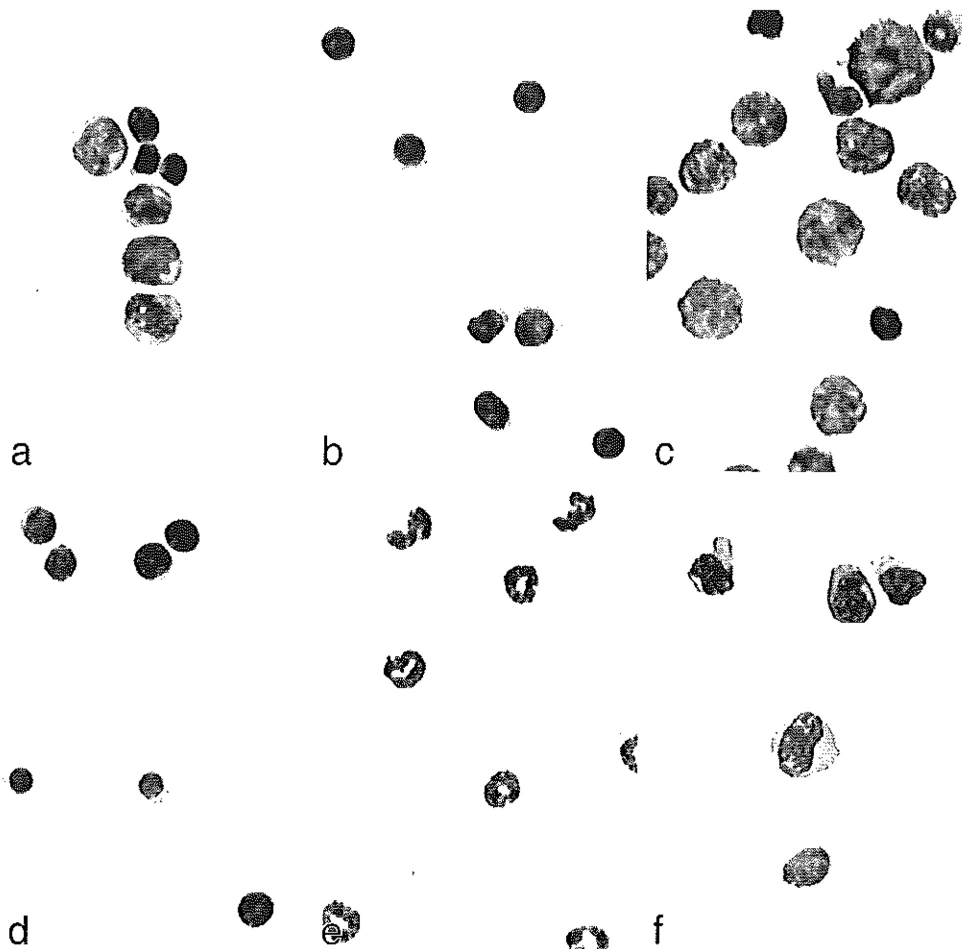

Figure 2. May-Grünwald/Giemsa stained cytocentrifuge preparations of bone marrow subsets sorted on the basis of ER-MP12 and ER-MP20 expression. (a) ER-MP12^{hi}20^{-} bone marrow subset; (b) ER-MP12^{med}20^{-} subset; (c) ER-MP12^{+}20^{+} subset; (d) ER-MP12^{-}20^{-} subset; (e) ER-MP12^{-}20^{med} subset; (f) ER-MP12^{-}20^{hi} subset. Magnification x 580.

six ER-MP12/20 bone marrow subsets. Therefore each subset was sorted, stained with May-Grünwald/Giemsa and differentially counted (Table 1 and Fig. 2). The smallest subset, ER-MP12^{hi}20^{-}, consisted predominantly of blast cells: morphologically undifferentiated blasts as well as recognizable blasts of the myeloid, erythroid and lymphoid lineages. The ER-MP12^{med}20^{-} subset was remarkably homogenous with predominantly mature lymphoid cells and a few undifferentiated blasts. The ER-MP12^{+}20^{+} subset contained a large proportion of morphologically undifferentiated blasts, together with recognizable precursors of the myeloid, erythroid and lymphoid lineages. The ER-MP12^{-}20^{-} subset consisted almost exclusively of erythroid cells, erythroblasts as well as more mature cells.

The ER-MP12^{-}20^{med} subset was highly enriched for granulocytes. Finally, the ER-MP12^{-}20^{hi} subset contained mainly monocytes and a few immature myeloid cells and undifferentiated blasts. Thus, on the basis of ER-MP12 and ER-MP20 antigen expression, mouse bone marrow can be separated into six morphologically distinct subsets with a relatively high cell type homogeneity.

M-CSF-responsive macrophage precursors have the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, or ER-MP12^{-}20^{hi} phenotype

The morphological analysis of the sorted subsets showed that putative macrophage precursors, i.e. morphologically undifferentiated blasts, immature myeloid cells and monocytes, are mainly present in the ER-MP12^{hi}20^{-}, ER-MP12^{med}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} subsets. To verify the presence of macrophage precursors in one or more of these subsets, and their absence from the other ER-MP12/20 subsets, all six bone marrow subsets were isolated by cell sorting and cloned in soft-agar in the presence of the macrophage-specific growth factor M-CSF. Only from the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} subsets could macrophage colonies and clusters be grown (Table 2). No macrophage colonies or clusters were obtained from the other three subsets.

In the ER-MP12^{hi}20^{-} subset one out of eight plated cells gave rise to primarily large macrophage colonies in response to M-CSF (Table 2), while in the ER-MP12^{+}20^{+} subset one out of nine plated cells gave rise to primarily clusters and small colonies. In the ER-MP12^{-}20^{hi} bone marrow subset, which consists predominantly of monocytes (cf. Table 1), an average of one out of two plated cells formed a small cluster of usually less than 5

Table 2 Presence of M-CSF-responsive macrophage precursors in bone marrow subsets sorted on the basis of ER-MP12 and ER-MP20 antigen expression

BM subset	No. of colonies/ 10^3 plated cells[a)]	No. of clusters/ 10^3 plated cells	Mean frequency of macrophage precursors within subset	Mean no. of macrophage precursors/ 10^4 NBMC (M-CFC/ M-clustFC)[b)]
ER-MP12^{hi}20^{-}	108 ± 9	12 ± 1	1:8	20 (18/2)
ER-MP12^{+}20^{+}	22 ± 8	94 ± 10	1:9	91 (17/74)
ER-MP12^{-}20^{hi}	0 ± 0	409 ± 95	1:2	188 (0/188)
Unfractionated BM[c)]	4 ± 1	13 ± 3	1:59	170 (36/134)

Macrophage colonies and clusters were determined after 12-14 days of M-CSF-stimulated culture in soft-agar. Colonies contain ≥ 50 cells; clusters < 50 cells. No macrophage colonies or clusters were obtained from ER-MP12^{med}20^{-}, ER-MP12^{-}20^{-}, and ER-MP12^{-}20^{med} bone marrow subsets. [a)]Data are the mean ± SD of three experiments. For each subset, two different concentrations of sorted cells were plated. Per concentration the number of colonies and clusters per 10^3 cells was calculated. The numbers shown in the table are the means of those calculated numbers. The numbers were corrected for the impurity of the sorted subsets; this was possible since contaminating cells were only from the subsets from which no macrophage colonies or clusters could be obtained. The means of the relative sizes of the bone marrow subsets in these three experiments were used to calculate the absolute numbers of macrophage precursors, and were 1.7% for the ER-MP12^{hi}20^{-} subset, 7.9% for the ER-MP12^{+}20^{+} subset and 4.6% for the ER-MP12^{-}20^{hi} subset. [b)] NBMC: nucleated bone marrow cells; M-CFC: macrophage colony-forming cell; M-clustFC: macrophage cluster-forming cell. [c)] BM: bone marrow.

cells, and occasionally a cluster of 15-20 cells. Taking into account the relative sizes of the sorted subsets in bone marrow, these data indicate that macrophage colony-forming cells reside in approximately equal numbers in both the ER-MP12^{hi}20$^-$ and ER-MP12$^+$20$^+$ subsets. Macrophage cluster-forming cells are mainly present in the ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} bone marrow subsets. Together, our data show that (i) M-CSF-responsive macrophage precursors reside in the ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} subsets, and (ii) the proliferative potential of the M-CSF-responsive cells, as indicated by the sizes of the colonies or clusters formed, decreases with a concomitant increase in ER-MP20 and decrease in ER-MP12 antigen expression.

The ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} bone marrow subsets differentially express the Mac-1 antigen

The differences in cellular composition and proliferative potential of the ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} bone marrow subsets suggested a difference in maturation stage between the macrophage precursors present in those subsets. To investigate this, we examined the expression of the macrophage maturation-related marker Mac-1[10,11] within the three subsets. As shown in Fig. 3, all ER-MP12^{hi}20$^-$ cells were Mac-1 negative/dull. In contrast, 40 ± 5% (n=3) of the ER-MP12$^+$20$^+$ bone marrow cells clearly expressed the Mac-1 antigen. This percentage correlates closely with the total content of myeloid cells in this subset as determined by the morphological analysis (Table 1). Finally, virtually all ER-MP12$^-$20^{hi} cells were Mac-1-positive. This observation was in accordance with the morphological data which showed that this subset almost exclusively contains monocytes and some immature myeloid precursors, which are all Mac-1-positive[10,12]. Thus, the increase in the proportion of Mac-1 positive cells and level of antigen expression observed from the ER-MP12^{hi}20$^-$ to the ER-MP12$^-$20^{hi} subset supports an increase in maturity of the macrophage precursors present in those subsets.

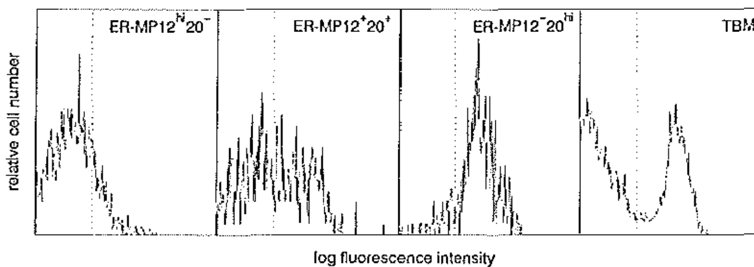

Figure 3. Cell surface expression of Mac-1 antigen by ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} nucleated bone marrow cells. Cells were triple-labeled as described in materials and methods with M1/70 (Mac-1), ER-MP12 and ER-MP20 monoclonal antibodies. Mac-1 expression within the ER-MP12/20 subsets was determined by flow cytometric analysis. The staining profile of total bone marrow (TBM) is also given. The dotted lines represent negative control values. Results are from one representative experiment out of three.

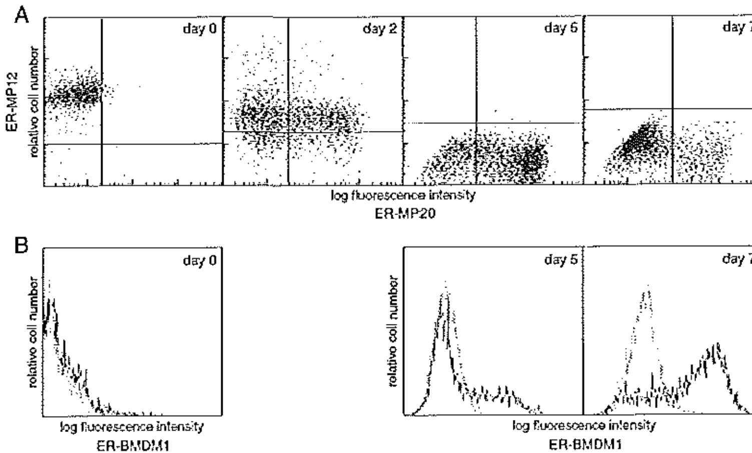

Figure 4. Phenotypic development of ER-MP12^{hi}20^{-} bone marrow cells cultured in the presence of M-CSF. ER-MP12^{hi}20^{-} bone marrow cells were sorted and cultured in the presence of M-CSF-containing conditioned medium. At day 2, 5, and 7 of culture the cells were collected and the expression of ER-MP12 and ER-MP20 antigens was determined in two-color flow cytometric analysis (A). Negative control limits are shown in each dot plot (vertical and horizontal lines). In addition, the expression of the macrophage maturation marker ER-BMDM1 was determined in one-color analysis (B). Negative controls (dotted lines) are shown in each histogram. Results are from one representative experiment out of two.

ER-MP12^{hi}20^{-} macrophage precursors successively express the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} phenotype during M-CSF-stimulated maturation in vitro

So far, the data obtained in this study suggest that the three macrophage precursor subsets represent successive stages in a linear maturation pathway, implying that the progeny of the putatively least mature subset, i.e. ER-MP12^{hi}20^{-}, should pass through the other two phenotypes upon macrophage maturation. To test this hypothesis directly, we sorted ER-MP12^{hi}20^{-} bone marrow cells and cultured the isolated cells in M-CSF-containing conditioned medium. After varying periods of culture the level of ER-MP12 and ER-MP20 antigen expression was determined. As a marker for mature macrophages, the expression of the ER-BMDM1 antigen was also examined. This latter antigen is expressed at increasing levels upon maturation from the monocytic stage onwards[5].

At day 2 of M-CSF-stimulated culture of ER-MP12^{hi}20^{-} cells, about one third of the developing cells expressed both the ER-MP12 and the ER-MP20 antigens (Fig. 4A), suggesting that many, if not all, mononuclear phagocytes indeed pass through the ER-MP12^{+}20^{+} stage upon *in vitro* maturation. The remaining ER-MP12^{+}20^{-} cells probably represented M-CSF-unresponsive cells from other hematopoietic lineages still present at this time of culture. At day 5 of culture, the cells had completely lost ER-MP12 antigen expression (Fig. 4A). Yet, 50-59% (n=2) of the cells expressed the ER-MP20 antigen at a high level, similar to that of ER-MP12^{-}20^{hi} monocytes in freshly isolated bone marrow (cf. Fig. 1). The ER-MP20 negative cells present after 5 days of culture represented mononuclear phagocytes beyond the monocytic stage of development as (i) at this day of culture 26-34% of the cells expressed the mature macrophage marker ER-BMDM1 (Fig.

4B) and (ii) the ER-MP20 negative cells showed a high level of autofluorescence (Fig. 4A, day 5 and 7) which is characteristic of more mature mononuclear phagocytes (ref. 11 and unpublished observations). Upon final maturation into mature macrophages (day 7) the expression of the ER-MP20 antigen was lost (Fig. 4A) and the large majority of the cells (66-75%) had become ER-BMDM1 positive (Fig. 4B).

In summary, these findings show that ER-MP12^{hi}20^{-} macrophage precursors successively express the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} phenotypes during M-CSF-stimulated maturation *in vitro*. Under these conditions ER-MP12^{-}20^{-} cells represent the final stage of macrophage development. The latter cells, however, are mature macrophages and do not represent the ER-MP12^{-}20^{-} subset found in normal mouse bone marrow.

Discussion

In the present study we focused on the identification of different subpopulations of macrophage precursors in the bone marrow of the mouse. To this end, the reactivity of the anti-macrophage precursor monoclonal antibodies ER-MP12 and ER-MP20 with bone marrow macrophage precursors was assessed in two-color flow cytometry. At least six phenotypically distinct bone marrow subsets can be discerned using these monoclonal antibodies. However, only three of these subsets, *i.e.* ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi}, gave rise to macrophage progeny after M-CSF-stimulated culture. Together these subsets comprise about 15% of nucleated bone marrow cells.

All data presented in this study support the notion that these precursors reflect different, successive stages of macrophage development in the murine bone marrow. The first indication for the existence of a maturational difference came from morphological analysis of the sorted subsets. The differential counts showed that potential macrophage precursor cell types, *i.e.* morphologically undifferentiated blasts, immature myeloid cells, and monocytes, were not evenly distributed among the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} subsets. Morphologically undifferentiated blasts were concentrated in the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{+} subsets, immature myeloid cells in the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} subsets, whereas monocytes were concentrated in the ER-MP12^{-}20^{hi} subset, thus suggesting differences in maturation stage among these subsets. A second indication was obtained from the differences in macrophage colony/cluster-forming capacities of the subsets. ER-MP12^{hi}20^{-} cells formed the largest colonies and therefore are presumably the most immature cells. ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} cells formed predominantly large and small clusters, respectively, and thus, most likely represent subsequent stages. In the third place, the expression of the Mac-1 antigen, which is expressed relatively late during macrophage maturation[10,11], follows the proposed maturation sequence of the precursor subsets. The Mac-1 antigen is not expressed in the ER-MP12^{hi}20^{-} subset, while about half of the ER-MP12^{+}20^{+} subset and all cells in the ER-MP12^{-}20^{hi} subset are Mac-1 positive. Finally, the most direct indication for the existence of a maturation sequence came from the phenotypic development of the, putatively youngest, ER-MP12^{hi}20^{-} precursors during M-CSF-stimulated culture. We found that the developing cells successively passed through ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} stages before final maturation into mature macrophages. Thus, the morphological data, the clonogenic data, the expression of Mac-1, and the pheno-

typic development during culture all indicate that the three phenotypically distinct M-CSF-responsive bone marrow macrophage precursor subsets most likely represent successive stages along a maturation pathway in the order ER-MP12^{hi}20^{-} → ER-MP12^{+}20^{+} → ER-MP12^{-}20^{hi}.

Interestingly, macrophage colonies could be generated from both the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{+} bone marrow subsets. Expressed in absolute numbers, about half of all bone marrow macrophage colony-forming cells resided in the ER-MP12^{hi}20^{-} subset and the other half in the ER-MP12^{+}20^{+} subset. MacVittie reported for C57Bl/6J mice an M-CFC frequency in bone marrow of approximately twice the frequency of GM-CFC[13]. Thus, of all macrophage colonies formed in their study, about two thirds was derived from an M-CFC and one third from a GM-CFC. Combining these data with our observations on ER-MP12/20 expression by macrophage colony-forming cells, we speculate that the ER-MP12^{hi}20^{-} macrophage precursors, which form the largest colonies, represent the more immature GM-CFC and part of the M-CFC, while the colony-forming macrophage precursors in the ER-MP12^{+}20^{+} subset might represent the majority of the M-CFC.

Our data showed that the earliest M-CFC in the bone marrow are ER-MP12^{hi}20^{-}. Recently splenic M-CFC were found to express the ER-MP20 antigen at a high level and thus differ from bone marrow M-CFC, which are ER-MP20^{-} or ER-MP20^{dim} [14]. It is unlikely that this difference in ER-MP20 expression reflects a maturational difference since the splenic ER-MP20^{hi} cells are, like the bone marrow ER-MP20^{-} and ER-MP20^{dim} cells, able to form large macrophage colonies in culture. Therefore, it will be interesting to study the ER-MP12 antigen expression of splenic M-CFC, as, in bone marrow, this is clearly related to colony forming capacity.

Both the differential counts and the macrophage precursor frequencies (cf. Tables 1 and 2) of the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{+} subsets indicate that these fractions do not solely contain precursors of the macrophage lineage. Morphological analysis of the subsets showed that precursors of the erythroid, lymphoid and granulocytic lineages are present in these subsets. In accordance with this observation, we recently reported that ER-MP12^{hi}20^{-} bone marrow cells gave rise to both myeloid cells as well as T and B cells upon intravenous (i.v.) transfer into irradiated recipients[15]. Thus, although the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{+} bone marrow subsets are both highly enriched for macrophage precursors, additional cell surface markers are required to separate early macrophage precursors from other hematopoietic progenitors.

A remarkable finding was that no macrophage colonies or clusters could be grown from ER-MP12^{med}20^{-} bone marrow cells in M-CSF-stimulated culture, although we recently reported that ER-MP12^{med}20^{-} bone marrow cells, upon i.v. transfer into irradiated recipients, were capable of both myeloid, and T and B cell repopulation[15]. This apparent contradiction can be explained by the presence of more immature progenitors and multipotent stem cells within the ER-MP12^{med}20^{-} subset[16]. Such cells do not yet respond in culture to M-CSF alone. *In vivo*, however, the cells most likely meet the appropriate microenvironments and will eventually become sensitive to M-CSF and form mature macrophage progeny.

To our knowledge, ER-MP12 and ER-MP20 are the first set of monoclonal antibodies described which positively identify discrete, successive macrophage precursor stages in the

bone marrow of the mouse. Furthermore, as the ER-MP12^{hi}20^{-} bone marrow subset was found to contain macrophage precursors as well as precursors of other lineages, it may be possible, using additional markers, to identify early branch points in hematopoiesis.

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4 ER-MP12 antigen, a new cell surface marker on mouse bone marrow cells with thymus-repopulating ability: II. Thymus-homing ability and phenotypic characterization of ER-MP12-positive bone marrow cells

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Abstract

Previously, we showed that six distinct subsets of bone marrow cells can by identified using the monoclonal antibodies ER-MP12 and ER-MP20 in two-color immunofluorescence analysis. Upon *intrathymic* transfer into sublethally irradiated mice, thymus-repopulating ability was restricted to ER-MP20$^-$ bone marrow cells expressing either high or intermediate levels of the ER-MP12 antigen (1-2% and ~30% of bone marrow nucleated cells, respectively). The highest frequency of thymus-repopulating cells was found in the minor subset of ER-MP12^{hi}20$^-$ bone marrow cells. In the present study we demonstrate that upon *intravenous* transfer, thymus-homing and -repopulating bone marrow cells are exclusively confined to the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ bone marrow subpopulations, the highest frequency being detected among ER-MP12^{hi}20$^-$ bone marrow cells. Analysis of the peripheral blood leukocytes of reconstituted mice showed that not only prothymocytes but also progenitor cells of the B cell lineage as well as the myeloid lineage were present within both subsets. Three-color flow cytometric analysis revealed that ER-MP12^{hi}20$^-$ bone marrow cells in particular were phenotypically heterogeneous with respect to the expression of the cell surface markers Thy-1, Sca-1, CD44, B220, and c-*kit*. Taken together our data demonstrate that ER-MP12 positively identifies bone marrow cells with the ability to home to and repopulate the thymus. The phenotypic heterogeneity displayed by the ER-MP12^{hi}20$^-$ bone marrow subset, containing the highest frequency of thymus-homing and -repopulating cells, provides a basis for further separation of prothymocyte activity from other hematopoietic activities in the bone marrow of the mouse.

Introduction

The thymus is the major site for T cell development. In this organ, complex and poorly understood selection processes, likely directed by an intricate network of heterogeneous stromal cell populations, shape the TCR specificity repertoire of the maturing thymocytes[1-6]. The mature, selected thymocytes descend from a minute population of intrathymic precursor cells[1,7,8] which undergo extensive proliferation and differentiation in the subcapsular zone of the thymic cortex. In adult life this intrathymic pool of T cell precursors is maintained by bone marrow-derived progenitor cells (prothymocytes)[9-11] which seed the thymus continuously at a low rate[12].

So far, the characterization of prothymocytes is being hampered by a low frequency of these cells in the bone marrow, the absence of distinguishing morphological features and, above all, the paucity of monoclonal antibodies detecting cell surface antigens specific for T cell progenitors. Hence, the phenotype of thymus-repopulating cells is still poorly characterized. It is not clear as yet whether these progenitor cells are either multipotent stem cells, lymphoid lineage-restricted progenitor cells or progenitor cells exclusively committed to the T cell lineage.

In the previous paper[13], we showed that six distinct subpopulations of bone marrow cells could be identified using the recently developed monoclonal antibodies ER-MP12 and ER-MP20 [14] in two-color immunofluorescence analysis. As assessed by *intrathymic* transfer thymus-repopulating ability appeared to be restricted to ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells. The highest frequency of thymus-repopulating cells was found in the subpopulation of ER-MP12^{hi}20^{-} bone marrow cells.

In the present study we transferred the six bone marrow subpopulations isolated on the basis of different levels of ER-MP12 and ER-MP20 antigen expression *intravenously* into sublethally irradiated Ly-5/Thy-1 congenic mice in order to determine whether the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets have the capacity to home to the thymus *in vivo* and thus contain the genuine prothymocytes. Our data indicate that bone marrow cells which express the ER-MP12 antigen but not the ER-MP20 antigen indeed meet these physiological criteria of prothymocytes. The highest frequency of thymus-repopulating cells was detected in the ER-MP12^{hi}20^{-} bone marrow subpopulation. Furthermore, phenotypic analysis of peripheral blood leukocytes of reconstituted mice revealed that not only thymus-repopulating cells but also progenitor cells of the B cell lineage and the myeloid lineage were present among ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells. Finally, three-color flow cytometric analysis revealed that ER-MP12^{hi}20^{-} bone marrow cells particularly were heterogeneous with respect to the expression of the cell surface antigens Thy-1, Sca-1, CD44, B220 and c-*kit*, indicating that this subpopulation can be further divided into smaller subsets. This phenotypic heterogeneity provides a basis for further attempts to separate prothymocytes from progenitor cells of other hematopoietic lineages.

Materials and Methods

Mice

C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2, Ly-5.1) mice (kindly provided by Dr I. L. Weissman, Stanford University, Stanford, CA), C57BL/Ka Bl-1 (Thy-1.1, Ly-5.2), CBA/Rij x C57BL/Ka [BCBA(F1)], BALB/c, B10.D2/n, BALB.k, and SJL/J mice were cesarean derived, foster reared, raised and maintained under clean conventional conditions with free access to food and water (acidified to pH 2.8) in the mouse facilities of the Department of Immunology.

Monoclonal antibodies and fluorescent reagents

The monoclonal antibodies used in the present study are listed in table 1. ACK2 (anti-c-*kit*) and A20-1.7 (anti-Ly5.1) were kindly provided by Dr S.-I. Nishikawa, Kumamoto University Medical School, Kumamoto, Japan and Dr S. Kimura, Sloan-Kettering Cancer Center, New York, NY, respectively. Monoclonal antibodies were used either (partially) purified, conjugated to biotin or FITC (Sigma, St. Louis, MO) by standard procedures or as culture supernatant. In some instances anti-Thy-1.2 monoclonal antibody conjugated to 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS, Boehringer Mannheim, Germany) according to the manufacturer's procedure was used. Goat anti-rat Ig absorbed with mouse Ig and conjugated to phycoerythrin (GαRa-PE) (Caltag Laboratories, San Franscisco, CA), rabbit anti-rat(Fab)'$_2$ fragments coupled to FITC (RαRa-FITC) (Cappel, Organon Teknika, Turnhout, Belgium), streptavidin-phycoerythrin (SAV-PE) (Caltag Laboratories), and streptavidin-Tri-Color (SAV-TC) (Caltag Laboratories) were used as second stage fluorescent reagents.

Table 1 Monoclonal antibodies

Monoclonal antibody	Antigen	Reference
ER-MP12	ER-MP12	14
ER-MP20	ER-MP20	14
59-AD2.2	Thy-1	15
H129.19	CD4	16
53-6.72	CD8	15
KT3	CD3	17
RA3 6B2	B220	18
RB6 8C5	Gr-1	19
M1/70	Mac-1	20
IM7.8.1	CD44	21
ACK2	c-*kit*	22
E13 161-7	Sca-1	23
30H12	Thy-1.2	15
A20-1.7	Ly-5.1	Dr. S. Kimura

Preparation of cell suspensions

Suspensions of bone marrow cells and thymuses were prepared as described[13] using Dutton's balanced salt solution (Gibco, Breda, The Netherlands) supplemented with 5% FCS (DBSS-FCS). Peripheral blood was obtained by heart puncture and collected in tubes containing heparin-coated glass beads. Erythrocytes were depleted by hypotonic lysis using a 17 mM Tris buffer (pH 7.2) supplemented with 0.144 M ammonium chloride (4 volumes buffer per 1 volume blood). After 10 min at 4°C, the cells were washed 3 times with PBS.

Immunofluorescence staining

Two-color immunofluorescence staining using conjugated monoclonal antibodies was performed as described before[13]. PBS supplemented with 0.5% BSA and 20 mM NaN_3 (PBS-BSA-NaN_3) was used for washing as well as diluting the reagents to optimal concentrations. When fluorescein-conjugated second stage anti-rat Ig were used in two- or three-color stainings the cells were first incubated with hybridoma culture supernatant, washed and subsequently incubated with RαRa-FITC (two-color stainings) or GαRa-PE (three-color stainings) supplemented with 2% normal mouse serum to avoid nonspecific binding. After two washes the cells were incubated and washed in the presence of 2% normal rat serum to block any free anti-rat Ig binding sites of the conjugate. Next the cells were incubated with biotin-conjugated monoclonal antibody, washed and finally incubated with SAV-PE (two-color stainings) or SAV-TC and FITC-conjugated monoclonal antibody (three-color stainings). For cell sorting experiments bone marrow cells were stained as previously described[13].

Flow cytometric analysis and cell sorting

Cell surface fluorescence was analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Erythrocytes and dead cells were excluded from analysis by electronic gating on the basis of light scatter characteristics. Cell sorting was performed using a FACS 440 (Becton Dickinson) as described previously[13].

Cell transfer and analysis of repopulation

C57Bl/Ka Bl-1 recipient mice (aged 10-15 weeks) were exposed to 7.5 Gy (unless otherwise stated) of γ-irradiation using two opposing ^{137}Cs sources (Gammacell 40 irradiator, Atomic Energy of Canada Ltd, Ottawa, Canada) at a dose rate of 1.01 Gy/min. Two hours after irradiation different numbers of unseparated or sorted congenic bone marrow cells were injected i.v. into mice via their tail veins. Three weeks after i.v. transfer the recipient mice were killed. Thymocytes were stained with directly conjugated anti-Thy-1.2 monoclonal antibody to detect donor-derived thymocytes. In one experiment recipient mice were irradiated at 8.0 Gy and reconstituted; after 4 weeks they were killed and their peripheral blood was analyzed for the presence of donor-derived T and B lymphocytes and myeloid cells. Peripheral blood leukocytes were dually labeled with either anti-B220 (B cells), anti-CD3 (T cells), or anti-Mac-1 (myeloid cells) (followed by RαRa-FITC as a second stage reagent) and donor-specific biotinylated anti-Ly-5.1 followed by SAV-PE. Cell surface fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson).

Cell suspensions with ≥ 1% donor-derived cells in thymus or peripheral blood were scored as positive for donor-type repopulation.

Results

Thymus-homing and -repopulating ability of bone marrow subpopulations sorted on the basis of a different expression of the ER-MP12 and ER-MP20 antigens

In the accompanying paper[13] we showed that bone marrow cells could be separated into six subpopulations on the basis of difference in ER-MP12 and ER-MP20 antigen expression. Using the *intrathymic* transfer assay thymus-repopulating ability appeared to be exclusively confined to ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ bone marrow cells, with the highest frequency of thymus-repopulating cells being found in the former subset (constituting 1-2% of total nucleated bone marrow cells).

In the present study we assessed the thymus-homing ability of the six bone marrow subpopulations after *intravenous* transfer in order to investigate whether functional prothymocyte activity is confined to the ER-MP12^{hi}20$^-$ and ER-12^{med}20$^-$ subsets or maybe one of these. The six bone marrow subpopulations, each characterized by a different expression of the ER-MP12 and ER-MP20 antigens, were isolated by fluorescence-activated cell sorting and transferred i.v. into sublethally irradiated Ly-5/Thy-1 congenic mice. Twenty-one days after cell transfer the thymuses of the recipient mice were analyzed for the presence of donor-derived (i.e. Thy-1.2$^+$) thymocytes (Table 2). As a control the thymus-repopulating ability of unseparated bone marrow cells was determined.

In nine out of 39 mice (23%) reconstituted with total bone marrow cells donor-derived thymocytes were detected after i.v. injection of 25,000 cells. In recipient mice injected i.v. with 25,000 separated bone marrow cells thymus-homing and -repopulating cells appeared to be restricted to the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations. In experiments 3 and 4, thymus-repopulating cells were only detected in the bone marrow subpopulation with a high level of ER-MP12 antigen expression, while in the second experiment, only a very low percentage (3.5% ± 1.6%) of donor-derived thymocytes was detected in eight out of 15 mice reconstituted with ER-MP12^{med}20$^-$ bone marrow cells. Re-analysis of the sorted bone marrow fractions revealed the same degree of purity (>96%) for the ER-MP12^{med}20$^-$ subset in each experiment (data not shown), indicating that the differences between the individual experiments cannot be explained by the presence of contaminating ER-MP12^{hi}20$^-$ cells.

Results were more clear-cut when only 5000 cells were injected i.v. Under these conditions thymus-repopulating ability was exclusively confined to ER-MP12^{hi}20$^-$ bone marrow cells (Table 2). These data demonstrate that: (i) bone marrow cells expressing the ER-MP12 antigen but not the ER-MP20 antigen are able to home to and repopulate the thymus of sublethally irradiated mice after i.v. transfer; (ii) the ER-MP12^{hi}20$^-$ bone marrow subset contains the highest frequency of thymus-homing and -repopulating bone marrow cells.

Table 2 Thymus-homing and -repopulating ability of sorted bone marrow cell subpopulations

Cell source	% of BM cells[a)]	Number of cells transfered	Exp. number	Positive mice/total mice[b)]	% Donor-derived thymocytes[c)]
total BM		25 x 10^3		9/39	29.6 ± 7.8
		5 x 10^3		0/10	
ER-MP12^{med}20$^-$	30.7 ± 4.9	25 x 10^3	1	4/10	45.3 ± 6.1
			2	8/15	3.5 ± 1.6
			3	0/9	
			4	0/14	
		5 x 10^3	3	0/10	
			4	0/10	
ER-MP12^{hi}20$^-$	2.1 ± 0.4	25 x 10^3	1	5/7	24.0 ± 10.2
			2	5/5	44.5 ± 15.7
			3	5/6	36.2 ± 11.6
		5 x 10^3	3	4/6	25.4 ± 11.5
			4	10/15	33.2 ± 8.3
ER-MP12$^+$20$^+$	9.2 ± 3.1	25 x 10^3		1/9	2.4
ER-MP12$^-$20^{med}	26.8 ± 6.6	25 x 10^3		0/18	
ER-MP12$^-$20^{hi}	4.8 ± 1.7	25 x 10^3		0/14	
ER-MP12$^-$20$^-$	28.6 ± 5.6	25 x 10^3		0/14	

C57BL/Ka Bl-1 (Ly-5.2, Thy-1.1) recipient mice were irradiated 7.5 Gy and injected i.v. with donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Thymus reconstitution was analyzed 21 days after cell transfer. [a)] The relative distribution of the subpopulation among nucleated bone marrow cells (mean percentage ± SEM of 14 experiments). [b)] Thymus suspensions with ≥ 1% donor-derived thymocytes were scored as positive. [c)] The mean percentage of donor-derived (Thy-1.2$^+$) cells ± SEM was calculated from four to 10 mice.

Multi-lineage reconstitution potential of ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ bone marrow cells

To assess the developmental potential of ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ bone marrow cells more extensively we i.v. transferred sorted ER-MP12^{hi}20$^-$ or ER-MP12^{med}20$^-$ bone marrow cells, or unseparated bone marrow cells into 8 Gy irradiated Ly-5/Thy-1 congenic recipient mice. Four weeks later peripheral blood nucleated cells were analyzed for the presence of donor-derived (i.e. Ly-5.1$^+$) cells. In each sample the percentage of T lymphocytes (CD3$^+$ cells), B lymphocytes (B220$^+$ cells), and myeloid (Mac-1$^+$) cells of donor origin was determined. These results are shown in Table 3. Donor-derived cells were detected in all recipient mice. The average percentage of Ly-5.1$^+$ peripheral blood nucleated cells appeared to be maximal after i.v. transfer of ER-MP12^{hi}20$^-$ bone marrow cells (e.g. 31.0% versus 13.5% after transfer of 5000 ER-MP12^{med}20$^-$ cells). The highest percentage of donor-derived T lymphocytes in peripheral blood was detected after transfer

of ER-MP12^{hi}20^{-} bone marrow cells (e.g. 6.2% versus 1.4% after transfer of 5000 ER-MP12^{med}20^{-} cells).

At this time point (i.e. 28 days after i.v. transfer) the percentage of donor-derived peripheral T cells was still low. Yet high percentages of donor-derived (Thy-1.2^{+}) *thymocytes* were detected after transfer of ER-MP12^{hi}20^{-} cells as well as ER-MP12^{med}20^{-} cells (Table 3). The considerable percentage of donor-derived thymocytes in mice reconstituted with 5000 ER-MP12^{med}20^{-} cells is in sharp contrast with the absence of donor-derived thymocytes at 21 days after transfer of these cells (Table 2). This apparent discrepancy may be explained by the presence of multipotent hematopoietic stem cells within the ER-MP12^{med}20^{-} subset. These cells most likely need additional maturational events in the bone marrow and may therefore need more time to mature into thymocytes (i.e. 28 days instead of 21 days).

Donor-derived cells belonging to the B cell lineage and myeloid lineage were also found after i.v. transfer of either subset (Table 3). The highest percentage of both donor-derived B lymphocytes and myeloid cells was found after transfer of ER-MP12^{hi}20^{-} bone marrow cells. Taken together our results show that both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subsets contain not only prothymocytes but also progenitor cells capable of differentiating into B lymphocytes and myeloid cells.

Cell surface phenotype of ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells

The cell surface phenotype of both bone marrow subsets was examined using three-color immunofluorescence analysis in order to investigate to what extent ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells are phenotypically heterogeneous and resemble previously described bone marrow subpopulations enriched for hematopoietic stem cells and progenitor activity[24-26]. The staining profiles of ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subpopulations with a panel of monoclonal antibodies against different cell surface antigens is shown in Fig. 1A and B respectively. For comparison the profiles of ungated nucleated bone marrow cells are shown (Fig. 1C).

We analyzed the expression of the mature lineage markers Gr-1, Mac-1, CD4, CD8 and B220 by ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells in order to compare the current progenitor fractions with those isolated by others using depletion of bone marrow cells for the mentioned mature markers[24,25,27,28]. Compared to unseparated bone marrow cells (Fig. 1C) both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subpopulations were depleted of cells that strongly expressed Gr-1 and Mac-1 (Fig. 1A and B, respectively). Lymphoid cells expressing high levels of CD4 and CD8, most likely mature T lymphocytes, were only detected in the ER-MP12^{med}20^{-} subset (Fig. 1B). In both subpopulations cells were detected which stained dimly for CD4. In this context it is noteworthy that low levels of CD4 were recently detected on pluripotent stem cells[29,30] and on the earliest subset of thymocytes detectable[7,8]. About 90% of the ER-MP12^{med}20^{-} bone marrow cells expressed B220 (Fig. 1B), suggesting that the majority of the cells in the ER-MP12^{med}20^{-} bone marrow subset belong to the B cell lineage. In contrast, ER-MP12^{hi}20^{-} cells differed strikingly from the ER-MP12^{med}20^{-} subset in that about 50% lacked the expression of B220 (Fig. 1A). Analysis of the forward light scatter against the fluorescence distribution (Fig. 2A) revealed that the B220^{-} cells in the ER-MP12^{hi}20^{-} subset were mainly blast-like

Table 3 Thymus and peripheral blood chimerism after i.v. reconstitution with sorted ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subpopulations

Cell source	Cell number (*n*)[a]	% donor-derived thymocytes (Thy-1.2^{+})[b]	% donor-derived cells (Ly-5.1^{+})[d]
ER-MP12^{hi}20^{-}	10 x 10^3 (7)	89.5 ± 1.9	66.5 ± 6.4
	5 x 10^3 (7)	39.9 ± 12.0	31.0 ± 8.0
ER-MP12^{med}20^{-}	25 x 10^3 (9)	72.3 ± 6.4	51.5 ± 6.8
	10 x 10^3 (6)	61.8 ± 10.3	34.7 ± 10.5
	5 x 10^3 (4)	24.9 ± 13.8	13.5 ± 4.8
total BM	25 x 10^3 (6)	49.9 ± 15.7	23.1 ± 6.8
	10 x 10^3 (2)	35.9	6.7

C57BL/Ka Bl-1 (Ly-5.2, Thy-1.1) recipient mice were irradiated 8.0 Gy and reconstituted i.v. with donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Thymus and peripheral blood chimerism was analyzed 28 days after cell transfer. [a] *n*: Number of mice analyzed. [b] The average percentage ± SEM of donor-derived thymo-

cells. Interestingly the ER-MP12^{hi}20^{-} bone marrow cells expressing the highest level of B220 antigen appeared to be large blast-like cells while ER-MP12^{hi}20^{-} cells with an intermediate level of B220 expression showed a scatter profile characteristic for lymphoid cells (Fig. 2A).

Both subsets contained cells that expressed low levels of Thy-1 as well as cells lacking detectable levels of this cell surface marker (Fig. 1A and B). Low levels of the Thy-1 antigen have been detected on bone marrow cells with thymus-repopulating ability and other hematopoietic progenitors and stem cells[24,31-33]. A small percentage of ER-MP12^{hi}20^{-} bone marrow cells (2.5-7%) expressed high levels of the Thy-1 antigen (Fig. 1A). Analysis of the forward light scatter against the fluorescence distribution revealed that the majority of the Thy-1^{hi} ER-MP12^{hi}20^{-} cells were large blast-like cells (Fig. 2B), suggesting that these cells are probably not T lymphocytes. In contrast, the majority of the Thy-1^{hi} cells in the ER-MP12^{med}20^{-} bone marrow subset (Fig. 1B) appeared to be small lymphoid cells (data not shown), most likely representing mature T cells.

It has been shown previously that thymus-repopulating ability is exclusively confined to bone marrow cells expressing Sca-1 (Ly-6A/E)[25]. Our data demonstrate that about 30% of both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells expressed Sca-1 (Fig. 1A and B, respectively) compared to about 10% of ungated bone marrow cells (Fig. 1C). The only other cells in bone marrow that expressed this cell surface marker were found in the ER-MP12^{+}20^{+} subset (data not shown). Thus, in accordance with the thymus-repopulating activity the expression of Sca-1 appeared to be restricted to bone marrow cells expressing the ER-MP12 antigen.

ER-MP12^{hi}20^{-} cells differed markedly from ER-MP12^{med}20^{-} cells in the expression of c-*kit*, the receptor for stem cell factor[34,35], which has been reported on hematopoietic

Table 3 Thymus and peripheral blood chimerism after i.v. reconstitution with sorted ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subpopulations (continued)

Peripheral blood		
% T cells (CD3^{+}) of donor origin (Ly-5.1^{+})$^{d)}$	% B cells (B220^{+}) of donor origin (Ly-5.1^{+})$^{d)}$	% myeloid cells (Mac-1^{+}) of donor origin (Ly-5.1^{+})$^{d)}$
16.4 ± 3.3	84.9 ± 3.0	83.6 ± 8.3
6.2 ± 1.8	76.5 ± 3.3	44.0 ± 15.3
5.9 ± 0.8	74.4 ± 3.2	74.1 ± 6.4
5.3 ± 1.7	52.7 ± 9.2	55.9 ± 13.7
1.4 ± 0.4	42.5 ± 2.6	23.0 ± 12.3
4.1 ± 1.6	48.6 ± 9.4	33.5 ± 10.4
1.2	23.0	8.6

cytes (Thy-1.2^{+}). $^{c)}$ The average percentage ± SEM of donor-derived (Ly-5.1^{+}) peripheral blood nucleated cells. $^{d)}$ The average percentage ± SEM of either peripheral blood T cells (CD3^{+}), B cells (B220^{+}), and myeloid cells (Mac-1^{+}) which were of donor origin (Ly-5.1^{+}).

stem cells and progenitor cells[26,36,37]. Two percent of the cells in ungated bone marrow and in the ER-MP12^{med}20^{-} subpopulation expressed high levels of c-*kit* (Fig. 1C and B, respectively). In contrast, 35-40% of the ER-MP12^{hi}20^{-} cells, mainly blast-like cells (data not shown), expressed this cell surface marker at high levels (Fig. 1A), suggesting a high frequency of progenitor cells in this latter subpopulation.

CD44 is differentially expressed by most hematopoietic cells[38]. Multipotent stem cells and progenitor cells, including thymus-repopulating cells, express intermediate levels of this surface marker[21,25,38] while the earliest intrathymic T cell precursors express high levels[7]. Our results show that all ER-MP12^{med}20^{-} bone marrow cells expressed intermediate levels of this antigen (Fig. 1B). Analysis of the ER-MP12^{hi}20^{-} subpopulation revealed cells expressing intermediate levels as well as high levels (more than 50%) of CD44 (Fig. 1A).

Taken together our data demonstrate that: (i) selection against ER-MP20 and for ER-MP12 antigen expression results in the depletion of bone marrow cells expressing high levels of the myeloid lineage differentiation markers Gr-1 and Mac-1; (ii) both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} cells are heterogeneous with respect to the expression of several cell surface markers; (iii) ER-MP12^{hi}20^{-} cells are phenotypically more heterogeneous than ER-MP12^{med}20^{-} bone marrow cells.

Expression of ER-MP12 and ER-MP20 antigens by different mouse strains

The potential usefulness of the ER-MP12 and ER-MP20 antigens for the enrichment of bone marrow cells with thymus-repopulating ability depends, partly, upon the expression of these cell surface markers by a wide variety of mouse strains. Therefore we examined the expression of the ER-MP12 and ER-MP20 antigens by bone marrow cells from mice of different haplotypes (Table 4). Our results show that both antigens are at least expressed

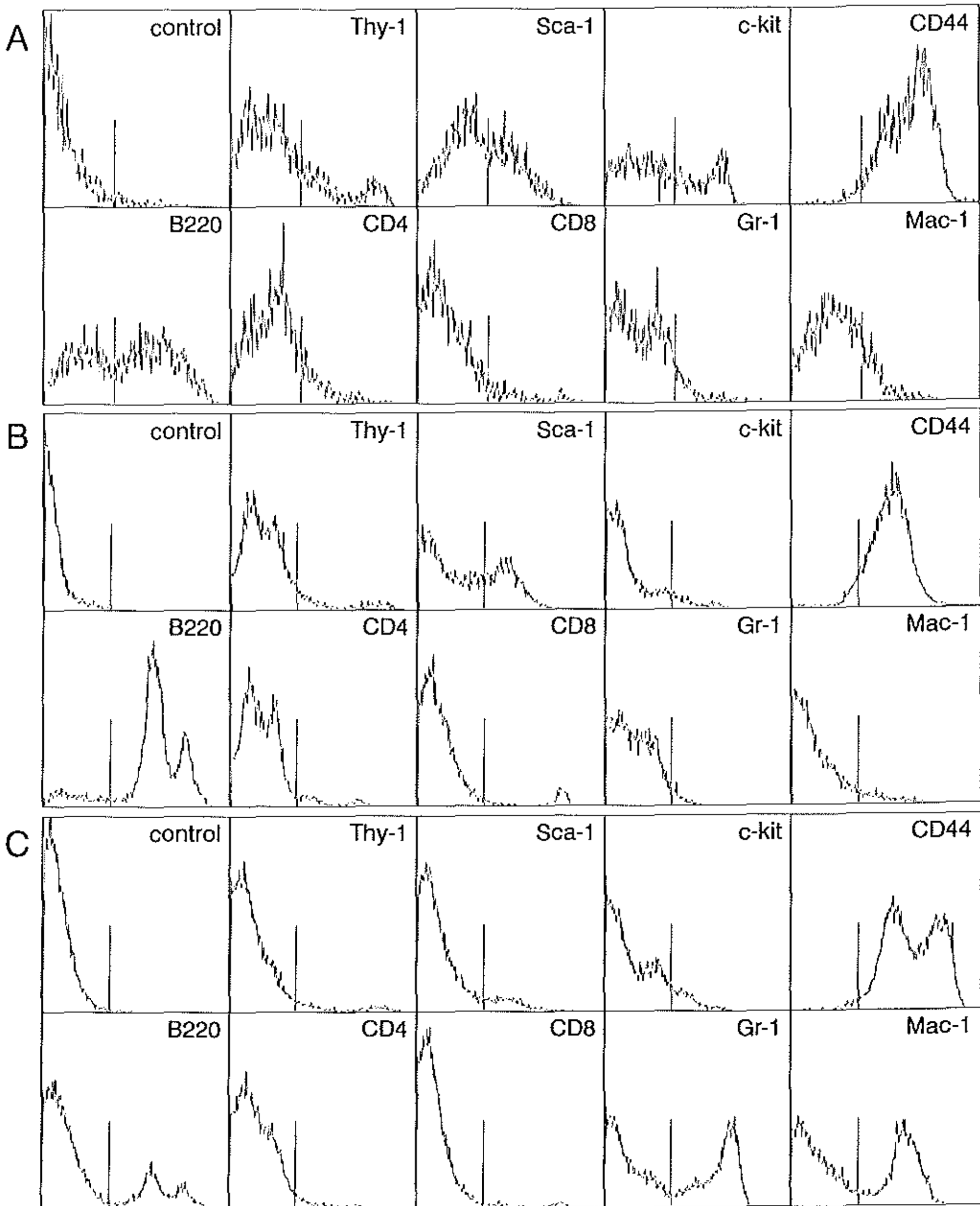

Figure 1. Cell surface phenotype of ER-MP12^{hi}20^{-} (A), ER-MP12^{med}20^{-} (B) and unseparated bone marrow cells (C). Bone marrow cells were stained with monoclonal antibodies specific for either of the indicated antigens (second stage reagent: GαRa-PE), FITC-conjugated ER-MP20, and biotinylated ER-MP12 (followed by SAV-TC). Control: isotype-matched control monoclonal antibody (IgG2a or IgG2b). Life gates were set on either ER-MP12^{hi}20^{-} (A) or ER-MP12^{med}20^{-} cells (B) and the expression of the indicated markers was analyzed. Histograms in panel A, B and C were generated from 4000, 10000, and 14000 events respectively. The results of one representative experiment are shown.

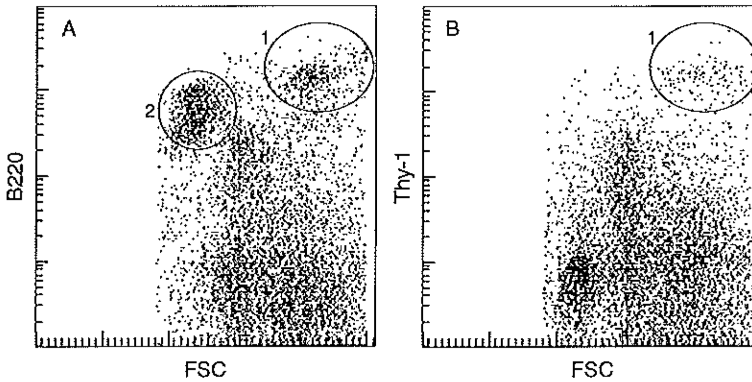

Figure 2. Forward scatter versus log fluorescence distribution of ER-MP12^{hi}20^{-} bone marrow cells stained with B220 (A) and Thy-1 (B). Bone marrow cells were stained as described in Methods (three-color staining). Life gates were set on ER-MP12^{hi}20^{-} cells and the expression of the third marker was analyzed. 1: blast-like cells expressing high levels of either B220 (A) or Thy-1 (B). 2: lymphoid cells expressing B220. Dot plots were generated from 8000 events and are the result of one representative experiment.

by mice of H-2^{b}, H-2$^{b/q}$, H-2^{d}, H-2^{k}, and H-2^{s} haplotypes. The distribution of cells among the bone marrow subsets characterized by different expression of the ER-MP12 and ER-MP20 antigens varied only slightly between the different mouse strains. Most importantly, approximately the same percentage (1.3-1.7% of total nucleated bone marrow cells) of ER-MP12^{hi}20^{-} bone marrow cells were detected in all tested strains.

Table 4 Relative distribution of bone marrow subsets characterized by distinct patterns of ER-MP12 and ER-MP20 antigen expression in various mouse strains

Mouse strain	H-2	12^{-}20^{-} [a]	12^{med}20^{-}	12^{hi}20^{-}	12^{-}20^{med}	12^{-}20^{hi}	12^{+}20^{+}
C57BL/6-Ly-5.1	b	26.6	26.8	1.7	29.5	5.2	10.2
C57BL/Ka BL-1	b	22.6	20.5	1.6	42.2	7.1	5.8
BCBA(F1)	b/q	29.7	16.2	1.3	40.7	5.7	6.4
BALB/c	d	22.4	30.9	1.5	36.2	4.5	4.5
BALB.k	k	28.5	21.5	1.3	40.6	4.0	4.4
SJL/J	s	26.9	32.7	1.5	27.5	5.5	6.2

Bone marrow cells were simultaneously stained with ER-MP12 and ER-MP20. The percentages of nucleated bone marrow cells among the six different subpopulations were determined by FACScan analysis. [a]ER-MP12^{-}20^{-} etc.

Discussion

In the previous paper[13] we showed that by using the monoclonal antibodies ER-MP12 and ER-MP20 in two-color immunofluorescence analysis six distinct subpopulations of bone marrow cells could be identified. Upon intrathymic transfer into sublethally irradiated recipient mice we found that thymus-repopulating ability was exclusively confined to bone marrow cells that expressed either high or intermediate levels of the surface antigen ER-MP12 but lacked the expression of the surface marker ER-MP20. The highest frequency of thymus-repopulating cells was found in the minor subset of ER-MP$12^{hi}20^{-}$ bone marrow cells (1-2% of total nucleated bone marrow cells). However, by means of intrathymic transfer the ability of the injected cells to home to the thymus, which is a prerequisite for prothymocytes, could not be assessed.

In the present study we transferred the six bone marrow subpopulations i.v. into sublethally irradiated mice in order to investigate whether thymus-homing and -repopulating cells (i.e. prothymocytes) are contained within the ER-MP$12^{hi}20^{-}$ and ER-MP$12^{med}20^{-}$ bone marrow subpopulations. Our results demonstrate that prothymocyte activity (assayed 21 days after i.v. transfer) is indeed restricted to the ER-MP$12^{hi}20^{-}$ and ER-MP$12^{med}20^{-}$ subset of bone marrow cells. The highest frequency of thymus-homing and -repopulating cells was detected in the ER-MP$12^{hi}20^{-}$ subpopulation.

Analysis of the peripheral blood leukocytes of mice reconstituted 28 days earlier with either ER-MP$12^{hi}20^{-}$ or ER-MP$12^{med}20^{-}$ cells revealed the presence of donor-derived T lymphocytes, B lymphocytes and myeloid cells, indicating that not only thymus-repopulating cells, but also progenitor cells of the B cell lineage as well as the myeloid lineage were present in both subpopulations.

At this later time point of analysis (i.e. 28 days) high percentages of donor-derived thymocytes were found after i.v. transfer of either 5000 ER-MP$12^{hi}20^{-}$ or 5000 ER-MP$12^{med}20^{-}$ cells, whereas at 21 days donor-derived thymocytes were only detected in mice reconstituted with the ER-MP$12^{hi}20^{-}$ subset. This apparent discrepancy is most likely explained by the presence of multipotent stem cells within the ER-MP$12^{med}20^{-}$ subset[39] which need to undergo additional maturational events in the bone marrow before acquiring the ability to home to the thymus. These cells therefore need more time to mature into thymocytes, resulting in the detection of donor-derived thymocytes at a later time point (i.e. 28 days after i.v. transfer). In contrast the thymus-repopulating cells in the ER-MP$12^{hi}20^{-}$ subpopulation probably already have acquired the ability to home to the thymus, and therefore will give rise to progeny at an earlier time point (i.e. 21 day after i.v. transfer).

Our results demonstrate that although the ER-MP$12^{hi}20^{-}$ subset of bone marrow cells contains a high frequency prothymocytes the separation of the pro-T cell activity from other hematopoietic activities will depend upon the use of additional cell surface markers.

Cell surface phenotyping revealed that both ER-MP$12^{hi}20^{-}$ and ER-MP$12^{med}20^{-}$ bone marrow cells were heterogeneous with respect to the expression of several cell surface markers. Both subpopulations lacked cells expressing high levels of Gr-1 and Mac-1, indicating that ER-MP20 is extremely suitable for depleting at least mature myeloid cells in the enrichment procedure of hematopoietic progenitor cells. As the Mac-1 antigen has been detected on a subset of thymus-repopulating cells[24,25,40] it remains to be tested

whether Mac-1^{lo} cells, constituting a small percentage of both ER-$MP12^{hi}20^-$ and ER-$MP12^{med}20^-$ cells, are capable of differentiating into the T cell lineage. Cells expressing high levels of CD4 were depleted of the ER-$MP12^{hi}20^-$ subset, indicating that mature T lymphocytes (at least $CD4^+CD8^-$ cells) are excluded by selecting for high levels of ER-MP12 antigen expression. In contrast mature T lymphocytes were detected among ER-$MP12^{med}20^-$ cells. Cells expressing low levels of CD4 were found in both subsets. Recent reports have shown that both hematopoietic stem cells and the earliest detectable thymocytes express CD4 at a low level[7,8,29,30]. Therefore the use of anti-CD4 monoclonal antibody in bone marrow purging protocols should be avoided because this may lead to the depletion rather than the enrichment of progenitors and stem cells.

Almost all of the ER-$MP12^{med}20^-$ bone marrow cells expressed B220. These cells therefore most probably belong to the B cell lineage. In contrast, half of the ER-$MP12^{hi}20^-$ bone marrow cells were $B220^-$. Surprisingly the ER-$MP12^{hi}20^-$ cells expressing high levels of B220 appeared to be blast-like cells. Further research is required to determine whether this $B220^{hi}$ subpopulation of ER-$MP12^{hi}20^-$ bone marrow cells is restricted to B cell lineage development. On the other hand the presence of thymus-repopulating cells in this $B220^{hi}$ subset may not be excluded, because at least some T cell progenitor activity has been detected in a subset of the Thy-1^{lo}Sca-1^+ bone marrow cells expressing B220 and Mac-1[40].

The majority of ER-$MP12^{hi}20^-$ and ER-$MP12^{med}20^-$ cells are Thy-1^-, while both subsets contain cells expressing low levels of Thy-1. Progenitor cells, including thymus-repopulating cells, and hematopoietic stem cells have been found to express low levels of Thy-1 [24,25,28]. However, it has recently been shown that only in Thy-1.1 genotype mouse strains was the stem cell activity restricted to Thy-1^{lo} bone marrow cells while in mouse strains expressing the Thy-1.2 allele (*i.e.* the common form of the Thy-1 locus, found in mice used in this study) this activity was detected in both Thy-1^- and Thy-1^{lo} subsets[41]. Although the thymus-repopulating ability of the Thy-1.2^- and Thy-1.2^{lo} cells was not assessed in that study[41] a low level of Thy-1 expression is maybe not a valid criterion for the isolation of prothymocytes from the bone marrow of Thy-1.2 genotype mice. Interestingly, some ER-$MP12^{hi}20^-$ bone marrow cells expressed high levels of Thy-1.2. These cells are most likely immature, because they have a blast-like appearance in the scatter profile. As about 50% of the natural killer cells express Thy-1 [42] the presence of these cells within the Thy-1^{hi} subset of ER-$MP12^{hi}20^-$ cells may not be excluded.

Cells expressing the Sca-1 antigen were present in both subsets. It has been shown previously that Sca-1 is expressed by thymus-repopulating cells and other hematopoietic progenitors and stem cells[25,28,40]. This cell surface marker however is not an ideal marker for the isolation of thymus-repopulating cells, because Sca-1 is constitutively expressed only by bone marrow cells of Ly-6^b haplotype mice[43]. In contrast our data show that the ER-MP12 and ER-MP20 antigens are expressed on a broad range of mouse strains with only slight differences in the size of the six bone marrow subpopulations.

Cells expressing high levels of the proto-oncogene c-*kit* were markedly enriched among ER-$MP12^{hi}20^-$ bone marrow cells. This cell surface marker has been detected on hematopoietic stem cells and progenitor cells[26,36-38], also including the earliest intrathymic

precursor cells[44]. Therefore separation of the ER-MP12^{hi}20^{-} bone marrow cells on the basis of c-*kit* expression may lead to the further purification of prothymocytes.

ER-MP12^{hi}20^{-} cells expressed either high or intermediate levels of CD44 while all ER-MP12^{med}20^{-} bone marrow cells expressed this cell surface marker at an intermediate level. An intermediate level of CD44 has been detected on pluripotent stem cells and Thy-1^{lo}Lin^{-}Sca-1^{+} bone marrow cells[25,38] while the earliest intrathymic T cell precursors express high levels of CD44 [7,8]. In this context separation of ER-MP12^{hi}20^{-} bone marrow cells on the basis of high levels of CD44 expression may lead to a further enrichment of thymus-repopulating cells.

Finally, cell surface markers such as Fall-3, which distinguishes pluripotent stem cells from B cell progenitors[45], or the heterogeneity of different stem cell and progenitor subsets for rhodamine 123 retention and wheat germ agglutinin affinity[39,46,47] may prove to be useful for the separation of the pro-T cell activity from other hematopoietic activities in the ER-MP12^{hi}20^{-} subset.

In summary our data demonstrate that progenitor cells with the capacity to home to and repopulate the thymus are confined to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets of bone marrow cells. The highest frequency of thymus-homing and -repopulating bone marrow cells was found among ER-MP12^{hi}20^{-} cells which heterogeneously expressed the cell surface markers Thy-1, Sca-1, CD44, B220, and c-*kit*. This phenotypic heterogeneity provides a basis for the further purification of thymus-homing and -repopulating cells from the ER-MP12^{hi}20^{-} subpopulation.

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5 High-level expression of the ER-MP58 antigen on mouse bone marrow hematopoietic progenitor cells marks commitment to the myeloid lineage

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Abstract

Studies on the early events in the differentiation of the non-specific immune system require the identification and isolation of myeloid-committed progenitor cells. Using the monoclonal antibodies ER-MP12 and ER-MP20, generated against immortalized macrophage precursors, we have shown previously that the earliest macrophage colony-stimulating factor (M-CSF)-responsive cells in the bone marrow have the ER-MP$12^{hi}20^{-}$ phenotype. In addition, we found that the ER-MP$12^{hi}20^{-}$ subset (comprising about 2% of total nucleated marrow) contains progenitor cells of all hematopoietic lineages. Aiming at the identification and purification of the myeloid progenitor cells within the ER-MP$12^{hi}20^{-}$ subset, we used ER-MP58, a marker expressed at high level by all M-CSF-responsive bone marrow progenitors. With this marker the ER-MP$12^{hi}20^{-}$ cell population could be divided into three subfractions: one with absent or low level ER-MP58 expression, one with intermediate and one with high ER-MP58 expression. These subfractions were isolated by fluorescence-activated cell sorting and tested *in vitro* and *in vivo* for their differentiation capacities. In addition, the expression of ER-MP58 on stem cell subsets was examined in the cobblestone area-forming cell (CAFC) assay. Our data indicate that in the ER-MP$12^{hi}20^{-}$ subpopulation myeloid-committed progenitors are characterized by high-level expression of the ER-MP58 antigen, whereas cells with other or broader differentiation capacities have an ER-MP58 negative/low or intermediate phenotype. These myeloid-committed progenitors have no significant repopulating ability *in vivo*, in contrast to the ER-MP58 intermediate cells. Primitive CAFC-28/35, corresponding to cells providing long-term hematopoietic engraftment *in vivo*, also did not express the ER-MP58 antigen at a high level. Thus, cells committed to the myeloid lineage can be separated from progenitor cells with other differentiation capacities by means of multiparameter cell sorting using ER-MP58 in combination with ER-MP12 and ER-MP20.

Introduction

Primitive hematopoietic stem cells differentiate into various lineage-committed progenitor cells. At present, it is largely unknown what the crucial events are in lineage commitment of hematopoietic stem cells. The study of the mechanisms involved in lineage commitment requires the identification of lineage-specific markers which permit the isolation of different subsets of committed progenitor cells.

The currently available cell separation protocols combine multiple cell characteristics to obtain bone marrow cell populations that are highly enriched in hematopoietic stem cell activity[1-4]. Although primitive hematopoietic stem cells can be separated from more committed progenitor cells in this way, the different classes of committed progenitor cells are still difficult to dissect[5]. Using density separation and counterflow centrifugal elutriation, a highly enriched population of granulocyte/macrophage colony-forming cells (GM-CFC) has been isolated from the bone marrow of cyclophosphamide-treated mice[6,7]. Isolation of committed progenitors on the basis of cell surface antigen expression, however, has the advantage that information on phenotypic changes associated with commitment may provide insight into molecules and, ultimately, the mechanisms crucial in lineage commitment.

In our search for cell surface markers that allow the identification of myeloid-committed progenitors, we previously generated a series of monoclonal antibodies which recognize cell surface antigens expressed by mouse hematopoietic progenitor cells[8,9]. Using two of these monoclonal antibodies, ER-MP12 and ER-MP20, we identified in mouse bone marrow three successive maturation stages of cells of the macrophage lineage[10]. The bone marrow subset containing the earliest macrophage colony-stimulating factor (M-CSF)-responsive progenitor cells, *i.e.* the ER-MP12^{hi}20^{-} (about 2% of nucleated bone marrow cells), was found to contain also cells with thymus-repopulating ability, cells with capacity to reconstitute all hematopoietic lineages in sublethally irradiated mice[11], as well as primitive hematopoietic stem cells[12].

In the present study, we searched for novel cell surface markers which distinguish the myeloid-committed progenitor cells present in this ER-MP12^{hi}20^{-} bone marrow subset from primitive hematopoietic stem cells and cells committed to other lineages. To this purpose, we employed the ER-MP58 monoclonal antibody, which was previously found to detect all M-CSF-responsive progenitors in total mouse bone marrow[8]. The ER-MP12^{hi}20^{-} population was fractionated on the basis of ER-MP58 expression using multiparameter flow cytometry. The resulting three subfractions were then tested *in vitro* for their content of macrophage colony-forming units (CFU-M), granulocyte/macrophage colony-forming units (CFU-GM), and CFU-pre-B, and *in vivo* for their potential to repopulate the thymus and to confer peripheral blood reconstitution of the myeloid, B lymphoid, and erythroid lineages. In addition, the expression of ER-MP58 on the various stem cell subsets was examined *in vitro* in the cobblestone area-forming cell (CAFC) assay. Our data indicate that early, ER-MP12^{hi}20^{-}, myeloid-committed cells can be separated from other hematopoietic stem and progenitor cells on the basis of their high level ER-MP58 expression.

Materials and methods

Mice

C57BL6/Ly5.1-Pep3b (Thy-1.2, Ly-5.1; breeding pairs originally provided by Dr I.L. Weissman, Stanford University, Stanford, CA), C57BL/Ka Bl-1 (Thy-1.1, Ly-5.2) and CBA/Rij x C57BL/Ka [BCBA(F1)] mice were kept under specific pathogen free conditions with free access to food and water in the mouse facilities of our department.

Monoclonal antibodies and conjugates

The monoclonal antibodies used in this study were ER-MP12 [8], ER-MP20 (anti-Ly6C)[8,13], ER-MP58 [8], A20-1.7 (anti-Ly-5.1; kindly provided by Dr S. Kimura, Sloan-Kettering Cancer Center, New York, NY), 30H12 (anti-Thy-1.2)[14], RA3 6B2 (anti-B220)[15], KT3 (anti-CD3)[16], and M1/70 (anti-Mac-1)[17]. Antibodies were used either as undiluted hybridoma culture supernatants (ER-MP58 and M1/70), or purified and conjugated to N-hydroxysuccinimidobiotin (Boehringer Mannheim, Mannheim, FRG; ER-MP12 and A20-1.7), fluorescein isothiocyanate isomer I (FITC, Sigma, St.Louis, MO; ER-MP20, RA3 6B2 and KT3), or 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FLUOS, Boehringer Mannheim; 30H12).

FITC-conjugated rabbit anti-rat IgG $F(ab)'_2$ fragments (Cappel, Organon Teknika, Turnhout, Belgium), R-phycoerythrin-conjugated goat-anti-rat IgG (mouse-adsorbed; Caltag Laboratories, San Francisco, CA), Tri-Color-conjugated streptavidin (Caltag Laboratories) and R-phycoerythrin-conjugated streptavidin (Caltag Laboratories) were used as second-stage reagents.

Cell suspensions

Bone marrow cell suspensions were prepared in PBS supplemented with 5% heat-inactivated fetal calf serum, 60 µg/ml penicillin and 100 µg/ml streptomycin as described previously[10]. Thymus suspensions were made in PBS supplemented with 0.5% bovine serum albumin (BSA) and 20mM NaN_3 by gently pressing thymic lobes through a 100-µm nylon sieve (Polymon PES, Kabel, Amsterdam, The Netherlands).

Immunofluorescent staining and cell sorting

For cell sorting experiments, 2 x 10^8 bone marrow cells were labeled as described[10], using PBS supplemented with 5% heat-inactivated fetal calf serum, 60 µg/ml penicillin and 100 µg/ml streptomycin as washing buffer. A FACS Vantage (Becton Dickinson, Sunnyvale, CA) was used for all cell sorting experiments. After sorting, the purity of the sorted cell populations exceeded 95%, unless stated otherwise. Viable cells were counted using a Bürker hemocytometer.

Immunofluorescent labeling for phenotypic analysis was performed as described[10,11], with the only modification that cells were incubated in each step for 10 min at room temperature (in the presence of NaN_3 to avoid antibody internalization) instead of 30 minutes on ice. In our hands, this modification does not influence staining levels or profiles (data not shown). All phenotypic analyses were performed with a FACScan cytofluorimeter (Becton Dickinson).

Conditioned media

Conditioned medium of L929 and CJ8/GM10.1 cells was used as a source of M-CSF and recombinant GM-CSF, respectively. The CJ8/GM10.1 cell line[18] was kindly provided by Drs. S.Green and J.Karn, Cambridge, GB. Conditioned media were prepared as described[19].

Enumeration of colony-forming units in culture

CFU-M/CFU-GM culture

The frequency of M-CSF- and GM-CSF-responsive cells in bone marrow (subsets) was assessed in soft-agar cultures. The medium used in these cultures was Iscove's modified Dulbecco's medium (IMDM) with Glutamax-I (Gibco BRL) supplemented with 20% FCS (heat-inactivated), penicillin (final concentration 60 μg/ml), streptomycin (100 μg/ml), β-mercaptoethanol (10^{-4}M; β-ME; Merck) and 20% of M-CSF-containing conditioned medium or 10% of GM-CSF-containing conditioned medium (which was previously assessed to be an optimal concentration for colony formation). The colony assay was performed as described[10]. After 12-14 days of culture, the number of colonies (≥ 50 cells) was determined by examining the plates using a phase contrast microscope.

CFU-pre-B culture

Pre-B cell colonies were grown as described by Lee et al.[20] with some modifications. Briefly, a single cell suspension of total or fractionated bone marrow was cultured in IMDM with Glutamax-I (Gibco) supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 10^{-4}M β-ME, 1% BSA, 15% FCS, 1.2% (w/v) methylcellulose (Methocel MC, Fluka Chemie, Buchs, Switzerland) and 20 ng/ml mouse IL-7 (R&D Systems, Abingdon, UK). IL-7 was shown to induce specifically the formation of pre-B cell colonies from fresh bone marrow[20]. After 6 days of culture at 37°C, 5% CO_2 in air, the number of colonies (≥ 50 cells) was counted.

Cell transfer and analysis of repopulation

For analysis of subset differentiation capacity *in vivo*, sorted bone marrow cells of C57BL6/Ly5.1-Pep3b mice were injected intravenously into sublethally (7.5 Gy) irradiated congenic C57BL/Ka Bl-1 mice (11 weeks old), as described by Slieker et al.[11]. Recipient mice were killed 1, 2, and 3 weeks after transfer and thymus (week 3) and peripheral blood (all time points) were isolated and analyzed for donor-type repopulation by FACS analysis[11]. Donor-derived reconstitution of the erythroid lineage was analyzed (week 3) by determining the hematocrit using a hematocrit centrifuge.

Cobblestone area-forming cell (CAFC) assay

For frequency analysis of hematopoietic stem cell subsets, sorted bone marrow cells were cultured in the limiting dilution-type long-term bone marrow culture as previously developed and described by Ploemacher et al. and van der Loo et al.[21-24]. Cultures were examined for 5 weeks at weekly intervals for the presence of cobblestone areas (hematopoietic clones) using an inverted phase-contrast microscope.

Results

ER-MP58 detects all morphologically recognizable myeloid progenitor cells in mouse bone marrow

Previously, we found that ER-MP58 is expressed by all M-CSF-responsive cells in mouse bone marrow[8], making this marker of potential interest for the purification of myeloid-committed cells from the ER-MP12^{hi}20$^-$ bone marrow subset. Therefore, we analyzed the reactivity pattern of ER-MP58 on normal bone marrow cells. Upon staining of bone marrow cells with ER-MP58, we sorted the bone marrow into two fractions: one characterized by a high ER-MP58 expression (ER-MP58^{hi}; 39 ± 4% of nucleated bone marrow; n=15; Fig. 1A) and one with absent to low level ER-MP58 expression (ER-MP58$^{-/lo}$), and analyzed their cellular composition on May-Grünwald/Giemsa-stained cytocentrifuge preparations (Table 1). The ER-MP58^{hi} bone marrow fraction appeared to be highly enriched for all stages of myeloid cells. The ER-MP58$^{-/lo}$ bone marrow fraction, on the other hand, contained virtually no myeloid cells: in this fraction almost all erythroid and lymphoid cells were found. Both bone marrow fractions contained blast cells without morphological lineage characteristics, with a majority in the ER-MP58$^{-/lo}$ subset. Thus, ER-MP58 distinctively recognizes all stages of myeloid development in the bone marrow. These data support the selection of ER-MP58 to purify further myeloid-committed progenitors from the ER-MP12^{hi}20$^-$ bone marrow subset.

Early M-CSF- or GM-CSF-responsive cells express the ER-MP58 antigen predominantly at a high level

To examine ER-MP58 expression on M-CSF- and GM-CSF-responsive cells present in the ER-MP12^{hi}20$^-$ bone marrow subset, bone marrow was triple-labeled with ER-MP12, ER-MP20 and ER-MP58 monoclonal antibodies. Flow cytometric analysis revealed that within the ER-MP12^{hi}20$^-$ bone marrow fraction, three subfractions could be identified on the basis of their ER-MP58 expression (Fig. 1C): 25±8% of the ER-MP12^{hi}20$^-$ cells had a ER-

Table 1 Morphological analysis of bone marrow subsets sorted on the basis of ER-MP58 expression

BM subset	Myeloid			Erythroid		Lymphoid	Undiff. blasts
	Imm. prog.	Band + segm.	Monocytes	Erythro-blasts	Polychr. + normoblasts		
ER-MP58$^{-/lo}$	0	3	0	30	27	18	22
ER-MP58^{hi}	17	58	12	2	0	5	6

For differential cell counts, bone marrow cells were sorted on their level of ER-MP58 antigen expression, spun onto microscopic slides and stained with May-Grünwald/Giemsa. For each fraction 100 cells were counted. Data represent the percentage of cells present in the ER-MP58 subsets. No megakaryocytes were found in these samples. Imm. prog.: immature myeloid progenitors; band + segm.: band form and segmented granulocytes; polychr.: polychromatophilic erythroblasts; undiff. blasts: morphologically undifferentiated blasts.

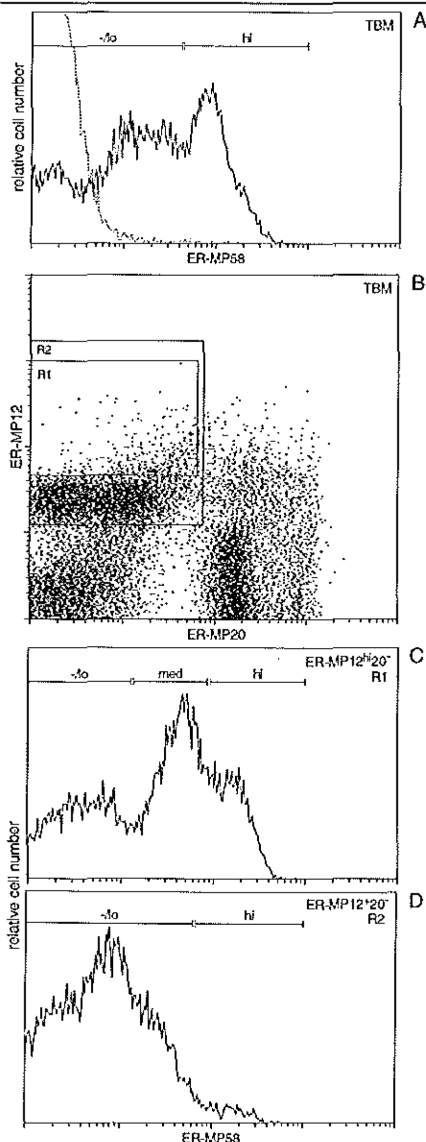

Figure 1. Cell surface expression of ER-MP58 antigen by (A) total nucleated bone marrow cells and by (C) ER-MP12^{hi}20^{-} and (D) ER-MP12^{+}20^{-} nucleated bone marrow cells (2.4 ± 0.3% (n=9) and 31 ± 7% (n=10) of total nucleated bone marrow, respectively; see (B)). Cells were triple-labeled with ER-MP58, ER-MP12 and ER-MP20 monoclonal antibodies and expression on total bone marrow and within the ER-MP12^{hi}20^{-} and ER-MP12^{+}20^{-} subsets was determined using a FACS Vantage flowcytometer. The negative control is shown as a dotted line. Windows as used for cell sorting are shown.

MP58^{hi} phenotype (n=8), 35±3% expressed the ER-MP58 antigen at an intermediate level (ER-MP58^{med}) and 40±8% showed no or low level expression of the antigen (ER-MP58$^{-/lo}$). To examine which of these subfractions contained myeloid progenitor cells, all three ER-MP58 subfractions of the ER-MP12^{hi}20^{-} population were isolated by cell sorting and cloned in soft-agar in the presence of the myeloid growth factors M-CSF or GM-CSF. Both in M-CSF- and GM-CSF-stimulated cultures, colonies could be grown primarily from the ER-MP12^{hi}20^{-}58^{hi} and, to some extent, from the ER-MP12^{hi}20^{-}58^{med} subfraction, but not from ER-MP12^{hi}20^{-}58$^{-/lo}$ bone marrow cells (Table 2). On the basis of their recovery, it was calculated that the large majority of the M-CFC (82-86%) and the majority of the GM-CFC (52-66%) reside within the ER-MP12^{hi}20^{-}58^{hi} bone marrow population.

Bone marrow cells with pre-B cell colony-forming capacity primarily express the ER-MP58 antigen at an intermediate level

To test for the presence of cells with the capacity to differentiate along the B cell lineage, the ER-MP12^{hi}20^{-} bone marrow cells were sorted on the basis of their differential ER-MP58 expression and tested *in vitro* in the CFU-pre-B assay. The large majority (87 ± 10%, *n*=3) of the pre-B cell colonies were generated from the ER-MP58^{med} subfraction, while only 13 ± 10% originated from the ER-MP58^{hi} subfraction (Table 3). No CFU-pre-B were found in the ER-MP58$^{-/lo}$ subfraction. Although the numbers of CFU-pre-B differed considerably in the three experiments, the relative distribution over the ER-MP58 subfractions was consistent.

Hematopoietic progenitor cells with early reconstitution potential in vivo express ER-MP58 at an intermediate level

To extend the *in vitro* results mentioned above, we assessed the early myeloid, B lymphoid and erythroid developmental potential of the three ER-MP12^{hi}20^{-} subfractions *in vivo*. For this purpose, we transferred the sorted subsets intravenously (i.v.) into 7.5-Gy sublethally irradiated congenic recipient mice for analysis of repopulation at day 7, 14 and 21 after transfer. The repopulating ability of the ER-MP12^{hi}20^{-}58$^{-/lo}$ subset was tested at day 21 only, as the data obtained *in vitro* from the colony assays (section 3.2 and 3.3) as well as data on ER-MP58 expression by primitive hematopoietic stem cells (section 3.6) indicated that this subset contains more primitive hematopoietic stem cells, not likely to give progeny *in vivo* after 1 or 2 weeks. For analysis of repopulation, nucleated peripheral blood cells were analyzed for the presence of donor-derived (Ly-5.1^{+}) cells. In addition, the percentage of myeloid (Mac-1^{+}) and B (B220^{+}) cells of donor origin was determined (peripheral T cell reconstitution cannot yet be determined at these time points; 25 and unpublished observations). Seven days after transfer, donor-derived repopulation was still low for all subsets tested (Table 4). Few myeloid cells, but no B cells of donor origin could be detected at this time point. At day 14, a substantial percentage of donor-derived cells was found only in mice injected with the ER-MP12^{hi}20^{-}58^{med} subset (46.6% compared to an average of 3.7% after transfer of equal numbers of ER-MP12^{hi}20^{-}58^{hi} bone marrow cells). Similar results were obtained at day 21 after transfer: only the mice reconstituted with the ER-MP12^{hi}20^{-}58^{med} subfraction showed substantial donor-derived repopulation (40% compared to 9% and 3% found after transfer of equal numbers of ER-MP12^{hi}20^{-}58$^{-/lo}$ and ER-MP12^{hi}20^{-}58^{hi} cells, respectively). Consequently, at these time points donor-derived cells belonging to the myeloid or B cell lineages were found particularly after i.v. transfer of ER-MP12^{hi}20^{-}58^{med} cells, and much less after transfer of the ER-MP58$^{-/lo}$ and ER-MP58^{hi} subfractions. To analyze the capacity of the ER-MP58 subfractions to repopulate the erythroid lineage, the hematocrit of donor-reconstituted mice was determined and compared with the hematocrit of non-reconstituted, endogenously repopulated mice (Table 4). Erythroid repopulation exceeding endogenous repopulation was only found in mice reconstituted with the ER-MP12^{hi}20^{-}58^{med} subfraction. Together, these data indicate that cells with the capacity to repopulate the myeloid, B lymphoid and erythroid lineages *in vivo* as early as 2-3 weeks after i.v. transfer reside essentially all in the ER-MP58^{med} subfraction of the ER-MP12^{hi}20^{-} bone marrow subset.

Table 2 Presence of M-CSF- and GM-CSF-responsive precursors in ER-MP12^{hi}20^{-} bone marrow subfractions sorted on the basis of ER-MP58 expression

BM fraction		M-CSF-stimulated culture					
	No. of colonies per 10^4 plated cells		% recovery		relative distribution		
Exp.	I	II	I	II	I	II
ER-MP12^{hi}20^{-} all	427	256	100	100		
ER-MP12^{hi}20^{-} 58$^{-/lo}$	–[a)]	–[b)]				
ER-MP12^{hi}20^{-} 58^{med}	250	250	21	40	14%	18%
ER-MP12^{hi}20^{-} 58^{hi}	1600	2000	127	179	86%	82%

Colonies were determined after 12-14 days of M-CSF- or GM-CSF-stimulated culture. Data are from two experiments. The recovery and relative distribution of the CFU-M/-GM in the three ER-MP58 subfractions was calculated using their frequencies of 34.0 and 22.9% for ER-MP58^{hi} (experiment I and II, respectively); 36.6 and

Bone marrow cells with thymus-repopulating ability have the ER-MP58$^{-/lo}$ and ER-MP58^{med} phenotype

To analyze the capacity of the ER-MP58 subfractions to repopulate the T cell lineage, we determined the thymus-repopulating ability of the ER-MP12^{hi}20^{-} 58$^{-/lo}$, 58^{med} and 58^{hi} subsets in the experiments described in the previous section. At 21 days after transfer, thymuses were analyzed for donor-derived (Thy-1.2^{+}) repopulation (Table 5). Cells with thymus-repopulating ability appeared to be restricted to the ER-MP58$^{-/lo}$ and ER-MP58^{med} subfractions. No donor-derived thymus-repopulation was observed in mice injected with ER-MP12^{hi}20^{-}58^{hi} cells. Comparing the experiments in which 5000 cells per mouse were injected, the highest frequency of thymus-repopulating cells was found in the ER-MP12^{hi}20^{-}58^{med} subfraction (53% of all mice reconstituted versus 23% of all mice given ER-MP12^{hi}20^{-}58$^{-/lo}$ cells).

Primitive hematopoietic stem cells are ER-MP58 negative/low

We also investigated the expression of the ER-MP58 antigen on various hematopoietic stem cell subsets *in vitro*. For this purpose, the stem cell content of bone marrow fractions sorted on the basis of differential ER-MP58 expression was determined using the CAFC assay, a limiting dilution-type long-term bone marrow culture. Previously we demonstrated that bone marrow stem cells represent a heterogeneous population of cells residing in both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subsets[12]. Hence we chose to combine these two bone marrow subsets (further denoted as ER-MP12^{+}20^{-}) and use the combined population as the starting-population for cell sorting on the basis of differential ER-MP58 expression. The expression of the ER-MP58 antigen within this ER-MP12^{+}20^{-} subset is shown in Fig. 1D. An average of 6 ± 3% (n=7) of the ER-MP12^{+}20^{-} cells expressed the ER-MP58 antigen at a high level (ER-MP58^{hi}). The other 94 ± 3% was

Table 2 Presence of M-CSF- and GM-CSF-responsive precursors in ER-MP12^{hi}20^{-} bone marrow subfractions sorted on the basis of ER-MP58 expression (continued)

BM fraction	GM-CSF-stimulated culture					
	No. of colonies per 10^4 plated cells		% recovery		relative distribution	
Exp.	I	II	I	II	I	II
ER-MP12^{hi}20^{-} all	283	300	100	100		
ER-MP12^{hi}20^{-} 58$^{-/lo}$	–[c]	3	–	0.4		0.3%
ER-MP12^{hi}20^{-} 58^{med}	375	417	48	57	34%	48%
ER-MP12^{hi}20^{-} 58^{hi}	767	800	92	61	66%	52%

40.7% for ER-MP58^{med} (exp. I and II, respectively) and 29.4 and 36.6% for ER-MP58$^{-/lo}$ (exp. I and II, respectively). [a] Less than 1 in 7500 plated cells; [b] less than 1 in 6000 plated cells; [c] less than 1 in 2100 plated cells.

characterized by absent or low level ER-MP58 expression (denoted ER-MP58$^{-/lo}$). These two subfractions were sorted and tested in the CAFC assay. Primitive CAFC, which give rise to cobblestone areas at day 28/35 of culture, were exclusively found in the ER-MP58$^{-/lo}$ fraction (Fig. 2A). These primitive CAFC-d28/35 have been shown to correspond with cells with long-term repopulating ability (LTRA) *in vivo*[21-24]. Less primitive CAFC, *i.e.* stem cells giving rise to cobblestone areas at day 7/10 of culture were enriched in the

Table 3 Presence of CFU-pre-B in ER-MP12^{hi}20^{-} bone marrow subfractions sorted on the basis of ER-MP58 expression

BM fraction	No. of colonies per 10^5 plated cells			% recovery			relative distribution		
Exp.	I	II	III	I	II	III	I	II	III
ER-MP12^{hi}20^{-} all	250	10	50	100	100	100			
ER-MP12^{hi}20^{-} 58$^{-/lo}$	–[a]	–[b]	–[b]						
ER-MP12^{hi}20^{-} 58^{med}	1350	67	150	198	273	105	94%	92%	76%
ER-MP12^{hi}20^{-} 58^{hi}	100	10	80	14	23	33	6%	8%	24%

Colonies (>50 cells) were determined after 6 days of IL-7-stimulated culture. Data are from three independent experiments with 2 dishes per group. Variability in CFU-pre-B assay has also been reported by others[32-34]. Recovery and relative distribution of CFU-pre-B was calculated as described in legend of Table 2. In the third experiment 20.8% of the ER-MP12^{hi}20^{-} cells were ER-MP58^{hi}, and 34.9% ER-MP58^{med}. [a] Less than one in 3000 plated cells; [b] Less than one in 10^4 plated cells.

Table 4 Early peripheral blood repopulation in the myeloid, B, and erythroid lineages after i.v. reconstitution with sorted ER-MP12^{hi}20^{-} bone marrow subfractions

BM subfraction	Days after i.v. transfer	No. of cells injected	% donor-derived cells[a]
ER-MP12^{hi}20^{-} all	7[c]	20000	1.6 ± 0.7
	14[c]	20000	28.9 ± 11.0
	21[d]	10000	75.6 ± 14.5
ER-MP12^{hi}20^{-} 58$^{-/lo}$	7	–[f]	–
	14	–	–
	21	5000	8.8 ± 3.4
ER-MP12^{hi}20^{-} 58^{med}	7	20000	1.0 ± 0.6
	14	20000	46.6 ± 11.3
	21	5000	39.9 ± 7.2
ER-MP12^{hi}20^{-} 58^{hi}	7	20000	1.7 ± 1.3
	14	20000	3.7 ± 3.3
	21	5000	2.9 ± 2.9[g]
none (irradiation control)[h]	21		

C57BL/KaBl-1 (Ly-5.2, Thy-1.1) recipient mice were sublethally irradiated (7.5 Gy) and injected i.v. with bone marrow cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Injected cell numbers were chosen on the basis of earlier transfer experiments involving the total ER-MP12^{hi}20^{-} bone marrow subset (11 and unpublished data). Peripheral blood chimerism was analyzed 7, 14 and 21 days after cell transfer. [a] Data are the means ± SD. [b] Results are from two experiments with 13 to 17 mice analyzed in total per group, except for the irradiation

ER-MP58^{hi} subset. For such CAFC a high correlation with CFU-S-7/12 has been reported[21-24]. These CAFC-d7/10 were also present in the ER-MP58$^{-/lo}$ subset. Considering the frequencies of the ER-MP58 subfractions within the ER-MP12^{+}20^{-} bone marrow population, our data show that about half of the less primitive CAFC were recovered from this ER-MP58$^{-/lo}$ subfraction (Fig. 2B). In conclusion, all primitive hematopoietic stem cells as well as part of the more mature stem cells do not, or only at a low level, express the ER-MP58 antigen.

Discussion

In this study we searched for cell surface markers allowing the separation of myeloid-committed cells from other hematopoietic progenitor cells in mouse bone marrow. Previously we generated a panel of monoclonal antibodies against immortalized macrophage progenitor cells from mouse bone marrow (reviewed in 9). With two of these monoclonal antibodies, ER-MP12 and ER-MP20, we previously identified a bone marrow population (ER-MP12^{hi}20^{-}; about 2% of nucleated bone marrow) containing various classes of hematopoietic progenitor cells, including the earliest M-CSF-responsive cells[10-12]. Here we report that early myeloid-committed cells can be separated from other

Table 4 Early peripheral blood repopulation in the myeloid, B, and erythroid lineages after i.v. reconstitution with sorted ER-MP12^{hi}20^{-} bone marrow subfractions (continued)

% myeloid cells (Mac-1^{+}) of donor origin[a)]	% B cells (B220^{+}) of donor origin[a)]	erythroid repopulation (hematocrit)[b)]
2.7 ± 0.6	ND[e)]	–
44.6 ± 14.1	64.3 ± 15.1	–
75.6 ± 14.5	52.7 ± 10.8	40 ± 3
–	–	–
–	–	–
37.9 ± 28.4	19.7 ± 7.4	10 ± 8
3.6 ± 2.4	ND	–
69.6 ± 13.8	67.3 ± 3.0	–
73.6 ± 15.8	54.8 ± 9.2	41 ± 3
1.7 ± 0.4	ND	–
8.0 ± 7.1	2.2 ± 1.2	–
11.4 ± 10.3	6.8 ± 4.7	14 ± 6
		14 ± 9

control group (n=3). Data are the means ± SD. [c)] Results are from 3 mice analyzed per group. [d)] Results are from one experiment out of two with similar results, with 7 or 8 mice analyzed per group. [e)] ND: donor B cells were not detectable at this time point. [f)] Not tested. [g)] Percentages are based on 5 donor-repopulated mice out of 7 injected; in the other groups all recipient mice were repopulated. [h)] These mice were used to determine the endogenous erythroid repopulation.

Table 5 Thymus-repopulating ability of ER-MP12^{hi}20^{-} bone marrow subfractions sorted on the basis of ER-MP58 expression

BM fraction	No. of cells injected	No. of positive mice/ total No. of mice[a)]	% donor-derived thymocytes[b)]
ER-MP12^{hi}20^{-} all	10000	10/14	47 ± 10
ER-MP12^{hi}20^{-} 58$^{-/lo}$	5000	3/13	44 ± 15
	10000	1/3	42
ER-MP12^{hi}20^{-} 58^{med}	5000	9/17	40 ± 11
ER-MP12^{hi}20^{-} 58^{hi}	5000	0/13	
	10000	0/4	

C57BL/KaBl-1 (Ly-5.2, Thy-1.1) recipient mice were sublethally irradiated (7.5 Gy) and injected i.v. with bone marrow cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Thymus reconstitution was analyzed 21 days after cell transfer. Results are pooled data from two experiments with similar results. [a)] Thymus suspensions with ≥ 1% donor-derived thymocytes were scored as positive. [b)] Data are the mean ± SEM.

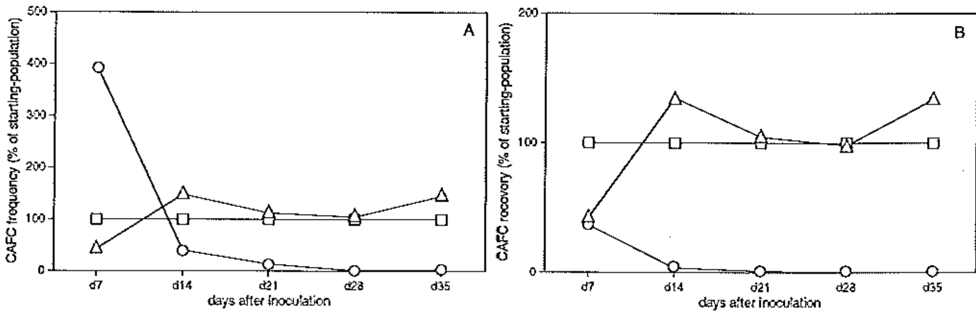

Figure 2. CAFC frequency and recovery in bone marrow subsets sorted on the basis of ER-MP58, ER-MP12 and ER-MP20 expression. In (A) the frequency of CAFC in the ER-MP12^{+}20^{-}58$^{-/lo}$ and ER-MP12^{+}20^{-}58^{hi} subfractions is expressed as percentage of the CAFC frequency in the total ER-MP12^{+}20^{-} starting-population. In (B) the recovery of CAFC is shown. (□) ER-MP12^{+}20^{-} (starting-population), (Δ) ER-MP12^{+}20^{-}58$^{-/lo}$, (O) ER-MP12^{+}20^{-}58^{hi} bone marrow subsets. Data represent the mean of two experiments.

hematopoietic progenitor and stem cells residing in the ER-MP12^{hi}20^{-} bone marrow population on the basis of their high level ER-MP58 expression. This conclusion is based on functional data obtained *in vitro* and *in vivo*. M-CSF- and GM-CSF-responsive cells were predominantly found in the subpopulation of ER-MP12^{hi}20^{-} cells with a high level ER-MP58 expression (at average 84% and 59% of all recovered M-CFC and GM-CFC, respectively). On the basis of this high ER-MP58 expression, these myeloid precursors could be separated from the large majority of cells with the capacity to develop along the B cell lineage (ER-MP58^{med}), along the T cell lineage (ER-MP58$^{-/lo}$ or ER-MP58^{med}), and along the erythroid lineage (ER-MP58^{med}). Primitive hematopoietic stem cells (CAFC-d28/35) were not found among cells with high level ER-MP58 expression.

For cells with myeloid differentiation potential, our data indicate that ER-MP12^{hi}20^{-}58^{med} cells are less mature than ER-MP12^{hi}20^{-}58^{hi} cells. This is suggested by the different distribution of MCFC versus GM-CFC in the ER-MP58^{hi} and ER-MP58^{med} subfractions (see Table 2). Furthermore, our finding that differentiation capacities for all hematopoietic lineages were contained in the ER-MP58^{med} subpopulation suggests the presence of multipotent stem cells within this fraction. An indication that such multipotent cells indeed were present in the ER-MP58^{med} subpopulation was obtained from the CAFC data: part of the less primitive CAFC-d7/10, correlating with CFU-S-d12 [22] *i.e.* multipotent stem cells with short-term repopulating ability *in vivo*, are characterized by a negative/low to intermediate ER-MP58 expression.

A seemingly contradictory finding was that the ER-MP12^{hi}20^{-}58^{hi} subset did not give rise to substantial donor-derived myeloid repopulation upon transfer into irradiated recipients, while the majority of ER-MP12^{hi}20^{-} myeloid-committed cells (M-CFC and GM-CFC) are present in this particular subpopulation. However, this finding is in accordance with a recent report showing that committed progenitors do not contribute to early hematopoietic reconstitution of irradiated recipients[26]. In earlier studies the role of committed progenitors in repopulation *in vivo* had already been questioned by Harrison et

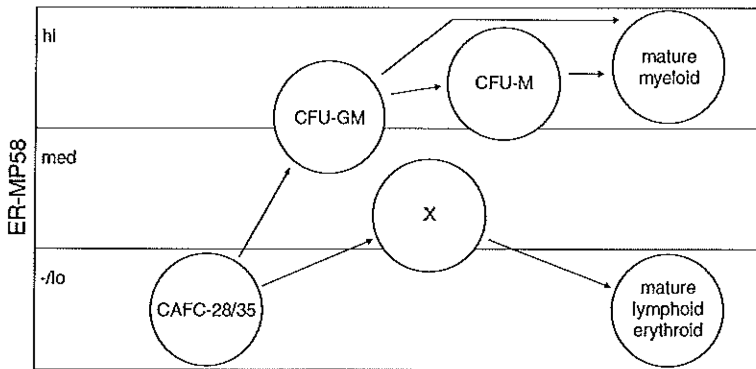

Figure 3. Schematic representation of ER-MP58 antigen expression during bone marrow hematopoiesis as determined in the present study. For clarity, the overlap between the hematopoietic subsets is not taken into account. X: progenitors committed to other than myeloid lineages and/or multi-potent progenitor/stem cells.

al. and Uchida et al., as these autors demonstrated that multilineage precursors, and Thy-1.1^{lo}Lin$^-$Sca-1$^+$ hematopoietic stem cells are capable of early hematopoietic repopulation[27,28]. It has been suggested that committed progenitors cannot contribute significantly to lineage reconstitution[28] because they are limited in the number of cell divisions they can undergo[29]. Alternatively, committed progenitors may be less efficient than stem cells in homing to the bone marrow upon i.v. transfer, and thus lack the appropriate micro-environment to produce mature blood cells[28]. In summary, our data on repopulation *in vivo* show that the ER-MP12^{hi}20$^-$58^{hi} myeloid-committed progenitor population is not contaminated by multipotent stem cells.

The ER-MP12^{hi}20$^-$58$^{-/lo}$ subset gave only limited repopulation of the myeloid, B and T cell lineage *in vivo*, and no CFU-C (unpublished data), CFU-M, CFU-GM, or CFU-pre-B activity *in vitro*. This subset probably contains more primitive hematopoietic stem cells that *in vitro* need additional growth factors, while *in vivo* a time-period exceeding 3 weeks is required to allow substantial peripheral repopulation. This possibility is supported by our finding that the most primitive stem cells in the ER-MP12$^+$20$^-$ subset are found in the ER-MP58$^{-/lo}$ subfraction.

To our knowledge, ER-MP58 is the only marker at present which differentiates between early myeloid-committed cells and other hematopoietic progenitor and stem cells in mouse bone marrow. Recently, Trevisan and Iscove[5] reported phenotypic similarity of progenitor cells detected in a variety of short-term assays (CFU-multi, CFU-E/Meg, CFU-G/M, as well as short-term repopulation *in vivo*) for the expression of H-2K, CD45, AA4.1, HSA, CD71 and the lineage markers B220, Mac-1, Gr-1 and CD8. This indicates that none of these markers allows for separation of myeloid-committed progenitor cells from other hematopoietic activities. In addition to the markers mentioned above, the macrophage marker F4/80 cannot be used for identification of myeloid-committed progenitor cells either, as bone marrow M-CFC do not express this antigen[30]. Recently, Antica et al.[31] reported that Lin$^-$Thy-1^{lo}HSA$^+$Sca-2$^+$ progenitor cells have the capacity to differentiate into the B and T and myeloid lineages. Thus, Sca-2 does not discriminate between early

myeloid and lymphoid progenitors either. So far, ER-MP58 seems to be the earliest expressed marker useful for identification and purification of myeloid-committed cells from mouse bone marrow.

The molecular identity of the ER-MP58 antigen is as yet unknown. Clearly, ER-MP58 is distinct from commonly used myeloid markers as Mac-1, F4/80 and Gr-1. The finding that high level ER-MP58 expression coincides with myeloid commitment might reflect a role for the ER-MP58 antigen in myeloid differentiation. However, support for this notion awaits the characterization of the ER-MP58 antigen.

In Fig. 3, a schematic representation of ER-MP58 antigen expression during hematopoietic differentiation in bone marrow is shown, summarizing the presently available data. While ER-MP58 is not or only faintly expressed on primitive hematopoietic stem cells, it is transiently expressed at an intermediate level during differentiation and maturation into the erythroid and lymphoid lineages. More mature erythroid and lymphoid cells are again characterized by a negative to low expression of this marker. In contrast, commitment to the myeloid lineage coincides with upregulation of ER-MP58 expression, with a continued high level of ER-MP58 on the most mature stages of myeloid development in bone marrow. Upon further maturation of monocytes into tissue macrophages, expression of ER-MP58 is downregulated again[8,9].

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6 Dendritic cell precursor subpopulations identified in mouse bone marrow: phenotypic identity with macrophage precursors

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Abstract

The lineage relationship of dendritic cells (DC) with other hematopoietic cell types has been extensively studied. So far, evidence in support of three distinct DC lineages has been reported. These are a myeloid, a lymphoid and a separate DC lineage. Previously we have generated two monoclonal antibodies, ER-MP12 and ER-MP20, which in two-color flow cytometry identify different maturation stages of M-CSF-responsive myeloid precursors. In the present study we examined in which of the ER-MP12/20 bone marrow subsets GM-CSF-responsive DC precursors are present. For this purpose, ER-MP12/20 bone marrow subsets were isolated using a cell sorter and cultured for 14 days in the presence of rGM-CSF. In these cultures DC development was assessed on the basis of morphology and MHC class II expression. At day 3 of culture, DC-like cells were exclusively present among the cells growing from the ER-MP$12^{+}20^{+}$ (myeloid blasts) and ER-MP$12^{-}20^{hi}$ (monocytes) bone marrow subsets. From day 6 onwards, DC-like cells were also found among cells grown from the ER-MP$12^{hi}20^{-}$ subset (consisting of early blast cells). No DC-like cells could be grown from the ER-MP$12^{med}20^{-}$, ER-MP$12^{-}20^{-}$, and ER-MP$12^{-}20^{med}$ bone marrow subsets, which consist of lymphoid, erythroid and granulocytic cells, respectively. Thus, DC precursors reside in the same ER-MP12/20-defined bone marrow subsets as macrophage precursors and are not found in the subsets containing the morphologically identifiable precursors for the other hematopoietic lineages. In addition, the difference in time of appearance in culture between DC grown from the ER-MP$12^{hi}20^{-}$ versus the ER-MP$12^{+}20^{+}$ and ER-MP$12^{-}20^{hi}$ bone marrow subsets suggests that DC precursors from the ER-MP$12^{hi}20^{-}$ subset are more immature than those from the other two bone marrow subsets. This is similar to the difference in maturation stage we previously reported for the ER-MP12/20-defined macrophage precursors. To approach the question whether DC share precursors with macrophages all along this ER-MP12/20-defined pathway, bone marrow subsets were first cultured for four or five days in the presence of M-CSF to induce development along the macrophage lineage. Subsequently the capacity of these M-CSF-derived cells to generate DC-like cells was tested in GM-CSF-stimulated cultures. We found that cells with the capacity to differentiate into DC-like cells are still present in the day 4 to 5 M-CSF-stimulated cultures. So far, the data obtained from these cultures support a common ER-MP12/20-defined developmental pathway for DC and macrophage precursors. In addition, our data suggest that macrophages and DC-like cells are derived from a common precursor population till at least the monocytic bone marrow stage.

Introduction

Dendritic cells (DC) are bone marrow-derived cells that have the unique capacity to initiate a primary immune response by efficient antigen presentation to naive T cells[1]. The dendritic cell system is widely distributed throughout the body and comprises Langerhans cells in the skin, veiled cells in the lymph, interdigitating cells in the lymphoid organs and interstitial DC in the connective tissue of non-lymphoid organs[2]. Many studies have addressed the lineage relationships of DC with other hematopoietic lineages. Three possible origins for DC have been suggested, which may exist next to each other. First, a considerable amount of experimental data supports a myeloid origin of DC (reviewed by Peters et al.[3], Lutz et al.[4], Thomas and Lipsky[5]). DC have been generated *in vitro* from different stages of myeloid development, ranging from early common progenitors for DC, granulocytes and macrophages in bone marrow to monocytes in peripheral blood[3,6-10]. In these experiments, the generation of DC from precursors was dependent on the presence of GM-CSF in the cultures. Second, several studies report on a lineage relationship between DC and lymphoid cells. Common progenitor populations for DC and lymphoid cells have been identified in bone marrow and thymus[11-16]. In these studies, no direct evidence was given for the existence of a common multipotent progenitor cell for T, DC, NK and B cells in either the thymic or bone marrow progenitor populations, due to a lack of suitable clonal assays. So far, only a common multipotent B/NK/DC progenitor could be demonstrated[14]. Yet, a recent study on mouse thymic DC precursors strongly suggests the existence of a common precursor of DC and T cells, next to myeloid-related DC, with CD8α as a marker of the lymphoid-related DC lineage[11]. Apart from the reported lineage relations with the myeloid and lymphoid lineages, a third possibility is that DC originate from a separate progenitor. In GM-CSF- and TNF-α-stimulated human bone marrow cultures, progenitor cells distinct from CFU-GM have been detected that give rise to pure DC colonies in culture (CFU-DC)[17]. The DC generated in such colonies resembled epidermal DC[17]. In addition, a separate precursor giving rise to Birbeck granule-positive DC has been detected in liquid cultures of human bone marrow and cord blood[9,18]. Together, these data are suggestive of the existence of a separate DC precursor for epidermal Langerhans cells[18,19].

In our studies on the early differentiation of the mononuclear phagocyte system in the mouse, we have previously generated two monoclonal antibodies, ER-MP12 and ER-MP20[20], which in two-color flow cytometry identify different maturation stages of M-CSF-responsive macrophage precursors. We showed that the most immature ER-MP12^{hi}20^{-} macrophage precursors develop via the ER-MP12^{+}20^{+} stage into ER-MP12^{-}20^{hi} monocytes[21]. Thus, in mouse bone marrow, development along the macrophage pathway is characterized by upregulation of ER-MP20 followed by downregulation of ER-MP12 antigen expression. The ER-MP12^{+}20^{+} bone marrow subset consists mainly of myeloid precursors (about 80% of the cells express Gr-1, unpublished observation), while the ER-MP12^{-}20^{hi} subset consists of monocytes[22]. The early ER-MP12^{hi}20^{-} progenitor population, however, was found to contain also cells with B, T, and erythroid differentiation capacities as well as hematopoietic stem cells[23,24]. Using a third cell surface marker, ER-MP58, we recently described that based on a high expression of ER-MP58 myeloid-committed cells

can be separated from all other hematopoietic capacities present in the ER-MP12^{hi}20^{-} subset (ER-MP58$^{-/lo}$ or ER-MP58^{med})[22].

In the present study we questioned in which of the ER-MP12/20/58-defined bone marrow subsets GM-CSF-responsive DC progenitors reside. For this purpose, bone marrow was fractionated by cell sorting on the basis of differential ER-MP12/20/58 antigen expression and the subsets were cultured in the presence of GM-CSF. After various periods of time, cultures were examined for the presence of DC-like cells by microscopic inspection of cellular morphology and immunocytochemical analysis of MHC class II expression. In addition, we asked till what ER-MP12/20-defined stage of development, DC-like cells can be derived from macrophage progenitors. To this end, sorted bone marrow subsets were cultured in the presence of M-CSF to induce development along the macrophage lineage. After four to five days of culture the stimulant was changed to GM-CSF and the capacity of the *in vitro* generated macrophage precursor cells to give rise to DC-like cells was determined. Our data support a similar ER-MP12/20/58-defined maturation pathway for DC and macrophage precursors in mouse bone marrow. In addition, our data are suggestive of a common progenitor of macrophages and DC till at least after the bone marrow monocytic stage.

Materials and Methods

Mice

Female C57BL6/Ly-5.1-Pep^{3b} mice between 11 and 13 weeks of age were used in this study. Animals were specific pathogen free, and were kept with free access to food and water.

Monoclonal antibodies and conjugates

In this study the following monoclonal antibodies were used: ER-MP12 (anti-CD31)[20,25], ER-MP20 (anti-Ly-6C)[20,26], ER-MP58 [20,22]; M5/114 (anti-MHC class II)[27]. Antibodies were used either as undiluted culture supernatants (M5/114) or purified and conjugated to FITC (fluorescein isothiocyanate, isomer I, Sigma Chemical Co., St. Louis, MO; ER-MP20) or biotin (N-hydroxysuccinimidobiotin, Boehringer Mannheim GmbH, Mannheim, FRG; ER-MP12). As second stage fluorescent reagents were used R-Phycoerythrin-conjugated or Tri-Color-conjugated streptavidin (SAV-PE and SAV-TC, respectively; Caltag Laboratories, San Francisco, CA), and R-phycoerythrin-conjugated goat-anti-rat IgG (mouse-absorbed; GαRa-PE; Caltag Laboratories).

Cell suspensions

Single cell suspensions of bone marrow, isolated from femora and tibiae, were prepared as described previously[21,22]. Cultured bone marrow cells were isolated from teflon culture bags (adherent as well as non-adherent cells) or from culture dishes or plates (non-adherent cells). Cells were washed in phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (FCS; heat inactivated), 60 μg/ml penicillin, and 100 μg/ml streptomycin when sterile suspensions were required. Prior to phenotypic analyses, cultured cells were washed

with PBS supplemented with 0.5% (v/v) bovine serum albumin (BSA, Organon Teknika, Boxtel, The Netherlands) and 20mM sodium azide.

Cell sorting and flow cytometric analysis

For cell sorting, bone marrow cells were labeled with two (ER-MP12 and ER-MP20) or three (ER-MP12, ER-MP20, and ER-MP58) monoclonal antibodies as described previously[21,22]. Prior to cell sorting, cell suspensions were filtered over a 30 μm sieve (Polymon PES, Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. A FACS Vantage equipped with Lysis II software was used for cell sorting (Becton Dickinson, Sunnyvale, CA). After sorting, the purity of the cell suspensions was checked by re-running sorted samples. Purity exceeded 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 ($\geq 2 \times 10^4$ cells) were pipetted into 96-microwell plates (round bottom, Nunc, Denmark) and labeled with antibodies as described before[21]. Samples were analyzed on a FACScan flow cytometer using Cell Quest 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 air for one hour 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 minutes at RT, and washed twice with PBS and once with PBS supplemented with 0.5% BSA. Cells were incubated with primary monoclonal antibody, followed by rabbit anti-rat IgG conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA)(both incubations 30 minutes, RT). Diaminobenzidine (DAB, Sigma, St.Louis, MO, USA) was used as substrate for the HRP. Between incubations, cell preparations were washed in PBS supplemented with 0.5% (v/v) Tween-20. After the DAB reaction was completed (3-5 minutes, RT), 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[28] was used as a source of mouse M-CSF. Conditioned medium was prepared as described elsewhere[29]. Recombinant mouse GM-CSF (rGM-CSF) was a kind gift of Dr. R. Coffman (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA).

rGM-CSF- and M-CSF-stimulated bone marrow cultures

Dendritic cells were generated by culturing total bone marrow or isolated subsets in 100 mm petri dishes or 24 well plates (Nunc) in DMEM supplemented with 5% FCS (heat inactivated; 0.22μm 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, up to 14 days.

Bone marrow-derived macrophages were generated by culturing total bone marrow or isolated subsets in Teflon culture bags[30] in IMDM (with glutamax I; Gibco) supplemented with 20% FCS (heat inactivated; 0.22 μm filtered), 15% LADMAC-conditioned medium as a source of M-CSF, 10^{-4}M β-mercaptoethanol, 60 μg/ml penicillin, 100 μg/ml streptomycin. Cells were cultured for 4 or 5 days at 37°C, 7% CO_2.

Results and Discussion

DC precursors reside within the same ER-MP12/20 bone marrow subsets as precursors of the macrophage lineage

To examine in which ER-MP12/20-defined bone marrow subset(s) DC precursors reside, bone marrow was labeled with ER-MP12 and ER-MP20 monoclonal antibodies (Fig. 1) and fractionated into 6 subsets using a cell sorter. The sorted subsets were cultured with GM-CSF in 24-well plates. After 3, 6, 10 and 14 days of culture, the development of DC-like cells was evaluated by examining the plates under an inverted light microscope (Table 1). At all time points tested, developing macrophages (from round non-adherent precursors to spindle shaped or round adherent cells) were the predominant cell type present. At day 3 of culture, DC-like cells (non-adherent round cells with cytoplasmic processes/veils) were observed only in the cultures of the ER-MP$12^{+}20^{+}$ and ER-MP$12^{-}20^{hi}$ bone marrow subsets. At day 6, few DC-like cells started to appear among cells grown from the ER-MP$12^{hi}20^{-}$ subset. By days 10 and 14, DC-like cells were clearly visible in cultures de-

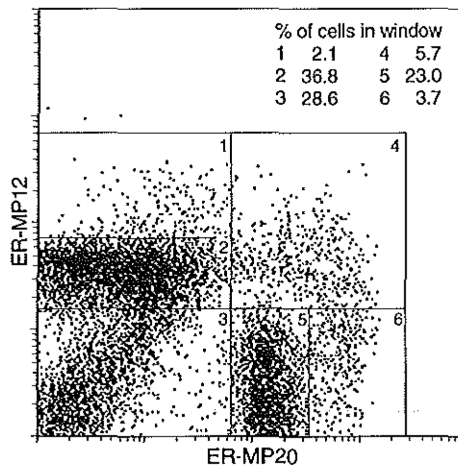

Figure 1. Dot plot of ER-MP12/20-labeled nucleated bone marrow cells. Upon double labeling of bone marrow cells with these monoclonal antibodies, six distinct subsets can be discerned. Gates used for cell sorting are shown. Percentages are the mean of five experiments.

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]				% of MHC II^{++} cells with DC morphology (immunocytochemistry)					
		day 3	day 6	day 10	day 14	day 6		day 10		day 14	
					Exp.	I	II	I	II	I	II
ER-MP12^{hi}20$^-$	+	–	±	+	+	6	ND[c]	15	9	13	ND
ER-MP12^{med}20$^-$	–	–	–	–	–	–	–	–	–	–	–
ER-MP12$^-$20$^-$	–	–	–	–	–	–	–	–	–	–	–
ER-MP12$^+$20$^+$	+	+	+	+	+	20	43	16	ND	18	ND
ER-MP12$^-$20^{med}	–	–	–	–	–	–	–	–	–	–	–
ER-MP12$^-$20^{hi}	+	+	+	+	+	23	26	40	33	ND	ND
TBM	+	+	+	+	+	19	ND	23	ND	28	ND

[a] During entire culture. [b] Results are for both experiment I and II, as they were identical. [c]ND: not enough cells present on the poly-L-lysine coated slides to determine percentage MHC II positive cells.

rived from this subset. No DC nor other cell types could be grown from the ER-MP12^{med}20$^-$, ER-MP12$^-$20$^-$ and ER-MP12$^-$20^{med} subsets. These bone marrow subsets consist of morphologically identifiable cells of the lymphoid, erythroid and granulocytic lineage, respectively[21,24].

At days 6, 10, and 14, cultures were also examined for the presence of DC-like cells by assessing MHC class II expression in immunocytochemistry. Cells with high level MHC class II expression and typical DC morphology were scored as DC-like cells (see Fig. 2). At day 6, the frequency of DC in the ER-MP12^{hi}20$^-$ culture was lower than that in the ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} cultures (an average of 6% versus 33% and 25%, respectively), analogous with the results from visual examination of the cultures. At days 10 and 14, the frequency of DC-like cells in the ER-MP12^{hi}20$^-$ subset had doubled (from 6% to 12 and 13%, respectively), but was still lower than that in the ER-MP12$^+$20$^+$ (16 and 18%) and ER-MP12$^-$20^{hi} (at average 37% at day 10) subsets.

Our results show that GM-CSF-responsive DC precursors reside in the same ER-MP12/20-defined bone marrow subsets as M-CSF-responsive macrophage precursors, i.e. ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} [21] and are not found in the subsets containing morphologically identifiable precursors for the other hematopoietic lineages. In addition, the difference in time of appearance in culture between DC-like cells grown from the ER-MP12^{hi}20$^-$ versus the ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} bone marrow subsets suggests that ER-MP12^{hi}20$^-$ DC precursors are less mature than ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} DC precursors. This notion was supported by the difference in proliferative capacity of these three subsets. GM-CSF-stimulated culture of the distinct subsets in semi solid medium demonstrated that from the ER-MP12^{hi}20$^-$ subset predominantly large colonies were generated, while from the ER-MP12$^+$20$^+$ subset predominantly clusters and some smaller colonies were grown. From the ER-MP12$^-$20^{hi} subset primarily small clusters were generated (data not shown). Thus, the proliferative potential of the GM-CSF-respon-

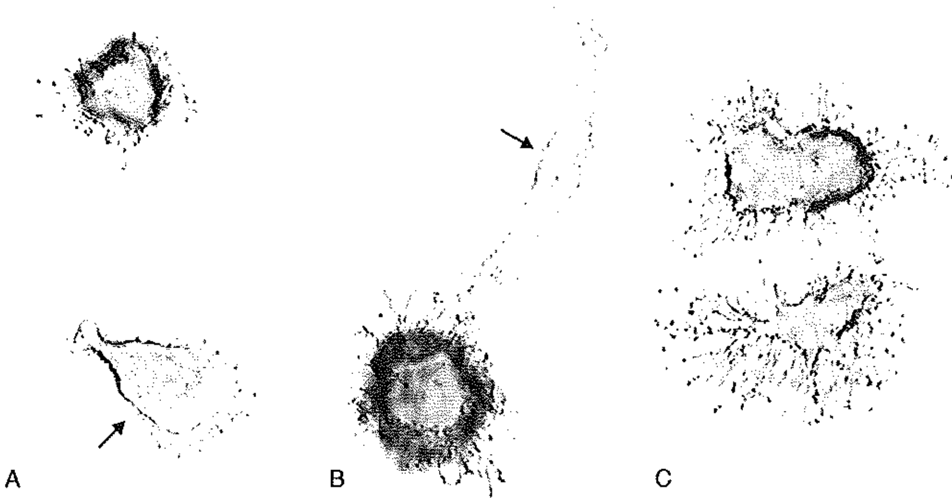

Figure 2. Immunocytochemical analysis of MHC class II expression on cells obtained from day 6 GM-CSF-stimulated cultures. DC-like cells (high level class II expression and typical dendritic morphology) from cultures grown from (A) ER-MP12^{hi}20^{-}, (B) ER-MP12^{+}20^{+}, and (C) ER-MP12^{-}20^{hi} bone marrow cells are shown. Macrophages were also present in the cultures (arrow) and differed from DC-like cells in morphology and class II expression (lower levels). Magnification x 590.

sive cells, as indicated by the sizes of the colonies/clusters formed, is the highest among ER-MP12^{hi}20^{-} cells and the lowest among ER-MP12^{-}20^{hi} cells. Together, our data suggest that GM-CSF-responsive bone marrow DC precursors follow a similar ER-MP12/20-defined developmental pathway as macrophage precursors, with the order ER-MP12^{hi}20^{-} $\rightarrow$ ER-MP12^{+}20^{+} $\rightarrow$ ER-MP12^{-}20^{hi}.

ER-MP12^{hi}20^{-} DC precursors show high level expression of ER-MP58

Previously we showed that the majority of ER-MP12^{hi}20^{-} early myeloid-committed cells can be purified from the other hematopoietic differentiation capacities present in this subset on the basis of differential expression of a third marker, ER-MP58[22]. Since we found that DC could also be generated from the ER-MP12^{hi}20^{-} bone marrow subset, we examined the ER-MP58 expression by these early DC precursors. For this purpose ER-MP12^{hi}20^{-} bone marrow cells were fractionated according to their level of ER-MP58 expression and cultured in the presence of GM-CSF. Cultures were examined by microscope for the presence of DC-like cells at days 5, 7 and 14. DC-like cells could only be detected in cultures of the ER-MP58^{hi} subset, starting from day 7 (Table 2). This onset of DC appearing in culture is similar to what is 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^{-} bone marrow subset are characterized by a high level ER-MP58 expression.

Table 2 DC-like cells generated from ER-MP12^{hi}20^{-} bone marrow subsets

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

[a)] During entire culture. [b)] From this subset only macrophages and immature cells were generated.

Bone marrow 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 bone marrow development, or, alternatively, follow a phenotypically similar, parallel developmental pathway, we set out to culture the ER-MP12^{hi}20^{-} bone marrow subset sequentially with M-CSF ("primary culture") and GM-CSF ("secondary culture"). In these cultures, M-CSF-responsive precursor cells are first induced to develop along the macrophage lineage *in vitro*[21]. The potential of these macrophage precursors to form DC-like cells was then assessed by changing the growth stimulus to GM-CSF. After 4 days of M-CSF-stimulated liquid culture of the ER-MP58^{hi} subset of ER-MP12^{hi}20^{-} bone marrow cells, about 90% of the cultured cells expressed the ER-MP20 antigen (Fig. 3A), confirming *in vitro* development along the macrophage lineage[21]. Changing the growth stimulus at this point to GM-CSF resulted in the appearance of DC-like cells in culture only 1 day later (Table 3). Apart from DC-like cells, macrophages (spindle-shaped or round plastic adherent cells) also developed in these cultures. After 9 days of GM-CSF-stimulated secondary culture, 28% of the cells expressed MHC class II molecules at a high level and had typical DC morphology, as determined in immunocytochemistry (Table 3, Fig. 3A). Thus, ER-MP12^{hi}20^{-}58^{hi} bone marrow cells developing for 4 days along the macrophage lineage in an M-CSF-stimulated culture still have the potential to generate DC-like cells.

When the ER-MP58^{med} subset of the ER-MP12^{hi}20^{-} bone marrow population was cultured for 4 days in the presence of M-CSF, about 70% of the cells expressed the ER-MP20 antigen (Fig. 3B), compared to 90% in cultures of the ER-MP58^{hi} cells of the ER-MP12^{hi}20^{-} subset (Fig. 3A). Upon subsequent GM-CSF-stimulated culture of the ER-MP12^{hi}20^{-}58^{med} cells, DC-like cells could be detected after 3 days of secondary culture (Table 3). After 9 days of GM-CSF-stimulated secondary culture, 28% of the cells had a high MHC class II expression and DC-like morphology (Table 3, Fig. 3B). Thus, after four days of primary culture of ER-MP12^{hi}20^{-}58^{med} cells in the presence of M-CSF, these cells also have the potential to generate DC-like cells. This is remarkable, as from ER-MP12^{hi}20^{-}58^{med} cells put directly into GM-CSF-stimulated culture no DC-like cells could be generated (Table 2). However, this discrepancy can be explained by taking into account the observed difference in maturation stage between ER-MP58^{med} and ER-MP58^{hi} cells of the ER-MP12^{hi}20^{-} subset (explained below).

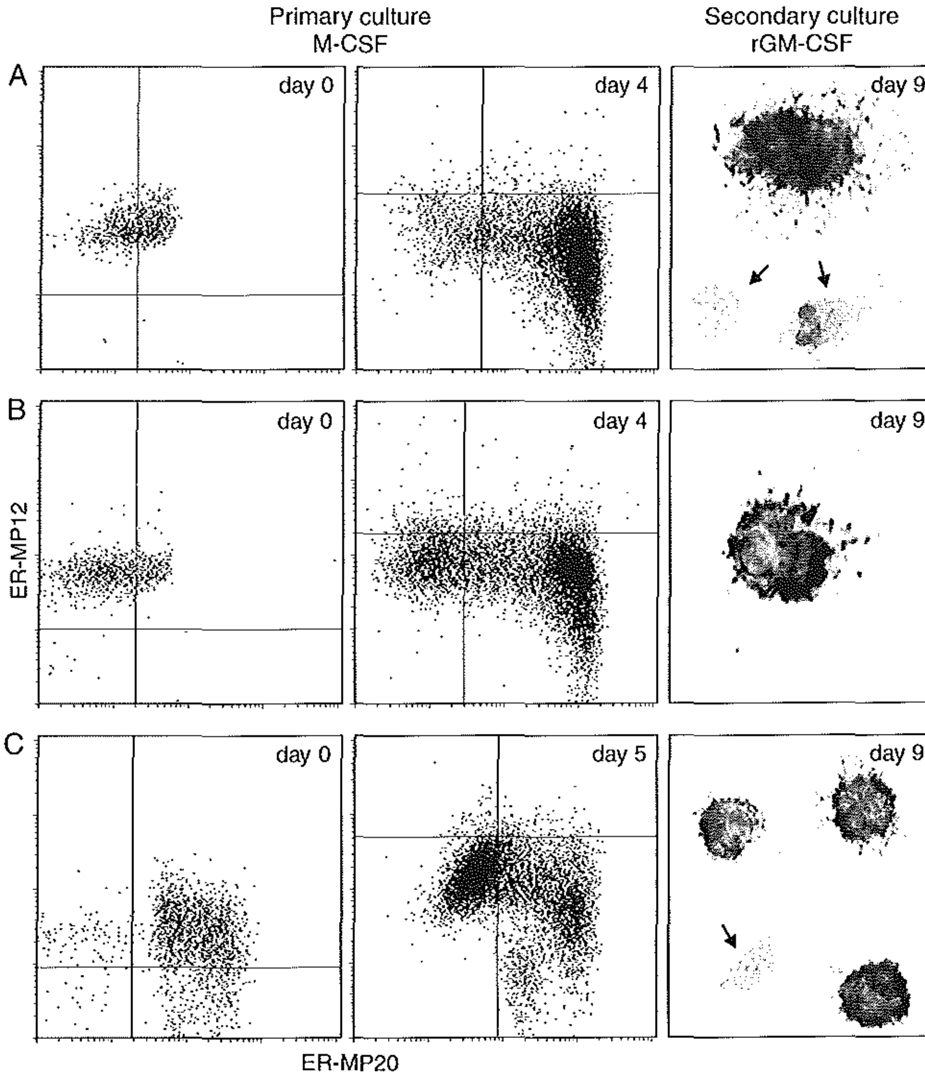

Figure 3. Development of (A) ER-MP12^{hi}20^{-}58^{hi}, (B) ER-MP12^{hi}20^{-}58^{med}, and (C) ER-MP12^{+}20^{+} bone marrow cells in primary M-CSF-stimulated and secondary GM-CSF-stimulated culture. Bone marrow subsets were sorted and cultured for 4 or 5 days in the presence of M-CSF-containing conditioned medium. 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. Negative control limits are shown in each dot plot (vertical and horizontal lines); upon culture, these control values increase due to increasing autofluorescence of the 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-like cells (high class II expression and dendritic morphology). Mononuclear phagocytes were also present in these secondary cultures (arrow). Magnification (A) and (C) x 670, (B) x 1000.

Several data indicate that the ER-MP12^{hi}20^{-}58^{med} cells that respond in these cultures are less mature than the ER-MP12^{hi}20^{-}58^{hi} cells. First, a previous study indicated that in the ER-MP12^{hi}20^{-} bone marrow subset, cells expressing ER-MP58 at a high level have differentiated further along the myeloid lineage than cells with intermediate ER-MP58 expression[22]. Second, the percentage of ER-MP20^{+} cells found in M-CSF-stimulated cultures of ER-MP58^{med} cells is lower than that in cultures of ER-MP58^{hi} cells (70 versus 90%, respectively). Since the ER-MP20 antigen is increasingly expressed upon maturation along the myeloid lineage[21,31], and culture conditions were identical for both the ER-MP58^{med} and ER-MP58^{hi} subsets, this difference in percentage positive cells supports the notion that ER-MP12^{hi}20^{-}58^{med} bone marrow cells are less mature than ER-MP12^{hi}20^{-}58^{hi} cells. As for optimal development along the monocyte/macrophage lineage about twice as much serum was required than for the generation of DC-like cells, we speculate that the higher serum concentrations, in combination with the M-CSF-containing conditioned medium used in the primary cultures induced maturation of early progenitor cells to or beyond the point that they first became responsive to pure rGM-CSF. This would explain the finding that ER-MP12^{hi}20^{-}58^{med} bone marrow cells do only respond to rGM-CSF after primary M-CSF-stimulated culture, while the more mature ER-MP12^{hi}20^{-}58^{hi} bone marrow cells respond in both rGM-CSF- and M-CSF-stimulated cultures.

The GM-CSF-stimulated secondary cultures of the ER-MP58^{med} cells differed from cultures of the ER-MP58^{hi} cells of the ER-MP12^{hi}20^{-} subset in the time of appearance of DC-like cells (day 3 versus day 1, respectively). This is in accordance with the notion that ER-MP12^{hi}20^{-}58^{med} cells lag behind in maturation compared to ER-MP12^{hi}20^{-}58^{hi} cells. Thus, cells obtained from ER-MP12^{hi}20^{-}58^{med} primary cultures need more time to generate DC-like cells upon secondary culture in GM-CSF. However, another explanation for the difference in time of appearance of DC-like cells could be that the ER-MP12^{hi}20^{-}58^{med} bone marrow subset contains less GM-CSF-responsive progenitors capable to develop into DC-like cells than the ER-MP12^{hi}20^{-}58^{hi} subset. This would also result in an extended culture period before detectable numbers of DC-like cells are reached. However, the finding that DC-like cells could not be generated directly from the ER-MP12^{hi}20^{-}58^{med} subset but only after M-CSF-stimulated primary culture argues against this explanation.

To test whether bone marrow precursors which had developed further along the macrophage pathway *in vitro* could still give rise to DC-like cells, we sorted the more mature ER-MP12^{+}20^{+} myeloid blast subset and cultured it for 5 days in the presence of M-CSF prior to secondary culture in GM-CSF. After M-CSF-stimulated culture approximately half of the cells expressed the ER-MP20 antigen (Fig. 3C). The other cells in culture were ER-MP20 negative. These ER-MP20^{-} cells represented mature macrophages, and not early immature cells like in the ER-MP12^{hi}20^{-} cultures, as (i) they were high in forward and perpendicular light scatter (data not shown), indicative of a complex cell type like the mature macrophage with its numerous vacuoles, lysosomes etc. and (ii) they showed a high autofluorescence level, also characteristic of mature macrophages. When the day 5 M-CSF-derived cells were further cultured in GM-CSF, DC-like cells could be detected after only one day of secondary culture and in increased numbers after three days of culture (Table 3). After 9 days of secondary culture, 71% of

Table 3 Development of DC-like cells from *in vitro* generated macrophage precursors

BM subset	Duration of primary M-CSF culture before switch to GM-CSF	DC-like cells visible in secondary GM-CSF culture (days after growth factor switch)		% of MHC II^{++} cells with DC morphology (immunocytochemistry)
		day 1	day 3	day 9
ER-$MP12^{hi}20^{-}58^{hi}$	4 days	±	+	28%
ER-$MP12^{hi}20^{-}58^{med}$	4 days	±?	+	28%
ER-$MP12^{+}20^{+}$	5 days	±	+	71%

the cells examined in immunocytochemistry had a high MHC class II expression and a morphology characteristic for DC (Table 3, Fig. 3C). Thus, also within a cell population that has developed further along the macrophage lineage, cells with the capacity to develop into DC-like cells are still retained. Remarkably, the percentage of DC-like cells derived from the M-CSF-stimulated ER-$MP12^{+}20^{+}$ cells is almost three times higher than that found with the ER-$MP12^{hi}20^{-}$ bone marrow subsets as starting populations (71% versus 28%). Most likely, this finding can be explained by the fact that the cells derived from the M-CSF-stimulated culture of the ER-$MP12^{+}20^{+}$ bone marrow subset are more mature than the cells derived from primary culture of the two ER-$MP12^{hi}20^{-}$ subsets. Upon secondary culture, the former cells cannot proliferate substantially anymore. Instead they either become adherent macrophages or non-adherent DC-like cells. This results in a relatively high percentage of DC-like cells in suspension. In contrast, most of the cells obtained from primary culture of the ER-$MP12^{hi}20^{-}$ subsets still have a high proliferative potential. Thus, in secondary cultures, many non-adherent progenitor cells are formed, next to non-adherent DC-like cells and adherent macrophages. Consequently, a lower percentage of DC-like cells is found starting from the ER-$MP12^{hi}20^{-}$ subset.

Summarizing, the data obtained in this study are in line with the hypothesis that over a prolonged stretch of mononuclear phagocyte development progenitors of the mononuclear phagocyte system may either develop into monocytes/macrophages or DC, depending on local concentrations of specific growth factors. However, additional clonal experiments are required to confirm this hypothesis. Furthermore, experiments verifying the DC nature of the DC-like cells generated in this study will have to be done, including the mixed lymphocyte reaction (MLR) and flow cytometric analysis of MHC class II expression and other markers such as NLDC-145. Yet, as the culture conditions used in our study are similar to those of studies in literature in which the MLR activity of the DC was demonstrated[32,33], we expect that the DC-like cells generated in our cultures will also prove potent stimulators in MLR. Finally, it would be interesting to test sorted ER-MP12/20/58 bone marrow subsets also for the presence of the lymphoid-related DC described by Wu et al.[11], as opposed to the presently studied myeloid DC.

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7 Bone marrow cellular composition in *Listeria monocytogenes* infected mice detected using ER-MP12 and ER-MP20 antibodies: a flow cytometric alternative to differential counting

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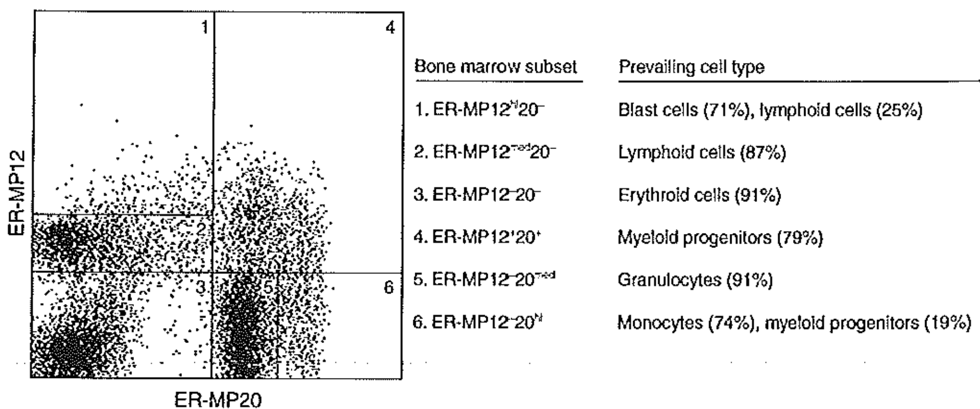
Abstract

Detailed assessment of bone marrow cellular composition is essential in evaluation of various experimental *in vivo* systems, such as expression of transgenes, null mutations and stimulation of host defence in infection. Traditional morphological analysis of mouse bone marrow is laborious, requires specific cytological expertise, and is somewhat subjective. As an alternative, we examined whether double labeling of bone marrow with the anti-precursor monoclonal antibodies ER-MP12 and ER-MP20 could be used for differential analysis by flow cytometry, as these antibodies divide mouse bone marrow into six relatively homogeneous cell populations. Following a sublethal infection of mice with *Listeria monocytogenes*, we monitored changes in cellular composition of the bone marrow at various time points by three methods: differential morphological count; single-color flow cytometric analysis using markers for the myeloid, erythroid and lymphoid lineages; and double labeling with ER-MP12 and ER-MP20. As expected, the bone marrow composition changed dramatically during infection, leading to an increase of myeloid cells which peaked after 1 week infection. Data determined by ER-MP12/20 flow cytometric analysis appeared to be in close agreement with both morphology and lineage marker analysis. In addition, ER-MP12/20 analysis provided more detailed information with regard to the presence of early myeloid precursors compared to lineage marker analysis. These data show that flow cytometric analysis of bone marrow using ER-MP12 and ER-MP20 monoclonal antibodies provides a relatively simple, rapid and objective method for assessment of the cellular composition in the bone marrow of the mouse.

Introduction

The bone marrow is the major site of adult hematopoiesis. Under steady state conditions, hematopoiesis is a continuous process in which a constant number of blood cells is generated each day. Perturbations in this process are reflected in the cellular composition of the bone marrow and in evaluation of various experimental systems, quantitative analysis of bone marrow composition is required. For instance, the expression of transgenes, null mutations or application of immunomodulatory agents may have profound effects on hematopoiesis[1-6]. Conventional methods for the assessment of bone marrow cellular composition are morphological analysis on May-Grünwald/ Giemsa stained bone marrow preparations or flow cytometric analysis of lineage-specific expression of cell surface molecules such as, in the mouse, B220 (B cells), CD3 (T cells), Gr-1 (myeloid cells) and TER-119 (erythroid cells). When more detailed information on the presence of early myeloid progenitor stages is required, differential counting has the advantage over lineage marker analysis. However, this particular method requires specific cytological expertise, is somewhat subjective and is time consuming.

In our search for novel markers for the identification of myeloid progenitor cells, we previously generated two monoclonal antibodies, ER-MP12 and ER-MP20, which in two-color flow cytometry identified different stages of myeloid progenitor cell development[7]. In addition, these monoclonal antibodies divided mouse bone marrow into six subsets with remarkable cell type homogeneity (Fig. 1)[7,8]. The ER-MP12^{hi}20^{-} subset consists primarily of blast cells, both morphologically undifferentiated blasts and blasts with lineage characteristics. The ER-MP12^{med}20^{-} subset contains mainly lymphoid cells and the ER-MP12^{-}20^{-} subset erythroid cells. The ER-MP12^{+}20^{+} population consists of myeloid blasts, morphologically undifferentiated blasts (most of them of the myeloid lineage as indicated by myeloid marker expression; unpublished observation) and few



Bone marrow subset	Prevailing cell type
1. ER-MP12^{hi}20^{-}	Blast cells (71%), lymphoid cells (25%)
2. ER-MP12^{med}20^{-}	Lymphoid cells (87%)
3. ER-MP12^{-}20^{-}	Erythroid cells (91%)
4. ER-MP12^{+}20^{+}	Myeloid progenitors (79%)
5. ER-MP12^{-}20^{med}	Granulocytes (91%)
6. ER-MP12^{-}20^{hi}	Monocytes (74%), myeloid progenitors (19%)

Figure 1. Cellular composition of ER-MP12/20 bone marrow subsets. Cytocentrifuge preparations of bone marrow subsets sorted on the basis of differential ER-MP12 and ER-MP20 expression were stained with May-Grünwald/Giemsa and prevailing cell type identified by differential cell counts[7,8].

vation) and few erythroid progenitors. Band forms and segmented cells of the granulocytic lineage are the prevailing cell types in the ER-MP12$^-$20^{med} subset, while monocytes form the vast majority of the ER-MP12$^-$20^{hi} bone marrow population. Together, these data suggested that ER-MP12/20 analysis of bone marrow could provide a new, simple, rapid and quantitative method for differential analysis of mouse bone marrow.

In the present study we aimed at validating the use of ER-MP12/20 subset analysis for assessment of cellular bone marrow composition in an experimental *in vivo* model. To this end, mice were infected with a sublethal dose of *Listeria monocytogenes* and at various time points during infection bone marrow was analyzed for ER-MP12/20 subset composition in two-color flow cytometry. These data were compared with lineage marker expression and differential morphological counts. Our results show that data on cellular composition obtained from ER-MP12/20 analysis correlate closely with data obtained from either lineage marker analysis or differential morphological counting. The advantage of ER-MP12/20 analysis over the conventional composition analyses is that it gives more detailed information on early precursor stages compared to lineage marker analysis, and that it is easier and faster than differential counting.

Materials and methods

Mice

Specified pathogen-free, 11 to 13 weeks old female C57BL/Ka mice were used (Harlan CPB, Austerlitz, The Netherlands). Experimental design and animal care were performed according to institutional guidelines.

Bacteria

Listeria monocytogenes strain EGD was used in this study (stored at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA), containing 10% (v/v) glycerol). Bacteria were grown in Todd-Hewitt broth for 16 hour at 37°C and preserved on ice. Prior to inoculating the mice, bacteria were washed twice in phosphate buffered saline (PBS).

Experimental infection caused by L. monocytogenes

Mice were infected by intravenous inoculation of $5x10^3$ CFU *Listeria monocytogenes*. Pilot experiments had indicated that this dose caused a severe, but sublethal infection. At t=0, 1 hour, 1, 2, 3, 7, 14, 21 and 35 days mice were killed by CO_2 exposure, and peripheral blood, spleen, liver and bone marrow were isolated to monitor the bacterial load in these organs. Peripheral blood samples were taken from the retro-orbital plexus. Serial 10-fold dilutions were prepared in phosphate-buffered saline (PBS) and from each dilution 0.2 ml was spread on a tryptone soya agar (TSA) plate (Oxoid Ltd., Basingstoke, England). Spleen and liver were homogenized in 20 ml PBS for 30 seconds at 10.000 rpm in a VirTis homogenizer (The VirTis Co. Inc., Gardiner, New York) as described earlier[9]. Serial 10-fold dilutions were made in PBS and volumes of 0.2 ml from each dilution and 2 ml of the undiluted homogenate were spread on TSA

plates. The remainder of the homogenate was poured into plastic dishes, together with an equal volume of double concentrated TSA. Bone marrow cell suspensions were prepared from both femora and tibiae as described[7]. Undiluted suspensions and serial 10-fold dilutions in PBS and undiluted suspensions were spread on TSA plates. After 48 hours of culture at 37°C, bacterial colonies were counted.

Phenotypic analysis of bone marrow composition (flow cytometry)

The monoclonal antibodies used in this study are listed in Table 1. MAbs were applied as hybridoma culture supernatants or as optimally titrated FITC (ER-MP20) or biotin (ER-MP12) conjugates[7]. Phenotypic analyses were performed as described previously[7]. In brief, for single-color analyses bone marrow cells were first incubated with hybridoma culture supernatant, washed, and subsequently incubated with rabbit anti-rat IgG $F(ab)'_2$ fragments conjugated to FITC (Cappel, Organon Teknika, Turnhout, Belgium). In two-color analyses, bone marrow cells were successively incubated with ER-MP12-biotin, followed by ER-MP20-FITC together with streptavidin-tricolor (Caltag Laboratories, San Francisco, CA). Incubations (10 minutes, room temperature) were performed in the presence of 20 mM NaN_3 to prevent antibody internalization. Between incubations cells were washed twice in PBS supplemented with 0.5% (v/v) bovine serum albumin (Boseral, Organon Teknika) and 20 mM NaN_3. Samples were analyzed using a FACScan (Becton Dickinson, Sunnyvale, CA) with CellQuest software (Becton Dickinson). For inter-experiment instrument standardization, microspheres of known fluorescence intensity (Flow Cytometry Standards, Research Triangle Park, NC) were measured in each individual experiment.

Table 1 Monoclonal antibodies and their specificities

Antibody	Antigen	Expression in bone marrow	Reference
ER-MP12	CD31, PECAM-1	Early hematopoietic progenitor and stem cells; lymphoid cells; no expression on late stages of erythroid, granulocytic and monocytic development	7,8,10,29-32
ER-MP20	Ly-6C	Increasingly expressed upon maturation of granulocytes and monocytes from colony-forming cell stages onwards; subset of T cells	21,29,33-36
RA3 6B2	B220	B cells and B precursors	37
KT3	CD3	T cells	38
TER-119	TER-119	Erythroid cells	39
RB6 8C5	Gr-1	Increasingly expressed upon maturation of granulocytes from the colony-forming stage; transiently expressed during monocytic development	36,40

Differential counts

Bone marrow cells were centrifuged onto microscopic slides in a cytocentrifuge. Preparations were air-dried and fixed in methanol (Labscan Ltd., Dublin, Ireland) at room temperature for 10 minutes. Afterwards, cytospins were stained with May-Grünwald/ Giemsa (Merck, Darmstadt, FDR) and differentially counted. Per time point, bone marrow preparations of two mice were analyzed morphologically.

Results

In the present study we examined whether two-color flow cytometric analysis of bone marrow with ER-MP12 and ER-MP20 monoclonal antibodies can be used as an alternative method for the assessment of cellular composition of mouse bone marrow. In an experimental infection with *Listeria monocytogenes*, bone marrow composition was determined by ER-MP12/20 analysis, analysis of lineage marker expression and differential counting. In the first part of this section, changes in ER-MP12/20 bone marrow subset composition during infection are shown. In the second part, ER-MP12/20 data are compared with lineage marker and morphological analysis, in order to validate the use of ER-MP12/20 analysis for assessment of bone marrow cellular composition.

Course of infection

To monitor the course of *Listeria monocytogenes* infection, peripheral blood, liver, spleen and bone marrow were screened for the presence of viable bacteria at various time points after i.v. inoculation with $5x10^3$ bacteria. Peripheral blood was sterile at all time points examined. The bacterial loads in liver, spleen and bone marrow are shown in Fig. 2. One hour after inoculation, bacteria were trapped in liver and spleen but were not yet present in bone marrow. The peak of infection was measured at 72 hours in all three organs. After 2 weeks the spleen was sterile again. In liver and bone marrow, bacteria were no longer detectable at week 5.

Extensive changes in bone marrow composition detected by ER-MP12/20 flow cytometric analysis during Listeria monocytogenes infection

Changes in bone marrow composition during infection, detected by ER-MP12/20 analysis, are shown in Fig. 3. One hour after injection of the bacteria, no changes in ER-MP12/20 profile were detected. At day 1, the percentage of ER-MP12^-20^{hi} cells, i.e. monocytes, was clearly diminished and the percentage of ER-MP12^-20^{med} cells, i.e. band and segmented granulocytes, also seemed decreased. This decrease in percentages of monocytes and granulocytes continued till day 3, with a final 70% decrease compared to control values. At the next time point measured, one week after infection, monocytes (ER-MP12^-20^{hi}) and late stages of granulocytic development (ER-MP12^-20^{med}) had returned, and comprised approximately half of the bone marrow. This return was preceded by an increase in the ER-MP12^+20^+ myeloid precursor population, from day 2 till day 7. Thus, one week after infection, bone marrow consisted almost

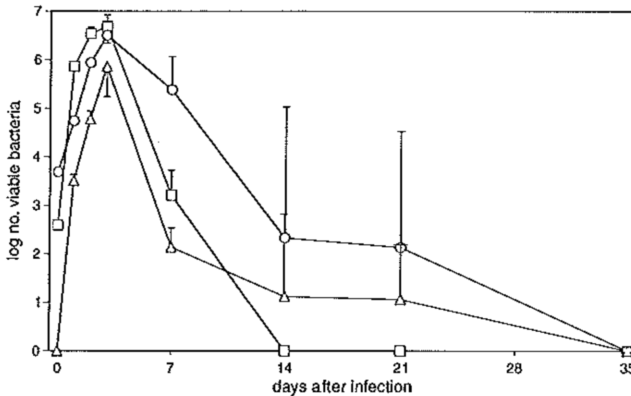


Figure 2. Load of *Listeria monocytogenes* in liver (O), spleen (□) and bone marrow (Δ). Mice were injected intravenously with 5 x 10^3 *Listeria monocytogenes*. At 1 hour and 1, 2, 3, 7, 14, 21 and 35 days after infection peripheral blood and organs were isolated to determine the number of viable bacteria present. Peripheral blood was found sterile at all time points tested. Data are the mean ± SD of 6 mice per time point. At day 14, the liver of 3 out of 6, and bone marrow of 4 out of 6 mice was sterile. At day 21, liver and bone marrow of 3 out of 6 mice were sterile.

exclusively of ER-MP12$^+$20$^+$, ER-MP12$^-$20^{med} and ER-MP12$^-$20^{hi} cells, i.e. immature and mature myeloid cells. Erythroid cells (ER-MP12$^-$20$^-$) and lymphoid cells (ER-MP12^{med}20$^-$) had disappeared within three and seven days after infection, respectively. Two weeks after infection a predominance of more mature myeloid cells (ER-MP12$^-$20^{med} and ER-MP12$^-$20^{hi}) was still seen, while the erythroid (ER-MP12$^-$20$^-$) and lymphoid (ER-MP12^{med}20$^-$) populations were being restored. Three weeks after infection, bone marrow composition was almost as in controls although an elevation in ER-MP12$^-$20^{med} granulocytes and ER-MP12$^-$20^{hi} monocytes was seen till 5 weeks after onset of the infection.

Comparison between ER-MP12/20 analysis and conventional analyses of bone marrow composition

To validate the use of flow cytometric ER-MP12/20 analysis as an alternative method for the assessment of cellular bone marrow composition, this method was compared with conventional composition analysis by lineage marker analysis and differential counting. For this purpose, bone marrow cells of each mouse were analyzed by flow cytometry also for the expression of the lineage markers Gr-1 (myeloid cells), B220 and CD3 (lymphoid cells), and TER-119 (erythroid cells). In addition, bone marrow of two mice per time point was differentially counted on May-Grünwald/Giemsa stained cytocentrifuge preparations. For comparison of the number of myeloid cells detected by each method, the total number of ER-MP12$^+$20$^+$, ER-MP12$^-$20^{med} and ER-MP12$^-$20^{hi} cells (representing primarily immature myeloid progenitors, band and segmented granulocytes, and monocytic cells, respectively) was compared with (i) the number of Gr-1$^+$ cells and (ii) the total number of myeloid cells as determined by differential

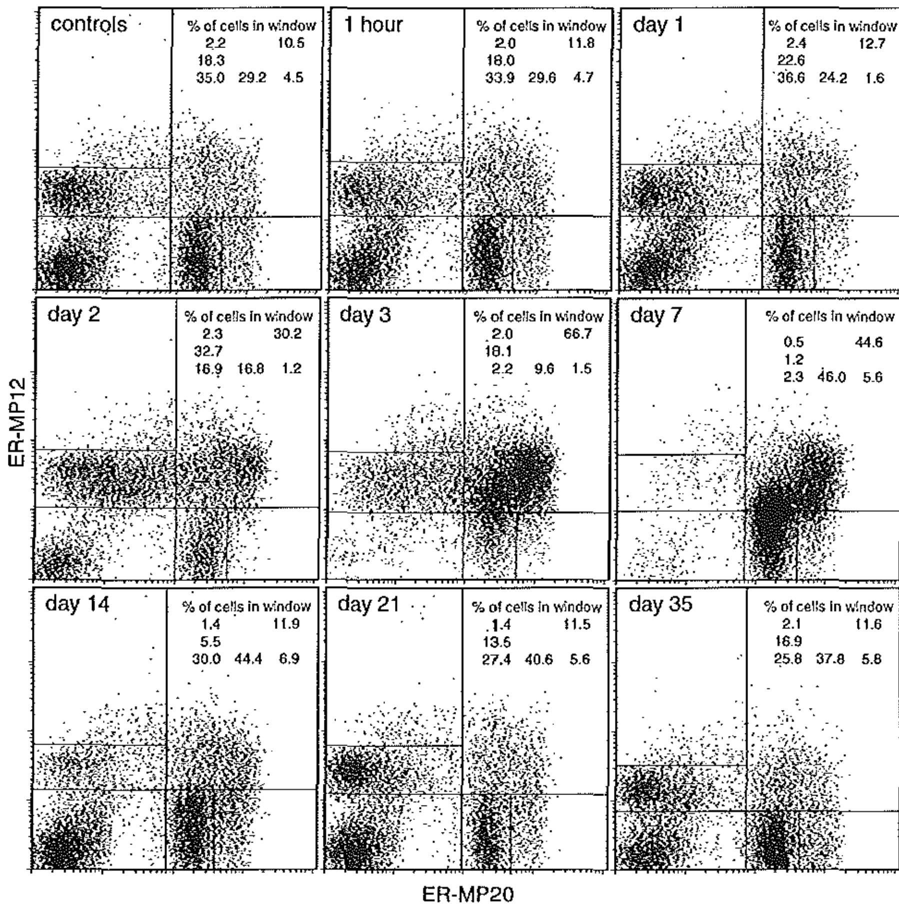


Figure 3. Flow cytometric analysis of ER-MP12 and ER-MP20 bone marrow subsets in the course of infection with *Listeria monocytogenes*. Gates used for analysis are shown; at each time point, gates were set on the basis of ER-MP12/20 expression in uninfected control mice. Percentages are the mean of 6 mice per time point.

counting (i.e. the total of immature myeloid progenitors, band form and segmented neutrophils, monocytes, and eosinophils). For comparison of the number of lymphoid cells, the total of ER-MP12^{med}20^{-} (mainly lymphoid cells) and ER-MP12^{hi}20^{-} cells (early blasts) was used. These ER-MP12^{hi}20^{-} cells were included in the lymphoid population as under steady state conditions more than half of these cells were found to express the B220 Ag, indicative of their B lineage identity[10]. In addition, this population was reported to contain progenitors of the B and T cell lineage[10-12]. The number of lymphoid cells obtained by ER-MP12/20 analysis was compared with (i) the total number of B220^{+} and CD3^{+} cells and (ii) the number of lymphoid cells determined by morphological examination of the bone marrow. For comparison of the

erythroid population size determined by the various methods, the number of ER-MP12$^-$20$^-$ cells was compared with (i) TER-119$^+$ cells and (ii) the number of morphologically identified erythroid cells.

Comparable bone marrow composition data obtained throughout infection using different analysis methods: In Fig. 4 the kinetics of myeloid, lymphoid and erythroid cell populations during infection are shown, as detected by each of the three analysis methods. Absolute cell counts were calculated from the myeloid, lymphoid and erythroid population frequencies and the total number of bone marrow cells obtained from each mouse (from 2 femora and 2 tibiae; Fig. 4A). Comparable changes in myeloid, lymphoid and erythroid cell numbers were detected by either ER-MP12/20 analysis, lineage marker analysis or differential counting (Fig. 4A-C). All three methods used showed an overall one-third decrease in total myeloid cells in bone marrow during

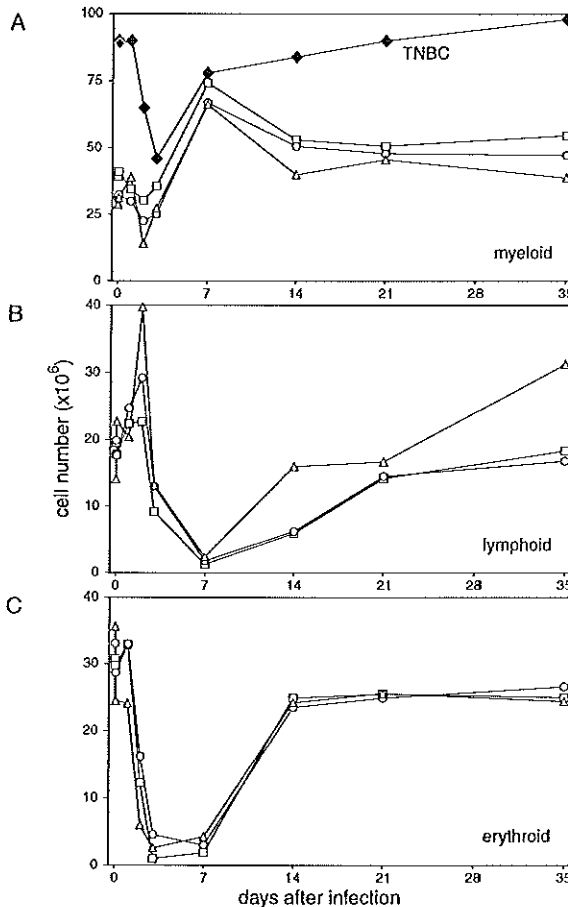


Figure 4. Changes in bone marrow composition in the course of infection with *Listeria monocytogenes* detected by ER-MP12/20, lineage markers, or differential counting. Myeloid (A), lymphoid (B) and erythroid (C) bone marrow population sizes as determined by ER-MP12/20 analysis (□), lineage marker analysis (○) and differential counting (Δ). Total nucleated bone marrow cell (TNBC) numbers are given at each time point for comparison in graph A (♦). Data are the mean of 6 mice per time point. Standard deviations ranged between 0.1 and 9.3 x 10^6 for subpopulation sizes and between 3 and 18 x 10^6 for total bone marrow counts (obtained from 2 femora and 2 tibiae of each mouse).

the first two days of infection (Fig. 4A). After this initial decrease, the number of myeloid cells increased and peaked at day 7 with approximately twice the control levels. All three methods showed that at this time point the bone marrow consists almost exclusively of myeloid cells (Fig. 4A-C). From day 7 onwards, myeloid cell numbers gradually decreased again, but were still slightly elevated at week 5 (Fig. 4A). After a strong decline at day 7, lymphoid cells had returned to control numbers by day 21 (Fig. 4B). Similarly, the number of erythroid cells, which was minimal at days 3 and 7, was largely restored again by day 14 (Fig. 4C). Thus, throughout the infection, data on bone marrow composition obtained from ER-MP12/20 analysis are comparable to the data obtained from lineage marker or morphological analysis.

High correlation between ER-MP12/20 analysis of bone marrow composition and lineage marker analysis or differential counting: Linear regression analysis showed a high and significant correlation between ER-MP12/20 analysis of bone marrow composition and data obtained by lineage marker analysis for all populations ($r > 0.9$, $p < 0.0001$; Fig. 5A). Percentages of myeloid, lymphoid, and erythroid cells obtained by differential counting also correlated strongly with percentages obtained by ER-MP12/20 bone marrow analysis ($r=0.87$, $p < 0.0001$; Fig. 5B). These data indicate that ER-MP12/20 bone marrow subset analysis can replace lineage marker analysis and differential counting in the measurement of total myeloid, lymphoid, and erythroid bone marrow cell populations.

Discussion

In the present study we aimed at validating the use of ER-MP12/20 two-color flow cytometric analysis as a simple, rapid and objective method for the assessment of cellular bone marrow composition in the mouse. For this purpose, bone marrow composition was determined by ER-MP12/20 subset analysis, lineage marker analysis and differential counting, under both steady state and experimental conditions. Sublethal *Listeria monocytogenes* infection was used as the experimental system as this is a well-characterized model in which significant shifts in myeloid progenitor cell numbers have been reported[13-16].

Major changes in both the relative and absolute numbers of myeloid, erythroid and lymphoid cells were observed in the first week of infection. In the first three days a decrease in ER-MP12^-20^{hi} monocytic and ER-MP12^-20^{med} granulocytic cells was seen. Previously, mobilization of monocytes and granulocytes into the circulation and tissues was demonstrated early in infection[17-20]. Thus, the decrease in ER-MP12^-20^{hi} and ER-MP12^-20^{med} bone marrow cells is probably due to an increased efflux of these cells into the bloodstream and peripheral tissues. The demand for myeloid cells in the periphery is reflected in the increased production of the immediate progenitors of these cells from day 2 to day 7 (ER-MP12^+20^+ expanded), leading to an almost complete myeloid bone marrow composition by day 7. This increase in myelopoiesis occurred initially at the expense of erythro- (day 2 to 7) and later also lymphopoiesis (day 3 to 7). From one week onwards, ER-MP12/20 bone marrow subset composition gradually

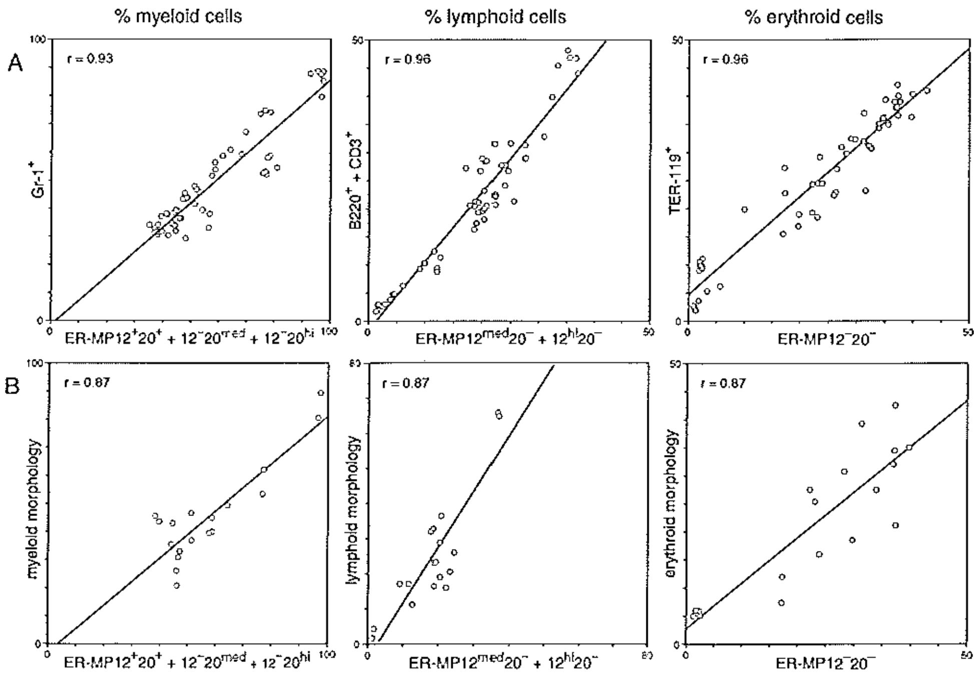


Figure 5. Diagrams showing linear regression analysis for the correlation between the percentages of myeloid, lymphoid, and erythroid cells determined by (A) ER-MP12/20 versus lineage marker analysis and (B) ER-MP12/20 analysis versus differential counting. Data are from a total of 54 (A) or 18 (B) mice. $p < 0.0001$ for all analyses.

returned to normal. Thus, in this model of *Listeria monocytogenes* infection, dramatic shifts in bone marrow composition are seen, allowing the examination of the validity of ER-MP12/20 subset analysis over a broad range of values.

Throughout the infection, data on myeloid, lymphoid, and erythroid population sizes obtained from ER-MP12/20 subset analysis were comparable to the data obtained from lineage marker and/or morphological analysis. This is substantiated by the high correlations found between ER-MP12/20 bone marrow subset and lineage marker analysis ($r > 0.9$), as well as ER-MP12/20 and morphological analysis ($r=0.87$). Together, these data validate the use of ER-MP12/20 bone marrow subset analysis as an alternative method to determine bone marrow composition. ER-MP12/20 bone marrow subset analysis has the advantage over lineage marker analysis that it gives additional information on shifts in myeloid progenitor subsets. For example, the decrease in the monocytic (ER-MP12$^-$20^{hi}) and granulocytic (ER-MP12$^-$20^{med}) subsets detected by ER-MP12/20 analysis at day 1 after infection could not be detected by analysis of Gr-1 expression. The advantage of ER-MP12/20 subset analysis over differential counting is that it does not require specific expertise on morphological recognition of mouse bone marrow cells and that it is less time consuming.

Small variations were observed between the myeloid and lymphoid population sizes determined either by ER-MP12/20 or lineage marker analysis. These variations are inherent to the methods used. Consistently, a slightly higher number of myeloid cells was obtained from ER-MP12/20 subset analysis compared to lineage marker analysis. This difference is most likely due to the fact that not all ER-MP12^+20^+ cells express the Gr-1 antigen (approximately 80% Gr-1-positive cells can be detected by flow cytometry, data not shown). In assessing the lymphoid population, ER-MP12/20 analysis occasionally results in lower numbers of lymphoid cells than does lineage marker analysis. This is especially the case early in infection, when a peak in lymphoid cells is seen (day 2). A conceivable explanation for this finding involves the nature of the ER-MP20 antigen, Ly-6C. Ly-6C has been shown to be expressed by a subset of T cells[21]. These Ly-6C (ER-MP20) expressing cells will not fall in the ER-MP$12^{hi}20^-$ and ER-MP$12^{med}20^-$ subsets used to determine the percentage of lymphoid cells. Thus, such T cells will be missed in the ER-MP12/20 analysis, but will be detected by CD3 expression. Although the percentage of Ly-$6C^+$ T cells is very low (total T cells already less than 4%) under steady state conditions, this percentage may increase under experimental conditions. This caveat should be kept in mind in cases where changes in T cell population sizes are expected.

In all three hematopoietic lineages, some variations were found between data obtained from ER-MP12/20 subset analysis versus differential counting during the first three days of infection. Later on, the lymphoid bone marrow population was overestimated compared to ER-MP12/20 subset and lineage marker analysis. Overall, data on bone marrow composition obtained on the basis of morphology differed more from ER-MP12/20 subset analysis than lineage marker analysis did. This is not surprising, as the latter two methods are based on the same technique, i.e. flow cytometric detection of cell surface markers. Compared to this technique, morphological analysis can lead to different results when for example the stage at which cells are first recognized differs between the methods.

The myeloid subsets distinguished by ER-MP12/20 analysis differ from the myeloid subsets distinguished in differential counting. In the latter method, myeloid cells were classified as either immature myeloid progenitors, band and segmented neutrophils, monocytes, or eosinophils. The ER-MP12^+20^+, ER-MP12^-20^{med} and ER-MP12^-20^{hi} subsets, however, represent phenotypically distinct developmental stages of the myeloid lineage. The ER-MP12^+20^+ subset was found to contain mainly immature cells (undifferentiated blasts and immature myeloid progenitors), as well as among others a few band and segmented cells and a few monocytes[7]. This subset contains clonable progenitors for both the granulocytic and monocytic lineages (ref. 7 and data not shown). The myeloid nature of the large majority of this subset was deferred from the finding that approximately 80% of the cells express the myeloid marker Gr-1 (unpublished observation). The ER-MP12^-20^{med} subset consists virtually exclusively of band and segmented neutrophils, end stages of neutrophilic development. No distinction can be made between band and segmented neutrophils on the basis of ER-MP12/20 expression levels. Cells of the monocytic/macrophage lineage, on the other hand, can be readily distinguished from granulocytic cells on the basis of their high level of ER-

MP20 expression (ER-MP12$^-$20^{hi}). Morphologically, approximately three quarters of this ER-MP12$^-$20^{hi} subset consist of monocytic cells and one quarter of immature myeloid progenitors. These immature myeloid progenitors are cells with a ring-shaped nucleus. Such cells (not found in humans) are traditionally assigned to the granulocytic lineage. However, recently a subtype of mouse bone marrow cells with ring-shaped nuclei was detected by H. Biermann, C. Sorg and C. Sunderkötter (personal communication). The morphological characteristic of this cell type is a small cytoplasmic center in the nucleus, with a diameter smaller than the width of the ring of nucleoplasm. Remarkably, several of such cells were found to express monocyte/ macrophage-related markers like ER-HR3, F4/80 and MHC class II. In contrast, these markers were absent from virtually all other cells with ring-shaped nuclei. These data suggest that (at least part of) this new cell subtype belongs to the monocyte/ macrophage lineage. Interestingly, examination of May-Grünwald/Giemsa stained cytospins of the sorted ER-MP12$^-$20^{hi} subset revealed that all cells with a ring-shaped nucleus present in this bone marrow fraction were of the mononuclear type with the small cytoplasmic center in the nucleus. This finding suggests that the ER-MP12$^-$20^{hi} subset contains progenitors of the monocytic lineage only. Taken together, the ER-MP12$^+$20$^+$, ER-MP12$^-$20^{med} and ER-MP12$^-$20^{hi} subsets represent different stages of myeloid development with a high lineage homogeneity rather than the different classes of myeloid progenitors identified by differential counting.

Remarkably, the ER-MP12^{hi}20$^-$ subset, which was also shown to contain early progenitors of the myeloid lineages[7], did not increase in the course of infection, while the other myeloid cell-containing subsets did. A possible explanation for this phenomenon could be that under these "hematopoietic stress" conditions, the ER-MP12^{hi}20$^-$ stage is a very short-lived stage, and that progenitors leave this stage shortly after commitment to the myeloid lineages. Increased production of myeloid cells would then only be reflected in an increase of myeloid progenitors at later stages in development. The observed increase in ER-MP12$^+$20$^+$ cells from day 2 to day 7 would fit with this hypothesis. Alternatively, under these inflammatory conditions, a phenotypically different route may be followed in which ER-MP12$^+$20$^+$ myeloid progenitors develop directly from ER-MP12^{med}20$^-$ [8] pluri- and multipotent stem cells. Confirmation of this notion awaits further experimentation.

How stable is the expression of ER-MP12 and ER-MP20 antigens by the various bone marrow subpopulations? Within the first two days of *Listeria* infection, significant increases of IFN-γ levels have been reported[22,23]. Increased levels of IFN-γ are known to have profound effects on the expression of certain cell surface markers. Thus, it could be argued that the observed decrease in the ER-MP12$^-$20^{hi} and ER-MP12$^-$20^{med} subsets in the first days of infection is not due to an efflux of these cells into the blood stream, but to an upregulated ER-MP12 expression as a result of increased IFN-γ levels. However, several findings argue against this possibility. First, at day 1 no increase in ER-MP12$^+$20$^+$ population size is observed, while this would have been expected if the total 8.5% decrease seen in the ER-MP12$^-$20^{hi} and ER-MP12$^-$20^{med} populations sizes would have been the result of elevated ER-MP12 expression levels. Second, in experiments using various ER-MP12-expressing cell lines we could not

upregulate ER-MP12 expression by either IFN-γ or several other stimuli (unpublished observations). Third, although ER-MP20 (Ly-6C) is readily inducible by IFN-γ on certain cell types[24,25], we did not observe increased expression of this marker on bone marrow cells in the present study. Together, these data argue against upregulation of ER-MP12 expression on bone marrow hematopoietic cells during infection with *Listeria monocytogenes*.

In several studies, ER-MP12 and/or ER-MP20 have been successfully used to detect changes in bone marrow composition under various experimental conditions[26-28]. To prevent inaccurate use of these antibodies we want to stress that only the combination of ER-MP12 and ER-MP20 in two-color flow cytometric analysis of bone marrow cell suspensions allows for the specific identification of (1) an early blast cell subset containing early progenitors for all hematopoietic lineages as well as some hematopoietic stem cell subsets (ER-MP$12^{hi}20^{-}$), (2) a lymphoid population, also containing hematopoietic stem cells (ER-MP$12^{med}20^{-}$), (3) an erythroid population (ER-MP$12^{-}20^{-}$), (4) a subset with mainly progenitors for both granulocytes and monocytes (ER-MP$12^{+}20^{+}$), (5) a granulocytic population (ER-MP$12^{-}20^{med}$) and (6) a monocytic (ER-MP$12^{-}20^{hi}$) population[7,8].

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8 Dietary n-3 fatty acids increase spleen size and post-endotoxin circulating tumor necrosis factor in mice; role of macrophages, macrophage precursors, and CSF-1

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Abstract

In experimental studies in mice, dietary supplementation with n-3 fatty acids (FA) alleviates inflammation and increases resistance to infection. Nevertheless, TNF production capacity was found to be increased in n-3 FA-fed mice. We previously found increased relative spleen weights in n-3 FA-fed mice. Here, the nature of this increased spleen size was further investigated. Spleen cellularity was significantly increased in mice fed n-3 FA (fish oil 15% w/w), compared to controls fed corn oil (15%) or normal lab chow ($p<0.05$). Experiments with T-cell deficient nude mice and experiments employing macrophage depletion through liposomal dichloromethylene-biphosphonate (Cl_2MDP), revealed that the increase in spleen cellularity is T-cell independent and largely due to macrophage accumulation in the spleen. Accumulation of marginal zone and red pulp macrophages was histologically and immunohistochemically confirmed. N-3 FA induced peripheral blood monocytosis and an aspecific increase in bone marrow cellularity. Post-endotoxin circulating TNF concentrations were significantly increased in n-3 FA-fed mice compared to controls. Splenectomy did not abolish this increase in circulating TNF. However, after macrophage depletion through liposomal Cl_2MDP, circulating TNF was not detectable after endotoxin challenge. Circulating concentrations of colony-stimulating factor-1 (CSF-1) did not differ between the various experimental groups. It is suggested that the cellular changes observed relate to increased constitutive production of TNF.

Introduction

N-3 fatty acids (FA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are poly-unsaturated FA in which the last double bond is located between the third and fourth carbon atom from the methyl end of the fatty acid chain. Dietary supplementation with n-3 FA results in modification of the fatty acid composition of the cell membranes, thereby reducing the production and biological activity of prostaglandins and leukotrienes[1]. More recently, dietary supplementation with n-3 FA has been associated with modulations in the production of the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), the principal polypeptide mediators of inflammation[2,3].

In experimental studies, dietary supplementation with n-3 FA has been shown to alleviate inflammation in various clinical disease states and in animal models[4-11]. Preliminary evidence indicates that dietary n-3 fatty acid supplementation may increase resistance to infection in multi-trauma patients[12]. N-3 FA increase survival following endotoxic shock or infections with live microorganisms in various animal models[13-18], and dietary n-3 FA increase IL-1α and TNF production capacity of mouse peritoneal cells[15,19,20]. In the course of our experiments in mice, we noticed a 1.5-fold increase in relative spleen weight in n-3 FA-fed mice compared to controls. Similar observations have been reported by other groups studying mice or rats[21-23].

To elucidate the mechanism of the enhanced resistance to infections and possibly relate this to the increased spleen weight in mice, we studied the effects of dietary n-3 FA on the cellular composition of spleens, peripheral blood and bone marrow. Also, the effects of dietary n-3 FA on circulating concentrations of endotoxin-induced TNF and steady-state colony-stimulating factor-1 (CSF-1) were investigated.

Materials and methods

Mice

In most experiments, 6 weeks old female Swiss mice, approximate weight 25 g were used. The animals were housed in plastic cages (type II and type III, Macrolon, Beyer en Eggelaar, the Netherlands), under specific pathogen free conditions at 23 ± 2°C and 7 ventilations/h. In some experiments, normal female Balb/c and T-cell deficient female Balb/c nude mice (nu/nu) were used. Nude mice were housed in cages covered with sterilized filter bonnets.

Diet

Mice were fed a fat-free standard reference diet as a dry powder, supplemented with 14% fish oil concentrate and 1 % corn oil (fish-oil group; FO), 15% corn oil (CO), or normal lab chow. The FO concentrate used was EPAX 3000 TG, a kind gift of Pronova, Bergen, Norway. The FO concentrate contained 37.1% n-3 FA, i.e. 17.7% EPA and 14.4% DHA (w/w). D-alpha tocopherol (0.67 mg/g) and a natural mix of tocopherols (0.525 mg/g) were added to avoid autooxidation. CO was a commercialy available preparation (Mazola). 1 % CO was added to the FO diet to avoid essential fatty acid deficiency. The FO concentrate was kept under strict anaerobic conditions until preparation of the diet to avoid

autooxidation. Diets were prepared at least twice per week, and kept at 4°C until administration.

Spleen analysis

The mice were anesthesized with ether and blood was taken from the retrobulbar vessels after eye extraction. The animals were killed by neck dislocation and spleens were removed and weighed. Total body weight was determined immediately before bleeding, and relative spleen weight was calculated afterwards. A suspension of spleen cells was made over a nylon filter, and cells were counted with a Coulter counter. The effect of dietary n-3 FA on spleen weight was determined in normal Swiss mice, normal Balb/c mice and T-cell deficient nude Balb/c mice (nu/nu).

Spleen cells were phenotypically characterized with flowcytometry using a set of monoclonal antibodies specific for mouse cells: 59-AD 2.2 detecting Thy 1 (T-lymphocytes), RA3 6B2 detecting B220 (B-lymphocytes), H129.19 detecting MT4 (CD4-positive T-lymphocytes), 53-6.72 detecting Ly-2 (CD8-positive T-lymphocytes), KT3 (CD3-positive T-lymphocytes), and F4/80 (macrophages). Details concerning these antibodies can be found in Leenen et al.[24].

After immunohistochemical staining of spleen sections with RA3 6B2 (anti-B220), a monoclonal antibody specific for mouse B-cells, the relative surface area of white pulp was quantitated using a Leitz Diaplan light microscope and a Videoplan image processing system.

To analyze the role of mature phagocytic macrophages in the increase in spleen size, mice were fed FO for 0, 2, or 4 weeks. Subsequently the animals were injected intravenously in the tail vein with 1 mg of liposomal dichloromethylene-biphosphonate (Cl_2MDP or clodronate), 24 h before LPS challenge, to deplete macrophages[25]. Control animals receiving the same diet were injected simultaneously with saline. Liposomal Cl_2MDP was prepared as described[25]. Spleen histology was studied in treated and untreated animals to verify macrophage depletion.

Peripheral blood cells

Peripheral blood cell count was performed by separately determining hematocrit and the total white blood cells count (using Coulter counter). Differential white blood cell count was performed by quantifying the light scatter profile of nucleated cells.

Bone marrow analysis

Bone marrow was obtained from both femora of each mouse by flushing the femur shafts with 3 ml culture medium (RPMI, Dutch modification, Flow, Irvine, Scotland). Nucleated cells were counted with a Coulter counter. Soft-agar cultures of bone marrow cells were stimulated with macrophage colony-stimulating factor (M-CSF) for 2 weeks and the number of macrophage precursors in the original preparation was quantitated by counting the number of macrophage colonies and clusters (<50 cells); macrophage colony forming cells (M-CFC), and macrophage cluster forming cells (M-clustFC) are given per 10^4 nucleated bone marrow cells (NBMC)[26]. NBMC were phenotypically characterized with flowcytometry using monoclonal antibodies detecting antigens of mouse macrophage and

granulocyte precursors. The monoclonal antibodies were produced at the Department of Immunology, Erasmus University, Rotterdam, Netherlands, as described[27]. The following monoclonal antibodies were used: ER-MP12 and ER-MP20, identifying subpopulations of macrophage precursors, ER-MP58, identifying myeloid cells, and ER-MP21, identifying transferrin-receptor expressing cells[28].

Post endotoxin circulating TNF

1.5 h after i.p. administration of 10 μg lipopolysaccharide (*E. coli*, serotype O55:B5, Sigma, St. Louis) (LPS), mice were anesthesized with ether and blood was taken from the retrobulbar vessels after eye extraction. Blood samples (approximately 1 ml) were mixed with 100 μl EDTA-solution (21.4 mg/ml EDTA in H_2O) and kept on ice until centrifugation at 1500 g during 5 min. Resultant plasma was isolated and stored at -20°C until assay.

TNFα was measured by ELISA using TN3, a hamster monoclonal antibody specific for murine TNFα and lymphotoxin, as described[29,30].

In separate experiments, the effect of splenectomy on post-endotoxin circulating TNF was studied. Splenectomy was performed after ether anesthesia in four week old mice, two weeks before the animals were started on the experimental diets.

CSF-1

CSF-1 was determined in unstimulated serum samples by specific RIA as described in detail elsewhere[31]. This assay is based on the competition by CSF-1 for the interaction between ^{125}I-labeled, purified mouse L cell CSF-1 glycoprotein with a rabbit polyclonal antibody to purified L cell CSF-1[32] and is more sensitive than the conventional CSF-1 bioassay based on bone marrow colony formation. Assays were carried out in duplicate on 20 μl samples. The concentration of CSF-1 in units/ml (1 unit=12 pg) was determined with reference to a standard curve prepared using a stable, partially purified L cell CSF-1 preparation.

Statistical analysis

Differences between groups were analyzed using the Kruskal-Wallis non-parametric ANOVA test, corrected for ties. Results were considered statistically significant at P-value $< .05$.

Results

Effects of FO supplementation on spleen cellularity

The effects of dietary n-3 FA on spleen, peripheral blood, and bone marrow cells are summarized in Table 1. The following experiments were done to elucidate the mechanisms of the increased spleen weight in FO fed mice. After four weeks of dietary supplementation with FO or CO, experimental Swiss mice were compared with control animals on normal lab chow. FO fed mice had significantly increased relative spleen weights ($P = 0.01$) and spleen cellularity ($P = 0.02$). In separate experiments, time-effect relationships

in these responses were investigated. Mice were studied simultaneously after 0, 2, 4, and 6 weeks of dietary FO supplementation. Increases in spleen weight were most prominent after 2 weeks and appeared somewhat blunted after 6 weeks (Fig. 1A). Similar results were obtained in normal Balb/c mice. To investigate whether the increase in spleen size was accounted for or mediated by T-cells, T-cell deficient female Balb/c mice were fed FO for 0, 2, or 4 weeks. Relative spleen weight was significantly increased from 2 weeks on, indicating that the increase in spleen weight was independent of the presence of T-cells (results not shown).

Table 1 Effect of dietary n-3 FA on spleen, peripheral blood, and bone marrow cells

	Diet group			
	Fish oil	Corn oil	Normal	P
Spleen				
Relative spleen weight (mg/g)	5.9 ± 0.5	4.8 ± 0.3	4.6 ± 0.2	0.01
Spleen cellularity (x 10^8)	2.7 ± 0.2	2.0 ± 0.2	1.4 ± 0.5	0.02
B220 positive spleen cells (%)	41 ± 4	36 ± 2	31 ± 6	0.04
Peripheral blood				
Hematocrit	43 ± 2.6	42 ± 3.2	43 ± 1.6	NS
White blood cell count (x 10^6/mL)	12.2 ± 4.0	9.1 ± 1.9	7.9 ± 1.3	NS
Lymphocytes (%)	55 ± 8	69 ± 4	64 ± 5	NS
Granulocytes (%)	34 ± 8	25 ± 4	31 ± 6	NS
Monocytes (%)	11 ± 2	6 ± 1	5 ± 1	0.02
Bone marrow				
Cellularity (x 10^6)	24 ± 5	21 ± 7	13 ± 5	0.02
M-CFC (# macrophage precursors/10^4 NBMC)	58 ± 15	51 ± 17	77 ± 20	NS
M-clustFC (# macrophage precursors/10^4 NBMC)	94 ± 22	80 ± 18	98 ± 27	NS
ER-MP21^{hi} (erythroid cells) (%)	22 ± 5	25 ± 3	22 ± 5	NS
ER-MP58^{hi} (myeloid cells) (%)	50 ± 7	50 ± 2	54 ± 6	NS
ER-MP12^{hi}20$^-$ (committed progenitors) (%)	1.2 ± 0.3	1.7 ± 0.2	1.6 ± 0.5	NS
ER-MP12$^+$20$^+$ (immature myeloid precursors) (%)	5.4 ± 1.2	5.6 ± 0.5	5.0 ± 1.2	NS
ER-MP12$^-$20^{hi} (monocytes) (%)	5.7 ± 0.9	6.2 ± 0.8	5.7 ± 1.2	NS

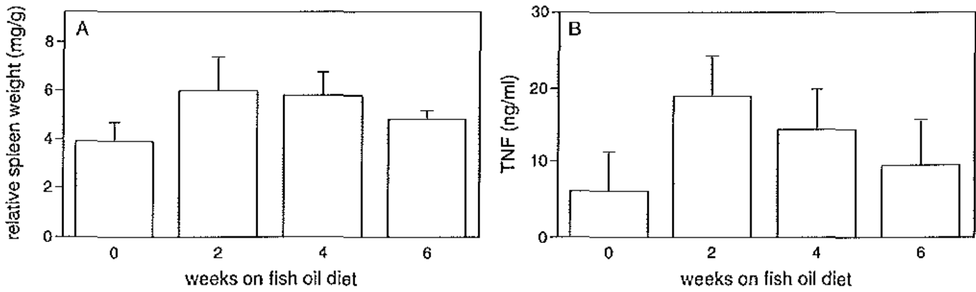

Figure 1. Time-effect relationships of dietary n-3 FA on relative spleen weight (A) and on post-endotoxin circulating TNF (B). Relative spleen weight was significantly increased from 2 weeks on fish-oil diet on, as was post-endotoxin circulating TNF (n=12).

Phenotypical characterization of spleen cells using a set of monoclonal antibodies, revealed no percentual differences in T-lymphocytes or macrophages between the various dietary groups. However, FO fed mice had a small but significant increase in B220 positive B-lymphocytes (Table 1).

Pretreatment with liposomal Cl_2MDP 24 h before endotoxin challenge did not significantly affect spleen weight. However, in mice that had been on FO diet for 2 or 4 weeks, liposomal Cl_2MDP resulted in a decrease in spleen weight (Fig. 2A). In these FO fed mice, treatment with liposomal Cl_2MDP resulted in spleen weights that were not significantly different from mice that had been on normal diet only. Depletion of marginal zone and red pulp macrophages was histologically confirmed but accurate quantitation of F4/80 positive macrophages was not possible in cell suspensions, where their numbers were equal in control and FO fed mice. However, for other reasons (see below), the results indicate that the increase in spleen weight in FO fed mice is mainly attributable to an increase in the splenic macrophage population that is depleted by pretreatment with lipo-somal Cl_2MDP.

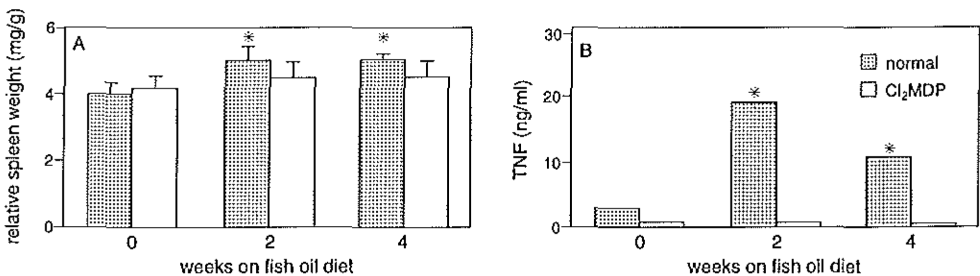

Figure 2. Balb/c mice were fed fish oil for 0, 2, or 4 weeks. At the end of the diet period, subsets of mice were injected i.v. with liposomal Cl_2MDP 24 h before LPS challenge. In contrast to mice injected with saline, animals injected with liposomal Cl_2MDP did not show a significant increase in relative spleen weight following 2 or 4 weeks of fish-oil supplementation (A). Post-endotoxin circulating TNF was not detectable in the liposomal Cl_2MDP-treated mice (B). * Indicates a statistically significant difference compared to mice that had no fish oil diet.

To a lesser extent, B-lymphocytes seem to contribute to the increased spleen cellularity in FO fed mice. Quantification of the relative surface area of red and white pulp in spleen sections, indicated a decrease in T and B-cell containing white pulp (Fig. 3). A significant number of B220-positive B-cells, however, is also present in the red pulp. We therefore conclude that FO fed mice had a significantly increased content of red-pulp area, containing mainly macrophages. F4/80 staining of spleen sections, identifying the red pulp areas, confirmed the relative increase of the red pulp area in the FO fed mice.

The effect of FO supplementation on peripheral blood cells

To investigate a possible relation between circulating blood cells and changes in spleen cellularity, peripheral blood cells were quantitated. Hematocrit did not differ between the various treatment groups. White blood cells count was higher in FO fed mice, but this difference did not reach statistical significance. Analysis of the light-scatter profile of peripheral blood cells showed a significant increase in percentage of monocytes in FO fed mice ($P = 0.02$, n=6).

The effect of FO supplementation on bone marrow cells

Possible relations between changes in spleen and peripheral blood cellularity and the cellular composition of bone marrow were investigated. Bone marrow cellularity was significantly increased in FO fed mice compared with mice fed CO or normal lab chow. This increase in bone marrow cellularity was equally distributed over the various hemopoietic lineages, since differential flow cytometric analysis of bone marrow cells, using ER-MP12 and ER-MP20 monoclonal antibodies, showed no significant differences between the various experimental groups (Table 1). Moreover, soft agar cultures of bone marrow cells did not reveal a *relative* increase in macrophage precursors, measured as M-CFC or M-clustFC.

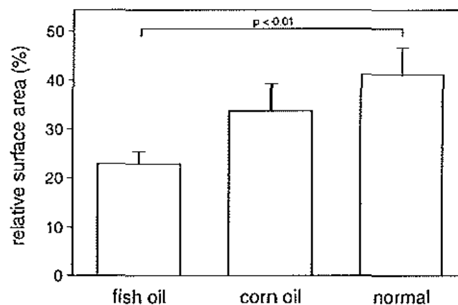

Figure 3. Spleen sections were stained for B220, an antigen specifically expressed by mouse B cells. The relative surface area of white pulp was quantitated using a Leitz Diaplan light microscope and a Videoplan image processing system. The boundaries of white pulp areas could be established easily since B 220 staining is confluent in the peripheral B cell zones of the white pulp but only scattered in the red pulp. Relative surface areas are expressed as mean ± SD.

The effect of FO supplementation on post-endotoxin TNF

To investigate the suggested increase in mononuclear phagocyte functionality, we measured circulating TNF concentrations after endotoxin challenge. Post-endotoxin concentrations of circulating TNF were significantly higher in FO-fed mice than in control mice. Post-endotoxin TNF was 10.4 ± 6.6 ng/ml in FO-fed mice, 2.2 ± 1.4 in CO-fed mice, and 2.7 ± 1.3 in mice fed normal diet (mean ± standard deviation, $P < 0.05$). In separate experiments, time-effect relationships in TNF production and FO diet were investigated. Similar to the increase in spleen weight, increases in post-endotoxin circulating TNF were most prominent after 2 weeks of FO diet, and appeared somewhat blunted after 6 weeks (Fig. 1B). Post-endotoxin TNF in T-cell deficient nude Balb/c mice was significantly increased from 2 weeks on, indicating that the increase in post-endotoxin circulating TNF was independent of the presence of T-cells (results not shown).

Splenectomy, performed 2 weeks before starting on the FO supplemented diet, did not influence the increase in post-endotoxin circulating TNF concentrations after 2 and 4 weeks of diet: at both timepoints, post-endotoxin circulating TNF concentrations were significantly increased compared to baseline (results not shown). At baseline and at 2 and 4 weeks of dietary FO supplementation, post-endotoxin circulating TNF was not detectable in liposomal Cl_2MDP-treated mice (Fig. 2B). These observations suggest that splenic macrophages, despite their accumulation after dietary FO supplementation, are not the most important contributors to the production of circulating TNF. On the other hand, the macrophage population that is depleted by liposomal Cl_2MDP and that does not reside in the spleen, appears to be the most important producer of post-endotoxin circulating TNF.

The effect of FO supplementation on circulating concentrations of CSF-1

The mononuclear phagocyte growth factor, CSF-1, is required for the development of the majority of mouse macrophages[33]. To accurately measure circulating concentrations of CSF-1, serum samples were subjected to a mouse CSF-1 specific RIA. In biological samples, this RIA detects only biologically active CSF-1[32,34], including both glycoprotein and proteoglycan forms[35]. Circulating concentrations of immunoreactive CSF-1 did not differ between the various diet group: 702 ± 89 U/ml in FO-fed mice, 753 ± 96 in CO-fed mice, and 802 ± 158 in mice fed normal diet (mean ± standard deviation, $p = 0.40$).

Discussion

The present study shows that dietary n-3 FA supplementation has a significant effect on the generation and distribution of mononuclear phagocytes in mice: n-3 FA induce a generalized increase in bone marrow cellularity, peripheral blood monocytosis, and accumulation of macrophages in the spleen leading to an increase in spleen size. What could be the mechanism of these n-3 FA-induced changes?

The aspecific increase in bone marrow cellularity without preferential stimulation of monocytopoiesis is in accordance with our observation on CSF-1: this specific monocyte growth factor was not increased in the n-3 FA fed mice. The generalized increase in bone marrow cellularity suggests that earlier, broad spectrum hematopoietic growth factors such

as interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are involved. The production of these factors is largely regulated by pro-inflammatory cytokines including IL-1[36,37]. We have previously shown that IL-1α production capacity is increased in mice after dietary n-3 FA[15]. Increased constitutive production of this cytokine may be involved in the increased bone marrow cellularity observed in the present study. Moreover, Pelus and others have shown that PGE_2 has an important modulatory effect on hematopoiesis in mice: exogenous administration of PGE_2 reduced nucleated bone marrow and splenic cellularity, while blockade of PGE_2 biosynthesis increased bone marrow and splenic cellularity, especially in the presence of IL-1α[38-41]. These observations may partly explain the results obtained in the present study, since dietary supplementation with n-3 FA in mice basically leads to increased IL-1α and a decreased PGE_2 production capacity.

Peripheral blood monocytosis induced by dietary n-3 FA has not been described before, but may be induced through a feed-back mechanism following the accumulation of macrophages in the red pulp of the spleen. It is apparently not due to increased concentrations of CSF-1. This is in accordance with the observation that the number of red pulp macrophages is only slightly reduced in CSF-1 deficient op/op mice[42]. Other factors, including IL-3, GM-CSF, and the pro-inflammatory cytokines may be considered. The results of our experiments with macrophage depletion using liposomal Cl_2MDP suggest that the observed increase in spleen size following dietary FO supplementation is largely due to accumulation of macrophages in the spleen. The immunohistochemical staining of spleen sections with B220 showed a decrease in the relative surface area of white pulp in the FO-fed mice, thereby supporting the concept of accumulation of red-pulp macrophages in FO-fed mice. As regulator cells, macrophages are responsible for the growth of lymphoid organs such as the spleen. Since TNF is an important growth factor for lymphoid organs, increased endogenous TNF production in FO-fed mice may contribute to this effect[43]. The phenotypical characterization of spleen cell suspensions using a set of monoclonal antibodies is not suitable for the assessment of the number of macrophages in the spleen, since especially tissue-fixed macrophages are lost in the process of making a cell suspension over a nylon filter. We therefore regard the concept that dietary FO supplementation leads to macrophage accumulation in the spleen as valid.

The increase in spleen size after dietary n-3 FA appeared to be accompanied by increased circulating concentrations of post-endotoxin TNF. We and others have previousy shown that peritoneal macrophages of mice fed n-3 FA have increased TNF production capacity at the level of the single cell[15,20,44]. Since splenectomy did not affect the increased TNF concentrations following dietary n-3 FA, macrophage accumulation in the spleen is not responsible for the increased TNF concentrations. Interestingly, macrophage depletion by liposomal Cl_2MDP completely abolished post-endotoxin circulating TNF. It might be expected that Kupffer cells of the liver, due to their sinusoidal location, might be a major target for the uptake of liposomal Cl_2MDP. In fact, recent evidence indicates that macrophage depletion in mice results in a 50-70% reduction in TNF mRNA in the liver following endotoxin challenge[45]. Therefore, the Kuppfer cells may be the most important contributors to the production of post-endotoxin TNF. Similar to peritoneal macrophages, Kuppfer cells may have increased TNF production capacity at the single cell level

following dietary n-3 FA. We did not assess the number of Kupffer cells. The small increase in relative liver weight in FO-fed mice does not rule out a substantial increase in the number of Kupffer cells.

In conclusion, dietary n-3 FA supplementation in mice induces an aspecific increase in bone marrow cellularity, peripheral blood monocytosis, accumulation of macrophages in the spleen, and increased post-endotoxin circulating concentrations of TNF. The mechanism of these changes remains to be elucidated.

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9 General discussion

Background of the study

Mature macrophages form a phenotypically and functionally heterogeneous population of cells, widely distributed throughout the body. In the concept of the mononuclear phagocyte system, the extensive heterogeneity of these cells is thought to be generated at the level of the monocyte entering the tissue microenvironments[1]. However, throughout the years data have accumulated in favor of additional origins of mononuclear phagocyte heterogeneity, such as the generation of separate, self-maintained macrophage lineages already in ontogeny, or the existence of macrophage sublineages at the level of precursors in bone marrow or monocytes in blood[2-4]. Data suggesting the existence of distinct differentiation lineages in the bone marrow, each giving rise to different types of mature macrophages, have originally been reported by Bursurker and Goldman[5,6]. However, studies to examine this notion in more detail, have been seriously hampered by the limited characterization and low frequency of macrophage precursors, and, in general, myeloid-committed progenitors in the bone marrow. One way to approach this difficulty is to identify cell surface markers that can be used to isolate macrophage progenitors from mouse bone marrow by fluorescence-activated cell sorting. For this purpose, a panel of monoclonal antibodies has been generated previously against immortalized macrophage progenitor cells[7,8]. One of these antibodies, ER-MP58, was found to detect all M-CSF-responsive bone marrow cells, while two others, ER-MP12 and ER-MP20, were shown to detect phenotypic heterogeneity among macrophage precursors[8]. It was not clear, however, whether the observed heterogeneity in macrophage precursor phenotype in bone marrow reflected the existence of macrophage sublineages, or different stages in maturation along a linear differentiation pathway. The aim of the studies described in this thesis was (i) to isolate phenotypically distinct macrophage precursor subsets from mouse bone marrow using the antibodies from the previously produced panel in order to investigate the nature of the observed phenotypic heterogeneity, and (ii) to separate and purify myeloid/macrophage progenitors from bone marrow cell suspensions with other or broader hematopoietic differentiation potential, in order to facilitate studies on the processes involved in commitment to the myeloid/mononuclear phagocyte lineage.

Identification of macrophage progenitor subsets in mouse bone marrow on the basis of differential ER-MP12, ER-MP20, and ER-MP58 monoclonal antibody binding

To identify different subpopulations of macrophage precursors in the bone marrow of the mouse, the reactivity of the ER-MP12 and ER-MP20 monoclonal antibodies with M-CSF-responsive macrophage precursors was assessed in two-color flow cytometry. Using these antibodies, six phenotypically distinct bone marrow subsets could be discerned[9]. In chapter 3 we showed that M-CSF-responsive macrophage progenitors were confined to three of these subsets, the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi}, in a frequency of 1:8, 1:9, and 1:2 plated cells, respectively. Together these subsets comprised about 15% of nucleated bone marrow cells. Interestingly, the ER-

MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} M-CSF-responsive macrophage precursors differed in their proliferative capacity. The highest proliferative potential was found among ER-MP12^{hi}20$^-$ cells, and loss of proliferative potential appeared to coincide with an upregulation of ER-MP20 expression. These data were suggestive of a difference in maturation stage between the ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} subsets. This notion was confirmed by assessing the phenotypic development of the putatively least mature ER-MP12^{hi}20$^-$ precursors at different time points during M-CSF-stimulated culture. The developing cells successively passed through ER-MP12$^+$20$^+$ and ER-MP12$^-$20^{hi} stages before final maturation into mature macrophages. Therefore, we concluded that the three phenotypically distinct M-CSF-responsive bone marrow macrophage progenitor subsets most likely represent successive stages along a maturation pathway in the order ER-MP12^{hi}20$^-$ $\rightarrow$ ER-MP12$^+$20$^+$ $\rightarrow$ ER-MP12$^-$20^{hi}. To our knowledge, this combination of ER-MP12 and ER-MP20 monoclonal antibodies provides the first positive identification of discrete, successive stages of M-CSF-responsive bone marrow mononuclear phagocyte development.

In addition to M-CSF-responsive macrophage progenitors, both the ER-MP12^{hi}20$^-$ and ER-MP12$^+$20$^+$ subsets also contain immature hematopoietic cells with other functionally defined differentiation capacities. In the ER-MP12^{hi}20$^-$ subset these are: primitive hematopoietic stem cells (determined both in the CAFC and LTRA assay)[10], CFU-S-day 12 [10], progenitor cells with thymus-repopulating potential (chapter 4), cells capable to confer early peripheral blood reconstitution in the erythroid[10], and myeloid, B and T cell lineages (chapter 4) upon intravenous transfer into irradiated recipients, and CFU-C[10]. It should be noted that these hematopoietic cells are not confined to the ER-MP12^{hi}20$^-$ subset; the ER-MP12^{med}20$^-$ bone marrow population also contains these stem and progenitor cell subsets (ref. 10 and chapter 4). In addition, the ER-MP12$^+$20$^+$ subset contains also some CFU-C, but no more primitive progenitors[10]. Morphological analysis of the ER-MP12$^+$20$^+$ subset further showed that it contains recognizable precursors of the granulocytic, erythroid, and lymphoid lineages (chapter 3). However, as about 80% of the ER-MP12$^+$20$^+$ cells express the myeloid antigen Gr-1 (unpublished observation), the majority of this subset belongs to the myeloid lineages. Thus, although the ER-MP12^{hi}20$^-$ and ER-MP12$^+$20$^+$ bone marrow subsets are both highly enriched for macrophage precursors, additional cell surface markers are required to separate early macrophage precursors from other hematopoietic progenitors. We consider the ER-MP12$^-$20^{hi} subset of high lineage homogeneity, as (i) only monocytic cells and their putative precursors are found in this subset (discussed in chapter 7) and (ii) virtually all cells express the mononuclear phagocyte marker F4/80 (unpublished observations), which has been reported on late stages of bone marrow mononuclear phagocyte development[11].

As stated above, of the three macrophage precursor-containing bone marrow subsets, only the ER-MP12^{hi}20$^-$ subset contained early progenitor cells with other or broader than myeloid differentiation capacities. Since the presence of in particular multi- or pluripotent cells can seriously interfere in studies on early macrophage development, we searched for additional cell surface markers allowing the separation of myeloid-committed cells from other hematopoietic stem and progenitor cells present in the ER-

MP12^{hi}20^{-} subset. In chapter 5 we showed that by using ER-MP58 monoclonal antibody previously found to detect all M-CSF-responsive progenitors in mouse bone marrow[8], early myeloid-committed cells can be separated from other hematopoietic progenitor and stem cells residing in the ER-MP12^{hi}20^{-} bone marrow population. This conclusion is based on functional data obtained *in vitro* as well as *in vivo*. M-CSF- and GM-CSF-responsive cells were predominantly found in the subpopulation of ER-MP12^{hi}20^{-} cells with a high level ER-MP58 expression (at average 84% and 59% of all recovered M-CFC and GM-CFC, respectively). On the basis of this high ER-MP58 expression, these myeloid precursors could be separated from the large majority of cells with the capacity to develop along the B cell lineage (ER-MP58^{med}), the T cell lineage (ER-MP58$^{-/lo}$ or ER-MP58^{med}), and the erythroid lineage (ER-MP58^{med}). Primitive hematopoietic stem cells (CAFC-d28/35) were not found among cells with high level ER-MP58 expression. Thus, with three-color cell sorting using ER-MP58, combined with ER-MP12 and ER-MP20 monoclonal antibodies, mouse bone marrow cells committed to the myeloid lineage can be separated from progenitor cells with other and/or broader differentiation capacities.

One might argue that the ER-MP58 monoclonal antibody used to label the bone marrow cells blocks the antigen and thereby abrogates differentiation in the lymphoid and erythroid lineages. Such a phenomenon could underlie the apparent absence of lymphoid and erythroid progenitors from the ER-MP58^{hi} subpopulation of ER-MP12^{hi}20^{-} cells. Although we cannot exclude this possibility formally, several data argue against it. First, ER-MP12^{hi}20^{-} progenitor cells with proven capacity to develop along the lymphoid and erythroid lineages do express the ER-MP58 antigen, albeit at an intermediate instead of a high level (chapter 5). Second, the light scatter characteristics of the ER-MP58 subsets of the ER-MP12^{hi}20^{-} population correspond with the observed differentiation capacities: ER-MP58$^{-/lo}$ cells appear to be a population of small lymphocytic cells, while the ER-MP58^{med} and ER-MP58^{hi} cells are characterized by more blast-like light scatter characteristics. The somewhat higher sideward scatter of the ER-MP58^{hi} cells compared to ER-MP58^{med} cells is suggestive of a myeloid nature of the ER-MP58^{hi} cells (data not shown). Third, in total bone marrow, morphologically recognizable stages of lymphoid and erythroid development are characterized by a negative to low ER-MP58 expression, whereas cells in different stages of myeloid development express the antigen at a high level (chapter 5). Together, these data make it very unlikely that cells with a lymphoid or erythroid differentiation capacity would reside among ER-MP58^{hi} cells in the ER-MP12^{hi}20^{-} subset. However, definitive proof on this issue requires the functional characterization of the ER-MP58 antigen.

ER-MP12 monoclonal antibody recognizes PECAM-1/CD31

Only recently, the ER-MP12 antigen has been identified as the platelet endothelial cell adhesion molecule PECAM-1/CD31 [12]. PECAM-1/CD31 is a transmembrane glycoprotein of the immunoglobulin gene superfamily of cell adhesion molecules[13-16]. Its expression has been studied most extensively in human (see for a review Watt et al.[17]), where it has been reported on platelets[13,18,19], endothelial cells[20,21], neutrophils[14,18,22,23],

monocytes[14,18,22], bone marrow macrophages[24], T lymphocyte subsets[25,26], granulocyte-macrophage precursors[22], and CD34$^+$ hematopoietic progenitor cells in bone marrow[27] and cord blood[28]. In an inflammation model in the mouse, PECAM-1/CD31 was shown to be essential for the transendothelial migration of leukocytes[29]. However, the expression of PECAM-1 on mouse hematopoietic cells is less well documented than in human[30,31]. The work described in this thesis, together with previous studies using ER-MP12 monoclonal antibody[8,32,33] provide a comprehensive picture of ER-MP12/PECAM-1 expression in mouse bone marrow hematopoiesis. The majority of the most primitive hematopoietic stem cells (CAFC-day 28/35 and LTRC) expresses ER-MP12/PECAM-1 at an intermediate level[10]. Upon stem cell maturation, ER-MP12/PECAM-1 expression increases to a high level on the majority of less primitive progenitor cells (CAFC-day 5)[10] and most *in vitro* clonable progenitors (chapter 3 and ref. 10). Late stages of myeloid development (monocytes and band/segmented neutrophils) as well as late stages of erythroid development (polychromatophilic erythroblasts and normoblasts) do not, or only at a low level express the antigen (chapters 3 and 7; ref. 10). In human, downregulation of PECAM-1 expression has also been reported for the postproliferative stages of granulocyte development, although in human PECAM-1 continues to be expressed on those cells[23]. In mouse T cell development, ER-MP12/PECAM-1 is expressed at a high level on cells with the capacity to home to and repopulate the thymus of irradiated mice (chapters 4 and 5), and at an intermediate level on mature T cells[10]. During B cell development, ER-MP12/PECAM-1 expression is transiently upregulated in the CFU-pre-B stage (chapter 5). However, the large majority of B220-positive cells in the bone marrow is characterized by an intermediate ER-MP12/PECAM-1 expression level (chapter 4 and ref. 10). In conclusion, the pattern of PECAM-1 expression in the mouse hematopoietic system as detected by ER-MP12 monoclonal antibody staining in general confirms and extends the data on PECAM-1 expression in human. Differences in epitopes recognized by anti-human and anti-mouse PECAM-1 monoclonal antibodies might explain discrepancies in expression levels detected on late stages of myeloid development in human versus mouse. Cell type-specific PECAM-1 epitope expression, as a result of differences in splice variant expression or glycosylation, have previously been reported[17]. In addition, such variations in epitope expression might also exist between human and mouse PECAM-1.

Based on the broad expression of this adhesion molecule among human hematopoietic and stromal bone marrow components, a central role for PECAM-1/CD31 in the regulation of hematopoiesis has been suggested[17,27]. First, PECAM-1 might be involved in the formation of the stromal cell - extracellular matrix network in which hematopoietic precursors develop[17]. Second, PECAM-1 could be involved in homotypic adhesion of hematopoietic progenitor cells to stromal macrophages which produce an array of hematopoietic regulatory molecules[34]. Alternatively, hematopoietic progenitors may bind in a heterotypic manner via PECAM-1 to heparan sulfate proteoglycans on stromal cells or in the extracellular matrix. Heparan sulfate is known to bind and thereby concentrate certain hematopoietic growth factors in the extracellular matrix[35,36]. Together, in this way PECAM-1 could be involved in guiding hematopoietic cells through the bone marrow microenvironmental niches that support early hematopoiesis.

It should be noted however, that in the mouse heterotypic adhesion has not yet been observed[15]. Third, PECAM-1 might be involved in regulating the release of mature hematopoietic progenitors from the bone marrow into the circulation[17,23]. Future experiments, e.g. *in vivo* antibody blocking studies or the generation of PECAM-1 knock out mice, will have to reveal the validity of these hypotheses.

ER-MP12 and ER-MP20 labeling of mouse bone marrow provides a flow cytometric alternative to differential counting

We found that the *combination* of ER-MP12 and ER-MP20 monoclonal antibodies divides mouse bone marrow into six subsets with remarkable cell type homogeneity (chapter 3 and ref. 10). These are (1) an early blast cell population containing early progenitors for all hematopoietic lineages as well as some hematopoietic stem cell subsets (ER-MP$12^{hi}20^{-}$), (2) a lymphoid population, which also contains hematopoietic stem cells (ER-MP$12^{med}20^{-}$), (3) an erythroid population (ER-MP$12^{-}20^{-}$), (4) a subset with mainly progenitors for both granulocytes and monocytes (ER-MP$12^{+}20^{+}$), (5) a granulocytic population (ER-MP$12^{-}20^{med}$) and (6) a monocytic (ER-MP$12^{-}20^{hi}$) population. This finding prompted us to examine whether ER-MP12/20 subset analysis could be used as an alternative method for the assessment of cellular bone marrow composition. In chapter 7 we demonstrated the validity of this method in an experimental *in vivo* model. Data on myeloid, lymphoid, and erythroid population sizes obtained from ER-MP12/20 subset analysis correlated strongly with data obtained from flow cytometric analysis of lineage marker expression or from morphological analysis of May-Grünwald/Giemsa stained bone marrow cytospins. The advantage of ER-MP12/20 subset analysis over differential counting is that it does not require specific expertise on morphological recognition of mouse bone marrow cells, that it is objective, and less time consuming. Compared to lineage marker analysis, ER-MP12/20 analysis gives additional information on subsets of myeloid progenitors, whereas this information is difficult to obtain by analysis using other markers, e.g. Gr-1.

The myeloid subsets discerned by ER-MP12/20 analysis differ from those distinguished by differential counting. By morphological analysis immature myeloid progenitors, band and segmented neutrophils, monocytes, and eosinophils are distinguished. The ER-MP$12^{+}20^{+}$, ER-MP$12^{-}20^{med}$ and ER-MP$12^{-}20^{hi}$ subsets however, represent phenotypically distinct developmental stages of the myeloid lineage, with a high lineage homogeneity in the ER-MP$12^{-}20^{med}$ (granulocytic) and ER-MP$12^{-}20^{hi}$ (monocytic) subsets, rather than the different classes of myeloid progenitors identified by differential counting. The ER-MP$12^{+}20^{+}$ subset contains colony- and cluster-forming cells of both the monocyte/macrophage and granulocyte lineages (chapter 3 and unpublished observations). As in the granulocytic ER-MP$12^{-}20^{med}$ subset no proliferation could be observed in GM-CSF-stimulated cultures (chapter 6), we conclude that this subset contains postproliferative (i.e. post-myelocyte) stages of granulocyte development only. Morphological analysis of this subset, revealing over 90% band and segmented neutrophils (chapter 3, ref. 10), supports this notion. In the monocytic ER-MP$12^{-}20^{hi}$ subset

only small (primarily < 5 cells) clusters were formed (chapter 3). As monoblasts have been reported to divide once to give rise to promonocytes, which in turn also divide once to give rise to monocytes[37], we conclude that the ER-MP12^{-}20^{hi} subset contains cells from the monoblast stage onwards, with the majority of this subset consisting of monocytic cells (chapter 3, ref. 10).

In chapter 8, ER-MP12/20 bone marrow subset analysis was used to determine cellular bone marrow composition in a study on the effects of dietary fish oil supplementation in mice. Dietary fish oil supplementation has been shown to enhance resistance to infections in various animal models[38]. To elucidate the mechanism of enhanced resistance to infections, the effect of dietary fish oil on the cellular composition of, among others, bone marrow was examined. An increase in bone marrow cellularity was observed in mice fed fish oil, compared with mice fed normal lab chow. However, no changes in bone marrow composition were detected by ER-MP12/20 or lineage specific marker analysis. Moreover, in agreement with the ER-MP12/20 subset analysis, M-CSF-stimulated soft agar cultures of bone marrow cells did not reveal changes in macrophage colony- or cluster-forming cell frequency. Thus, dietary fish oil supplementation in mice induced an aspecific increase in bone marrow cellularity, without changing its composition. It should be mentioned that ER-MP12/20 subset analysis (as well as lineage marker and morphological analysis) relies on phenotypic characteristics of bone marrow cells. Thus, when in an experimental model no changes in cellular composition can be detected by ER-MP12/20 (or conventional) bone marrow analysis, this does not formally exclude functional alterations of the cells. Nevertheless, the data on bone marrow composition obtained in chapter 8 extend and support the use of ER-MP12/20 subset analysis as an alternative method to determine cellular bone marrow composition in experimental mouse models.

Does mononuclear phagocyte heterogeneity originate already at the level of precursor cells in the bone marrow?

To approach the question whether mononuclear phagocyte heterogeneity is generated also at the level of precursor cells in the bone marrow, we set out to identify macrophage precursor cells in mouse bone marrow. In this study, we showed the existence of three phenotypically distinct macrophage precursor subsets, detected on the basis of their differential ER-MP12 and ER-MP20 antibody binding. Upon examination of the nature of the observed phenotypic heterogeneity, it was found that these subsets reflect successive stages of M-CSF-responsive mononuclear phagocyte development (chapter 3). Thus, the phenotypic heterogeneity detected among macrophage precursors with ER-MP12 and ER-MP20 monoclonal antibodies in two-color flow cytometry, does not reflect distinct precursor subsets which will give rise to different lineages of mature macrophages. However, these data do not imply that such precursor subsets are nonexistent. Rather, the ER-MP12 and ER-MP20 monoclonal antibodies provide new tools to search for macrophage precursor heterogeneity within three discrete, successive stages of bone marrow mononuclear phagocyte development. In a preliminary study, we

have detected in three-color flow cytometry additional phenotypic heterogeneity among the monocytic ER-MP12^{-}20^{hi} subset, using several of the mononuclear phagocyte markers described in chapter 2 (unpublished data). It will be interesting to extend this study and investigate whether separate precursors, e.g. for DC, can be identified in this way. In analyses of the ER-MP12^{hi}20^{-} subset, the ER-MP58 monoclonal antibody should be included to separate the myeloid-committed progenitors from multi- or pluripotent progenitor and stem cells present in this population (chapter 5). This is important as the presence of such multi- or pluripotent cells may influence the outcome of functional experiments with isolated bone marrow subsets.

Given the vast heterogeneity in the mononuclear phagocyte system, it is difficult to define strictly separated subsets of mature end cells, which is a prerequisite for unambiguous identification of lineage-specific precursors. However, the DC forms a good starting point to explore the existence of separate differentiation lineages within the mononuclear phagocyte system as this mature cell can be readily distinguished from other end stages of mononuclear phagocyte development on the basis of its characteristic "dendritic" morphology, high MHC class II expression and T cell-stimulating activity in the mixed leukocyte reaction.

So far, three possible precursors for DC have been suggested, which appear to exist next to each other. First, a lineage relationship with the myeloid lineage has been shown by identification of a common class II-negative granulocyte/macrophage/DC progenitor[39]. Beyond this stage, a lineage relation with the mononuclear phagocyte system was demonstrated by identification of bipotent macrophage/DC precursors along the myelomonocytic pathway[40-43]. Second, a separate DC progenitor has been reported in human[41,42,44]. This progenitor was shown to express CD1a as a specific marker and has been suggested to give rise to Langerhans cells[41,42]. Such a progenitor has not yet been identified in the mouse. Third, a lymphoid origin for DC has been reported[45,46]. In the mouse, lymphoid DC were recently shown to differ from myeloid DC in that they express CD8α[46]. These CD8α-positive DC were found to induce only a limited T cell proliferation compared to CD8α-negative DC[47] and may even induce apoptosis in CD4^{+} T cells[48]. Thus, DC are specialized end cells of the mononuclear phagocyte system. In addition, other pathways for DC development exist for specific types of mature DC.

As different stages of myeloid development can be isolated from other hematopoietic cells on the basis of ER-MP12/20/58 fractionation of bone marrow, we were interested in examining in which of the ER-MP12/20/58-defined bone marrow subsets GM-CSF-responsive DC precursors reside (chapter 6). Microscopic inspection of cellular morphology and immunocytochemical analysis of MHC class II expression revealed that DC-like cells were generated from bone marrow subsets containing precursors of the macrophage lineage (i.e. ER-MP12^{hi}20^{-}58^{hi}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi}, while no such cells could be grown from the lymphoid, erythroid, or granulocytic subsets. These data suggest that bone marrow DC and macrophage precursors follow the same ER-MP12/20/58-defined maturation pathway. Indeed we showed that cells with the capacity to differentiate into DC-like cells are still present in day 4 to 5 M-CSF-stimulated cultures of sorted ER-MP12/20 bone marrow subsets. These experiments are in line with the hypothesis that over a prolonged developmental

stretch precursors of the mononuclear phagocyte system may either develop into monocytes/macrophages or DC, depending on local concentrations of specific growth factors. However, as no clonal assays were applied in this study, it is also possible that the DC-like cells are (in part) generated from a separate DC-precursor with an ER-MP12/20 phenotype identical to the developing mononuclear phagocytes. Conclusive data on this issue require further experimentation.

Early ER-MP12^{hi}20^{-} GM-CSF-responsive DC precursors can be separated from lymphoid progenitors present in that subset on the basis of differential ER-MP58 expression (chapters 5 and 6). Therefore it will be interesting to test the ER-MP58$^{-/lo}$, ER-MP58^{med} and ER-MP58^{hi} subsets of the ER-MP12^{hi}20^{-} bone marrow population for the presence of lymphoid-related DC precursors[46], as opposed to the currently studied myeloid-related DC precursors. Possibly, progenitors for lymphoid-related DC can be separated from those of myeloid-related DC on the basis of differential ER-MP58 expression.

Conclusion and future directions

The studies conducted in this thesis have advanced the phenotypic identification of early stages of mononuclear phagocyte development. On the basis of differential ER-MP12 and ER-MP20 expression three successive stages in development can currently be isolated from mouse bone marrow: *i.e.* ER-MP12^{hi}20^{-} $\rightarrow$ ER-MP12^{+}20^{+} $\rightarrow$ ER-MP12^{-}20^{hi}. In addition, with three-color cell sorting using ER-MP58, combined with ER-MP12 and ER-MP20 monoclonal antibodies, early *in vitro* colony-forming myeloid-committed cells can be separated on the basis of differential ER-MP58 expression from progenitor cells with other or broader differentiation capacities. To our knowledge, the combination of ER-MP12 and ER-MP20 monoclonal antibodies provides the first positive identification of discrete, successive stages of M-CSF-responsive bone marrow mononuclear phagocyte development. In addition, including ER-MP58 in the ER-MP12/20 combination allows for the separation of early myeloid-committed cells from other hematopoietic progenitors. We expect that the ER-MP12, ER-MP20 and ER-MP58 antibodies will prove valuable tools in (i) further elucidating the origins of mononuclear phagocyte heterogeneity, and (ii) studies on the processes involved in commitment to the myeloid/mononuclear phagocyte lineage. In addition, as bone marrow is divided into six subsets with remarkably high lineage homogeneity in two-color flow cytometry using ER-MP12 and ER-MP20 monoclonal antibodies, the combination of these two antibodies can be used as a relatively simple, rapid and objective method for the determination of cellular bone marrow composition. In Fig. 1 a summary of presently available data on ER-MP12, ER-MP20, and ER-MP58 antigen expression during hematopoietic differentiation in mouse bone marrow is presented.

Sublineages of the mononuclear phagocyte system

Multi-parameter flowcytometric analysis of bone marrow with ER-MP12/20/58 in com-

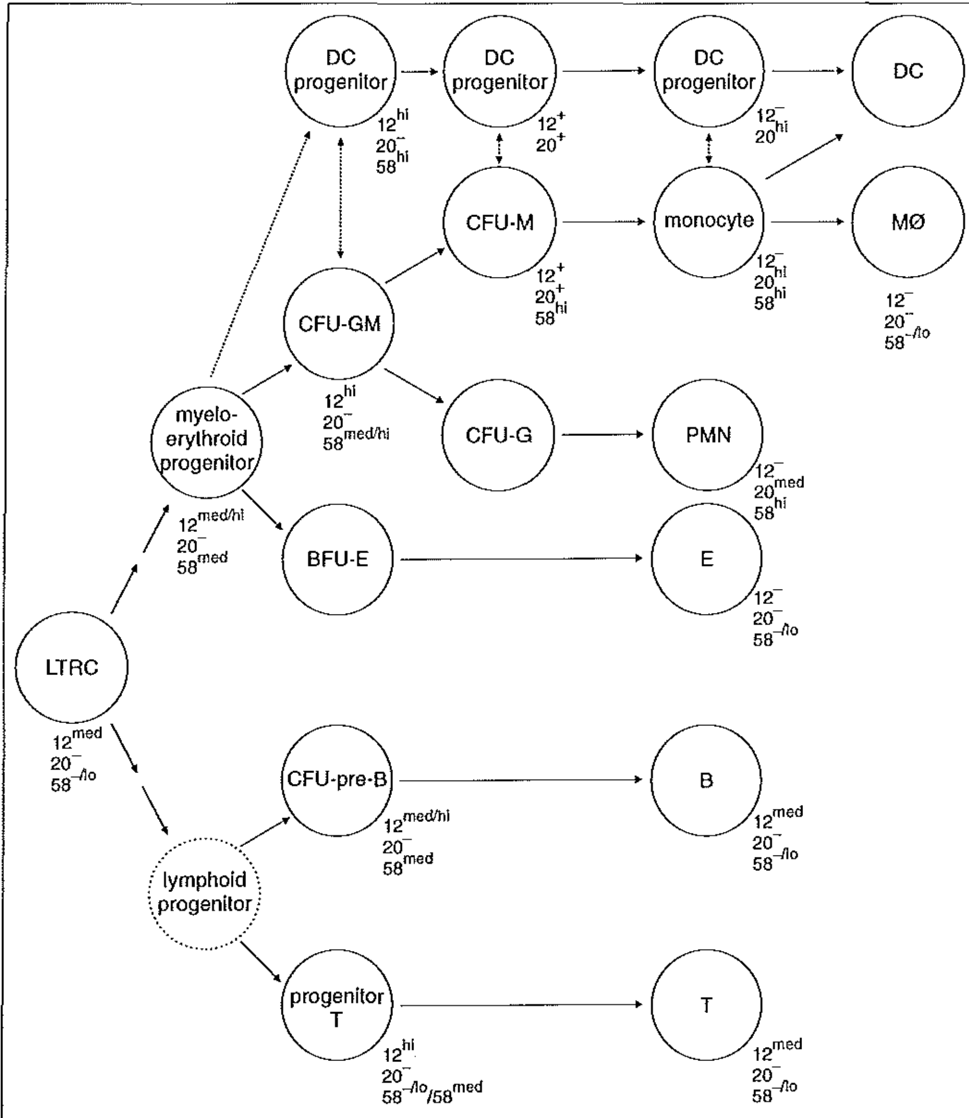

Figure 1. Schematic representation of differential ER-MP12, ER-MP20, and ER-MP58 expression during hematopoiesis in adult bone marrow, summarizing currently available data. LTRC: long-term repopulating cell; DC: dendritic cell; CFU-GM: colony-forming unit-granulocyte/macrophage; CFU-M: colony-forming unit-macrophage; Mϕ: macrophage; CFU-G: colony-forming unit-granulocyte; PMN: polymorphonuclear granulocyte; CFU-E: colony-forming unit-erythroid; E: erythrocyte; CFU-pre-B: colony-forming unit pre-B. CFU-pre-B could be generated from ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow subsets only. From the other ER-MP12/20 subsets no CFU-pre-B were grown (unpublished data).

bination with other, sublineage specific antibodies may lead to the identification of precursors of hematopoietic cells related to the mononuclear phagocyte system, e.g. precursors of DC[49,50], osteoclasts[51], natural killer cells[52-54], and CD5-positive B cells[55,56]. Identification of such precursor cells would facilitate studies on the processes involved in sublineage commitment, e.g. the role of specific cytokines and differentially expressed genes. Likewise, it will be interesting to search for separate precursors for bone marrow-derived macrophages which differ in e.g. antigen presentation, phagocytic capacity, cytotoxicity, cytokine production, or *in vivo* localization pattern after intravenous transfer into conditioned recipient mice[57,58].

In recent years many mutant mice have been described which show a defect in the mononuclear phagocyte system[59,60]. In several of these mice specific mononuclear phagocyte subpopulations are affected. For instance in the M-CSF -/- *op/op* mouse, peritoneal macrophages, osteoclasts and spleen methallophils are almost completely absent, while other macrophage populations are only partially or not at all affected[61,62]. Furthermore, mice with a null mutation in the *c-fos* proto-oncogene completely lack osteoclasts due to a block early in osteoclast differentiation[63]. Phenotypic examination of the bone marrow of these and other mutant mice may aid in the identification of possible sublineage specific precursor cells, as particular precursor subsets may be absent from such mice.

Myeloid commitment

We showed that on the basis of three-color cell sorting using ER-MP58, combined with ER-MP12 and ER-MP20 monoclonal antibodies, cells committed to the myeloid lineage can be separated from progenitor cells with other or broader differentiation capacities. At present, it is largely unknown what the crucial events are in lineage-commitment of hematopoietic progenitor cells. Therefore, identification of genes which are differentially and specifically expressed by ER-MP58^{hi} myeloid-committed cells versus other ER-MP58$^{-/lo}$ and ER-MP58^{med} progenitor cells, may shed light on the fate determining steps in commitment of progenitor cells. In addition, this approach probably will provide new functional markers for monitoring the differentiation of myeloid cells.

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Summary

Mature macrophages form a functionally and phenotypically heterogeneous population of cells widely distributed throughout the body. Several mechanisms contributing to mature macrophage heterogeneity have been suggested. These are (i) induction of heterogeneity by local conditions or stimuli encountered in peripheral tissues; (ii) the existence of self-maintained, bone marrow-independent macrophage populations generated early in ontogeny; and (iii) heterogeneity resulting from differences at the macrophage precursor level. At present, many questions still exist concerning the precise contribution of each of these mechanisms to mature macrophage heterogeneity.

In depth studies on the existence of distinct macrophage precursors in bone marrow have been seriously hampered by limited characterization and low frequency of macrophage precursors, and, in general, myeloid-committed progenitors in mouse bone marrow. Therefore, the aims of this thesis were (i) to identify different stages of bone marrow mononuclear phagocyte development using anti-macrophage precursor monoclonal antibodies previously generated in our laboratory, and (ii) to separate early myeloid-committed progenitor cells from other hematopoietic progenitors in order to facilitate studies on the processes involved in commitment to the myeloid/mononuclear phagocyte lineage.

In **chapter 2** a selection of the currently available anti-mouse mononuclear phagocyte antibodies is reviewed. These markers can be divided into four categories: (i) markers expressed by immature mononuclear phagocytes, (ii) markers expressed by mature macrophages in general, (iii) markers expressed by macrophage subsets, and (iv) markers expressed by IFN-γ-stimulated macrophages. Since very few antibodies are fully specific for one stage of macrophage development, combinations of these antibodies are often required to determine from the cellular phenotype whether macrophages are immature, mature, belong to a particular subset, or are stimulated or not. A more precise determination of the developmental stage of macrophages depends on a better insight into the developmental relationships between the various members of the mononuclear phagocyte system and the phenotypic hallmarks that identify related cells.

To extend the identification of macrophage precursors in mouse bone marrow we examined in **chapter 3** (i) whether the anti-macrophage precursor monoclonal antibodies ER-MP12 and ER-MP20 detected phenotypic heterogeneity among macrophage precursors in two-color flow cytometric analysis of mouse bone marrow, and, if so, (ii) whether that phenotypic heterogeneity reflected different maturation stages or the existence of macrophage sublineages. Upon double labeling of bone marrow with ER-MP12 and ER-MP20 antibodies, six phenotypically distinct bone marrow subsets can be discerned. Fluorescence-activated cell sorting followed by M-CSF-stimulated culture in soft agar revealed that macrophage precursors were confined to the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} bone marrow subsets. Moreover, we found evidence that the macrophage precursors present in these subsets represent successive stages along a maturation pathway with the order ER-MP12^{hi}20^{-} $\rightarrow$ ER-MP12^{+}20^{+} $\rightarrow$ ER-MP12^{-}20^{hi}.

In **chapter 4** we examined the ER-MP12/20 phenotype of cells with the capacity to home to and repopulate the thymus upon intravenous transfer into sublethally irradiated recipient mice. Our data indicated that cells with the capacity to repopulate the thymus within three weeks after transfer, reside in the ER-MP12^{hi}20^{-} subset and are to a lesser

extent found among ER-MP12^{med}20^{-} bone marrow cells. The other ER-MP12/20 bone marrow subsets do not contain such thymus repopulating progenitor cells. Analysis of peripheral blood reconstitution in mice transferred with the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets revealed that multipotent hematopoietic stem cells and/or progenitor cells of the B and myeloid lineages are also present among ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} bone marrow cells.

In **chapter 5** we examined whether the myeloid-committed cells found in the ER-MP12^{hi}20^{-} subset can be distinguished and purified from the other hematopoietic stem and progenitor cells residing in this subset. For this purpose, bone marrow cells were triple labeled with ER-MP12/20 and ER-MP58, an antibody detecting all M-CSF-responsive progenitors in mouse bone marrow. With this monoclonal antibody the ER-MP12^{hi}20^{-} population could be divided into three subfractions. On the basis of functional data obtained *in vitro* and *in vivo* we concluded that early myeloid-committed cells can be separated from other hematopoietic progenitor and stem cells on the basis of their high level ER-MP58 expression.

On the basis of the experiments described in chapters 3, 4 and 5, we concluded that the combination of ER-MP12/20 and ER-MP58 monoclonal antibodies can be used to isolate successive stages of mononuclear phagocyte development from mouse bone marrow. As GM-CSF-responsive DC precursors are at least in part linked to the monocyte/macrophage lineage, we investigated the ER-MP12/20/58 phenotype of bone marrow DC precursors. In **chapter 6** we showed that DC precursors responsive to GM-CSF *in vitro*, indeed have the same ER-MP12/20/58-defined phenotype as progenitors of the macrophage lineage. Moreover, we found that DC precursors follow the same ER-MP12/20-defined maturation pathway as macrophage precursors. The data obtained in this study are in line with the hypothesis that over a prolonged stretch of mononuclear phagocyte development, progenitors of the mononuclear phagocyte system may either develop into monocytes/macrophages or DC, depending on local concentrations of specific growth factors. However, additional clonal experiments are needed to confirm this hypothesis.

From the initial experiments described in chapter 3 we had learned that the six ER-MP12/20-defined bone marrow subsets show a remarkable hematopoietic lineage homogeneity. Therefore we set out to examine whether the combination of these monoclonal antibodies in two-color flow cytometry can be used as an alternative method to determine the cellular composition of mouse bone marrow under both steady state and experimental conditions (**chapter 7**). Changes in bone marrow cellular composition were monitored in a sublethal infection with *Listeria monocytogenes*. Data obtained from ER-MP12/20 bone marrow subset analysis were compared with data obtained from analysis of lineage marker expression and data from conventional morphological analysis. Bone marrow composition determined by ER-MP12/20 analysis appeared to be in close agreement with morphology and lineage marker analysis, indicating that ER-MP12/20 subset analysis provides a relatively simple, rapid and quantitative alternative for assessment of the cellular composition in the bone marrow of the mouse.

In **chapter 8**, ER-MP12/20 bone marrow subset analysis was used to determine cellular bone marrow composition in a study on the effects of dietary fish oil supple-

mentation in mice. Fish oil consumption has been shown previously to enhance resistance to infections in various animal models. Examination of the cellular composition of bone marrow was part of the analyses performed to shed light onto the mechanism(s) behind the enhanced resistance. An increase in bone marrow cellularity was observed in mice fed fish oil, compared with mice fed normal lab chow. However, no changes in bone marrow composition were detected by ER-MP12/20 or lineage specific marker analysis. Moreover, in agreement with the ER-MP12/20 subset analysis, M-CSF-stimulated soft agar cultures of bone marrow cells did not reveal changes in macrophage colony- or cluster-forming cell frequency. Thus, the data on bone marrow composition obtained in chapter 8 extend and support the use of ER-MP12/20 subset analysis as an alternative method to determine cellular bone marrow composition in experimental mouse models.

Samenvatting

Macrofagen vormen een functioneel en fenotypisch heterogene populatie cellen, die wijd is verspreid door het lichaam. Mechanismen die verondersteld worden bij te dragen aan het genereren van deze brede diversiteit zijn o.a. (i) inductie van heterogeniteit door lokale omstandigheden of stimuli in de perifere weefsels, (ii) het bestaan van zichzelf instandhoudende, van beenmerg onafhankelijke macrofaagpopulaties die al tijdens de embryonale ontwikkeling gegenereerd zijn, en (iii) vorming van verschillende subpopulaties macrofaagvoorlopercellen die uitgroeien tot verschillende typen rijpe macrofagen.

Diepgaand onderzoek naar het bestaan van verschillende typen macrofaagvoorlopercellen werd tot dusver bemoeilijkt door een beperkte fenotypische karakterisering en een lage frequentie van deze voorlopercellen, en meer algemeen van myeloïd-gecommitteerde voorlopercellen in het beenmerg. Het doel van dit proefschrift was derhalve (i) om in het beenmerg verschillende stadia in de ontwikkeling van mononucleaire fagocyten te identificeren met behulp van in ons laboratorium geproduceerde monoklonale antistoffen tegen macrofaagvoorlopercellen, en (ii) om vroege myeloïd-gecommitteerde voorlopercellen te scheiden van andere hematopoietische voorlopercellen ten behoeve van studies naar de processen betrokken bij "commitment" naar de myeloide/macrofaag differentiatierichting.

In **hoofdstuk 2** is een selectie van de thans beschikbare antistoffen beschreven, gericht tegen muis mononucleaire fagocyten. De markers, gedetecteerd door deze antistoffen kunnen worden onderverdeeld in vier categorieën: (i) markers, karakteristiek voor onrijpe mononucleaire fagocyten, (ii) markers voor rijpe macrofagen in het algemeen, (iii) markers voor macrofaagsubpopulaties, en (iv) markers aanwezig op IFN-γ-gestimuleerde macrofagen. Aangezien slechts enkele van deze antistoffen volledig specifiek zijn voor één bepaald stadium van macrofaagontwikkeling, zijn combinaties van deze antistoffen nodig om uit het cellulaire fenotype te kunnen afleiden of macrofagen onrijp dan wel verder ontwikkeld zijn, of ze behoren tot een bepaald subtype, of al dan niet gestimuleerd zijn. Een meer exacte bepaling van het stadium van ontwikkeling vereist een beter inzicht in de voorloper - nakomeling relaties tussen de verschillende cellen van het mononucleaire fagocytensysteem en in de fenotypische karakteristieken van gerelateerde cellen.

Voor een verdere identificatie van macrofaagvoorlopercellen in het beenmerg van de muis hebben we in **hoofdstuk 3** onderzocht of (i) de anti-macrofaagvoorlopercel antistoffen ER-MP12 en ER-MP20 in twee-kleuren-flowcytometrische analyse fenotypische heterogeniteit detecteren onder macrofaagvoorlopercellen en, indien dit het geval is, (ii) of deze fenotypische heterogeniteit een afspiegeling is van verschillende ontwikkelingsstadia van mononucleaire fagocyten of een reflectie van verschillende macrofaagsublijnen. Zes fenotypisch verschillende celpopulaties kunnen worden onderscheiden na dubbelkleuring van beenmerg met ER-MP12 en ER-MP20 antistoffen. Fluorescentiegeactiveerde celsortering, gevolgd door M-CSF-gestimuleerde kweek in soft-agar, toonde aan dat macrofaagvoorlopercellen slechts aanwezig zijn in de ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, en ER-MP12^{-}20^{hi} beenmergpopulaties. Onze data wezen er verder op dat de macrofaagvoorlopercellen, die aanwezig waren in deze subpopulaties, opeen-

volgende stadia vertegenwoordigen in een maturatiesequentie met de volgorde ER-MP12^{hi}20^{-} → ER-MP12^{+}20^{+} → ER-MP12^{-}20^{hi}.

In **hoofdstuk 4** hebben we het ER-MP12/20 fenotype onderzocht van hematopoietische voorlopercellen die het vermogen hebben om na intraveneuze injectie de thymus van sublethaal bestraalde muizen te repopuleren. De data toonden aan dat cellen die in staat zijn om binnen drie weken de thymus te repopuleren voornamelijk te vinden zijn in de ER-MP12^{hi}20^{-} beenmergcelpopulatie en in mindere mate in de ER-MP12^{med}20^{-} populatie. De overige ER-MP12/20 beenmergpopulaties bevatten geen thymusrepopulerende cellen. Analyse van perifeer bloed van met ER-MP12^{hi}20^{-} en ER-MP12^{med}20^{-} beenmergcellen geïnjecteerde muizen liet zien dat multipotente hematopoietische stamcellen en/of voorlopercellen voor de B en myeloïde differentiatielijnen eveneens aanwezig zijn in de ER-MP12^{hi}20^{-} en ER-MP12^{med}20^{-} beenmergcelpopulaties.

In **hoofdstuk 5** hebben we onderzocht of de myeloïd-gecommitteerde cellen aanwezig in de ER-MP12^{hi}20^{-} subpopulatie kunnen worden onderscheiden en gesepareerd van de overige hematopoietische stam- en voorlopercellen in deze beenmergcelpopulatie. Hiertoe werden beenmergcellen gekleurd met zowel ER-MP12/20 als met ER-MP58, een antistof die alle M-CSF-responsieve voorlopercellen herkent in het beenmerg van de muis. Met de ER-MP58 antistof kon de ER-MP12^{hi}20^{-} populatie worden onderverdeeld in drie subpopulaties. Functionele data verkregen uit *in vitro* en *in vivo* testsystemen toonden aan dat vroege ER-MP12^{hi}20^{-} myeloïd-gecommitteerde voorlopercellen op basis van hun hoge ER-MP58 expressie kunnen worden gescheiden van andere hematopoietische voorloper- en stamcellen.

De conclusie van de studies beschreven in de hoofdstukken 3 tot en met 5 luidde dat met de combinatie van ER-MP12/20 en ER-MP58 monoklonale antistoffen opeenvolgende stadia van mononucleaire fagocytontwikkeling kunnen worden geïsoleerd uit het beenmerg van de muis. Aangezien ook GM-CSF-responsieve dendritische cel (DC) voorlopers voor tenminste een deel tot de monocyt/macrofaag differentiatielijn behoren, hebben we het ER-MP12/20/58 fenotype onderzocht van DC voorlopercellen. In **hoofdstuk 6** hebben we aangetoond dat DC voorlopercellen, die voor hun *in vitro* ontwikkeling afhankelijk zijn van GM-CSF, hetzelfde ER-MP12/20/58 fenotype hebben als voorlopercellen van de macrofaagdifferentiatielijn. Bovendien doorlopen deze cellen in beenmerg dezelfde ER-MP12/20-gedefinieerde maturatiesequentie. De data verkregen in deze studie ondersteunen de hypothese dat voorlopercellen van het mononucleaire fagocytensysteem zich gedurende een langere periode van hun ontwikkeling kunnen uitgroeien tot monocyten/macrofagen enerzijds of DC anderzijds, afhankelijk van lokale concentraties specifieke groeifactoren. Bevestiging van deze hypothese vereist echter aanvullende klonale experimenten.

De zes ER-MP12/20 beenmergsubpopulaties worden gekenmerkt door een opmerkelijke homogeniteit in hematopoietische differentiatierichting (hoofdstuk 3). Dit vormde de aanleiding tot onderzoek naar de bruikbaarheid van ER-MP12/20 flowcytometrische beenmerganalyse als alternatieve methode voor het bepalen van cellulaire beenmergcompositie (**hoofdstuk 7**). Hiertoe zijn veranderingen in cellulaire beenmergcompositie gevolgd in muizen met een sublethale infectie met *Listeria monocytogenes*. Data verkregen uit analyse van ER-MP12/20 beenmergsubpopulaties

werden vergeleken met data verkregen uit analyse van differentiatielijnspecifieke markers en uit conventionele morfologische analyse. Beenmergcompositiedata afgeleid uit ER-MP12/20 analyse bleken sterk te correleren met data uit zowel differentiatielijnspecifieke markeranalyse als ook morfologische analyse. Op grond van de gevonden data concludeerden we dat ER-MP12/20 flowcytometrische analyse van beenmerg een relatief eenvoudig, snel, en kwantitatief alternatief is voor analyse van de cellulaire beenmergcompositie in de muis.

Hoofdstuk 8 bevat een studie naar de effecten van visolieconsumptie op het immuunsysteem in de muis. Hierin is ER-MP12/20 analyse gebruikt voor het bepalen van de cellulaire beenmergcompositie. In het verleden is in verschillende experimentele diermodellen aangetoond dat visolieconsumptie de weerstand tegen infecties verhoogt. Analyse van de cellulaire beenmergcompositie vormde onderdeel van het onderzoek naar de mechanismen die ten grondslag liggen aan deze verhoogde weerstand. Muizen op visoliedieet vertoonden een toename in beenmergcelaantallen vergeleken met controlemuizen. Echter, noch ER-MP12/20 analyse, noch differentiatielijn-specifieke markeranalyse detecteerde veranderingen in beenmergcompositie. Functionele analyse van macrofaagvoorlopercellen in M-CSF-gestimuleerde beenmergkweken liet eveneens geen veranderingen zien in aantallen kolonie- of clustervormende voorlopercellen. De data bevestigen en ondersteunen het gebruik van ER-MP12/20 beenmerganalyse als alternatieve methode voor het vaststellen van beenmergcompositie in experimentele muismodellen.

Abbreviations

AGM	aorta-gonads-mesonephros
BM	bone marrow
BSA	bovine serum albumin
CAFC	coblestone area-forming cell
CFC	colony-forming cell
CFU-C	colony-forming unit in culture
CFU-GM	colony-forming unit-granulocyte/macrophage
CFU-M	colony-forming unit-macrophage
CFU-pre-B	colony-forming unit-pre-B
CFU-S	colony-forming unit-spleen
Cl_2MDP	dichloromethylene-biphosphate
CO	corn oil
CFC	colony-forming cell
GM-CFC	granulocyte/macrophage colony-forming cell
M-CFC	macrophage colony-forming cell
CSF	colony-stimulating factor
DAB	diaminobenzidine
DC	dendritic cell
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
EPA	eicosapentaenoic acid
FA	fatty acid
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fo	Forssman glycolipid antigen
FO	fish oil
HSC	hematopoietic stem cell
HPP-CFC	high proliferative potential colony-forming cell
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
i.v.	intravenous
LCM	L cell-conditioned medium
Lin	lineage-specific cell surface markers
LPS	lipopolysaccharide
LTRC	long-term repopulating cell
kDa	kilo Dalton
mAb	monoclonal antibody
MESF	molecules equivalent to soluble FITC
MHC	major histocompatibility complex

MLR	mixed leukocyte reaction
MRA	marrow-repopulating ability
NK	natural killer
NMS	normal mouse serum
NOD	non-obese diabetic
P	probability
PBS	phosphate-buffered saline
PI-linked	phospho-inositol-linked
PHSC	pluripotent hematopoietic stem cell
r	correlation coefficient
RT	room temperature
SAV-PE	streptavidin-conjugated phycoerythrin
SAV-TC	streptavidin-conjugated tricolor
SD	standard deviation
SEM	standard error of the mean
STRC	short-term repopulating cell
TCR	T cell receptor
TNF	tumor necrosis factor
TSA	tryptone soya agar

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