THYMUS-REPOPULATING STEM AND PROGENITOR CELLS IN MOUSE BONE MARROW

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Thymus-repopulating stem and progenitor cells /
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Rotterdam : Department of Immunology, Erasmus University
Rotterdam. - Ill.
Thesis Erasmus Universiteit Rotterdam. - With ref. - With
summary in Dutch.
ISBN 90-73436-25-7
NUGI 743
Subject headings: T cell commitment / thymus-repopulating
cells / pro-thymocytes.

THYMUS-REPOPULATING STEM AND PROGENITOR CELLS IN MOUSE BONE MARROW

Thymus-repopulerende stamcellen en voorlopercellen in het beenmerg van de muis

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof. dr. P.W.C. Akkermans M.A.
en volgens besluit van het college voor promoties.
De openbare verdediging zal plaatsvinden op
vrijdag 20 oktober 1995 om 13.30 uur

door

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geboren te Rotterdam

PROMOTIECOMMISSIE

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The studies described in this thesis were performed at the Department of Immunology, in collaboration with the Institute of Hematology, Erasmus University Rotterdam.

Illustrations : Tar van Os.
Printing : Ridderprint B.V., Ridderkerk.

Aan mijn ouders

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CHAPTER 1

General introduction

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1 Introduction

The adult bone marrow (BM) contains a minute population of progenitor cells which have the capacity to differentiate into mature T lymphocytes. Unlike the progenitor cells of the other hematopoietic lineages which mainly differentiate in the BM, the progenitor cells destined to become mature T cells must leave the BM and migrate to the thymus to complete their differentiation under the influence of the thymic microenvironments. The nature of the progenitor cells from BM that seed the thymus is still a matter of debate. Ultimately, T cells are derived from multipotent hematopoietic stem cells (HSC), as are all other cells of the hematopoietic system. To characterize the pre-thymic stages of T cell development, it is important to establish the lineage relationships between multipotent HSC and the progenitor cells that actually home to and colonize the thymus. As yet, there is no concrete evidence for the existence in the BM of progenitor cells that are exclusively committed to the T cell lineage (i.e. pro-thymocytes). Therefore, the site and developmental stage at which commitment to the T cell lineage occurs, as well as the molecular mechanisms that govern this process, remain to be determined. One approach to unravel the pre-thymic stages of T cell development is to identify the cell surface phenotype of the BM cells with thymus-repopulating ability (TRA). As yet, no cell surface markers are available that are uniquely expressed by thymus-repopulating progenitor cells. This study aimed at identifying new cell surface antigens that permit the identification and isolation of different subsets of thymus-repopulating BM cells, among which maybe progenitor cells that are committed to differentiate solely along the T cell lineage. This chapter reviews the current understanding on multipotent HSC and thymus-repopulating progenitor cells in mouse BM. In sections 2 and 3 attention is paid to the functional heterogeneity of the HSC compartment. This heterogeneity has become increasingly clear with the recent advances in HCS purification procedures and the concomitant development of new stem cell assays. Identification of functionally distinct hematopoietic stem and progenitor cell subsets is pivotal for the delineation of successive stages of hematopoietic development. Sections 4 and 5 focus on the identification and characterization of thymus-repopulating progenitor cells in the BM of the mouse. Some aspects of thymus colonization are addressed and assays are discussed that assess the capacity of purified hematopoietic stem and progenitor cell subsets to differentiate along the T cell lineage. In section 6 the cell surface antigens which so far have been employed for the isolation of hematopoietic stem and progenitor cells are reviewed. Section 7 considers the currently available data regarding the pre-thymic stages of T cell development and discusses the evidence that allude to the existence of lineage-restricted and T cell lineage-committed progenitor cells. The chapter is concluded with an introduction to the experimental work.

2 Hematopoietic stem cells

All mature blood cells have a limited life span. Vital functions, such as gas transport, hemostasis, and immunity, are extremely dependent on the continuous production of new hematopoietic cells. Life-long maintenance of the hematopoietic system is guaranteed by a small population of multipotent HSC which, in the adult animal, are mainly located in the BM [1-3]. The estimated frequency of the most primitive HSC is 1 per 10^4-10^5 BM cells [1,4]. Such primitive HSC are characterized by an extensive self-renewal capacity as well as the capacity to generate the mature progeny of all hematopoietic cell lineages over extended periods of time (i.e. multi-lineage long-term repopulating ability (LTRA)).

The first indication for the existence of multipotent HSC came from experiments in which BM cells from healthy mice were grafted into lethally irradiated mice and assessed for their radioprotective ability (RPA) [5]. Evidence for a common HSC for all hematopoietic cell lineages was initially derived from studies in patients with chronic myelogenous leukemia [6]. In these patients the Philadelphia chromosome could be detected in every cell lineage of the hematopoietic system, suggesting that the cells belonging to these lineages were derived from a single leukemic stem cell. The existence in normal adult BM of multipotent HSC was established unequivocally by analyzing the clonal progeny of transplanted mouse BM cells carrying either unique radiation-induced chromosomal abnormalities [7,8] or unique retroviral integration sites [9-13].

3 Functional characterization of hematopoietic stem cells

The study of the earliest stages of hematopoiesis has been seriously hampered by the very low frequency of HSC in normal BM and the lack of markers that permit a direct identification of these cells. With the recent advances in HSC purification procedures and the concomitant development of new assays to assess the developmental potential of isolated BM cell populations, it has become evident that the HSC compartment is extremely heterogeneous. It is now generally acknowledged that the most primitive cells within this compartment give rise to mature blood cells via a hierarchically concatenated series of progenitor cell stages which are characterized by decreasing self-renewal capacity and proliferation potential, increasing restriction of developmental potential, and increasing frequency and turnover rate [14-22] (Figure 1). Identification of functionally different classes of hematopoietic stem and progenitor cells is crucial for a better understanding of stem cell maturation and lineage commitment. In the following paragraphs attention will be paid to various stem cell assays that are used for the functional characterization of hematopoietic stem and progenitor subsets.

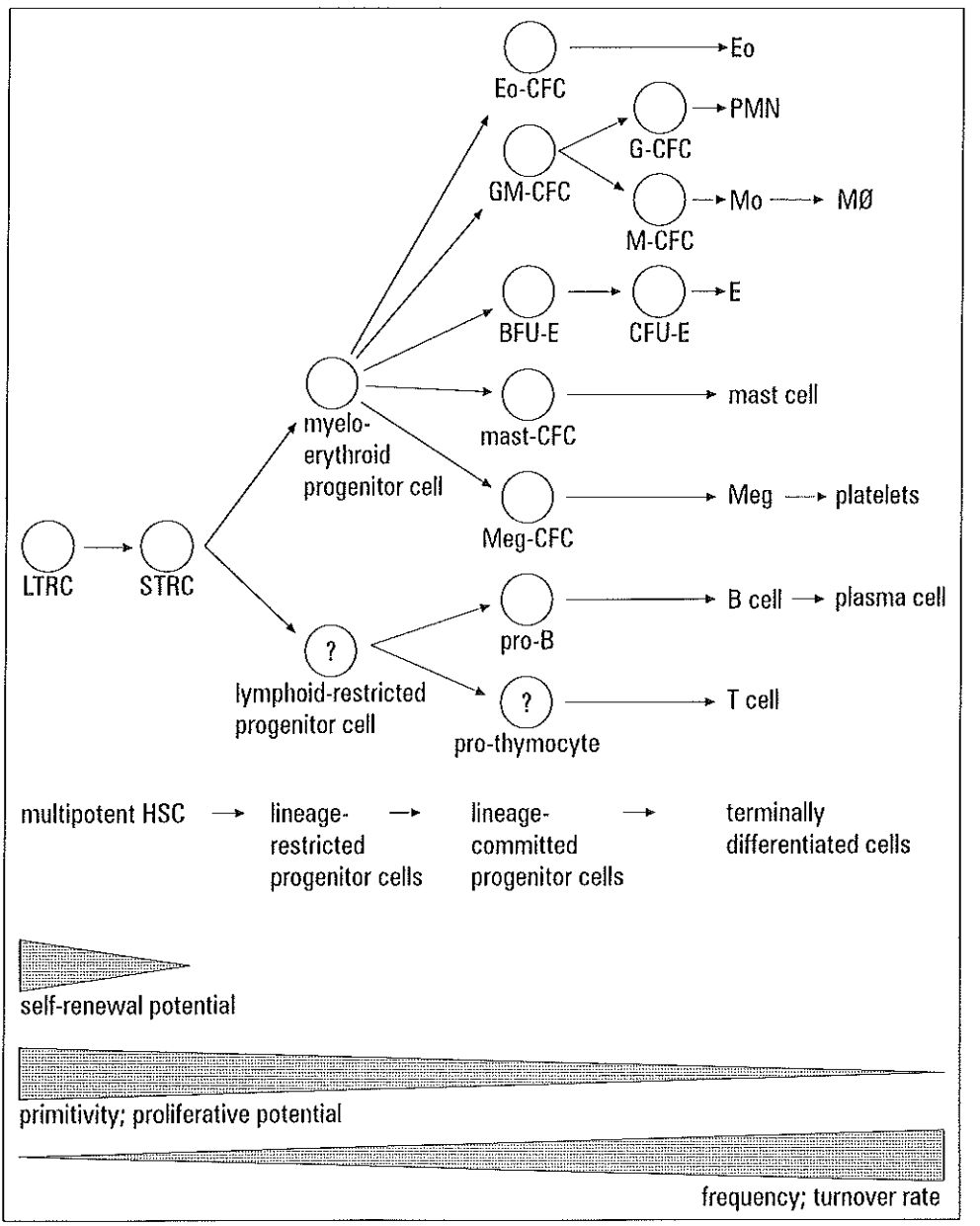


Figure 1. Schematic representation of hematopoietic development. LTRC: long-term repopulating cell; STRC: short-term repopulating cell; CFC: colony-forming cell; Eo: Eosinophil; GM: granulocyte/macrophage; G: granulocyte; PMN: polymorphonuclear phagocyte; M: macrophage; Mo: monocyte; MØ: macrophage; 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.

3.1 *In vivo* stem cell assays

The first *in vivo* assay for the quantitation of mouse HSC was described by Till and McCulloch in 1961 [5]. In this assay, hematopoietic cells are tested for their ability to generate macroscopic spleen colonies after intravenous (i.v.) transfer into lethally irradiated recipient mice. By injecting BM cells carrying unique radiation-induced chromosomal markers, it has been demonstrated that each spleen colony originates from a single cell, i.e. the CFU-S (colony-forming unit in the spleen) [23,24].

Since 1963 it has become increasingly clear that CFU-S are heterogeneous with respect to self-renewal ability, proliferative capacity, differentiation potential, cell cycle status, and cell surface phenotype. Cells isolated from spleen colonies present at 12-14 days (CFU-S d12-14) after transfer of BM cells have a much greater ability to generate new spleen colonies (i.e. self-renewal ability) in secondary lethally irradiated recipient mice compared to cells isolated from spleen colonies present at 7-9 days (CFU-S d7-9) after BM cell transplantation [25]. Furthermore, early-appearing spleen colonies mainly consist of one predominant hematopoietic lineage (usually erythroid), whereas most of the late-appearing colonies are multilineal (containing erythroid and myeloid lineage cells) [26].

Until the discovery that almost all CFU-S d7-9 have a transient nature [27,28] it was generally believed that CFU-S d12-14 merely represented a further developmental stage of the spleen colonies detected at earlier time points. However, CFU-S d12-14 appear to be relatively more resistant to the cytotoxic effects of cell cycle-active drugs such as 5-fluorouracil (5-FU) and hydroxyurea than CFU-S d7-9, indicating that CFU-S d12-14 are more primitive than CFU-S d7-9 [17-19,29,30]. Moreover, using 5-FU it was demonstrated that CFU-S d12-14 are not the most primitive HSC, since a subpopulation of BM cells was detected which were even more resistant to the cytotoxic effects of this agent. These 5-FU-resistant HSC repopulated primarily the irradiated marrow cavity with new CFU-S d12 rather than colonizing the spleen, leading to the assumption that cells with marrow-repopulating ability (MRA) are the precursor cells of CFU-S (i.e. pre-CFU-S) [18,19,30].

The existence of functionally different stem cell subsets in normal, unperturbed BM has been confirmed using cell separation procedures based on combinations of physical and phenotypic parameters, which include cell size, buoyant density, light scatter, lectin binding, membrane potential, uptake of fluorescent supravital dyes, and cell surface antigen expression [31].

By staining BM cell suspensions with fluorescent supravital dyes followed by fluorescent-activated cell sorting (FACS), it has been demonstrated that the kinetics and metabolic differences between BM cells can be exploited to isolate distinct hematopoietic stem and progenitor cell subsets [32,33]. For example,

the DNA-binding dye Hoechst 33342 stains quiescent CFU-S d12 and *in vitro* clonable progenitors less brightly than CFU-S d8 [33], while CFU-S d8 are brightly stained by the calcium-binding antibiotic tetracycline [32]. BM cells with MRA (i.e. pre-CFU-S activity) can be physically separated from the majority of the CFU-S d12-13 on the basis of differences in their level of retention of the fluorochrome rhodamine 123 (Rh123) by mitochondrial membranes [21,34-36]. Pre-CFU-S differ from the majority of CFU-S d12 in that they retain very low levels of Rh123. These $Rh123^{dull}$ cells are extremely capable of generating new CFU-S d12 and cells that rescue mice from irradiation-inflicted death [21,37]. CFU-S d12, on the other hand, exhibit a heterogeneous affinity for Rh123, some of them retaining the same amounts of Rh123 as the more mature CFU-S d8 [34,38]. Differences in Rh123 retention have been shown to correlate with functional heterogeneity in that CFU-S d12 with a low affinity for Rh123 show a higher RPA and MRA than CFU-S d12 with a high affinity for this supravital dye [34,38]. Likewise, the affinity for the lectin wheat germ agglutinin (WGA) conjugated to fluorescein isothiocyanate (FITC) has been used as a parameter for discriminating between distinct stem cell subsets [37,39,40]. By combining counterflow centrifugal elutriation (CCE), a procedure that separates cells on the basis of size and density, with plastic adherence and labeling with WGA-FITC, Ploemacher and Brons [39] showed that CFU-S d12, on average, differ from CFU-S d7 by a higher affinity for WGA and a higher perpendicular light scatter (PLS). By preparing BM cell suspensions greatly differing in the relative contributions of CFU-S d7 and CFU-S d12, they demonstrated that the proportion of CFU-S d12 but not CFU-S d7 correlated with RPA [39]. BM fractions with a low affinity for WGA, on the other hand, were enriched for pre-CFU-S as well as the progenitor cells of RPA cells, but were found to contain significantly less CFU-S d12 and RPA cells than BM cells with a high affinity for WGA [37,40].

Taken together, these studies conclusively confirm that CFU-S d12 are indeed not the most primitive cells in the HSC hierarchy. As pre-CFU-S do not form spleen colonies in primary lethally irradiated recipient mice, the CFU-S assay cannot be used for the quantification of multipotent HSC. The same conclusion holds true for the RPA assay, which measures the ability of cells to prevent recipient mice from irradiation-inflicted death over a period of 30 days. Only the more advanced cells of the stem cell compartment, such as CFU-S d12, are capable of generating sufficient numbers of mature cells to rescue lethally irradiated recipients in this relatively short period of time.

At present, the only way of identifying the most primitive cells in the HSC compartment is based on the functional quality that distinguishes these cells from all other hematopoietic cells, namely their multi-lineage LTRA. It is however still uncertain how long it takes before the progeny of long-term repopulating cells (LTRC) can be detected. Studies with retrovirally marked BM cells have indicated that reconstitution by LTRC can take two to five months

[2,3,12]. Although most of the cells with short-term repopulating ability (STRA) cannot function for more than three months, in some instances their progeny can be detected up to five months following transplantation [2,3]. Therefore, the evaluation of the functional activity of LTRC (i.e. the LTRA assay) must span at least six months.

Using CCE, Jones and co-workers [41] showed that a fraction of large cells, enriched for less primitive progenitor cells, only gave rise to an early and transient reconstitution, whereas a fraction of small, dense, lymphoid-like cells, which was virtually depleted of less primitive cells, gave a delayed, but sustained reconstitution of all hematopoietic lineages. This latter fraction was therefore enriched for primitive multipotent HSC (i.e. LTRC). This study clearly demonstrated that primitive HSC with LTRA are physically separable from less primitive short-term repopulating cells (STRC), which include CFU-S, *in vitro* colony-forming cells (see section 3.2.1), and RPA cells. As the slowly proliferating LTRC are unable to contribute to an early engraftment, which is necessary to rescue the lethally irradiated recipient mice from initial aplasia, they must be transplanted together with a source of STRC. The findings of Jones and co-workers [41] have been confirmed by a recent study of van der Loo *et al.* [42], who showed that the majority of CFU-S d12 are STRC that can be separated from LTRC on the basis of differences in WGA affinity or Rh123 retention. Other studies have also supported the notion that reconstitution of hematopoiesis in lethally irradiated mice occurs in two successive phases, of which only the second one is responsible for long-term reconstitution [2,3,22,43-45].

To identify and quantify LTRC, *in vivo* assays have been developed in which the survival of the lethally irradiated recipient is independent of the cell population under investigation. These so-called competitive repopulation assays are based upon the ability of histocompatible, but genetically distinguishable "test" cells to compete with either "unmanipulated" BM cells of the irradiated host [46,47], or BM cells of recipient mice whose LTRA have been compromised by two previous cycles of serial transplantation and regeneration [48,49]. Both normal and compromised BM cells serve to provide the necessary short-term support for the marrow-ablated recipient mice. LTRC can also be quantified *in vivo* by curing genetically mutant mice, such as mutant W mice or α-thalassemic mice, from their anemia with limiting numbers of wild-type BM cells [4,42]. The hematopoietic cells of these genetically mutant mice are compromised as a result of mutations in the c-*kit* gene [50,51] and the α-globin genes [52], respectively, giving the normal transplanted BM cells a growth advantage without the need of prior lethal irradiation.

3.2 *In vitro* stem cell assays

The *in vivo* quantification of hematopoietic stem and progenitor cells in a given suspension of BM cells is very cumbersome and requires many animals. Over the years, several types of *in vitro* culture systems have been developed for the quantification of immature hematopoietic cells. They can be divided into two groups, i.e. stroma-independent assays (section 3.2.1) and stroma-dependent assays (section 3.2.2).

3.2.1 Stroma-independent assays

Nearly all stroma-independent assays are based on "semi-solid" cultures in which the culture medium is made highly viscous by the inclusion of agar, plasma clot or methylcellulose. As a consequence, the progeny of individual hematopoietic cells are detected as colonies (CFU in culture, CFU-C) which can be enumerated and analyzed morphologically [53]. The cells giving rise to these colonies are called colony-forming cells (CFC). Colony formation is strictly dependent on one or multiple growth factors and/or cytokines added to the culture medium. The majority of these CFU-C assays allow the quantification of relatively mature, committed progenitor cells after an incubation period of 7-14 days [53]. Such assays, however, do not assess more primitive multipotent HSC such as MRA cells or LTRC.

Only a few stroma-independent assays detect more primitive cells of the hematopoietic system. Nahakata and Ogawa [54] have described an *in vitro* assay (the Ogawa blast assay) in which small colonies are formed containing undifferentiated blast cells after an extended incubation time of 16 days. In the Ogawa blast assay, blast cell colony formation appears to be supported by the actions of IL-6, G-CSF, IL-11, stem cell factor (SCF), and IL-12 in synergy with IL-3 [55]. These blast colonies have the capacity, upon replating, to form many secondary multi-lineage colonies and new blast colonies, suggesting extensive self-renewal activity within the primary colony. The progenitor cells giving rise to these blast colonies are relatively resistant to *in vivo* treatment with 5-FU, indicating that they are not actively cycling *in vivo* [56].

Another *in vitro* assay detects progenitor cells that are able to form macroscopic colonies containing at least 50.000 cells in 12-14 days [57]. Like the blast CFC, these high proliferative potential CFC (HPP-CFC) are relatively resistant to 5-FU treatment *in vivo* [57,58] and require multiple growth factors for survival and colony growth [59]. HPP-CFC are heterogeneous with respect to their growth factor requirements, and proliferative and differentiative capacity [59,60]. Recent studies have indicated that functionally distinct subpopulations of HPP-CFC can be isolated on the basis of differences in 5-FU sensitivity [60], Rh123 retention [36], and cell size and/or density [61]. These

subpopulations of HPP-CFC can be hierarchically ordered according to their growth factor requirements and proliferative and differentiative capacity [59]. HPP-CFC responsive to IL-1α, CSF-1, IL-3, and/or SCF are probably closely related, if not identical to CFC in the Ogawa blast assay. Both types of *in vitro* clonable progenitor cells are among the most primitive hematopoietic cells yet identified *in vitro*, having many functional characteristics of primitive HSC, such as pre-CFU-S activity and MRA [59].

3.2.2 Stroma-dependent assays

The other group of *in vitro* clonal assays is based on the Dexter-type stroma-dependent long-term BM culture (LTBMC) in which hematopoiesis can be maintained for several months in close association with a complex, plastic-adherent layer of BM-derived stromal cells [62]. These stromal cells support the growth and maintenance of HSC and progenitor cells via direct cell-cell contact, involving extracellular matrix components and adhesion molecules, and the production of multiple cytokines [63,64]. The endogenous hematopoietic activity of LTBMC can be eradicated by high doses of γ irradiation, without affecting the capacity of the stromal layers to support hematopoiesis when overlaid with fresh BM cells [65-67].

Ploemacher and co-workers [68-70] have developed a limiting dilution type LTBMC which permits *in vitro* frequency analysis of both primitive and more mature HSC. BM cells at limiting dilutions are seeded onto pre-established, irradiated stromal layers in microtiter wells and the presence of microscopically visible hematopoietic clones or "cobblestone areas" (CA) beneath the stroma in each well is scored at several time points after inoculation. By testing cell suspensions enriched for distinct stem cell subsets simultaneously *in vivo* and in the cobblestone area-forming cell (CAFC) assay, it was demonstrated that the CAFC frequency at day 28 (CAFC-28) correlates with MRA, while the CAFC frequency at day 10 (CAFC-10) corresponds with CFU-S d12 [40,69]. In addition, these studies revealed that CA formation around day 35 highly correlates with the presence of LTRC in BM cell suspensions [40]. Recently, it was shown that the heterogeneous BM-derived stromal layer can be replaced efficiently by a clonal BM stromal cell line (GBI/6), reducing both the number of mice and the time required to establish the feeder layers for the CAFC assay [71].

It should be noted that, although primitive hematopoietic cells with *in vivo* multi-lineage reconstituting ability are maintained in Dexter-type LTBMC [72-74], only mature cells of the myeloid lineage are produced [75]. Erythropoiesis can be induced by adding erythropoietin to the cultures [76] or transferring cDNA of this growth factor into BM stromal cells [77]. By modifying the culture conditions of the Dexter-type LTBMC, Whitlock and Witte [78] developed a

stroma-dependent assay permitting long-term B lymphopoiesis (the so-called Whitlock-Witte culture). Stromal cell lines have been cloned which support B lymphopoiesis as good as complex BM-derived stromal layers [79,80], and which can be used as inductive stroma in limiting dilution Whitlock-Witte cultures to quantify precursor B cells [81,82].

4 Thymus-repopulating hematopoietic progenitor cells

Whereas the BM provides the suitable microenvironmental niches for the development of myeloid cells as well as B cells, it is not capable of supporting the entire differentiation program of the T cell lineage. Instead, the inductive microenvironments for the generation of mature, immunocompetent T cells are provided by the thymus gland [83-89]. To complete their differentiation pathway, progenitor cells destined to become T lymphocytes must leave the BM and migrate to the thymus, which therefore functions as a primary lymphoid organ.

4.1 Thymus seeding in adult life

The existence of hematopoietic progenitor cells with thymus-repopulating ability (TRA) in the BM of adult mice has been firmly established by injecting intravenously chromosomally or antigenically distinguishable BM cells into irradiated recipients [90-96]. Thymus reconstitution in radiation BM chimeras exhibits a biphasic pattern in which an early but transient wave of thymocytopoiesis, initiated from radioresistant intrathymic precursor cells, is succeeded by a second but permanent wave, originating from the injected BM cells [96-103]. Besides the presence of thymus-repopulating cells in BM, hematopoietic progenitor cells with TRA have also been detected in the spleen [95,104,105] and the thymus [104-106], although the frequency of thymus-repopulating cells in these organs is much lower.

The entry of BM cells is not confined to the irradiated thymus, as thymus chimerism has also been observed in mice that received BM cells after irradiation of only the lower part of their body [107-109], or after irradiation of the whole body except the thymus [110]. In these part-body radiation BM chimeras the degree of thymus chimerism, though only evident several weeks after BM transplantation, correlates well with the degree of chimerism in BM [111], suggesting that thymocytopoiesis is maintained by extrinsic progenitor cells. In contrast, the level of thymus chimerism in non-irradiated mice injected with high doses of BM cells is extremely low (i.e. 1-3%) [111-113]. Higher levels of thymus chimerism, though far less than the expected 50%, have been detected in non-irradiated, antigenically distinguishable mice joined by parabiosis

[114-116]. Together with the data obtained with non-irradiated, BM-injected mice these results have been interpreted to indicate that thymocytopoiesis in normal (i.e. non-irradiated) animals is largely independent of the inflow of blood-borne progenitor cells. However, Donskoy and Goldschneider [117] recently provided evidence demonstrating that host- and donor-derived thymus-repopulating progenitor cells are not randomly distributed in the blood of the parabiotic partners and that the degree of thymus chimerism is in fact in equilibrium with the donor- and host-derived progenitor cells in blood. Furthermore, only a transient reconstitution of the thymus is observed after transfer of high numbers of thymocytes into irradiated recipient mice [106,113,118-120], indicating that the thymus itself does not accommodate a pool of self-replicating progenitor cells. Taken together, these data suggest that intrathymic T cell differentiation in adult life is maintained by extrinsically derived progenitor cells which, under normal circumstances, seed the thymus continuously at a very low rate [113].

4.2 Thymus colonization during ontogeny

By joining chromosomally distinguishable chick embryos in parabiosis it has been demonstrated that T cell development during embryogenesis depends upon the colonization of the thymic rudiments by blood-borne progenitor cells [121]. The extrinsic origin of fetal thymus-repopulating progenitor cells has been established unequivocally by the construction of chick-quail chimeras in which cells derived from quail tissue can be distinguished from chick cells by the presence of one large central mass of heterochromatic DNA in their interphase nuclei [122,123]. By grafting thymic primordia of different developmental stages into the somatopleura of the other species it was shown that the embryonic and early postnatal avian thymus is colonized by at least three successive waves of thymus-repopulating cells, separated from each other by "refractory" intervals during which almost no precursor cell influx occurs [124,125].

In the mouse the first hematopoietic progenitor cells have been shown to enter the embryonic thymus between day 10 and 11 of gestation [126,127]. Thymus-repopulating progenitor cells have been detected in the yolk sac and the aorta-gonad-mesonephros region between day 8 and 11 of gestation, whereas the first thymus-repopulating progenitor cells in fetal liver have been found around day 14-15 post coitum [128-131]. As yet, no conclusive data exist demonstrating that the mouse fetal thymic rudiments are colonized in a cyclic fashion. However, by grafting fetal thymus lobes under the kidney capsule of adult mice it has been shown that adult-type progenitor cells rapidly differentiated into mature thymocytes upon entry into thymic rudiments isolated

from 10 to 13 day-old embryos, whereas progenitor cells invading 13 to 15 day-old fetal thymic grafts only started to differentiate after a lag period of 11 to 13 days [132,133]. The progeny of these slow-starting progenitor cells eventually completely replaced that of the first cohort of rapidly differentiating progenitor cells. These data indirectly suggest that the mouse fetal thymus is colonized by (at least) two successive waves of progenitor cells which, under the influence of the developing fetal thymic microenvironments and with different kinetics, give rise to two different cohorts of thymocytes.

5 Functional characterization of thymus-repopulating progenitor cells

Apart from the low frequency in BM and the absence of distinguishing morphologic and phenotypic features, the identification of thymus-repopulating progenitor cells has been hindered by a lack of clonal assays. The three-dimensional relationship between the various cell types of the thymic microenvironment appears to be extremely important for the generation of mature thymocytes, as *in vitro* cultures utilizing monolayers of either heterogeneous thymic stromal cells or thymus-derived stromal cell lines are largely incapable of supporting the entire pathway of T cell differentiation. As yet, thymus-repopulating progenitor cells can only be identified by their ability to reconstitute either the thymus of an irradiated mouse (*in vivo* thymus reconstitution) or fetal thymus organ cultures depleted of endogenous lymphoid cells by treatment with deoxyguanosine (dGuo) (*in vitro* thymus reconstitution).

5.1 *In vivo* thymus reconstitution assays

The capacity of progenitor T cells to migrate to and repopulate the thymus of irradiated recipient mice upon i.v. transfer has been used extensively as an *in vivo* assay for the detection and quantification of these cells [94-96,105,110,113,134-138]. With the availability of mouse strains that express different allelic forms of the Thy-1 antigen on the cell surface of thymocytes and peripheral T lymphocytes (i.e. Thy-1 congenic mice), it has become relatively easy to distinguish between donor- and host-derived cells by using monoclonal antibodies (mAb) that recognize only one form of the Thy-1 antigen.

Only a few thymus-repopulating progenitor cells are required to reconstitute the thymus of an irradiated mouse [94-96,136,137]. In thymus lobes of irradiated mice reconstituted with graded numbers of Thy-1 congenic BM cells, distinct clones of donor-derived thymocytes can be discerned immunohistochemically for more than six months after i.v. injection, confirming the clonal nature of thymus reconstitution [136,137]. Hence, by transferring cells under

limiting dilution conditions into irradiated mice, the frequency of thymus-repopulating cells can be estimated using Poisson statistics. By employing an *in vivo* competitive limiting dilution approach, in which limiting numbers of congenic BM cells are mixed with a competing dose of syngeneic BM cells, Spangrude and Weissman [137] estimated the frequency of thymus-repopulating cells in unseparated BM to be $1/3.3x10^4$. This frequency, however, is probably an underestimation, as the efficiency of thymus homing of intravenously injected thymus-repopulating cells is not known.

Thymus-repopulating progenitor cells can also be identified by injecting a source of these cells *directly* into the thymus of sublethally irradiated mice (*in vivo* intrathymic (i.t.) reconstitution assay) [105,113,139]. The i.t. reconstitution assay does not depend upon the ability of the transferred cells to home to the thymus. It therefore allows the detection of all stem and progenitor cells that have the capacity to differentiate into thymocytes under the influence of the thymic microenvironment, including those which, under normal circumstances, would not migrate to the thymus [139]. As a consequence, the i.t. reconstitution assay is much more sensitive than the i.v. assay. Since BM colonization does not occur after i.t. injection of progenitor cells [139,140] (or at least not in the vast majority of reconstituted animals [113]), all donor-derived thymocytes descend from the i.t. transferred cells.

Using the i.t. reconstitution assay, it has been shown that injection of more than 200 thymus-repopulating progenitor cells does not result in a higher number of donor-derived thymocytes [139,141]. This observation suggests that the thymus contains only a finite number of microenvironmental sites or niches in which thymus-repopulating progenitor cells can lodge and differentiate into thymocytes, stressing the importance of measuring the TRA of any cell population under limiting dilution conditions.

Although frequency estimates determined by means of the i.t. reconstitution assay are not influenced by the seeding efficiency of thymus-repopulating progenitor cells, studies with radiolabeled BM cells have revealed that only about one-third of the injected BM cells can be found within the thymus one hour after i.t. transfer [105]. As it is not clear whether these cells are selectively retained by the thymic microenvironment or merely represent one-third of all transferred cells, the accurate frequency of BM-derived thymus-repopulating progenitor cells still remains to be established.

5.1.1 Thymus seeding after irradiation and BM transplantation

It has been difficult to ascertain at what time after irradiation and BM transplantation BM-derived thymus-repopulating progenitor cells start to enter the thymus, since the detection of donor-derived cells expressing high levels of the Thy-1 antigen is preceded by a period of 10 to 12 days in which no BM-

derived thymocytes can be detected [95,96,101,107,113,139]. By labeling BM cells either with a fluorochrome or a supravital dye it has been shown that 0.1% of the injected BM cells enters the thymus of an irradiated mouse as early as three hours after i.v. transfer [104,142,143]. Although about one-third express T cell markers shortly upon their arrival in the thymus [104,142], it is not clear whether the thymus is indeed reconstituted by descendants of this minute population of early thymus-homing cells. To determine the time of entry of thymus-repopulating progenitor cells into the thymus, Mulder and co-workers [144] re-irradiated thymus glands at various times after total body irradiation and BM cell transfer and subsequently analyzed the kinetics of appearance of donor-derived thymocytes. Thymus reconstitution by donor-derived cells was delayed if re-irradiation of the thymus was performed 48 hours or later after initial irradiation and BM transplantation, whereas thymus reconstitution was not affected if the thymus was re-irradiated either 1 hour or 24 hours after BM cell transfer. These results suggest that thymus-repopulating progenitor cells do not seed into the thymus directly upon BM transplantation, but instead enter the thymus at the second day after i.v. transfer of BM cells. However, although statistically significant, the differences in the kinetics of appearance of donor-derived thymocytes in this particular study [144] were only small. A more sensitive approach to study the time of entry of thymus-repopulating progenitor cells into the thymus of irradiated mice was used by Spangrude and Weissman [137]. These investigators combined the competitive limiting dilution assay, in which thymus reconstitution by congenic progenitor cells is an "all or nothing" event (see section 5.1), with a pulse-chase experiment. The chase, i.e. an excess of syngeneic BM cells, was given either simultaneously with a mixture of a limiting number of congenic BM cells and a competing dose of syngeneic BM cells, or four hours later. Thymus reconstitution was analyzed six weeks after BM cell transfer. Only when given at the same time as the congenic BM cells could a chase of syngeneic BM cells prevent thymus reconstitution by congenic BM cells, indicating that thymus-repopulating progenitor cells seed into the irradiated thymus within at least four hours after i.v. transfer [137]. In contrast, thymus reconstitution by congenic BM cells could not be prevented by an excess of lymph node cells, which lack the capacity to home to the thymus [104], suggesting that BM-derived thymus-repopulating progenitor cells utilize a specific homing mechanism to recognize and enter the thymus.

The lag period of 10 to 12 days which precedes the appearance of donor-derived thymocytes after irradiation and BM transplantation does not seem to result from BM colonization prior to thymus seeding, as a similar delay in the appearance of donor-derived thymocytes is seen after direct transfer of BM cells into the thymus of sublethally irradiated mice [113,139,145]. By using more sensitive methods for the detection of donor-derived cells in the thymus (see below), it has recently been shown that BM-derived progeny can be detected as early as 6-7 days after either i.v. or i.t. transfer of BM cells [141,146].

Together with the fact that only a small number of BM-derived thymus-repopulating progenitor cells participate in thymus reconstitution [94-96,136,137,139,141], these findings strongly suggest that the observed lag period is largely attributable to detection limits. Interestingly, the delay in the appearance of donor-derived thymocytes is an intrinsic feature of thymus-repopulating progenitor cells from BM, as it is not observed after transfer of thymus-derived progenitor cells [103,106,113,118-120,145].

As discussed above, a high level of Thy-1 antigen expression is only observed on thymocytes and peripheral T cells. Therefore, Thy-1 congenic mice cannot be used to evaluate the percentage of donor-derived non-T cells after irradiation and BM transplantation. The availability of Ly-5 congenic mouse strains has made it possible to study thymocytopoiesis before the expression of high levels of Thy-1, as well as the generation of donor-derived B cells and myeloid cells, since the Ly-5 antigen (CD45; common leukocyte antigen) is constitutively expressed by all nucleated hematopoietic cells [147]. The proportion of donor-derived T cells, B cells and myeloid cells is determined by staining cell suspensions also with a T cell marker, B cell marker and myeloid markers, respectively.

Analysis of thymus lobes injected directly with a BM cell suspension enriched for stem and progenitor cells unexpectedly revealed that the vast majority of donor-derived cells which are found in the thymus 6-10 days after i.t. transfer belonged to the myeloid lineage [141]. The number of donor-derived myeloid cells remained relatively constant ($\sim 5 \times 10^5$ cells/thymus lobe between six to 18 days after transfer) and appeared to be independent of the number of cells transferred [141]. Donor-derived B cells were only occasionally observed. Donor-derived non-T cells were also found after i.t. injection of only five purified cells. This minimizes the possibility that contaminating BM-derived stromal cells could have played a role in the induction of differentiation along the myeloid or B cell lineage in these reconstituted thymus lobes. Comparable results were obtained after i.t. transfer of unseparated BM cells, although donor-derived B cells were found in the thymus more frequently than after i.t. transfer of the enriched population of BM cells [148]. In addition to demonstrating that donor-derived thymocytes can be detected as early as 6-7 days after transfer of BM cells, these studies show that the irradiated thymic microenvironment does not exclusively support T cell development.

5.2 *In vitro* thymus reconstitution assay

Jenkinson and co-workers [149] have developed an *in vitro* organ culture system in which fetal thymus lobes isolated on day 14 of gestation are depleted of endogenous lymphoid cells by a culture period in the presence of deoxy-

guanosine (dGuo). These organ-cultured alymphoid lobes retain the capacity to attract thymus-repopulating progenitor cells, as well as the ability to induce subsequent T cell development and thus provide an *in vitro* approach to study the TRA of isolated cell populations. Reconstitution of dGuo-treated fetal thymus lobes is achieved either by allowing them to make contact with thymus-repopulating progenitor cells in hanging drop cultures [150-154] or by transferring cell suspensions containing these progenitor cells directly into the fetal thymus lobes using a microinjector [155-157]. Reconstitution by donor-derived cells is evaluated after culturing the fetal thymus lobes for 2-3 weeks in dGuo-free medium.

The advantage of the *in vitro* thymus reconstitution assay over the *in vivo* assays is that on average more than 10 fetal thymus lobes can be obtained from one pregnant mouse, making limiting dilution analyses more feasible. In addition, the sensitivity of the *in vitro* system is much higher than the *in vivo* assay, since the cells that proliferate in the dGuo-treated fetal thymus lobes are almost exclusively of donor origin. Using this *in vitro* approach it has been demonstrated unequivocally that a single thymic precursor cell can give rise to phenotypically distinct populations of thymocytes [150] which have undergone multiple T cell receptor (TcR) gene rearrangements under the influence of the thymic microenvironment [151].

6 Cell surface characteristics of hematopoietic stem and progenitor cells

As discussed in the previous sections, intrathymic T cell differentiation throughout life is maintained by BM-derived progenitor cells which under normal conditions seed the thymus continuously at a low rate [113,117]. However, the nature of the progenitor cells that migrate from the BM to the thymus is still not elucidated and it remains to be determined whether the thymus is seeded by 1) multipotent HSC, 2) progenitor cells with a lymphoid lineage-restricted developmental potential, or 3) precursor cells that are committed to differentiate solely along the T cell lineage (i.e. pro-thymocytes).

One approach to study the pre-thymic stage(s) of T cell development in BM is to identify and isolate different classes of thymus-repopulating cells on the basis of their cell surface characteristics and to establish the lineage relationships between these cell populations by carefully analyzing their developmental potential *in vivo* and *in vitro*. As yet there are no cell surface markers available that are uniquely expressed by either multipotent HSC (which by definition are capable of differentiating into T cells) or less primitive thymus-repopulating progenitor cells. The currently available protocols for the isolation of hematopoietic stem and progenitor cells therefore combine multiple cell surface characteristics in order to obtain BM cell populations that are highly enriched in

hematopoietic stem and/or progenitor cell activity. In general two approaches are employed simultaneously, i.e. (1) negative selection, in which unwanted cells (e.g. mature hematopoietic cells) are depleted from BM cell suspensions using mAb against cell surface antigens that are not expressed by the cells of interest, and (2) positive selection, which mainly relies on the availability of mAb against cell surface antigens that are differentially expressed during stem cell maturation. Whereas negative selection can be done using either FACS or magnet-activated cell sorting (MACS), positively selection is largely done by FACS.

In the following paragraphs a summary is given of the cell surface antigens which so far have been used for the identification and characterization of hematopoietic stem and progenitor cells, including those with TRA.

6.1 Thy-1, Sca-1 and "lineage-specific" cell surface antigens

One of the first cell surface antigens identified on hematopoietic stem and progenitor cells is Thy-1, a phosphatidylinositol-linked glycoprotein encoded by a member of the immunoglobulin gene superfamily [158,159]. Whereas this antigen is highly expressed on murine thymocytes and T lymphocytes [160], a low level of expression (defined as Thy-1^{lo}) has been demonstrated on CFU-S [161-163], the *in vitro* clonable HPP-CFC [58,164], thymus-repopulating progenitor cells [138,161], precursor B cells [81], and multipotent HSC [81,164-167].

Weissman and co-workers have shown that in mouse BM most of the hematopoietic progenitor activity is found in a subpopulation of BM cells that express about ten-fold less Thy-1 molecules on their cell surface than mature T cells and which lack the expression of cell surface markers characteristic for mature and maturing stages of the B cell lineage (i.e. B220), T cell lineage (i.e. CD4 and CD8), and myeloid lineages (i.e. Gr-1 and Mac-1) (hereafter referred to as lineage (Lin) markers) [81,138,165,166]. This Thy-1^{lo}Lin$^-$ subset (comprising ~0.2% of total BM cells) could be further fractionated using a mAb directed against Ly-6A/E (Sca-1), a glycoprotein encoded by a member of the Ly-6 multigene family [168-170]. Like Thy-1, Sca-1 is anchored to the cell membrane through a phosphatidylinositol linkage [171]. *In vivo* transfer studies have revealed that primitive HSC, i.e. multi-lineage LTRC, as well as cells with the capacity to repopulate secondary lethally irradiated mice, are exclusively found among Thy-1^{lo}Lin$^-$ cells that express high levels of Sca-1 antigen [167,172,173]. This Thy-1^{lo}Lin$^-$Sca-1^+ population, which represents about 0.05% of whole BM, was also shown to be extremely enriched for the less mature CFU-S d12 and cells with RPA [167,170], as well as for cells capable of homing to and repopulating the thymus of irradiated mice [174]. It remains to be determined, however, whether the thymus-repopulating activity displayed

by this Thy-1^{lo}Lin$^-$Sca-1$^+$ population is solely attributable to multipotent HSC or to the presence of lineage-restricted, or even T cell lineage-committed progenitor cells in this BM subset. Finally, almost all cells capable of generating late-appearing colonies in Dexter-type LTBMC are present in the Thy-1^{lo}Lin$^-$Sca-1$^+$ population of BM cells [175].

Initially, three other Thy-1^{lo} subsets, characterized by the expression of high levels of either Mac-1 or B220 or both, were reported to contain rapidly proliferating, lineage-restricted progenitor cells that gave rise to either myeloid cells (Thy-1^{lo}Mac-1$^+$B220$^-$), B cells (Thy-1^{lo}Mac-1$^-$B220$^+$), or both myeloid and lymphoid cells (Thy-1^{lo}Mac-1$^+$B220$^+$) [176-178]. Based upon these putative lineage-restricted Thy-1^{lo} progenitor subsets, a model for stem cell development was proposed in which the most primitive multipotent HSC (Thy-1^{lo}Lin$^-$Sca-1$^+$) differentiate into multipotent progenitor cells (Thy-1^{lo}Mac-1$^+$B220$^+$) capable of short-term reconstitution only, which subsequently give rise to rapidly proliferating lineage-committed (Thy-1^{lo}Mac-1$^+$B220$^-$ and Thy-1^{lo}Mac-1$^-$B220$^+$) progenitor cells [176-178]. However, by employing more rigorous cell sorting procedures, Morrison *et al.* [179] recently demonstrated that the restricted developmental activities ascribed to these Thy-1^{lo} subsets were largely due to the presence of contaminating multipotent stem and progenitor cells, leaving no evidence for the above mentioned model of stem cell differentiation.

Whereas high levels of Lin antigens are apparently not expressed by hematopoietic progenitor cells, low levels of at least some Lin markers have been demonstrated on these cells. Low levels of Mac-1 antigen have been detected on multipotent progenitor cells with STRA [179]. Likewise, low CD4 levels have been found on various functionally defined classes of immature hematopoietic cells, including *in vitro* clonable myeloid precursor cells, CFU-S, RPA cells, and thymus-repopulating cells [180,181]. However, not all progenitor cells express low levels of CD4; most of the early B cell precursors which respond to stroma in Whitlock-Witte cultures have been found among CD4$^-$ BM cells [182].

Using CCE in combination with Lin marker expression, Orlic and co-workers [183] showed that in normal BM the majority of LTRC, which are the most primitive cells in the HSC compartment, do not express detectable levels of CD4 antigen. However, it should be noted that the detection of low levels of CD4, or any other cell surface antigen, greatly depends upon the experimental conditions that are used. For example, Wineman *et al.* [182] showed that CD4 can be detected on LTRC provided that high concentrations of anti-CD4 mAb are used. In this context it is noteworthy that LTRC isolated from BM of 5-FU-injected mice have been shown to express the CD4 and Mac-1 antigens as well as other Lin markers [184,185], suggesting that the expression of these cell

surface antigens, and maybe also that of other cell surface markers, become upregulated upon exposure of HSC to 5-FU [186].

The most immature progenitor cells within the thymus probably represent the direct descendants of BM-derived thymus-homing and -populating progenitor cells. To be able to establish the developmental lineage relationship, it is therefore crucial to identify the cell surface phenotype of these most primitive intrathymic progenitor cells. By using *in vivo* thymus reconstitution assays it has been shown that the earliest precursor cells so far identified in the adult thymus are characterized by low levels of Thy-1 and CD4, and high levels of Sca-1 [187,188]. Other T cell differentiation antigens, including CD2, CD3, or CD8, have not been detected on these intrathymic precursor cells, which represent ~0.05% of all thymocytes, nor have been any of the other Lin markers. Thus, the earliest identifiable intrathymic precursor cells (referred to as $CD4^{lo}$ intrathymic precursor cells in the following paragraphs) resemble BM-derived HSC with regard to the expression of Thy-1, Sca-1, and Lin antigens.

6.2 Sca-2 antigen

Approximately one third of the Thy-1^{lo} cells in BM are characterized by the expression of the Sca-2 antigen [174]. By transferring Thy-1^{lo}Sca-2^{+} BM cells intravenously into irradiated mice it has been shown that this BM subset, like the subpopulation of Thy-1^{lo}Lin^{-}Sca-1^{+} BM cells (which do not express the Sca-2 antigen), contains thymus-repopulating progenitor cells. The thymus-repopulating activity could be depleted by selecting against Sca-1 antigen expression, indicating that Thy-1^{lo}Sca-2^{+} thymus-repopulating progenitor cells also express the Sca-1 antigen. As about 80% of the Thy-1^{lo}Sca-2^{+} BM cells express the B cell Lin marker B220, but no detectable levels of any other Lin antigen, it has been suggested that (at least the majority of) these BM cells are at a later stage of differentiation than Thy-1^{lo}Lin^{-}Sca-1^{+} BM cells [174]. It remains however to be established whether TRA is indeed confined to the major Lin^{+} fraction of Thy-1^{lo}Sca-2^{+} cells. Evidence suggesting that the Sca-2 antigen is indeed expressed at a later stage of hematopoietic development comes from a study in which Thy-1^{lo}Lin^{-}Sca-1^{+} BM cells were injected directly into the thymus of irradiated mice [141]. The first change in cell surface phenotype that could be detected was the acquisition of the Sca-2 antigen approximately one week after i.t. transfer. In contrast, phenotypic changes characteristic of later stages of the T cell maturation pathway were observed at later time points [141].

Within the thymus, Sca-2 antigen expression is confined to large cycling thymic blast cells in the cortex, whereas in peripheral tissues Sca-2 is expressed

predominantly by non-T cells [189]. More than 90% of the $CD4^{lo}$ intrathymic precursor cells are characterized by the expression of Sca-2 antigen [188]. Using the i.t. reconstitution assay, it has been demonstrated that TRA in the $CD4^{lo}$ intrathymic precursor population is confined to the Sca-2^+ subset [188]. Interestingly, Sca-2 antigen expression is the only phenotypic difference between the intrathymic $CD4^{lo}$ precursor population and the BM-derived Thy-$1^{lo}Lin^{-/lo}$Sca-1^+ subset containing multipotent hematopoietic stem and progenitor cells.

Recently, the gene encoding the Sca-2 antigen has been cloned [190]. Sca-2 is a cysteine-rich cell surface glycoprotein of 82 amino acids anchored in the cell membrane by a glycosyl-phosphatidylinositol moiety. Like the Sca-1 antigen, the Sca-2 antigen belongs to the Ly-6 multigene family, a group of cell surface molecules differentially expressed in several hematopoietic lineages. Members of this family have been implicated to play a role in signal transduction and cell activation.

6.3 Fall-3 antigen

By immunizing rats with $Lin^{-/lo}$ BM cells, a new mAb, Fall-3, has been raised which divides the Thy-$1^{lo}Lin^{-/lo}$ population into a Fall-3^+ and a Fall-3^- subset [82]. Functional characterization of these subpopulations has demonstrated that almost all LTRC and RPA cells are confined to the Fall-3^+ subset (comprising about 30% of total BM cells), whereas most of the early B cell precursors that respond to stromal cells in Whitlock-Witte cultures are found in the Fall-3^- subset. CFU-S d12 and IL-3-responding CFU-C, on the other hand, appeared to be present in both subsets of Thy-$1^{lo}Lin^{-/lo}$ cells [82]. Two-color immunofluorescence analysis has established that only 1-2% of all BM cells express Fall-3 and Sca-1 simultaneously, suggesting that the Fall-3 defines a novel marker on hematopoietic stem cells and progenitor cells.

6.4 MHC class I cell surface antigens

The differential expression of cell surface antigens encoded by the major histocompatibility complex (MHC) class I loci has also been used for the isolation of hematopoietic stem and progenitor cells, usually in combination with other parameters [48,185,191-196].

Primitive hematopoietic cells with competitive LTRA have been identified among BM cells expressing a relatively high density of H-$2K^b$ molecules [48,185]. CFU-S d9-12 were reported to express higher levels of H-$2K^k$ antigens than CFU-S d8 and thymus-repopulating BM cells [192-194]. Very high

levels of H-2K^b molecules were also detected on CD4^{lo} intrathymic precursor cells [187]. It has been shown that CFU-S d12 are separable from the more mature CFU-S d8 on the basis of a higher density of Qa-m2 molecules on their cell surface [195], while differential expression of Qa-m7 cell surface antigens has been exploited to separate HPP-CFC from more restricted *in vitro* clonable progenitor cells [196].

6.5 CD44 antigen

Trowbridge and co-workers [197] showed that a polymorphic cell surface glycoprotein of 95 kD, termed Pgp-1 or CD44, is expressed by CFU-S and thymus-repopulating progenitor cells. Subsequent studies have established that these classes of progenitor cells are characterized by the expression of intermediate levels of CD44 antigen [174, 198]. In contrast, more committed *in vitro* CFC are found among BM cells expressing relatively higher levels of CD44 [198].

Whereas CD44 is found on nearly all BM cells, only a small proportion of the cells within the thymus (5-10%) is characterized by the expression of CD44 [197]. Thymus reconstitution experiments have revealed that this intrathymic population of CD44^{hi}-expressing cells contains the majority of the thymus-homing progenitor cells which can transiently repopulate the thymus of irradiated mice [199,200]. This population of CD44^{hi} cells is enriched for cells that have not yet undergone TcR β-chain gene rearrangement [201], affirming their immaturity. In addition, CD4^{lo} intrathymic precursor cells have been shown to express high levels of CD44 [187]. Treatment of intrathymic CD44^{hi} cells with anti-CD44 mAb inhibits their ability to reconstitute the thymus upon i.v. transfer [199]. However, it does not interfere with their ability to reconstitute the thymus following their injection directly into the irradiated thymus [202], suggesting that CD44 is involved in thymus homing rather than in the subsequent early stages of intrathymic T cell development. In contrast, addition of anti-CD44 mAb to Dexter-type LTBMC or Whitlock-Witte cultures completely abolishes the production of myeloid cells and B lineage cells, respectively [203], implying that CD44 is involved in the development of at least the myeloid lineage and the B cell lineage. It remains to be established, however, whether this treatment inhibits the formation of thymus-homing BM cells in these cultures.

Although CD44 is encoded by a single gene, alternative splicing of its multiple exons and cell type-specific posttranslational modifications generate a diverse family of molecules [204]. One of the ligands for CD44 is hyaluronic acid, which is a common component of extracellular matrices and extracellular fluids [205]. However, a recent study has demonstrated that CD44 molecules

expressed by intrathymic $CD4^{lo}$ precursor cells are not capable of binding to hyaluronate [202], suggesting that early intrathymic precursor cells probably utilize another ligand.

6.6 c-*kit* receptor

The proto-oncogene c-*kit*, encoding a transmembrane tyrosine kinase receptor [206], has been mapped to the mouse dominant white spotting (W) locus [50,51]. Mutations within this gene severely affect hematopoiesis, gametogenesis, and melanogenesis. Transplantation studies have demonstrated that the hematopoietic abnormalities of W mutant mice, which die perinatally of severe anemia, result from intrinsic defects of the primitive HSC [207]. The gene encoding the ligand for the c-*kit* receptor has been localized within the mouse Steel (Sl) locus [208-210]. Mice carrying mutations at the Sl locus have aberrant microenvironments for the development of hematopoietic cells, germ cells and melanocytes, resulting in a phenotype similar to that of W mutant mice [207,211]. In combination with other hematopoietic growth factors, the ligand for c-*kit* (known as steel factor (SLF), stem cell factor (SCF), mast cell growth factor (MGF), and kit ligand (KL)) has been shown to stimulate the proliferation of primitive progenitor cells *in vitro* [212-219]. Similar effects have been reported after *in vivo* administration of SLF either or not in combination with other factors [220-222], although it has been shown more recently that *in vivo* SLF treatment results in a redistribution of progenitor cells rather than in their expansion [223]. Nevertheless, together these studies suggest that c-*kit*/SLF interactions play an important role in hematopoiesis.

Between five to 10% of the cells in BM are characterized by the expression of c-*kit* [224-227]. About one third of these cells express high levels of c-*kit* [224]. c-*kit*-expressing BM cells are enriched for a variety of hematopoietic stem and progenitor cells, including nearly all *in vitro* clonable progenitor cells, CFU-S, pre-CFU-S, thymus-repopulating progenitor cells, and primitive multi-lineage LTRC [183,224-229]. By utilizing a CCE procedure that allows an almost complete separation of LTRC from more mature, STRC such as CFU-S d12 [41], Orlic and co-workers [183] have clearly demonstrated that primitive multi-lineage LTRC express high levels of the c-*kit*.

In vivo injection of a noncytotoxic anti-c-*kit* mAb (ACK2) results in a depletion of most of the *in vitro* clonable progenitor cells and an inhibition of *in vivo* CFU-S activity [224,225]. Morphologic examination of BM cells isolated from ACK2-treated mice revealed a complete absence of myeloid and erythroid cells, indicating that the c-*kit* receptor is of functional significance for at least *in vivo* myelopoiesis and erythropoiesis [224,230]. In contrast to the suppressive effects on the myeloid and erythroid lineages, *in vivo* treatment with the

same anti-c-*kit* antibody actually results in a stimulation of B cell development, a phenomenon which may be explained by a diminished interlineage competition for stromal growth factors or microenvironmental niches [224,230]. Phenotypic characterization of precursor B cell populations has shown that pro-B cells, but not pre-B cells, express relatively low levels of c-*kit* [230], implying that c-*kit*-mediated signals are not crucial for B lymphopoiesis *in vivo*. Addition of ACK2 to cocultures of cloned stromal cell lines and BM cells does not affect the *in vitro* maintenance and proliferation of BM cells capable of long-term multilineage reconstitution, indicating that interactions other than c-*kit* and its ligand play an important role in the survival and proliferation of primitive HSC [231,232]. Recently, it has been shown that ACK2 inhibits the adhesion of mainly early *in vitro* colony-forming cells to a stromal cell line, demonstrating that the c-*kit* receptor is also involved in the adhesion of at least part of the hematopoietic progenitor cells to stromal cells [233].

In the thymus, c-*kit* expression has been demonstrated on the earliest $CD4^{lo}$ precursor cells as well as subsets of $CD3^-CD4^-CD8^-$ (triple negative (TN)) thymocytes [234-237]. The highest level of c-*kit* expression is found on $CD4^{lo}$ precursor cells and the least mature ($CD44^+CD25^-$ and $CD44^+CD25^+$) stages of TN thymocytes, after which the c-*kit* expression is down-regulated. Addition of ACK2 to dGuo-treated fetal thymic lobes inhibits thymus reconstitution by fetal liver (FL)-derived hematopoietic progenitor cells and completely abrogates the repopulation starting from adult $CD44^+CD25^-$ TN thymocytes, indicating that interactions between c-*kit* and its ligand are pivotal for early intrathymic T cell development [235]. Slightly different results have been reported by Matsuzaki and colleagues [157] who showed that *in vitro* treatment with ACK2 completely abolished thymus reconstitution by directly injected adult BM-derived $Thy\text{-}1^{lo}Lin^{-/lo}$c-$kit^+$ cells, whereas T cell differentiation starting from adult thymus-derived $Thy\text{-}1^{lo}Lin^{-/lo}$c-$kit^+$ cells was less dependent on c-*kit*-mediated signals. However, this apparent discrepancy may be due to the use of a five-fold lower concentration of blocking mAb by Matsuzaki and co-workers [157].

6.7 AA4.1 antigen

The mAb AA4.1 has been used successfully for the isolation of hematopoietic stem and progenitor cells from FL [238]. The cell surface antigen recognized by AA4.1 is expressed by 0.5-1.0% of the FL cells at d14 of gestation. Both STRC and LTRC are confined to this $AA4.1^+$ subpopulation of FL cells [238,239]. Further characterization of $AA4.1^+$ FL cells has established that almost all FL-derived LTRC express low levels of at least one Lin marker, while many less primitive hematopoietic cells, such as *in vitro* CFC and CFU-S, are defined by the expression of high levels of Lin antigens [238,240]. In

addition to the expression of either low or high levels of Lin markers, FL-derived AA4.1$^+$ HSC and progenitor cells have been shown to express Sca-1 [240].

In adult mice, the highest frequency of AA4.1$^+$ cells is found in BM [241]. About half of AA4.1$^+$ BM cells express either cytoplasmic or surface IgM molecules, indicating their commitment to the B cell lineage [241]. In contrast to FL-derived LTRC, which are all characterized by the expression of the AA4.1 antigen, LTRC in adult BM have been found among AA4.1$^+$ as well as AA4.1$^-$ cells [183], making the AA4.1 antigen a less ideal marker for the isolation of HSC from normal adult BM. It should be noted, however, that the majority of LTRC in BM of mice treated four days earlier with 5-FU, express medium to high levels of AA4.1 [185]. As most of the HSC in 5-FU-treated mice are actively cycling [242], which is also a feature of embryonic HSC, it has been suggested that AA4.1 antigen expression is restricted to HSC in cell cycle.

6.8 JORO 37-5, JORO 75, and JORO 30-8 antigens

Palacios and co-workers [243,244] have described three mAb (JORO 37-5, JORO 75, and JORO 30-8) that recognize cell surface antigens expressed by BM- and fetal thymus-derived "pro-T" cell lines but not by cell lines representing more mature stages of the T cell lineage or other hematopoietic cell lineages. In BM of young adult mice, JORO$^+$ cells are only detectable after depletion of the vast majority of the cells using cytotoxic mAb against B220, HSA, Ia (MHC class II), and GM1.2 (which recognizes granulocytes). When injected intravenously into sublethally irradiated severe combined immunodeficient (SCID) mice both JORO 37-5$^+$ and JORO 75$^+$ BM cells give rise to mature T cells but not B cells. In contrast, JORO 30-8$^+$ BM cells are able to differentiate into T cells as well as B cells [244]. Phenotypic characterization of JORO$^+$ BM cells revealed that these cells express CD44 and low levels of Thy-1. They do not express detectable levels of CD3, CD4, CD8, B220, F4/80 or MHC class II antigens [244]. As only the donor-derived lymphoid cells of the reconstituted SCID mice were analyzed, it remains to be determined whether freshly isolated JORO$^+$ BM cells are capable of differentiating along the myeloid lineage.

Recently, Palacios and Samaridis [245] established nontransformed cell lines from purified JORO 30-8$^+$ BM cells by culturing these cells in the presence of IL-3, IL-4, and IL-6. Phenotypic analysis showed that these cell lines express Thy-1, CD44, MHC class I, c-*kit*, B220, Mac-1, and JORO 30-8 antigens, but not other cell surface markers, such as CD3, CD4, CD8, JORO 37-5 or JORO 75. In BM of young adult mice cells with an identical phenotype could be identified, constituting less than 0.2% of the mononucleated cells. Upon transfer into sublethally irradiated SCID mice, the cell lines were able to differentiate into T cells, B cells, and myeloid cells. However, they did not have the capacity to protect lethally irradiated mice from radiation-inflicted death nor

were they capable of giving rise to CFU-S d8 spleen colonies. In addition, these cell lines were not able to generate erythroid colonies *in vitro*. It was concluded that the cell lines generated from JORO 30-8$^+$ BM cells represent an intermediate stage of development between multipotent HSC and lineage-restricted progenitor cells, such as those identified by JORO 37-5 and JORO 75. However, it should be emphasized that additional experiments are required to determine whether 1) BM cells characterized by the expression of JORO 37-5 and JORO 75 are indeed exclusively committed to the T cell lineage, and, if so whether 2) JORO 37-5 and JORO 75 detect all progenitor cells committed to the T cell lineage.

Within the thymus of normal adult mice very few JORO$^+$ cells are detectable [244]. Nearly all JORO$^+$ cells are found in the subcapsular area and outer cortex but not in the medulla. Immunohistochemical analysis of thymus lobes of mice that were sublethally irradiated two days earlier, revealed the presence of significant numbers of JORO$^+$ cells, of which the majority localized in the subcapsular area and outer cortex. Four to six days after irradiation, JORO$^+$ cells tended to localize in the mid and deep cortex. At later time points, post-irradiation JORO$^+$ cells were no longer detectable. These results suggest that JORO$^+$ cells enter the thymus two days after irradiation. However, it cannot be ruled out that the post-irradiation JORO$^+$ cells are the progeny of resident radiation-resistant JORO$^+$ thymocytes.

During ontogeny JORO 37-5$^+$ cells are confined to the liver and thymus [246]. The first JORO 37-5$^+$ cells are found at day 9 of gestation in the liver primordium. The highest percentage of JORO 37-5$^+$ FL cells (i.e. 3-5%) is found at day 14-15 of gestation, after which the percentage of JORO 37-5$^+$ cells declines to ~1% in the newborn. The developmental potential of these JORO 37-5$^+$ FL cells remains to be studied. In the developing thymus the first JORO 37-5$^+$ cells are detected at day 10 of gestation, correlating well with the time at which hematopoietic cells start to colonize the thymic rudiment [121,126,127]. At day 14 of gestation about 90% of fetal thymocytes express the JORO 37-5 antigen. This percentage declines to less than 1% at birth.

In summary, although the data look very promising, further studies are required to determine whether JORO$^+$ FL cells and JORO$^+$ thymocytes are committed to differentiate along the T cell lineage.

6.9 CD34 antigen

The cell surface glycoprotein CD34 (MW 105-120 kD) is currently one of the most important markers for the identification and isolation of hematopoietic stem and progenitor cells from human BM [247-254]. A human CD34 cDNA clone has been successfully used for the isolation of the murine homologue of

the CD34 gene [255]. Transcription studies have identified two mRNA splice variants encoding for a full length (100 kD) and a truncated (90 kD) form of the CD34 protein, respectively [256,257]. Detectable levels of murine CD34 mRNA expression have been observed in hematopoietic tissues and a number of hematopoietic progenitor cell lines. In addition, high levels of CD34 transcripts have been found in several embryonic fibroblast cell lines and in tissues of non-hematopoietic origin, such as brain. As vascular endothelial cells have been shown to express the CD34 antigen [258-260], the CD34 mRNA expression detected in these non-hematopoietic tissues may be due to vascular endothelial cells. It has been shown that under the proper conditions the vascular form of CD34 can act as a ligand for L-selectin [259,260], raising the possibility that vascular CD34 may function as an adhesion molecule for hematopoietic stem and progenitor cells. The function of CD34 on hematopoietic stem and progenitor cells, however, remains to be clarified.

Recently, polyclonal antibodies have been raised against the extracellular (Ab 1202) and intracellular domain (Ab 1241) of murine CD34 [257]. Using these antisera, it was demonstrated that CD34 is expressed on the cell surface either as a full length form or as a truncated form. Staining of unseparated murine BM cells with Ab 1202 revealed a wide range of CD34 antigen expression, comparable to what has been reported for CD34 on human BM cells [247]. Selection of either the 15% or 3% highest CD34-expressing BM cells resulted in the enrichment of *in vitro* CFC, CFU-S, RPA cells, as well as BM cells capable of reconstituting all hematopoietic cell lineages for at least 60 days [257], indicating that the CD34 antigen may be useful for the identification and isolation of murine hematopoietic stem and progenitor cell subsets. Further characterization of CD34 antigen expression in mouse BM has to await the development of mAb against murine CD34.

7 Lineage relationships, lineage restriction and commitment to the T cell lineage

The nature of the progenitor cells from BM that home to and colonize the thymus is still not elucidated. It is as yet not clear whether the earliest identifiable precursor T cells within the thymus are the progeny of either 1) multipotent HSC, 2) progenitor cells restricted to the lymphoid lineages, or 3) progenitor cells that are committed to differentiate solely along the T cell lineage (i.e. pro-thymocytes) (Figure 2). Evidence alluding to the latter two possibilities has come from *in vivo* studies in which the developmental potential of genetically marked BM cells was assessed [3,8-11]. In some reconstituted mice, a unique (i.e. clonotypic) marker was only detected in the lymphoid lineages or even solely in the T cell lineage but not in any of the other hematopoietic lineages. However, it cannot be excluded that genetically marked

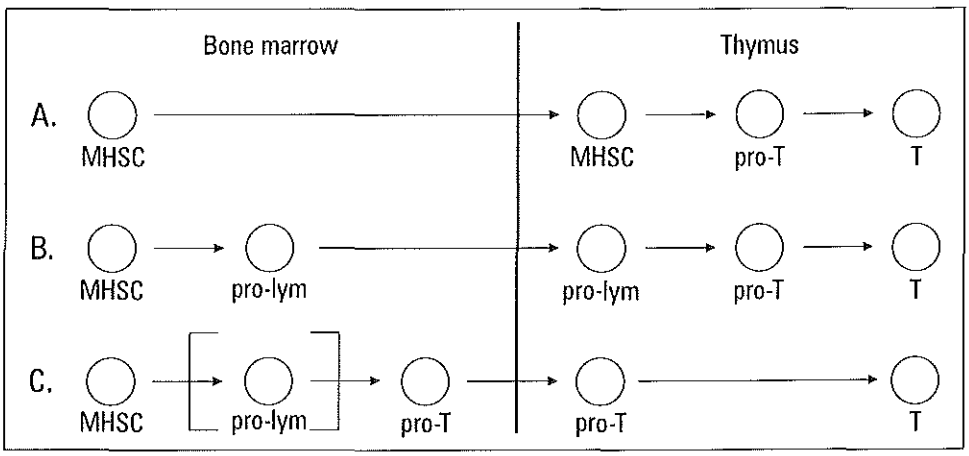


Figure 2. Hypothetical pathways of the development of T cells from multipotent HSC. MHSC: multipotent hematopoietic stem cell; pro-lym: lymphoid-restricted progenitor cell; pro-T: T cell lineage-committed progenitor cell (pro-thymocyte); T: mature T cell. Pathway **A** suggests that the thymus is seeded by multipotent HSC. Irreversible commitment to the T cell lineage occurs under the influence of the thymic microenvironments. In pathway **B**, the thymus is populated by lymphoid-restricted progenitor cells, which further develop along the T cell lineage within the thymic microenvironments. In pathway **C**, T cell commitment occurs within the BM microenvironment prior to thymus seeding [pro-thymocytes may be generated via a hypothetical lymphoid-restricted progenitor cell].

multipotent HSC directly have migrated to the thymus, where they were driven to differentiate along the T cell lineage under the inductive thymic microenvironments. In addition, sequential analyses of hematopoietic tissues and peripheral blood from mice reconstituted with genetically marked BM cells have demonstrated that in the first six months after BM transfer dramatic clonal fluctuations can be observed, including shifts in the contribution of other cell lineages [11,12,261]. Therefore, further research is required to clarify the nature of thymus-repopulating progenitor cells. To that end it is crucial to establish the lineage relationships between multipotent HSC and progenitor cells capable of colonizing the thymus. Characterization of the successive stages of hematopoietic development may permit the identification of the developmental stage at which lineage restriction and/or definite commitment to the T cell lineage takes place. In the next paragraphs, the currently available data on lineage relationships, lineage restriction and T cell commitment will be discussed.

Boersma and Mulder and their co-workers have investigated the lineage relationships between functionally different hematopoietic stem and progenitor cell subsets and the putative pro-thymocyte by comparing the growth kinetics

of donor-derived thymocytes in irradiated mice reconstituted with BM cell suspensions differing in the relative contributions of pre-CFU-S, CFU-S d12 and CFU-S d8-9 [38,96,135,192-194,262]. In their studies a delay in thymus reconstitution relative to the reconstitution of the thymus by normal, unseparated BM cells was interpreted to indicate that the cell suspension under investigation was depleted of "early" thymus-repopulating progenitor cells, which were considered to be committed to the T cell lineage. Thymus regeneration was delayed with 2-4 days in mice reconstituted with BM cells from 5-FU-treated mice, low density $WGA^{+}H\text{-}2K^{hi}$ BM cells [192], and $Rh123^{dull}$ BM cells [38]. Compared to normal BM, these BM cell suspensions were depleted of CFU-S d8 and enriched for CFU-S d12 or even pre-CFU-S (i.e. BM from 5-FU-treated mice). A more pronounced delay of 8-10 days was observed after transplantation of BM cells cultured in the presence of IL-3, which are highly enriched for CFU-S d8 [192]. Thymus reconstitution was not delayed in mice reconstituted with BM cells that either expressed relatively low levels of H-2K antigens [192] or retained high levels of Rh123 [38]. Both these BM cell populations were relatively enriched for CFU-S d8. In addition, the $Rh123^{bright}$ BM cell population was relatively depleted of pre-CFU-S, but still contained considerable numbers of CFU-S d12.

From these studies it was concluded that progenitor cells committed to the T cell lineage differ from pre-CFU-S, CFU-S d12 and CFU-S d8-9. A model was proposed in which pre-CFU-S either directly or via CFU-S d12 give rise to pro-thymocytes [194] (Figure 3). In this model it was hypothesized that CFU-S d8-9, most likely the direct progeny of CFU-S d12, have lost the potential to differentiate into progenitor cells capable of "early" thymus reconstitution. Additional support for this model was obtained from studies that demonstrated that CFU-S d8 and CFU-S d12 differ from "early" thymus-repopulating progenitor cells in their cell cycling status and their sensitivity to γ-irradiation [194,263]. It should however be mentioned that it cannot be excluded that a subset of CFU-S is capable of "early" thymus reconstitution, since both BM cell suspensions that reconstituted the thymus without any delay still contained considerable numbers of CFU-S. To investigate this possibility, additional experiments are required using BM cell suspensions that are either highly purified for CFU-S (in particular CFU-S d12) or severely depleted of these cells.

Although the lineage relationships postulated in the above mentioned model may be correct, explicit proof demonstrating that "early" thymus-repopulating progenitor cells are indeed committed to the T cell lineage has not been provided. It therefore still remains to be determined whether pro-thymocytes exist in the BM of the mouse.

As already mentioned at the beginning of section 6, one approach to unravel the pre-thymic stages of T cell development is to characterize different classes of thymus-repopulating progenitor cells according to their cell surface

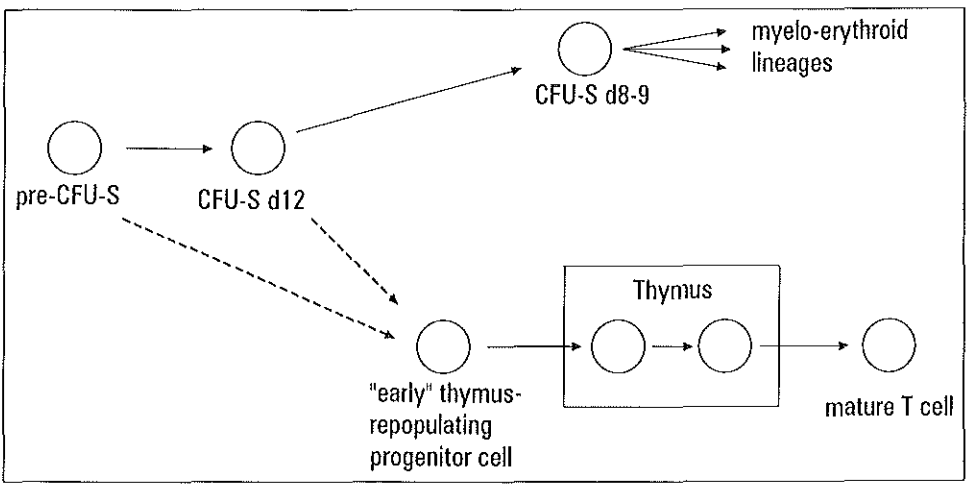


Figure 3. Putative lineage relationships between various classes of functionally defined hematopoietic progenitor cells (adapted from Mulder [194]). "Early" thymus-repopulating progenitor cells reconstitute the thymus of an irradiated mouse with the same kinetics as unseparated normal BM cells, whereas pre-CFU-S and CFU-S d12 exhibit a delayed thymus reconstitution. CFU-S d8-9 have lost the ability to differentiate into T cells. It is as yet not clear (indicated by broken lines) whether pre-CFU-S directly give rise to "early" thymus-repopulating progenitor cells or via CFU-S d12.

characteristics. However, using the currently available cell surface markers it has not (yet) been possible to identify and isolate either lymphoid-restricted or T cell lineage-committed progenitor cells. So far, TRA has been found to co-segregate with a variety of other hematopoietic activities, including RPA, CFU-S d12 activity, pre-CFU-S activity, multilineage LTRA, and *in vitro* colony-forming activity [38,170,193,226,264,265].

One of the hematopoietic stem and progenitor cell populations that have been extensively studied is the subset of Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells (see section 6.1). Recently, it has become increasingly evident that this population is by no means functionally homogeneous, as has been suggested earlier [170]. Analysis of lethally irradiated mice reconstituted with limiting numbers of Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells revealed the presence of both STRC and LTRC in this phenotypically defined subset of BM cells [172,173]. This observation has initiated the search for additional markers that may permit a further fractionation of the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cell population. Indeed, the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM subset has been further fractionated on the basis of the expression of the c-*kit* receptor [225,266,267]. However, this did not result in a segregation of hematopoietic activities, as all stem and progenitor cells were found among the cells that expressed the c-*kit* receptor. Fractionation on the basis of Rh123 retention, on the other hand, has demonstrated for the first

time that the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} population indeed contains functionally heterogeneous cells [268]. Pre-CFU-S and BM cells with a high proliferative potential were found to be highly enriched in the Rh123^{lo} subset but not in the Rh123^{hi} subset of Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells, whereas RPA cells and CFU-S d13 were present in both subsets. Using Lin$^{-/lo}$Sca-1^{+} BM cells, which display the same phenotypic and functional features as Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells [264], it was shown that most, if not all LTRC, are confined to the Rh123^{lo} fraction [267,268]. In contrast, the Rh$123^{med/hi}$ but not the Rh123^{lo} fraction of Lin$^{-/lo}$Sca-1^{+} BM cells, appeared to contain *in vitro* CFC capable of responding to IL-3 alone, suggesting that the progenitor cells in the Lin$^{-/lo}$Sca-1^{+}Rh$123^{med/hi}$ subset are relatively more mature than the progenitor cells in the Lin$^{-/lo}$Sca-1^{+}Rh123^{lo} population. The Lin$^{-/lo}$Sca-1^{+}Rh$123^{med/hi}$ subset also contained a higher frequency of *in vitro* CFC that could be stimulated by a wider range of cytokine combinations. However, both populations of Lin$^{-/lo}$Sca-1^{+} BM cells were equally capable of protecting lethally irradiated mice from radiation-inflicted death and were similarly enriched for CFU-S d13.

The Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} population has also been fractionated into functionally different subsets on the basis of cell cycle status using Hoechst 33342 [269]. Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} cells in the S/G2/M phase of the cell cycle (comprising ~20% of the total Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} population) appeared to be less capable of radioprotection and long-term multilineage reconstitution than Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} cells in the G0/G1 phase of the cell cycle. Finally, the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} subset has been separated according to the expression of low levels of the Mac-1 and/or CD4 antigens [267]. Three BM subpopulations could be identified: Mac-1^{-}CD4^{-}, Mac-1^{lo}CD4^{-}, and Mac-1^{lo}CD4^{lo} BM cells. Only the first subset, which constitutes 0.01% of total BM, was found to be highly enriched for LTRC. Both Mac-1^{lo} subsets, on the other hand, were relatively enriched for STRC. RPA cells and CFU-S were found in all three subsets. None of the other Lin markers were expressed at a level that allowed a further separation of the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} population.

Taken together, the above-mentioned studies indicate that at least a part of the stem and progenitor cell activities associated with the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cell population is separable from other hematopoietic activities on the basis of phenotypic differences. Further separation of the hematopoietic activities on the basis of cell surface phenotype is however critically dependent on the availability of additional markers.

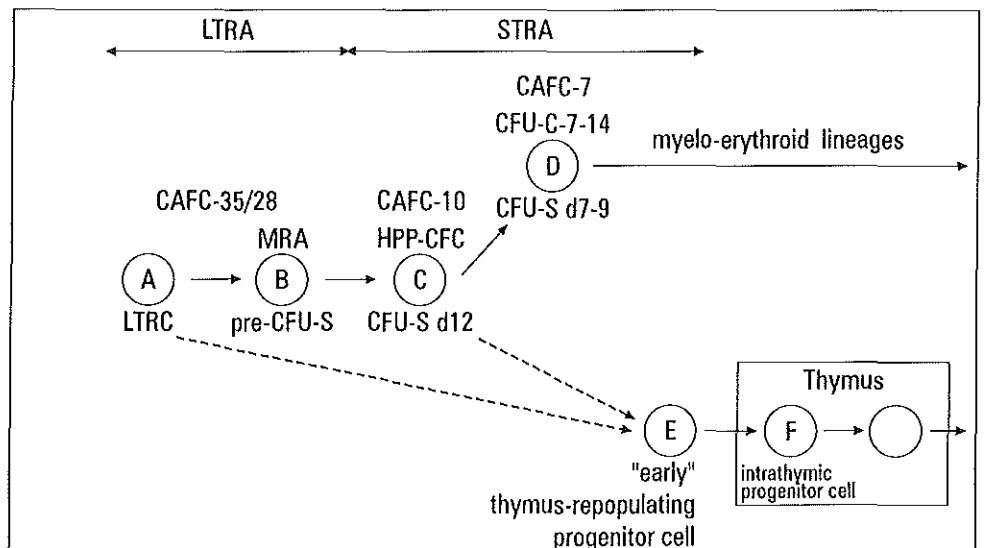
Recently, two functionally different classes of thymus-repopulating progenitor cells have been identified on the basis of cell surface characteristics and differences in the level of Rh-123 retention [270]. Lin^{-}Sca-1^{+}c-*kit*$^{+}$Rh123^{lo} BM cells, which are highly enriched for LTRC [268], appeared to be relatively incapable of giving rise to significant levels of donor-derived thymocytes at three weeks after i.v. transfer when compared with unseparated BM cells,

suggesting that this population of BM cells is depleted of "early" thymus-repopulating progenitor cells [270]. $Lin^-Sca\text{-}1^+c\text{-}kit^+Rh123^{med/hi}$ BM cells, on the other hand, were relatively more capable of repopulating the thymus at three weeks after i.v. transfer. In contrast to $Lin^-Sca\text{-}1^+c\text{-}kit^+Rh123^{lo}$ BM cells, this latter subset is depleted of LTRC but enriched for cells capable of short-term multilineage reconstitution [268]. These data indicate that "early" thymus-repopulating progenitor cells are separable from primitive LTRC, which reconstitute the thymus with a delayed kinetics. The relatively poor "early" TRA of $Lin^-Sca\text{-}1^+c\text{-}kit^+Rh123^{lo}$ BM cells is most likely due to an inadequate thymus seeding, as similar numbers of donor-derived thymocytes were found at two and three weeks after i.t. transfer of either population of $Lin^{-/lo}Sca\text{-}1^+c\text{-}kit^+$ BM cells [270]. However, it should be mentioned that the rate of T cell differentiation by intrathymically transferred $Lin^{-/lo}Sca\text{-}1^+c\text{-}kit^+Rh123^{med/hi}$ BM cells appeared to be faster than that of $Lin^{-/lo}Sca\text{-}1^+c\text{-}kit^+Rh123^{lo}$ BM cells.

Figure 4 summarizes the phenotypic and functional characteristics of the several, functionally defined classes of hematopoietic stem and progenitor cells in mouse BM. The putative lineage relationships between the various types of cells are shown. Further characterization of the pre-thymic stages of T cell development depends on the availability of additional markers that permit a further characterization of the "early" thymus-repopulating progenitor cells which may include the putative T cell lineage-committed progenitor cells.

8 Introduction to the experimental work

As discussed in the previous sections, the identity of the BM cells that actually home to and populate the thymus remains to be established. Further characterization of the pre-thymic stages of T cell development on the basis of cell surface characteristics requires the identification of new cell surface antigens that contribute to the separation of "early" thymus-repopulating progenitor cells from hematopoietic stem and progenitor cells exhibiting other and broader hematopoietic activities. In this thesis, we set out to identify new cell surface antigens which: 1) are differentially expressed by thymus-repopulating progenitor cells in the BM of the mouse, 2) permit the separation of functionally different classes of thymus-repopulating progenitor cells, and 3) contribute to the understanding of the progenitor-progeny relationships of the pre-thymic stages of T cell development. We have focused our attention to two recently developed mAb, ER-MP12 and ER-MP20. Both mAb, which were originally raised against macrophage precursor hybrid cell lines [272], have been shown to recognize cell surface antigens on subsets of macrophage progenitor cells, as well as other, not yet characterized progenitor cells in BM [273]. Immunohis-



marker	A	B	C	D	E	F
Thy-1	low	low	low	low	low	low
Lin	–/CD4^{low}	–/low	–/low	–/low	–/low	CD4^{low}
Sca-1	+	+	+	+	+	+
Sca-2	–	–	+*	ND	+*	+
c-*kit*	+	+	+	+	+	+
Fall-3	+	+	– or +	– or +	ND	ND
H-2K	+	+	+	+	+	+
CD44	+	+	+	+	+	+
CD34	ND	+	+	ND	ND	ND
Joro 37-5	ND	ND	ND	ND	+	+
Joro 75	ND	ND	ND	ND	+	+
Rh123	low	low	low/high	high	high	high
WGA	low	low	high	intermediate	ND	ND

Figure 4. Functional and phenotypic characteristics of several classes of functionally defined hematopoietic stem and progenitor cells. Putative lineage relationships are shown. *At least part of CFU-S d12 and "early" thymus-repopulating progenitor cells express the Sca-2 antigen. Cells lacking Sca-2 antigen expression, however, were not evaluated [271]. ND: not determined.

tochemical analysis of fetal thymus sections revealed that the cell surface markers recognized by ER-MP12 and ER-MP20 are also expressed by developing fetal thymocytes. We therefore set out to study the expression of the ER-MP12

and ER-MP20 antigens on BM cells with TRA.

In **chapter 2** we show that, when ER-MP12 and ER-MP20 are used simultaneously in two-color flow cytometric analysis, six subpopulations of BM cells can be identified on the basis of a differential expression of the ER-MP12 and ER-MP20 antigens. FACS followed by i.t. transfer of these BM subsets into sublethally irradiated recipient mice reveals that thymus-repopulating progenitor cells are exclusively confined to two BM subsets (comprising 1-2% and ~30% of total nucleated BM cells, respectively) that differentially express the ER-MP12 antigen but that do not express the ER-MP20 antigen. Subsequent limiting dilution experiments demonstrate that the highest frequency of thymus-repopulating cells is found in the minor subset of BM cells that express the highest level of ER-MP12 antigen.

In **chapter 3** the thymus-homing and -repopulating ability of the six phenotypically defined BM subpopulations is assessed upon i.v. transfer into irradiated recipient mice. Our data demonstrate that progenitor cells with the capacity to home to and repopulate the thymus within four weeks after i.v. transfer are confined to the two ER-MP20$^-$ BM subsets that express either high or intermediate levels of the ER-MP12 antigen. However, ER-MP12^{hi}20$^-$ BM cells are relatively more capable of "early" thymus reconstitution (measured at three weeks after i.v. transfer) than ER-MP12^{med}20$^-$ BM cells. It is hypothesized that this latter BM subset contains more primitive hematopoietic stem or progenitor cells that need to undergo additional maturational events before acquiring the capacity to home to the thymus, whereas progenitor cells present in the ER-MP12^{hi}20$^-$ BM subset already have acquired this capacity. Analysis of peripheral blood leucocytes of reconstituted mice furthermore reveals that both subsets contain multipotent hematopoietic stem and/or progenitor cells that are capable of giving rise to T and B lymphocytes, as well as myeloid cells. Finally, we show that ER-MP12^{hi}20$^-$ BM cells, in particular, are phenotypically heterogeneous with respect to the expression of Thy-1, Sca-1, CD44, c-*kit*, and the B cell differentiation antigen B220. This phenotypic heterogeneity provides a basis for a further purification and characterization of the progenitor cells with "early" TRA.

The experiments described in chapter 3 suggest that different classes of multipotent hematopoietic stem and progenitor cells may be identified by a differential expression of the ER-MP12 antigen. We therefore investigated the expression of this marker on several hematopoietic stem and progenitor cell subsets, including *in vivo* LTRC and CFU-S d12, *in vitro* CAFC, and *in vitro* clonable progenitor cells (CFC). In **chapter 4** we show that most of the LTRC, CAFC-28, and 75% of the CFU-S d12 can be detected in the ER-MP12^{med}20$^-$ subset, whereas the majority of the CAFC-5 and 25% of the CFU-S d12 are

found among ER-MP12^{hi}20^{-} BM cells. In addition, 80-90% of the *in vitro* CFC appear to express high levels of the ER-MP12 antigen, of which 10 to 20% also express the ER-MP20 antigen. These findings demonstrate that the ER-MP12 antigen is indeed differentially expressed within the hematopoietic stem and progenitor cell compartment. They support the hypothesis that less primitive progenitor cells, expressing high levels of ER-MP12 antigen, are responsible for an "early" thymus repopulation, while more primitive hematopoietic stem and/or progenitor cells, characterized by the expression of intermediate levels of ER-MP12 antigen, need more time to acquire the capacity to home to and repopulate the thymus, in which they probably progress through an developmental stage at which ER-MP12 antigen expression becomes upregulated.

In **chapter 5** we use dGuo-treated FTOC to evaluate the *in vitro* thymus reconstitution potential of the six BM subsets that are defined by a differential expression of the ER-MP12 and ER-MP20 antigens. The results show that, in agreement with the *in vivo* thymus reconstitution studies, TRA is confined to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets of BM cells. However, whereas *in vivo* the majority of the thymocytes generated from ER-MP12^{hi}20^{-} BM cells express both CD4 and CD8, most of the thymocytes derived from ER-MP12^{hi}20^{-} BM cells *in vitro* are still in the CD4^{-}CD8^{-} stage. ER-MP12^{med}20^{-} BM cells, on the other hand, give *in vitro* rise to mainly CD4^{-}8^{+} thymocytes and a five-fold higher percentage of mature (CD3^{+}) thymocytes compared with ER-MP12^{hi}20^{-} BM cells. The *in vitro* thymus reconstitution studies suggest that ER-MP12^{hi}20^{-} BM cells need more time to differentiate into mature thymocytes than ER-MP12^{med}20^{-} BM cells. However, the studies described in chapters 3 and 4 provide data arguing against this possibility. An alternative explanation for the *in vitro* experiments is presented which suggests that the dGuo-treated FTOC lacks microenvironments required for a full maturation of ER-MP12^{hi} thymus-repopulating progenitor cells.

In **chapter 6** we demonstrate that on the basis of differential ER-MP12 and ER-MP20 antigen expression three distinct M-CSF-responsive macrophage precursor subsets can be identified in mouse BM. We provide evidence that these phenotypically defined subsets represent successive stages along the following maturation pathway: ER-MP12^{hi}20^{-} $\rightarrow$ ER-MP12^{+}20^{+} $\rightarrow$ ER-MP12^{-}20^{hi}. Although the ER-MP12^{med}20^{-} BM subset gives rise to both lymphoid and myeloid cells upon i.v. transfer into irradiated recipient mice, individual cells in this subset are not yet capable of differentiating into myeloid cells upon *in vitro* stimulation with M-CSF alone. This latter finding is in good agreement with the data presented in chapter 4, in that the ER-MP12^{med}20^{-} BM subset is enriched for yet unrestricted, primitive hematopoietic stem and progenitor cells. Together with the data presented in chapters 2, 3, and 4, we propose that lineage restriction (i.e. loss of T cell lineage developmental

potential) and commitment to the macrophage lineage coincides with the upregulation of ER-MP20 antigen expression.

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CHAPTER 2

ER-MP12 antigen, a new cell surface marker on mouse bone marrow cells with thymus-repopulating ability

I. Intrathymic repopulating ability of ER-MP12-positive bone marrow cells

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International Immunology 1993;5:1093-1098

ER-MP12 antigen, a new cell surface marker on mouse bone marrow cells with thymus-repopulating ability: I. Intrathymic repopulating ability of ER-MP12-positive bone marrow cells

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Key words: bone marrow subpopulation, ER-MP20, intrathymic cell transfer, prethymic, prothymocyte, T cell development

Abstract

We searched for new cell surface markers that allow a positive identification of thymus-repopulating cells in the bone marrow (BM) of the mouse. Recently we raised two rat monoclonal antibodies (ER-MP12 and ER-MP20) that recognize cell surface antigens expressed by mouse haematopoietic progenitor cells, among which are progenitor cells of the macrophage lineage. Here we show that the ER-MP12 antigen, but not the ER-MP20 antigen, is also expressed by BM cells with thymus-repopulating ability. Using ER-MP12 and ER-MP20 in two-colour immunofluorescence analysis six subpopulations of BM cells can be identified. The thymus-repopulating ability of each BM subpopulation was assessed after fluorescence-activated cell sorting and subsequent intrathymic injection into sublethally irradiated Thy-1 congenic recipient mice. Thymus-repopulating activity appeared to be exclusively confined to two subsets of BM cells expressing either high or intermediate levels of the ER-MP12 antigen, but lacking ER-MP20 antigen expression. These BM subsets comprised 1 – 2% and 30% of total nucleated BM cells respectively. The frequency of thymus-repopulating cells was maximal in the minor BM subpopulation with the highest level of ER-MP12 antigen expression. We conclude that ER-MP12 detects a hitherto unknown cell surface marker expressed by BM cells with thymus-repopulating ability.

Introduction

Over the past few years studies focusing on intrathymic T cell development have led to a better understanding of the precursor – progeny relationships among thymocytes and the phenotypic stage at which TCR gene rearrangements, selection and lineage commitment occur (see, for reviews, 1 – 4). Considerable progress has been made in the phenotypic identification of the earliest T cell precursors within the thymus (5 – 11). In the adult mouse this intrathymic pool of T cell precursors is maintained by thymus-homing haematopoietic progenitor cells (prothymocytes) which seed the thymus continuously at a low rate (12). The majority of the progenitor cells of the T cell lineage reside in the bone marrow (BM) (13 – 16). Analysis of the prethymic stages of T cell development, however, has been hampered by the low frequency of prothymocytes in the BM (approximately 1 per 3×10^4 BM cells, as determined by i.v. transfer under limiting dilution conditions) (17), the absence of distinguishing morphological features of these cells, and the lack of mAb against specific cell surface antigens allowing their direct identification. As a consequence prothymocytes are still poorly defined.

Employing different combinations of parameters, highly enriched populations of haematopoietic stem cells can be isolated from mouse BM (reviewed in 18). Spangrude *et al.* have shown that BM cells with thymus-repopulating ability were enriched in two phenotypically defined BM subpopulations (17,19). Both subpopulations express low levels of the Thy-1 antigen and high levels of the Ly-6-A.2 (Sca-1) molecule. One of these sub-

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Transmitting editor: T. Hünig

Received 30 December 1992, *accepted* 28 May 1993

populations (0.05% of total BM) does not express detectable levels of lineage-specific antigens (designated as LIN$^-$), while the other subpopulation (0.1% of total BM) expresses at least one of the lineage-specific antigens (designated as LIN$^+$). These BM subpopulations however are not exclusively restricted to T cell lineage development, but are capable of short-term reconstitution of other haematopoietic cell lineages as well (20 – 23). Moreover, LIN$^-$Thy-1^{lo}Sca-1$^+$ BM cells also have the capacity for long-term multilineage repopulation and self-renewal (21 – 23). Thus further separation of these BM subpopulations using mAb against other (unknown) surface markers may lead to the separation of BM cells with prothymocyte activity from the other haematopoietic activities displayed by these BM subpopulations.

In this context we set out to search for new cell surface markers allowing a direct identification of BM cells with thymus-repopulating ability. In a parallel study in our laboratory in which we studied the early steps of macrophage differentiation we recently isolated two mAb, ER-MP12 and ER-MP20. These mAb recognize cell surface antigens of 140 kDa and 14 kDa respectively present on subpopulations of macrophage progenitor cells in the BM of the mouse (24). However the antigens detected by ER-MP12 and ER-MP20 are not restricted to progenitor cells of the macrophage lineage, but are also expressed by developing thymocytes early in ontogeny (W. A. T. Slieker and W. van Ewijk, unpublished results). Here we show that using the ER-MP12 and ER-MP20 mAb simultaneously six subpopulations of BM cells can be identified by two-colour immunofluorescence analysis. As assessed by intrathymic injection of the subpopulations isolated using a fluorescence-activated cell sorter (FACS) thymus-repopulating cells appeared to be exclusively confined to two subpopulations expressing the ER-MP12 antigen but not the ER-MP20 antigen. Moreover, the highest frequency of the thymus-repopulating cells was found in the BM subpopulation with the highest level of ER-MP12 antigen expression. We conclude that ER-MP12 detects a novel cell surface antigen which can be used as a positive marker for the isolation of thymus-repopulating cells in the BM of the mouse.

Methods

Mice

The C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2, Ly-5.1) mice (breeding pairs kindly provided by Dr I. L. Weissman, Stanford University, CA) and the C57BL/Ka 8l-1 (Thy-1.1, Ly-5.2) mice were cesarean derived, foster reared, bred, and maintained under clean conventional conditions with free access to food and water at the Animals Center and in the mouse facilities of the Department of Immunology. The drinking water was acidified to pH 2.8.

Monoclonal antibodies and fluorescent reagents

The production and initial characterization of the rat mAb ER-MP12 and ER-MP20 (both IgG2a isotypes) have been previously described (24). Briefly, spleen cells from a Lewis rat immunized with macrophage precursor hybrid cells (25) were fused with Y3 myeloma cells. Resulting hybridomas were screened for secretion of mAb selectively recognizing precursor stages in various models of macrophage maturation (24).

Other mAb used in the present study were: 30H12 (anti-Thy-1.2) (26); H129.19 (anti-CD4) (27); 53-6.72 (anti-CD8) (26). Phycoerythrin (PE) conjugated to GK1.5 (anti-CD4) (28) was purchased from Becton-Dickinson (Mountain View, CA).

mAb were purified either from ascites by sodium sulphate precipitation or from hybridoma culture supernatants by precipitation with ammonium sulphate or affinity chromatography using an anti-rat Ig κ chain mAb (29). Sodium and ammonium sulphate precipitates were dissolved in PBS and desalted by extensive dialysis against PBS. Partly purified ER-MP12 and anti-CD4 mAb were biotinylated using *N*-hydroxysuccinimidobiotin (Sigma, St Louis, MO) while the mAb ER-MP20, 30H12 and 53.6.72 were conjugated to FITC (isomer I; Sigma) by standard procedures. In some experiments 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer, Mannheim, Germany) was coupled to 30H12 according to the manufacturer's procedure. PE conjugated to streptavidin and streptavidin-TRICOLOR were obtained from Caltag Laboratories (San Francisco, CA).

Preparation of cell suspensions

Mice were killed by CO_2 exposure. All cell suspensions were made in Dutton's balanced salt solution (GIBCO, Breda, The Netherlands) supplemented with 5% FCS (DBSS – FCS). For cell sorting experiments BM cells were harvested from two or three CB57BL/6-Ly-5.1-Pep^{3b} mice aged 4 – 10 weeks. Cell suspensions were prepared as described previously (30) with slight modifications. Briefly, femora and tibiae were cleaned of muscles and tendons and ground in a mortar using DBSS – FCS. Single cell suspensions were obtained by aspiration through a 22 gauge needle into a 2 ml syringe, followed by sieving the cell suspension twice over nylon filters (mesh size 100 and 30 μm respectively; Polymon PES, Kabel, Amsterdam, The Netherlands). Thymus cell suspensions were prepared by pressing thymic lobes gently through a nylon sieve (mesh size 100 μm) in the presence of DBSS – FCS.

Immunofluorescence staining

For phenotypic analysis aliquots of 10^6 cells were placed in 96-well microwell plates (round bottom; Nunc, Denmark), spun down (250 *g*, 1 min, 4°C) and resuspended in PBS supplemented with 0.5% BSA and 20 mM NaN_3 (PBS – BSA – NaN_3) containing the optimal concentration of the appropriate fluorescein-conjugated mAb or fluorescent reagent. All incubations were carried out on ice for 30 min followed by three washes with PBS – BSA – NaN_3 at 4°C. In two-colour staining procedures the cells were first incubated with biotinylated mAb, washed and subsequently incubated with FITC-conjugated mAb and streptavidin-PE simultaneously. CD4 and CD8 antigen expression by donor-derived thymocytes was analysed using three-colour immunofluorescence. To that order thymocytes were first incubated with biotin-conjugated anti-CD8, washed, and subsequently incubated with FITC-conjugated anti-Thy-1.2, PE-conjugated anti-CD4, and streptavidin-TRICOLOR simultaneously.

For cell sorting experiments 10^9 BM cells were resuspended in 2 ml DBSS – FCS containing biotinylated ER-MP12. After 30 min on ice the cells were washed three times with large volumes of DBSS – FCS and subsequently resuspended in 2 ml DBSS – FCS containing both FITC-conjugated ER-MP20 and streptavidin – PE. After an incubation of 30 min on ice the cells were washed three times and resuspended in PBS supplemented

with 1% BSA and D-glucose (4.5 g/l; Merck, Amsterdam, The Netherlands) to a final concentration of 3×10^6 cells/ml.

Flow cytometric analysis and fluorescence-activated cell sorting

Cell surface fluorescence was analysed using a FACScan flow cytometer (Becton-Dickinson). Erythrocytes and dead cells were excluded from analysis by electronic gating on the basis of light scatter characteristics.

Cell separation was performed using a FACS 440 (Becton-Dickinson) at a sorting speed of 2500 – 3000 cells/s. Before cell sorting the cells were filtered through a 30 μm mesh nylon sieve. Deflected drops were collected in FCS-coated conical polypropylene tubes (Falcon; Becton-Dickinson). After washing the sorted cells were resuspended in DBSS – FCS and viable cells were counted using a Bürker haemocytometer. The purity of the sorted BM subpopulations was assessed by FACScan analysis.

Intrathymic transfer assay

The intrathymic injection assay for the detection of cells with thymus-repopulating ability was originally developed by Goldschneider *et al.* (31). Four- to seven-week-old CB57BL/Ka Bl-1 recipient mice were sublethally irradiated (5 Gy) at approximately 1.0 Gy/min using two opposing ^{137}Cs sources (Gammacell 40 irradiator; Atomic Energy of Canada Ltd, Ottawa, Canada). Two hours after irradiation the mice were anaesthetized with ether and the thymus was exposed surgically. Cells in a volume of 10 μl were injected directly into one of the thymic lobes using a Hamilton syringe with a 25 gauge needle. After injection the incision was closed with surgical wound clips (Becton-Dickinson). Thymocyte suspensions were prepared 20 – 21 days after intrathymic transfer and stained with FITC- or FLUOS-conjugated 30H12, recognizing the Thy-1.2 molecule expressed by donor-derived thymocytes. The percentage of Thy-1.2$^+$ donor-derived thymocytes was determined by FACScan analysis. Thymocyte suspensions with ≥ 1% donor-derived thymocytes were scored as positive for donor type repopulation. In some cases the cells were also stained with PE-conjugated anti-CD4 and biotinylated anti-CD8 (using streptavidin-TRICOLOR as second stage reagent) in order to analyse the distribution of donor-derived thymocytes within the four major thymocyte subsets.

Results

Two-colour ER-MP12 and ER-MP20 immunofluorescence analysis of BM cells

Two-colour immunofluorescence was used to analyse simultaneously the expression of the ER-MP12 and ER-MP20 antigens by BM cells. To that end BM cells were labelled with biotinylated ER-MP12 and visualized by PE-conjugated streptavidin and FITC-conjugated ER-MP20. By two-colour analysis at least six subpopulations with different phenotypes could be distinguished in a BM cell suspension (Fig. 1 and Table 1). Approximately one-third of the nucleated BM cells lacked the expression of both cell surface markers (i.e. ER-MP12$^-$20$^-$ BM cells). Cells lacking the ER-MP20 antigen but expressing the ER-MP12 antigen could be divided into a major subpopulation expressing intermediate levels of the ER-MP12 antigen (i.e. ER-MP12$^+$20$^-$) and a minor subpopulation with a high level of ER-MP12 antigen expression (i.e. ER-MP12^{++}20$^-$). Likewise, cells lacking the ER-MP12 antigen but expressing the ER-MP20 antigen could be clearly divided into a major subpopulation with an intermediate ER-MP20 antigen expression (i.e. ER-MP12$^-$20$^+$) and a minor subpopulation expressing high levels of the ER-MP20 antigen (i.e. ER-MP12$^-$20^{++}). Finally, cells expressing both surface markers simultaneously (i.e. ER-MP12$^+$20$^+$ cells) constituted a minor BM subpopulation.

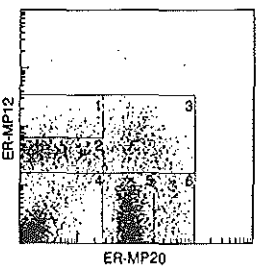

Fig. 1. Two-colour flow cytometric analysis of ER-MP12 and ER-MP20 antigen expression by total BM cells. Boxes indicate the six phenotypically distinct subpopulations: (1) ER-MP12^{++}20$^-$; (2) ER-MP12$^+$20$^-$; (3) ER-MP12$^+$20$^+$; (4) ER-MP12$^-$20$^-$; (5) ER-MP12$^-$20$^+$; (6) ER-MP12$^-$20^{++}. The average percentages of the subpopulations are listed in Table 1. The dot plot was generated from 5500 events.

Table 1. Distribution of the different nucleated BM cell subpopulations

BM subpopulation	Mean % of nucleated BM cells (±SEM)
ER-MP12$^-$20$^-$	28.6 ± 5.6
ER-MP12$^+$20$^-$	30.7 ± 4.9
ER-MP12^{++}20$^-$	2.1 ± 0.4
ER-MP12$^-$20$^+$	26.8 ± 6.6
ER-MP12$^-$20^{++}	4.8 ± 1.7
ER-MP12$^+$20$^+$	9.2 ± 3.1

C57BL/6-Ly-5.1-Pep^{3b} BM cells were simultaneously stained with ER-MP12 and ER-MP20. The percentages of the different subpopulations among the nucleated BM cells were determined by FACScan analysis. The average percentages ± SEM were calculated from the results of 14 experiments.

Thymus-repopulating ability of the sorted BM subpopulations

In order to assess the thymus-repopulating ability of the distinct subpopulations C57BL/6-Ly-5.1-Pep^{3b} BM cells stained with ER-MP12 and ER-MP20 simultaneously were separated into these six subpopulations by FACS. The sorted BM subsets were analysed using a FACScan flow cytometer, revealing that BM cell fractions could be obtained with a purity of 80 – 99%, depending on the subpopulation sorted. As an example the FACScan analyses of sorted ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ BM cells are shown in Fig. 2.

Next the sorted BM subpopulations were transferred intrathymically into sublethally irradiated C57BL/Ka Bl-1 recipient mice. As a control unseparated BM cells were transferred.

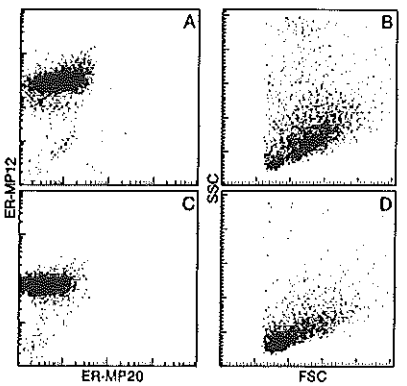

Fig. 2. Analysis of sorted ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells. Fluorescence profile [(A) and (C)] and scatter profile [(B) and (D)] of ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells respectively. Dot plots were generated from 5000 events.

Table 2. Intrathymic-repopulating ability of sorted BM cell subpopulations

Cell source	Number of cells transferred	Ratio (no. positive/no. recipient mice)	Mean % of Thy-1.2^{+} cells (± SEM)
Total BM cells	5000	3/16	41.6 ± 18.7
ER-MP12^{+}20^{-}	5000	21/47	22.7 ± 4.6
	2500	11/33	28.5 ± 7.4
	1250	0/26	
ER-MP12^{++}20^{-}	5000	15/18	31.2 ± 5.1
	2500	6/12	27.3 ± 9.8
	1250	17/22	32.6 ± 5.9
	625	6/7	40.0 ± 9.9
	78	2/8	19.1
ER-MP12^{+}20^{+}	5000	2/12	10.0
ER-MP12^{-}20^{+}	5000	1/15	53.6
ER-MP12^{-}20^{++}	5000	0/15	
ER-MP12^{-}20^{-}	5000	0/7	

C57BL/Ka BL-1 (Thy-1.1) recipient mice were irradiated sublethally (5 Gy) and reconstituted intrathymically with donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2) mice. Thymus reconstitution was analysed 20–21 days after intrathymic transfer. The average percentages ± SEM of Thy-1.2^{+} thymocytes in positive mice were calculated from three to 21 mice. The table is generated from the results of seven separate experiments.

Thymus reconstitution by donor-derived cells was analysed 20–21 days after intrathymic transfer using the expression of the Thy-1.2 allele as a marker for donor-derived thymocytes.

The combined results of seven separate experiments are summarized in Table 2. After injection of 5000 unseparated BM cells donor-derived thymocytes were detected in a small percentage of recipient mice. Virtually no prothymocytes were detected in the subpopulations expressing the ER-MP20 antigen nor in the subpopulation lacking both cell surface markers (i.e. the ER-MP12^{-}20^{-} subpopulation). BM cells with thymus-repopulating ability were exclusively confined to the populations expressing the ER-MP12 antigen. After titration of the cell dose it appeared that the frequency of thymus-repopulating cells was highest in the small subpopulation expressing high levels of the ER-MP12 antigen (i.e. the ER-MP12^{++}20^{-} subpopulation). In six out of seven mice reconstituted with as few as 625 ER-MP12^{++}20^{-} BM cells donor-derived cells accounted for ~40% of all thymocytes. Moreover, after transfer of only 78 ER-MP12^{++}20^{-} cells two out of eight mice showed donor-derived thymocytes (35.4% and 2.6% respectively).

Light scatter characteristics of ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells

Both ER-MP12 single positive subpopulations displaying thymus-repopulating activity are heterogeneous with respect to their light scatter characteristics [Fig. 2(B) and (D)]. The majority of the ER-MP12^{++}20^{-} BM cells are medium-sized blast-like cells [Fig. 2(A) and (B)]. Conversely, ER-MP12^{+}20^{-} BM cells are mainly small-sized lymphoid-like cells; only a minority of medium-sized cells are found within this BM subpopulation [Fig. 2(C) and (D)].

Phenotype of the donor-derived thymocytes

In order to investigate the differentiation potential of the injected BM cells in the host thymic microenvironment we analysed the CD4 and CD8 expression by the donor-derived thymocytes by means of three-colour immunofluorescence analysis. Thymocyte suspensions were stained with biotinylated anti-CD8 followed by TRICOLOR-conjugated streptavidin, and subsequently with PE-conjugated anti-CD4 and FITC-conjugated anti-Thy-1.2. After gating for Thy-1.2^{+} thymocytes the CD4 and CD8 expression was analysed (Table 3). Thymocytes of non-irradiated and non-reconstituted C57BL/6-Ly-5.1-Pep^{3b} mice served as a control. As shown in Table 3, ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells indeed differentiated into mature CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes under the influence of the thymus microenvironment.

Discussion

In this study we set out to search for new cell surface markers allowing a positive identification of thymus-repopulating cells in the BM of the mouse. Recently we isolated two mAb, ER-MP12 and ER-MP20, which recognize cell surface antigens present on progenitor cells in the BM of the mouse (24). We questioned whether BM cells with thymus-repopulating ability could be identified amongst ER-MP12^{+} or ER-MP20^{+} BM cells.

Two-colour flow cytometric analysis of BM cells stained with ER-MP12 and ER-MP20 revealed six phenotypically distinct subpopulations. Fluorescence-activated cell sorting followed by intrathymic injection of these BM subsets into sublethally irradiated Thy-1 congenic recipient mice showed that T cell progenitor activity was exclusively confined to two BM fractions expressing the ER-MP12 antigen but lacking ER-MP20 antigen expression. Moreover, titration of the cell dose showed that the frequency of thymus-repopulating cells was maximal in the minor BM subpopulation (1–2% of total nucleated BM cells) with the highest level of ER-MP12 antigen expression. BM cells with thymus-

Table 3. Phenotype of donor-derived thymocytes 3 weeks after intrathymic injection of sorted BM cells

Cell source	%CD4$^-$CD8$^-$	%CD4$^+$CD8$^+$	%CD4$^+$CD8$^-$	%CD4$^-$CD8$^+$
ER-MP12^{++}20$^-$	1.5	86.8	8.0	3.1
ER-MP12$^+$20$^-$	9.6	79.9	9.8	2.0

C7BL/Ka Bl-1 (Thy-1.1) recipient mice were irradiated sublethally (5 Gy) and reconstituted intrathymically with either ER-MP12^{++}20$^-$ or ER-MP12$^+$20$^-$ C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2) BM cells. After 3 weeks donor-derived thymocytes were analysed for CD4 and CD8 expression. Total thymocytes of normal C57BL/6-Ly-5.1-Pep^{3b} mice served as a control for the triple staining. The thymocyte subset distribution of these control mice was 2.9%, 85.2%, 10.7%, and 1.1% respectively. The results of two independent experiments are shown.

repopulating ability were absent from the other four BM subsets.

Both BM subpopulations containing thymus-repopulating cells are heterogeneous with respect to their light scatter characteristics. ER-MP12^{++}20$^-$ BM cells are mainly medium-sized blast-like cells, while some small-sized lymphoid-like cells are also present. Conversely, the majority of ER-MP12$^+$20$^-$ BM cells are small lymphoid-like cells, while a minority display a scatter profile of medium-sized blast-like cells. It would be interesting to determine whether the small-sized or the medium-sized BM cells are responsible for the thymus-repopulating activity of these two BM subsets. In this context it is noteworthy that the Thy-1^{lo}LIN$^-$Sca-1$^+$ BM cells, which contain T cell progenitor activity (together with other haematopoietic activities), are medium-sized lymphoid-like cells (20). Therefore, further separation of the ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ BM subsets on the basis of their light scatter characteristics may result in BM cell fractions with higher frequencies of thymus-repopulating cells.

ER-MP12 may be useful for further unravelling phenotypically the functional heterogeneity displayed by highly enriched BM populations. In this context it is noteworthy that Müller-Sieburg recently showed that a novel mAb, Fall-3, could be used successfully to separate primitive haematopoietic stem cells (Fall-3$^+$) from B cell precursors (Fall-3$^-$), both contained within the Thy-1^{lo}LIN$^-$ subset of BM cells (32). The antigen recognized by the mAb Fall-3 is, like the ER-MP12 antigen, expressed by about 30% of the BM cells. ER-MP12 however is different from Fall-3, since the Fall-3 antigen is expressed only on a few cells in the peripheral lymphoid organs (32). Furthermore, Li and Johnson (33) showed that Thy-1^{lo}LIN$^-$Sca-1$^+$ BM cells could be further separated into two functionally different subsets on the basis of rhodamine 123 retention. The rhodamine 123 dull subset engrafted both myeloid and lymphoid lineages while the rhodamine 123 intermediate/bright subset mainly repopulated the lymphoid lineages (33), demonstrating that it is indeed feasible to unravel the functional heterogeneity of highly enriched BM subpopulations either physically or phenotypically.

Although the ER-MP20 antigen is not expressed by thymus-repopulating cells it clearly contributes to the purification of these T cell progenitors by excluding at least 40% of the nucleated BM cells. As suggested earlier (24), the ER-MP20 antigen is most likely identical to the Ly-6C antigen (34), since both antigens are 14 kDa single-chain (glyco)proteins and are expressed by granulocytes (intermediate levels) and monocytes (high levels) in the BM and by endothelial cells in small blood vessels. Like Ly-6C, the ER-MP20 antigen is inducible with IFN-γ (34, P. J. M. Leenen, personal communication).

In the present study we show that under the influence of the thymic microenvironment intrathymically injected ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ BM cells are able to differentiate into the various thymocyte subsets. However, by definition, prothymocytes must be able to 'home' into the thymus. Using the intrathymic transfer assay no information can be obtained concerning the thymus-homing ability of the isolated BM subpopulations (31). Therefore in order to prove conclusively that prothymocytes are indeed identified by ER-MP12 we injected the sorted BM cell fractions i.v. into irradiated congenic mice. The results of these experiments are presented and discussed in the accompanying paper (35).

In summary, in this report we show that mouse BM cells with thymus-repopulating ability are exclusively confined to two BM subpopulations expressing the ER-MP12 antigen but lacking ER-MP20 antigen expression. The highest thymus-repopulating activity was found in the BM subpopulation (1 – 2% of total nucleated BM cells) with the highest level of ER-MP12 antigen expression.

Acknowledgements

We thank Mr T. M. van Os for the preparation of the figures, and Mr J. Brandenburg and Ms E. van Bodegom for animal care. We gratefully acknowledge Drs B. Backx and I. Touw of the Department of Cell Culture of the Dr Daniel den Hoed Cancer Centre in Rotterdam for the use of and their assistance with the FACS440 cell sorter.

Abbreviations

BM	bone marrow
DBSS – FCS	Dutton's balanced salt solution supplemented with 5% FCS
FACS	fluorescence-activated cell sorter
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccimide ester
FSC	forward scatter
LIN	lineage-specific antigens
mAb	monoclonal antibody
PBS – BSA – NaN_3	PBS supplemented with 1% BSA and 20 mM NaN_3
PE	phycoerythrin
SSC	side scatter

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CHAPTER 3

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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International Immunology 1993;5:1099-1107

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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Key words: bone marrow subpopulation, ER-MP20, intravenous cell transfer, prethymic, prothymocyte, T cell development

Abstract

In the accompanying paper we showed that six distinct subsets of bone marrow (BM) cells can be identified using the mAb ER-MP12 and ER-MP20 in two-colour immunofluorescence analysis. Upon *intrathymic* transfer into sublethally irradiated mice thymus-repopulating ability was restricted to ER-MP20$^-$ BM cells expressing either high or intermediate levels of the ER-MP12 antigen (1–2% and ~30% of BM nucleated cells respectively). The highest frequency of thymus-repopulating cells was found in the minor subset of ER-MP12^{++}20$^-$ BM cells. In the present study we demonstrate that upon *intravenous* transfer, thymus-homing and -repopulating BM cells are exclusively confined to the ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ subpopulations, the highest frequency being detected among ER-MP12^{++}20$^-$ BM cells. Analysis of the peripheral blood leucocytes 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-colour flow cytometric analysis revealed that ER-MP12^{++}20$^-$ BM 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 BM cells with the ability to home to and repopulate the thymus. The phenotypic heterogeneity displayed by the ER-MP12^{++}20$^-$ BM subset, containing the highest frequency of thymus-homing and -repopulating cells, provides a basis for further separation of prothymocyte activity from other haematopoietic activities in the BM 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 (BM)-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

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Transmitting editor: T. Hünig

Received 30 December 1992, *accepted* 28 May 1993

by a low frequency of these cells in the BM, the absence of distinguishing morphological features and, above all, the paucity of mAb 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 accompanying paper (13), we showed that six distinct subpopulations of BM cells could be identified using the recently developed mAb ER-MP12 and ER-MP20 (14) in two-colour immunofluorescence analysis. As assessed by *intrathymic* transfer thymus-repopulating ability appeared to be restricted to ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells. The highest frequency of thymus-repopulating cells was found in the subpopulation of ER-MP12^{++}20^{-} BM cells.

In the present study we transferred the six BM 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^{++}20^{-} and ER-MP12^{+}20^{-} subsets have the capacity to home to the thymus *in vivo* and thus contain the genuine prothymocytes. Our data indicate that BM 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^{++}20^{-} BM subpopulation. Furthermore, phenotypic analysis of peripheral blood leucocytes 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^{++}20^{-} and ER-MP12^{+}20^{-} BM cells. Finally, three-colour flow cytometric analysis revealed that ER-MP12^{++}20^{-} BM 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 haematopoietic lineages.

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 × 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.

mAb and fluorescent reagents

The mAb 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. mAb 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 mAb conjugated to 5(6)-carboxy-fluorescein-*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 Francisco, 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-TRICOLOR (SAV-TRICOLOR) (Caltag Laboratories) were used as second stage fluorescent reagents.

Table 1. Monoclonal antibodies

mAb	Antigen	Reference
ER-MP12	ER-MP12 Ag	14
ER-MP20	ER-MP20 Ag	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 BM cells and thymi 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 three times with PBS.

Immunofluorescence staining

Two-colour immunofluorescence staining using conjugated mAb 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-colour stainings the cells were first incubated with hybridoma culture supernatant, washed and subsequently incubated with RαRa – FITC (two-colour stainings) or GαRa – PE (three-colour stainings) supplemented with 2% normal mouse serum to avoid non-specific 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 mAb, washed and finally incubated with SAV – PE (two-colour stainings) or SAV-TRICOLOR and FITC-conjugated mAb (three-colour stainings).

Table 2. Thymus-homing and repopulating ability of sorted BM cell subpopulations

Cell source (% of BM cells)[a]	% of BM cells	Number of cells transferred	Experiment number	Positive mice/ total mice[b]	% Donor-derived thymocytes[c]
Total BM		25×10^3		9/39	29.6 ± 7.8
		5×10^3		0/10	
ER-MP12$^+$20$^-$	30.7 ± 4.9	25×10^3	1	4/10	45.3 ± 6.1
			2	8/15	3.5 ± 1.6
			3	0/9	
			4	0/14	
		5×10^3	3	0/10	
			4	0/10	
ER-MP12^{++}20$^-$	2.1 ± 0.4	25×10^3	1	5/7	24.0 ± 10.2
			2	5/5	44.5 ± 15.7
			3	5/6	36.2 ± 11.6
		5×10^3	3	4/6	25.4 ± 11.5
			4	10/15	33.2 ± 8.3
ER-MP12$^+$20$^+$	9.2 ± 3.1	25×10^3		1/9	2.4
ER-MP20$^-$20$^+$	26.8 ± 6.6	25×10^3		0/18	
ER-MP12$^-$20^{++}	4.8 ± 1.7	25×10^3		0/14	
ER-MP12$^-$20$^-$	28.6 ± 5.6	25×10^3		0/14	

C57BL/KaBl-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^{3c} (Ly-5.1, Thy-1.2) mice. Thymus reconstitution was analysed 21 days after cell transfer.

[a]The relative distribution of the subpopulation among nucleated BM 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.

For cell sorting experiments BM cells were stained as previously described (13).

Flow cytometric analysis and cell sorting

Cell surface fluorescence was analysed 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 BM 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 mAb 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 analysed for the presence of donor-derived T and B lymphocytes and myeloid cells. Peripheral blood leucocytes were dually labelled 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 analysed 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 BM 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 BM 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^{++}20$^-$ and ER-MP12$^+$20$^-$ BM cells, with the highest frequency of thymus-repopulating cells being found in the former subset (constituting 1–2% of total nucleated BM cells).

In the present study we assessed the thymus-homing ability of the six BM subpopulations after *intravenous* transfer in order to investigate whether functional prothymocyte activity is confined to the ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ subsets or maybe one of these. The six BM 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 thymi of the recipient mice were analysed for the presence of donor-derived (i.e. Thy-1.2$^+$) thymocytes (Table 2). As a control the thymus-repopulating ability of unseparated BM cells was determined.

In nine out of 39 mice (23%) reconstituted with total BM cells donor-derived thymocytes were detected after i.v. injection of 25,000 cells. In recipient mice injected i.v. with 25,000 separated BM cells thymus-homing and -repopulating cells appeared to be restricted to the ER-MP12^{++}20$^-$ and ER-MP12$^+$20$^-$ subpopulations. In experiments 3 and 4 thymus-repopulating cells were only detected in the BM subpopulation with a high level of ER-MP12 antigen expression, while in the second experiment

Table 3. Thymus and peripheral blood chimerism after i.v. reconstitution with sorted ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM subpopulations

Cell source	Cell number (*n*)[a]	% donor-derived thymocytes (Thy-1.2^{+})[b]	Peripheral blood			
			% donor-derived cells (Ly-5.1^{+})[c]	% 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]
ER-MP12^{+}20^{-}	10 × 10^{3} (7)	89.5 ± 1.9	66.5 ± 6.4	16.4 ± 3.3	84.9 ± 3.0	83.6 ± 8.3
	5 × 10^{3} (7)	39.9 ± 12.0	31.0 ± 8.0	6.2 ± 1.8	76.5 ± 3.3	44.0 ± 15.3
ER-MP12^{+}20^{-}	25 × 10^{3} (9)	72.3 ± 6.4	51.5 ± 6.8	5.9 ± 0.8	74.4 ± 3.2	74.1 ± 6.4
	10 × 10^{3} (6)	61.8 ± 10.3	34.7 ± 10.5	5.3 ± 1.7	52.7 ± 9.2	55.9 ± 13.7
	5 × 10^{3} (4)	24.9 ± 13.8	13.5 ± 4.8	1.4 ± 0.4	42.5 ± 2.6	23.0 ± 12.3
Total BM	25 × 10^{3} (6)	49.9 ± 15.7	23.1 ± 6.8	4.1 ± 1.6	48.6 ± 9.4	33.5 ± 10.4
	10 × 10^{3} (2)	35.9	6.7	1.2	23.0	8.6

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 analysed 28 days after cell transfer.

[a] *n*: number of mice analysed.

[b] The average percentage ± SEM of donor-derived thymocytes (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 T cells (CD3^{+}), B cells (B220^{+}) or myeloid cells (Mac-1^{+}) which were of donor origin (Ly-5.1^{+}).

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^{+}20^{-} BM cells. Re-analysis of the sorted BM fractions revealed the same degree of purity (>96%) for the ER-MP12^{+}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^{+}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^{+}20^{-} BM cells (Table 2). These data demonstrate that: (i) BM 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^{+}20^{-} BM subset contains the highest frequency of thymus-homing and repopulating BM cells.

Multi-lineage reconstitution potential of ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM cells

To assess the developmental potential of ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM cells more extensively we i.v. transferred sorted ER-MP12^{+}20^{-} or ER-MP12^{+}20^{-} BM cells or unseparated BM cells into 8 Gy irradiated Ly-5/Thy-1 congenic recipient mice. Four weeks later peripheral blood nucleated cells were analysed 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^{+}20^{-} BM cells (e.g. 31.0% versus 13.5% after transfer of 5000 ER-MP12^{+}20^{-} cells). The highest percentage of donor-derived T lymphocytes in peripheral blood was detected after transfer of ER-MP12^{+}20^{-} BM cells (e.g. 6.2% versus 1.4% after transfer of 5000 ER-MP12^{+}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^{+}20^{-} cells as well as ER-MP12^{+}20^{-} cells (Table 3). The considerable percentage of donor-derived thymocytes in mice reconstituted with 5000 ER-MP12^{+}20^{-} cells is in sharp contrast to 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 haematopoietic stem cells within the ER-MP12^{+}20^{-} subset. These cells most likely need additional maturational events in the BM 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^{+}20^{-} BM cells. Taken together our results show that both ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM subsets contain not only prothymocytes but also progenitor cells capable of differentiating into B lymphocytes and myeloid cells.

Cell surface phenotype of ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM cells

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

We analysed the expression of the mature lineage markers Gr-1, Mac-1, CD4, CD8 and B220 by ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM cells in order to compare the current

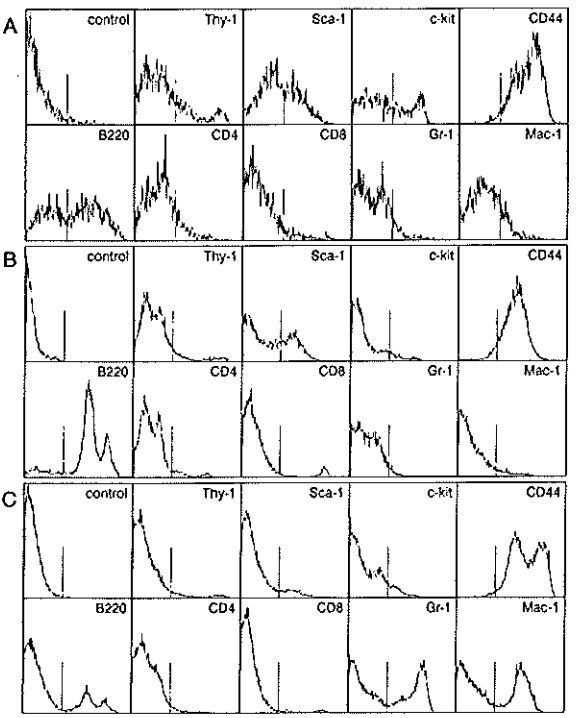

Fig. 1. Cell surface phenotype of ER-MP12^{+}20^{-} (A), ER-MP12^{+}20^{-} (B) and unseparated BM cells (C). BM cells were stained with mAb specific for either of the indicated Ag (second stage reagent: GαRa – PE), FITC-conjugated ER-MP20 and biotinylated ER-MP12 (followed by SAV-TRICOLOR). Control: isotype-matched control mAb (IgG2a or IgG2b). Life gates were set on either ER-MP12^{+}20^{-} (A) or ER-MP12^{+}20^{-} cells (B) and the expression of the indicated markers was analysed. Histograms in panels A, B and C were generated from 4000, 10,000 and 14,000 events respectively. The results of one representative experiment are shown.

progenitor fractions with those isolated by others using depletion of BM cells for the mentioned mature markers (24,25,27, 28). Compared to unseparated BM cells [Fig. 1(C)] both ER-MP12^{+}20^{-} and ER-MP12^{+}20^{-} BM subpopulations were depleted of cells that strongly expressed Gr-1 and Mac-1 [Fig. 1(A) and (B) respectively]. Lymphoid cells expressing high levels of CD4 and CD8, most likely mature T lymphocytes, were only detected in the ER-MP12^{+}20^{-} subset [Fig. 1(B)]. 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^{+}20^{-} BM cells expressed B220 [Fig. 1(B)], suggesting that the majority of the cells in the ER-MP12^{+}20^{-} BM subset belong to the B cell lineage. In contrast, ER-MP12^{+}20^{-} cells differed strikingly from the ER-MP12^{+}20^{-} subset in that about 50% lacked the expression of B220 [Fig. 1(A)]. Analysis of the forward light scatter against the fluorescence distribution revealed that the B220^{-} cells in the ER-MP12^{+}20^{-} subset were mainly blast-like cells [Fig. 2(A)]. Interestingly the ER-MP12^{+}20^{-} BM cells expressing the highest level of B220 antigen appeared to be large blast-like cells while ER-MP12^{+}20^{-} cells with an intermediate level of B220 expression showed a scatter profile characteristic for lymphoid cells [Fig. 2(A)].

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

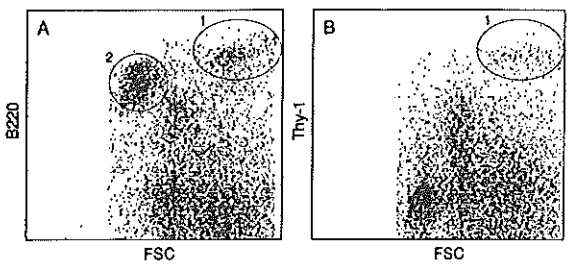

Fig. 2. Forward scatter versus log fluorescence distribution of ER-MP12^{++}20^{-} BM cells stained with B220 (A) and Thy-1 (B). BM cells were stained as described in Methods (three-colour staining). Life gates were set on ER-MP12^{++}20^{-} cells and the expression of the third marker was analysed. 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.

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

Mouse strain	MHC H-2	ER-MP12^{-}20^{-}	ER-MP12^{+}20^{-}	ER-MP12^{++}20^{-}	ER-MP12^{-}20^{+}	ER-MP12^{-}20^{++}	ER-MP12^{+}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

BM cells were simultaneously stained with ER-MP12 and ER-MP20. The percentages of nucleated BM cells among the six different subpopulations were determined by FACScan analysis.

in the ER-MP12^{+}20^{-} BM subset [Fig. 1(B)] 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 BM cells expressing Sca-1 (Ly-6A/E; 25). Our data demonstrate that about 30% of both ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} BM cells expressed Sca-1 [Fig. 1(A) and (B) respectively] compared to about 10% of ungated BM cells [Fig. 1(C)]. The only other cells in BM 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 BM cells expressing the ER-MP12 antigen.

ER-MP12^{++}20^{-} cells differed markedly from ER-MP12^{+}20^{-} cells in the expression of c-*kit*, the receptor for stem cell factor (34,35), which has been found on haematopoietic stem cells and progenitor cells (26,36,37). Two percent of the cells in ungated BM and in the ER-MP12^{+}20^{-} subpopulation expressed high levels of c-*kit* [Fig. 1(C) and (B) respectively]. In contrast, 35–40% of the ER-MP12^{++}20^{-} cells, mainly blast-like cells (data not shown), expressed this cell surface marker at high levels [Fig. 1(A)], suggesting a high frequency of progenitor cells in this latter subpopulation.

CD44 is differentially expressed by most haematopoietic 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^{+}20^{-} BM cells expressed intermediate levels of this antigen [Fig. 1(B)]. Analysis of the ER-MP12^{++}20^{-} subpopulation revealed cells expressing intermediate levels as well as high levels (more than 50%) of CD44 [Fig. 1(A)].

Taken together our data demonstrate that: (i) selection against ER-MP20 and for ER-MP12 antigen expression results in the depletion of BM cells expressing high levels of the myeloid lineage differentiation markers Gr-1 and Mac-1; (ii) both ER-MP12^{++}20^{-} and ER-MP12^{+}20^{-} cells are heterogeneous with respect to the expression of several cell surface markers; (iii) ER-MP12^{++}20^{-} cells are phenotypically more heterogeneous than ER-MP12^{+}20^{-} BM 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 BM 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 BM cells from mice of different haplotypes (Table 4). Our results show that both antigens are at least expressed 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 BM 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 BM cells) of ER-MP12$^+$20$^-$ BM cells were detected in all tested strains.

Discussion

In the previous paper (13) we showed that by using the mAb ER-MP12 and ER-MP20 in two-colour immunofluorescence analysis six distinct subpopulations of BM cells could be identified. Upon intrathymic transfer into sublethally irradiated recipient mice we found that thymus-repopulating ability was exclusively confined to BM 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-MP12$^+$20$^-$ BM cells (1 – 2% of total nucleated BM 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 BM 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-MP12$^+$20$^-$ and ER-MP12$^+$20$^-$ BM subpopulations. Our results demonstrate that prothymocyte activity (assayed 21 days after i.v. transfer) is indeed restricted to the ER-MP12$^+$20$^-$ and ER-MP12$^+$20$^-$ subset of BM cells. The highest frequency of thymus-homing and -repopulating cells was detected in the ER-MP12$^+$20$^-$ subpopulation.

Analysis of the peripheral blood leucocytes of mice reconstituted 28 days earlier with either ER-MP12$^+$20$^-$ or ER-MP12$^+$20$^-$ 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-MP12$^+$20$^-$ or 5000 ER-MP12$^+$20$^-$ cells, whereas at 21 days donor-derived *thymocytes* were only detected in mice reconstituted with the ER-MP12$^+$20$^-$ subset. This apparent discrepancy is most likely explained by the presence of multipotent stem cells within the ER-MP12$^+$20$^-$ subset (J. C. M. van der Loo *et al.*, in preparation) which need to undergo additional maturational events in the BM 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-MP12$^+$20$^-$ 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 days after i.v. transfer).

Our results demonstrate that although the ER-MP12$^+$20$^-$ subset of BM cells contains a high frequency of prothymocytes, the separation of the pro-T cell activity from other haematopoietic activities will depend upon the use of additional cell surface markers.

Cell surface phenotyping revealed that both ER-MP12$^+$20$^-$ and ER-MP12$^+$20$^-$ BM 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 haematopoietic progenitor cells. As the Mac-1 antigen has been detected on a subset of thymus-repopulating cells (24, 25,39) it remains to be tested whether Mac-1^{lo} cells, constituting a small percentage of both ER-MP12$^+$20$^-$ and ER-MP12$^+$20$^-$ cells, are capable of differentiating into the T cell lineage. Cells expressing high levels of CD4 were depleted of the ER-MP12$^+$20$^-$ 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$^+$20$^-$ cells. Cells expressing low levels of CD4 were found in both subsets. Recent reports have shown that both haematopoietic stem cells and the earliest detectable thymocytes express CD4 at a low level (7,8,29,30). Therefore the use of anti-CD4 mAb in BM 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$^+$20$^-$ BM cells expressed B220. These cells therefore most probably belong to the B cell lineage. In contrast half of the ER-MP12$^+$20$^-$ BM cells were B220$^-$. Surprisingly the ER-MP12$^+$20$^-$ cells expressing high levels of B220 appeared to be blast-like cells. Further research is required to determine whether this B220$^+$ subpopulation of ER-MP12$^+$20$^-$ BM cells is restricted to B cell lineage development. On the other hand the presence of thymus-repopulating cells in this B220$^+$ 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$^+$ BM cells expressing B220 and Mac-1 (39).

The majority of ER-MP12$^+$20$^-$ and ER-MP12$^+$20$^-$ cells are Thy-1$^-$, while both subsets contain cells expressing low levels of Thy-1. Progenitor cells, including thymus-repopulating cells, and haematopoietic 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} BM 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 (40). Although the thymus-repopulating ability of the Thy-1.2$^-$ and Thy-1.2^{lo} cells was not assessed in that study (40) a low level of Thy-1 expression is maybe not a valid criterion for the isolation of prothymocytes from the BM of Thy-1.2 genotype mice. Interestingly, some ER-MP12$^+$20$^-$ BM 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 (41) the presence of these cells within the Thy-1^{hi} subset of ER-MP12$^+$20$^-$ 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 haematopoietic progenitors and stem cells (25,28,39). 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 BM cells of Ly-6^b haplotype mice (42). 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 BM subpopulations.

Cells expressing high levels of the proto-oncogene c-*kit* were markedly enriched among ER-MP12$^+$$^+20^-$ BM cells. This cell surface marker has been detected on haematopoietic stem cells and progenitor cells (26,36–38), also including the earliest intrathymic precursor cells (43). Therefore separation of the ER-MP12$^+$$^+20^-$ BM cells on the basis of c-*kit* expression may lead to the further purification of prothymocytes.

ER-MP12$^+$$^+20^-$ cells expressed either high or intermediate levels of CD44 while all ER-MP12$^+$20$^-$ BM cells expressed this cell surface marker at an intermediate level. An intermediate level of CD44 has been detected on pluripotent stem cells and Thy-1^{lo}LIN$^-$Sca-1$^+$ BM cells (25,38) while the earliest intrathymic T cell precursors express high levels of CD44 (7,8). In this context separation of ER-MP12$^+$$^+20^-$ BM 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 (44), or the heterogeneity of different stem cell and progenitor subsets for rhodamine 123 retention and wheat germ agglutinin affinity (45,46, J. C. M. van der Loo *et al.*, in preparation) may prove to be useful for the separation of the pro-T cell activity from other haematopoietic activities in the ER-MP12$^+$$^+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$^+$$^+20^-$ and ER-MP12$^+$20$^-$ subsets of BM cells. The highest frequency of thymus-homing and -repopulating BM cells was found among ER-MP12$^+$$^+20^-$ cells which heterogeneously expressed the cell surface markers Thy-1, Sca-1, CD44, B220 and c-*kit*. This phenoptyic heterogeneity provides a basis for the further purification of thymus-homing and -repopulating cells from the ER-MP12$^+$$^+20^-$ subpopulation.

Acknowledgements

We thank Mr J. Brandenburg and Ms E. van Bodegom for animal care and Mr T. M. van Os for the preparation of the figures.

Abbreviations

BM	bone marrow
DBSS – FCS	Dutton's balanced salt solution supplemented with 5% FCS
FACS	fluorescence-activated cell sorter
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester
GαRA – PE	goat anti-rat Ig conjugated to phycoerythrin
LIN	lineage-specific antigens
PBS – BSA – NaN_3	PBS supplemented with 1% BSA and 20 mM NaN_3
PE	phycoerythrin
RαRa – FITC	rabbit anti-rat(Fab)$_2$ conjugated to FITC
SAV – PE	streptavidin – phycoerythrin
SAV-TRICOLOR	streptavidin-tricolor

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CHAPTER 4

Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12

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Blood 1995;85:952-962

Identification of Hematopoietic Stem Cell Subsets on the Basis of Their Primitiveness Using Antibody ER-MP12

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Monoclonal antibody ER-MP12 defines a novel antigen on murine hematopoietic stem cells. The antigen is differentially expressed by different subsets in the hematopoietic stem cell compartment and enables a physical separation of primitive long-term repopulating stem cells from more mature multilineage progenitors. When used in two-color immunofluorescence with ER-MP20 (anti-Ly-6C), six subpopulations of bone marrow (BM) cells could be identified. These subsets were isolated using magnetic and fluorescence-activated cell sorting, phenotypically analyzed, and tested in vitro for cobblestone area-forming cells (CAFC) and colony-forming units in culture (CFU-C; M/G/E/Meg/Mast). In addition, they were tested in vivo for day-12 spleen colony-forming units (CFU-S-12), and for cells with long-term repopulating ability using a recently developed α-thalassemic chimeric mouse model. Cells with long-term repopulating ability (LTRA) and day-12 spleen colony-forming ability appeared to be exclusively present in the two subpopulations that expressed the ER-MP12 cell surface antigen at either an intermediate or high level, but lacked the expression of Ly-6C. The ER-MP12^{med}20^{-} subpopulation (comprising 30% of the BM cells, including all lymphocytes) contained 90% to 95% of the LTRA cells and immature day-28 CAFC (CAFC-28), 75% of the CFU-S-12, and very low numbers of CFU-C. In contrast, the ER-MP12^{hi}20^{-} population (comprising 1% to 2% of the BM cells, containing no mature cells) included 80% of the early and less primitive CAFC (CAFC-5), 25% of the CFU-S-12, and only 10% of the LTRA cells and immature CAFC-28. The ER-MP12^{hi} cells, irrespective of the ER-MP20 antigen expression, included 80% to 90% of the CFU-C (day 4 through day 14), of which 70% were ER-MP20^{-} and 10% to 20% ER-MP20$^{med/hi}$. In addition, erythroblasts, granulocytes, lymphocytes, and monocytes could almost be fully separated on the basis of ER-MP12 and ER-MP20 antigen expression. Functionally, the presence of ER-MP12 in a long-term BM culture did not affect hematopoiesis, as was measured in the CAFC assay. Our data demonstrate that the ER-MP12 antigen is intermediately expressed on the long-term repopulating hematopoietic stem cell. Its level of expression increases on maturation towards CFU-C, to disappear from mature hematopoietic cells, except from B and T lymphocytes.

THE BONE MARROW (BM) hematopoietic stem cell compartment contains a hierarchically organized continuum of stem cell subsets, ranging from pluripotent hematopoietic stem cells to in vitro clonable multilineage progenitors.[1,2] The most immature hematopoietic stem cells are functionally defined by their extensive self-renewal capacity and ability to provide multilineage long-term repopulation (LTRA) in sublethally or lethally irradiated animals, which was studied using retrovirally marked stem cell clones.[3-7] It has been shown that LTRA cells can be physically separated from the large majority of day-12 spleen colony-forming units (CFU-S-12) on the basis of cell size and density using centrifugal counterflow elutriation (CCE).[8,9] However, the lack of stem cell-specific markers makes it difficult to highly enrich LTRA cells and simultaneously separate them from the short-term repopulating cells. Separation would benefit the search for stem cell specific genes,[10,11] and aid in the development of protocols for more efficient gene transfer in hematopoietic stem cells.

To achieve high enrichments, techniques such as CCE, density centrifugation, and magnetic sorting for lineage marker-negative cells, had to be combined with fluorescence-activated cell sorting (FACS).[12,13] The different protocols included separation on the basis of rhodamine-123 (Rh123) retention,[14-19] Hoechst 33342 fluorescence,[19,20] and wheat germ agglutinin (WGA) affinity.[21-24] This resulted in a 850- to 2,000-fold enrichment for multilineage long-term repopulating stem cells with relatively low numbers of copurified CFU-S-12.[19,22,25,26] However, the most primitive and more mature hematopoietic stem cell subsets could not be discriminated on the basis of cell surface antigens. Apart from stem cell purification, antigens that are selectively expressed by the most primitive hematopoietic stem cells could show information on the complex interactions of these cells with their specific microenvironment. Therefore, we set out to find cell surface markers that are differentially expressed on the different subsets of the hematopoietic stem cell compartment.

Recently, we produced two novel rat monoclonal antibodies (MoAbs), ER-MP12, and ER-MP20,[27,28] showing six distinct subpopulations of murine BM cells when used in two-color immunofluorescence.[29] ER-MP12 recognizes a yet unknown 140 kD single-chain glycoprotein on murine hematopoietic cells. ER-MP20 was shown to be directed against differentiation antigen (Ag) Ly-6C.[30] After sorting the different subsets on the basis of ER-MP12 and ER-MP20 Ag expression, the highest frequency of thymus-seeding and repopulating cells was found in the subpopulation (1% to 2% of BM cells) that lacked Ly-6C, but expressed the ER-MP12 Ag at a high level (ER-MP12^{hi}20^{-}).[29,31] After intravenous transfer, however, both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations generated B lymphocytes and myeloid cells in addition to T lymphocytes, indicating the presence of pluripotent hematopoietic cells. Although fre-

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Submitted June 3, 1994; accepted October 13, 1994.

Supported by the Netherlands Organization for Scientific Research (NWO), Project No. 900-508-242.

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0006-4971/95/8504-0011$3.00/0

quencies and recoveries of these pluripotent cells were not assessed, it showed that ER-MP12 could possibly be used as a tool in dissecting the hematopoietic stem cell compartment.

In the present report, we investigated the expression of the ER-MP12 Ag on the various hematopoietic stem cell and progenitor cell subsets in the BM. Using magnetic and FACS, BM cells were separated into six subpopulations on the basis of their differential expression of the ER-MP12 and ER-MP20 antigens. The subpopulations were phenotypically analyzed and subsequently tested for cobblestone-area forming cells [CAFC]-7 through CAFC-35[32,33] and long-term in vivo repopulating cells, using an α-thalassemic chimeric mouse model as was recently described.[23] In addition, the subsets were tested for CFU-S-12 and for in vitro clonable progenitors, including macrophage, granulocyte, erythroid, megakaryocyte, and mast cell colony-forming units.

Our data demonstrate that the ER-MP12 Ag is differentially expressed by the various subsets in the BM stem cell compartment, with LTRA cells expressing the ER-MP12 Ag at an intermediate level. With development towards colony-forming units in culture (CFU-C), the Ag expression gradually increases to disappear from most lineages in the course of their final maturation, except from T and B lymphocytes. The present results identify the ER-MP12 Ag as a novel positive marker on hematopoietic stem cells with a level of expression that is inversely related to the primitiveness of the cells in the stem and progenitor cell compartment.

MATERIALS AND METHODS

Animals. Male inbred BALB/cAnCrlRij mice, and female heterozygous α-thalassemic BALB/c ($Hba^{th/+}$) mice,[34] were bred at the former Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands. C57BL/6-Ly5.1-pep^{b} (Thy-1.2, Ly-5.1) and (CBA♂ × C57Bl♀)F1 mice were bred at the Central Animal Department of Erasmus University. All mice, 12 to 25 weeks of age, were bred, maintained under specific pathogen free conditions, and received acidified water (Ph 2.8) and food pellets ad libitum. The C57BL/6-Ly5.1-pep^{b} breeding pairs were kindly provided by Dr I.L. Weissman (Stanford University, Stanford, CA).

Monoclonal antibodies and conjugates. Rat MoAbs ER-MP12 and ER-MP20 (both IgG2a isotypes) were purified from culture supernatants by ammonium sulphate precipitation. ER-MP20 was conjugated to fluorescein isothiocyanate (FITC; Isomer I; Sigma, St Louis, MO; ER-$MP20^{FITC}$), whereas ER-MP12 was biotinylated (ER-$MP12^{bio}$) using N-hydroxy-succinimidyl-biotin (Sigma) according to standard procedures.[35] Streptavidin-conjugated R-phycoerythrin (SAV-PE) and streptavidin-conjugated TRICOLOR (SAV-TRI) were obtained from Caltag Laboratories (South San Francisco, CA). Hybridoma supernatants were used for MoAbs RA3-6B2 (anti-B220),[36] RB6-8C5 (anti-GR-1),[37] and M1/70 (anti-Mac-1).[38] Erythroid lineage specific MoAb TER-119[39] was kindly provided by Dr T. Kina, Kyoto University, Kyoto, Japan. MoAb PH2-99, an anti-*E. coli*-β-galactosidase (rat IgG2a) was made at the Department of Immunology, Erasmus University, Rotterdam, The Netherlands. As a second stage antibody R-phycoerythrin-conjugated mouse-adsorbed goat antirat IgG (H + L) (GαRa-PE; Caltag) was used. Antibodies and conjugates were titrated for optimal staining of mouse BM cells.

Preparation of cell suspensions. Preparation of the BM cells, and (in specific experiments) buoyant density centrifugation, using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient, was performed as previously described.[33] Cells with a density of 1.069 to 1.075 g/mL (2% to 6% of total BM) were collected from the interphase, washed in phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS), and maintained on ice throughout the staining and purification procedure. For long-term repopulation experiments, low-density BM cells were depleted of ER-MP20-positive cells by magnetic separation using the MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany), before FACS. Cells were incubated for 30 minutes with ER-$MP20^{bio}$ in PBS containing 0.01% (wt/vol) NaN_3, washed, and subsequently incubated for 15 minutes with streptavidin-conjugated MACS microbeads (Miltenyi Biotec) in PBS containing 0.01% NaN_3 and 5 mmol/L EDTA (Titriplex III; Merck, Darmstadt, Germany). After incubation, the cells were washed in PBS supplemented with 0.01% NaN_3, 5 mmol/L EDTA and 1% (wt/vol) bovine serum albumin (BSA; Fraction V; Sigma) and separated using the MACS column B2 (Miltenyi Biotec) at a flow rate of 0.2 to 0.3 mL/min. MACS beads were sterilized by filtration through a 0.22 μm filter. The nonmagnetic, ER-MP20–negative population (ER-$MP20^{-}$) was collected and maintained on ice in PBS containing 5% FCS.

Immunofluorescence staining and cell sorting. For two-color immunofluorescence staining, unseparated or low-density BM cells (5×10^6 cells per 50 μL) were incubated with ER-$MP12^{bio}$, washed in PBS containing 0.01% NaN_3 and 0.5% BSA, and subsequently incubated with ER-$MP20^{FITC}$ and SAV-PE. Washing buffer was used for all dilutions. For three-color analysis, the cells were first incubated with hybridoma supernatant (MoAbs, see above). This was followed by an incubation with Gαra-PE containing 2% normal mouse serum to avoid nonspecific binding. The cells were subsequently washed with buffer containing 2% normal rat serum to block any free binding sites, and incubated with ER-$MP12^{bio}$ followed by ER-$MP20^{FITC}$ and SAV-TRI. For in vivo experiments, low-density BM cells were first depleted of ER-MP20-positive cells by MACS and then stained with ER-$MP12^{bio}$ and SAV-PE for further sorting. All incubations were performed for 30 minutes at 0°C. Cells were analyzed using a FACScan flowcytometer (Becton Dickinson, Mountain View, CA) or sorted on either a FACS 440 or a FACS Vantage (Becton Dickinson) at a rate of 2,500 cells per second using a single argon ion laser tuned at 488 nm (100 mW). Viable cells were counted using a hemocytometer.

Colony assay. The number of in vitro clonable progenitors CFU-C was determined by culturing either 1 to 2×10^4 unseparated BM cells or varying numbers of sorted cells in 1 mL cultures. The culture medium consisted of the α-modification of Dulbecco's modified Eagle's medium (DMEM), (GIBCO, Grand Island, NY) at an osmolarity of 280 mOsm/kg. The medium was supplemented with 1.2% (wt/vol) methylcellulose (Methocel MC; Fluka Chemie, Buchs, Switzerland), 20% horse serum (GIBCO), 1% BSA, 80 μg/mL penicillin, 80 μg/mL streptomycin, 3.2 mmol/L L-glutamine (Merck, Darmstadt, Germany), 8×10^{-5} mol/L sodium selenite (Merck) and 8×10^{-5} mol/L β-mercaptoethanol (at final concentrations). The cultures were either stimulated by 10% (vol/vol) pokeweed mitogen-stimulated mouse spleen-conditioned medium (PWM-MSCM) and 2 U/mL recombinant human erythropoietin (Epo; Merckle, Ulm, Germany), or contained 2 U/mL Epo, 50 U/mL murine interleukin-3 (IL-3), 15 U/mL murine steel factor (SF), 50 U/mL human IL-11, and 2 ng/mL murine IL-12. The recombinant murine cytokines IL-3, SF, IL-11, and IL-12 were kindly provided by Dr S. Neben of the Genetics Institute (Cambridge, MA). The cultures were kept at 37°C, 5% CO_2, and 100% humidity. Colonies containing 50 cells or more were counted after 4 to 14 days of culture.

CAFC-assay. Long-term BM cultures were established in 96-well plates for limiting dilution analysis of CAFC as previously described.[32,40] To determine the effect of ER-MP12 on hematopoiesis in vitro, unseparated BM cells were tested in the CAFC assay in the presence or absence of 5 μg/mL ER-MP12 (rat IgG2a) or control MoAb PH2-99 (rat anti-*Escherichia coli*-β-galactosidase IgG2a).

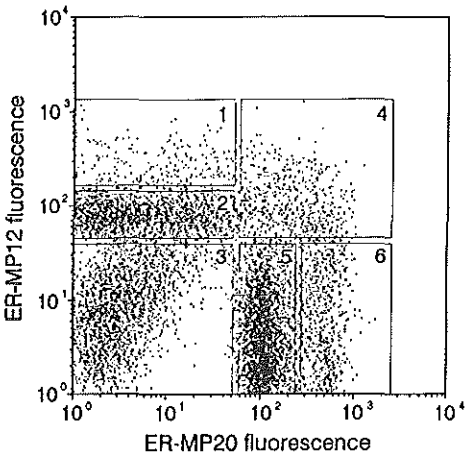

Fig 1. Two-color immunofluorescence analysis of mouse BM cells stained with MoAbs ER-MP12 and ER-MP20. Window 1 (ER-MP12^{hi}20^{-}) contained 2.1% ± 0.1% of the cells, window 2 (ER-MP12^{med}20^{-}) 30.0% ± 1.9%, window 3 (ER-MP12^{-}20^{-}) 28.1% ± 2.0%, window 4 (ER-MP12^{+}20^{+}) 9.2% ± 1.2%, window 5 (ER-MP12^{-}20^{med}) 25.6% ± 1.8%, and window 6 (ER-MP12^{-}20^{hi}) 5.0% ± 0.2% (average ± 1 SEM; based on 10 experiments).

For this purpose, BM cells were first labeled with one of the MoAbs and then inoculated. Half of the medium, including the MoAbs, were changed every 3 to 4 days. CAFC frequencies were determined over a 4-week period.

CFU-S assay. The number of CFU-S-12 in unseparated and sorted BM cells was determined by intravenous injection into eight Gy-irradiated BALB/c recipients (eight to 12 mice per group).[41] At day 12 the spleens were excised and fixed in Telleyesniczky's solution. The macroscopic colonies were counted.

LTRA. Unseparated BM and sorted cells, derived from male BALB/c donor mice, were intravenously injected into five Gy-irradiated female α-thalassemic BALB/c (Hba$^{dc/-}$) mice (six to eight mice per group). Red blood cell chimerism was determined from 6 weeks up to 1 year after transplantation. Using the forward light-scatter as a measure of erythrocyte size, the distribution of normal-sized (donor-type) and microcytic thalassemic (recipient-type) erythrocytes was analyzed using a small drop of peripheral blood as previously described.[42,43]

RESULTS

Phenotypic characterization of ER-MP12 and ER-MP20 labeled BM subsets. To study the distribution of the hematopoietic lineages, murine BM cells were sorted into six subpopulations, based on the expression of the ER-MP12 and ER-MP20 antigens in a two-color immunofluorescence analysis (Fig 1).[29] Four of the subpopulations expressed one of the two antigens, at either an intermediate or a high level. The ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets contained 2% and 30% of the nucleated cells, respectively, whereas the ER-MP12^{-}20^{med} and ER-MP12^{-}20^{hi} subsets included 26% and 5%, respectively. The subset that expressed both antigens, irrespective of the level of expression, was designated ER-MP12^{+}20^{+} and contained 9% of the BM cells. The remaining population that lacked both antigens and contained 28% of the cells was denoted as ER-MP12^{-}20^{-}. The distribution of the hematopoietic subsets was calculated by taking into account the sizes of the subpopulations in unseparated BM (Table 1). All early myeloid cells expressed the ER-MP20 Ag, whereas the early erythroid cells were found predominantly in the ER-MP12^{-}20^{-}, and for a smaller part in the ER-MP12^{+}20^{+} population. Although the ER-MP12^{hi}20^{-} subpopulation consisted of 93% blast cells, of which 50% undifferentiated (data not shown), undifferentiated blasts could quantitatively be recovered from all subpopulations. Interestingly, all late erythroid cells (98%), mature granulocytes (97%), lymphocytes (96%), and the majority of the monocytes (76%), could be recovered from the ER-MP12^{-}20^{-}, ER-MP12^{-}20^{med}, ER-MP12^{med}20^{-}, and ER-MP12^{-}20^{hi} subpopulations, respectively.

To verify the presence of the morphologically identified lymphocytes, granulocytes, and monocytes, the subpopulations were tested in a three-color immunofluorescence analysis for the expression of B220, Mac-1, or Gr-1. Erythroblasts were labeled with TER-119 (Fig 2). The ER-MP12^{med}20^{-} subset almost completely consisted of B220^{+} lymphocytes. Also, cells expressing high levels of CD4 and CD8 were exclusively found in this subset (data not shown).[31] All ER-MP12^{-}20^{-} cells appeared to express the TER-119 Ag, while the ER-MP12^{-}20^{med} cells all expressed Gr-1. The majority of the cells in the ER-MP12^{-}20^{hi} subpopulation, containing a high frequency of morphologically recognizable monocytes (Table 1), expressed Mac-1. Therefore, these data confirm the morphological analyses and show that the mature hematopoietic lineages in murine BM represent distinct cell classes on the basis of their expression of the ER-MP12 and ER-MP20 antigens.

In the following report, we successively studied the distribution of the ER-MP12 and ER-MP20 antigens on the different CAFC subsets, in vivo long-term repopulating stem cells, CFU-S-12 and in vitro clonable progenitors, respectively.

CAFC subsets differentially express the ER-MP12 Ag. Using the CAFC assay, we determined the frequency of hematopoietic precursors in the six subpopulations (Fig 1). We previously showed that CAFC frequencies determined at 10 days after inoculation (CAFC-10) correlate highly with the number of CFU-S-12, while frequencies determined after 4 weeks (CAFC-28/35) correlate highly with the long-term in vivo repopulating ability of a graft.[22-24,33,44] Compared with unseparated BM, the ER-MP12^{hi}20^{-}, ER-MP12^{med}20^{-}, and ER-MP12^{+}20^{+} subsets were enriched for CAFC (Fig 3A). The ER-MP12^{hi}20^{-} subset contained the highest frequency of early, as well as late CAFC (Fig 3A). Although this subpopulation quantitatively included 80% of the early CAFC, it contained only 10% of the more immature CAFC-28 (Fig 3B). In contrast, the ER-MP12^{med}20^{-} subpopulation contained only 5% of the more mature progenitors, but included nearly 90% of the CAFC-28. The ER-MP12^{+}20^{+} subpopulation contained only transient CAFCs that disappeared within the first 10 days. Therefore, CAFC-10 (CFU-S-12), as well as the more immature CAFC-28 (LTRA cells), were found only in the subpopulations that expressed the ER-MP12 Ag

Table 1. Distribution of Hematopoietic Lineages in Six Subpopulations Defined on the Basis of ER-MP12 and ER-MP20 Ag Expression

Population*	Frequency in NBMC (%)	Granuloid†		Erythroid		Lymphoid	Monocytes	Blasts
		Early	Late	Early	Late			
ER-MP12^{hi}20^{-}	2.1	2.0	0	6.4	0.3	1.7	0.4	10.0
ER-MP12^{med}20^{-}	30.0	0	0	1.4	0	96.2	7.8	20.5
ER-MP12^{-}20^{hi}	5.0	21.9	0.1	0.6	0.1	0	76.3	3.2
ER-MP12^{-}20^{med}	25.6	30.6	96.8	0	0	0	5.3	8.6
ER-MP12^{+}20^{+}	9.2	45.5	2.7	28.1	1.8	1.8	10.2	33.8
ER-MP12^{-}20^{-}	28.1	0	0.4	63.5	97.8	0.3	0	23.9

* BM was sorted into six different subpopulations on the basis of their ER-MP12 and ER-MP20 Ag expression.

† Sorted cells were stained with May-Grünwald/Giemsa and 400 cells per group were differentiated by microscope. Figures show the percentage recovery of the hematopoietic lineages with columns amounting to 100%. Myeloblasts and myelocytes were classified as early, bands and segmented granulocytes as late granuloid cells. Early erythroid cells included pro- and basophilic erythroblasts; late erythroid cells represent polychromatophils and normoblasts. Undifferentiated blast-like cells are denoted as blasts. Megakaryocytes are left out as their frequency was very low.

but lacked the expression of ER-MP20. More specifically, most immature stem cells (CAFC-28) expressed ER-MP12 at an intermediate level, whereas upon maturation from CAFC-28 up to CAFC-5, an increasing percentage was found in the subpopulation expressing ER-MP12 at a high level.

CAFC subsets in low-density and in unseparated BM are equally distributed with respect to ER-MP12 Ag expression. To achieve high numbers of purified cells for combined in vitro and in vivo studies, BM had to be pre-enriched by density centrifugation. Figure 4 shows the forward angle light-scatter against the ER-MP12 expression in unseparated BM, low-density BM, and MACS-depleted ER-MP20^{-} low-density BM. The procedure very effectively enriched for blast-like cells with a high and intermediate ER-MP12 expression. Furthermore, the ER-MP12^{-}20^{-} subpopulation was almost depleted by the density cut (Fig 4C), as it mainly consisted of erythroblast and normoblasts (Table 1 and Fig 2). To relate the distribution of the LTRA cells in low-density BM (see next section) to the other data, sorted cells from low-density BM were also tested in the CAFC assay, in parallel with the in vivo experiments. Low-density BM was approximately 10.5-fold enriched for all CAFC compared with unseparated BM (data not shown). As in unseparated BM, the low-density ER-MP12^{hi}20^{-} subset contained the more mature precursors that gave rise to an early CA-formation, whereas 90% to 95% of the CAFC-28 could be recovered in the ER-MP12^{med}20^{-} subpopulation (data not shown). Therefore, the distribution of the most immature CAFC subsets with respect to the expression of the ER-MP12 Ag had not changed by the pre-enrichment procedure.

Majority of in vivo *long-term repopulating stem cells express ER-MP12 intermediately.* To investigate whether the above defined subpopulations contained long-term or transiently in vivo repopulating stem cells, the subsets were intravenously injected into sublethally irradiated α-thalassemic mice. Donor-type repopulation was defined by the percentage of normal-sized erythrocytes in the peripheral blood

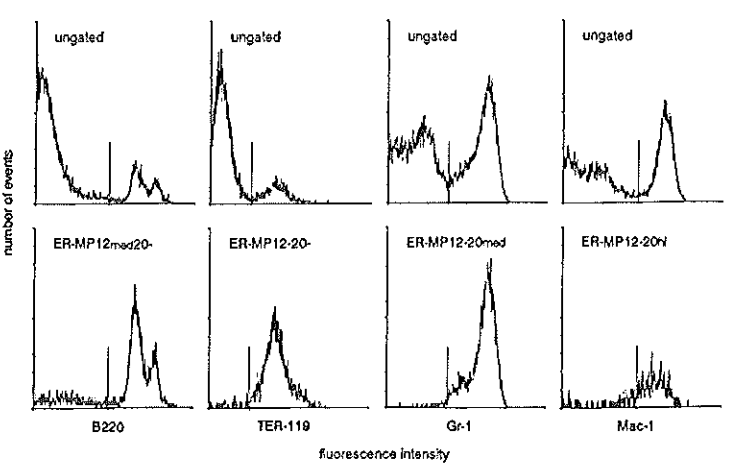

Fig 2. Expression of B220, TER-119, Mac-1, and Gr-1 on total BM cells (ungated), and BM cells gated for different ER-MP12/ER-MP20 subpopulations. For three color-immunofluorescence analysis, cells were first incubated with hybridoma supernatants subsequently followed by Gora-PE, ER-MP20^{FITC}, ER-MP12^{Bio}, and SAV-TRI. Subpopulations were gated according to the windows in Fig 1. Background fluorescence, as determined with an isotype-matched control MoAb, is indicated for each marker.

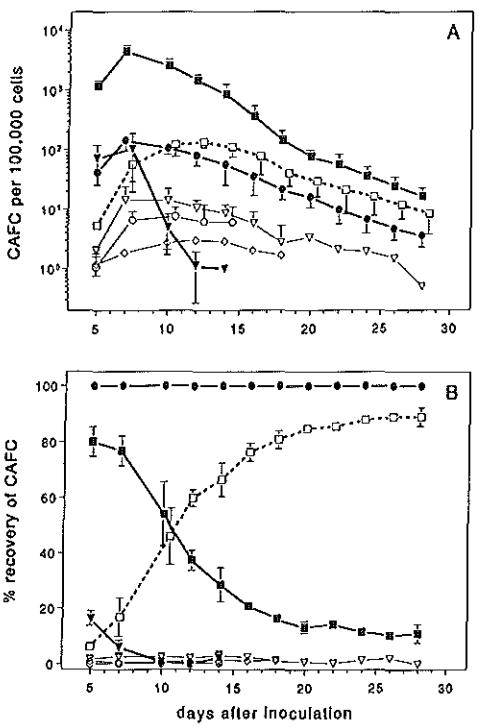

Fig 3. (A) CAFC frequencies (mean ± 1 SD) of cells sorted on the basis of ER-MP12 and ER-MP20 antibodies: (●) unseparated BM cells, (■) ER-MP12^{hi}20^{-}, (□) ER-MP12^{med}20^{-}, (◇) ER-MP12^{-}20^{-}, (▼) ER-MP12^{+}20^{+}, (▽) ER-MP12^{-}20^{med}, (○) ER-MP12^{-}20^{hi} (average of three experiments). (B) Relative distribution (mean ± 95% confidence limits) of the CAFC subsets in the sorted populations (symbols as in Fig 3A). Total recovery in the three experiments at different time points varied from 80% to 140%. Recoveries in individual experiments were normalized at 100% and then averaged.

of the microcytic recipients as previously described.[23,42] Different numbers of unseparated BM cells (Fig 5A) and low-density ER-MP12^{med}20^{-} and ER-MP12^{hi}20^{-} BM cells (Figs 5B and 5C, respectively), were transplanted per mouse. Chimerism was followed up to a year after transplantation. All fractions induced an initial period (0 to 4 months) of transient repopulation. On average, the ER-MP12^{med}20^{-} subset induced a higher level of stable chimerism (4 to 12 months) when compared with the level of transient repopulation than the ER-MP12^{hi}20^{-} subset, indicating it contained more LTRA cells relative to the number of STRA cells. The frequencies of the LTRA unit responsible for 20% donor-type repopulation at one year after transplantation were estimated by extrapolation (Fig 6). Total BM contained one LTRA unit per 55,000 cells, the low-density ER-MP12^{hi}20^{-} subset one per 40,000, and the ER-MP12^{med}20^{-} subset one per 8,000 cells. The ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets were, therefore, 1.4-fold and 6.9-fold enriched over total BM, respectively, and thus contained 7% and 93%, respectively, of the LTRA cells in low-density BM. This result corresponds with the CAFC data in that both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets contained in vivo LTRA cells, and that 90% to 95% of the stem cells (CAFC-28/35 and LTRA cells) were found within the subset expressing intermediate levels of the ER-MP12 Ag.

CFU-S-12 are heterogeneous with respect to ER-MP12 Ag expression. The distribution of CAFC-10 suggested that CFU-S-12 would be present in both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations (Fig 3B). To calculate the enrichment and recovery of CFU-S-12, the two subpopulations were injected into eight Gy-irradiated animals and tested for their day-12 spleen colony-forming ability (Table 2). The other subpopulations contained no significant number of CAFC-10 (Fig 3A) and were not tested for CFU-S-12. Compared with unseparated BM, the ER-MP12^{hi}20^{-} subpopulation was 11.4-fold enriched for CFU-S-12. On the basis of their recovery, it was calculated that the ER-MP12^{hi}20^{-} subset contained about 25% of the CFU-S-12, whereas 75% was found in the ER-MP12^{med}20^{-} subpopulation. Apparently, labeling and sorting of the cells did not affect their spleen colony-forming ability. Therefore, the data show that all BM CFU-S-12 can be recovered from the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets, while the majority expressed the ER-MP12 Ag at an intermediate level.

Myeloid progenitors all highly express the ER-MP12 Ag. To determine the number of mature progenitors in the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets, the subpopulations were tested for day 7 and day 14 CFU-C in standard methylcellulose cultures (Table 3). The ER-MP12^{hi}20^{-} subset was 30-fold enriched when compared with unseparated BM, and contained 67% to 71% of the CFU-C. The ER-MP12^{med}20^{-}, on the other hand, was not enriched and contained 14% to 20% of the CFU-C. The ER-MP12^{-}20^{-} subset contained less than 1% of the CFU-C. Significant differences between day 7 and day 14, with respect to recovery and enrichment of CFU-C, were not observed.

Part of the more mature progenitors, day 7 CFU-C (data not shown) and very early but transient CAFC (Fig 3B), have been found to express intermediate or high levels of the ER-MP20 Ag. To determine the distribution of the ER-MP12 Ag on all myeloid progenitors, BM was separated on the basis of ER-MP12 Ag expression alone. The cultures were stimulated by Epo, IL-3, SF, IL-11, and IL-12; a combination of cytokines that has shown to specifically support multilineage colony formation.[45,46] On average, 80% to 90% of the day 7 and day 14 CFU-C expressed ER-MP12 at a high level (Table 4). To determine the distribution of specific progenitors, colonies were individually picked after 14 days of culture, were stained with May-Grünwald/Giemsa and differentiated by microscope. Nearly all individual CFU-C colonies contained cells of the monocyte-macrophage lineage (Table 4). Although differences between the recoveries of individual lineages were small, the ER-MP12^{med} subpopulation gave rise to an overall higher percentage of colonies containing granulocytes, megakaryocytes, and erythroblasts. After counting the number of lineages per colony, irrespec-

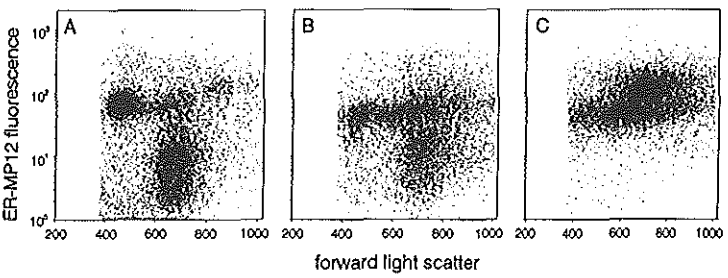

Fig 4. Dotplots of the forward light-scatter against the ER-MP12 fluorescence of (A) unseparated BM cells, (B) low-density (1.069 to 1.075 g/mL) BM cells, (C) low-density BM cells depleted of ER-MP20-positive cells by MACS (ie, ER-MP20$^-$). The ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subsets comprised 26% and 69% of the low-density cells, respectively.

tive of the type of lineage, 47% of the colonies grown from the ER-MP12^{med} subpopulation contained more than one lineage (Table 4). This is different from the other subpopulations that contained only 18% to 21% multilineage CFU-C, which indicates that the ER-MP12^{med} subpopulation contains the more immature progenitors. Taken together, our data show that 80% to 90% of all BM CFU-C, including both multilineage and lineage-restricted progenitors, express the ER-MP12 Ag at a high level.

Stroma-associated hematopoiesis in vitro could not be blocked by ER-MP12. To investigate whether the ER-MP12 Ag plays a role in hematopoiesis we studied the effect of MoAb ER-MP12 on cobblestone area formation. Unseparated BM cells were labeled with ER-MP12 or with rat isotype control MoAb PH2-99 (anti-*E. coli*-β-galactosidase) and tested in the CAFC assay for 4 weeks, in the presence of 5 μg/mL MoAb (ER-MP12 or PH2-99). Antibodies were added every 3 to 4 days by replacing half of the medium. Compared with control cultures, there was no significant effect on the CAFC frequencies (data not shown). A change

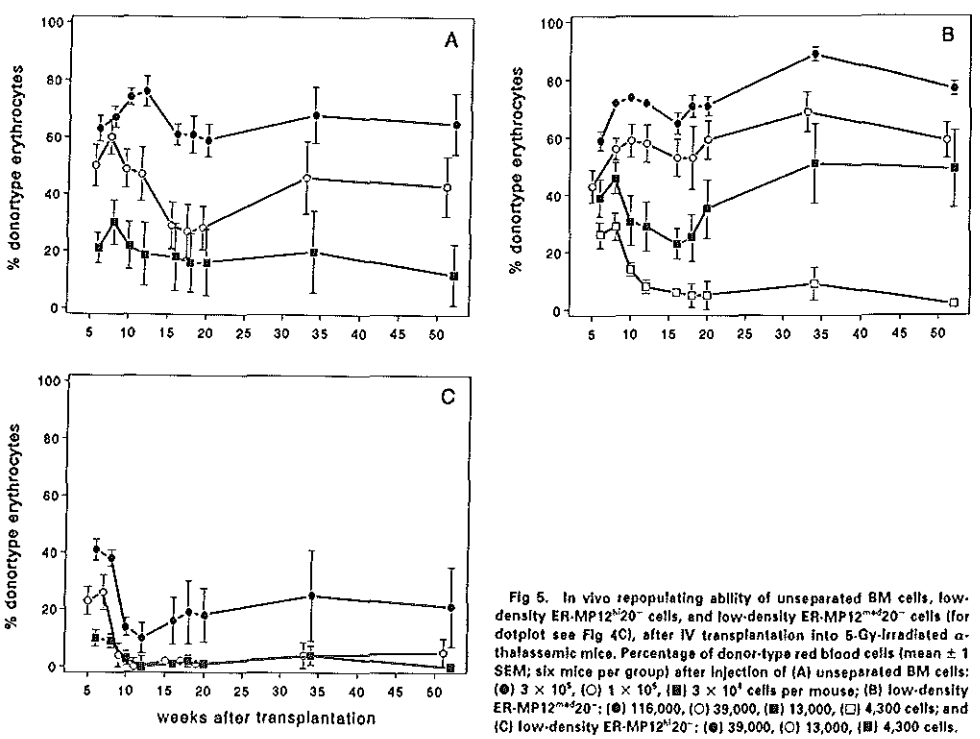

Fig 5. In vivo repopulating ability of unseparated BM cells, low-density ER-MP12^{hi}20$^-$ cells, and low-density ER-MP12^{med}20$^-$ cells (for dotplot see Fig 4C), after IV transplantation into 5-Gy-irradiated α-thalassemic mice. Percentage of donor-type red blood cells (mean ± 1 SEM; six mice per group) after injection of (A) unseparated BM cells: (●) 3 × 10^5, (○) 1 × 10^5, (■) 3 × 10^4 cells per mouse; (B) low-density ER-MP12^{med}20$^-$: (●) 116,000, (○) 39,000, (■) 13,000, (□) 4,300 cells; and (C) low-density ER-MP12^{hi}20$^-$: (●) 39,000, (○) 13,000, (■) 4,300 cells.

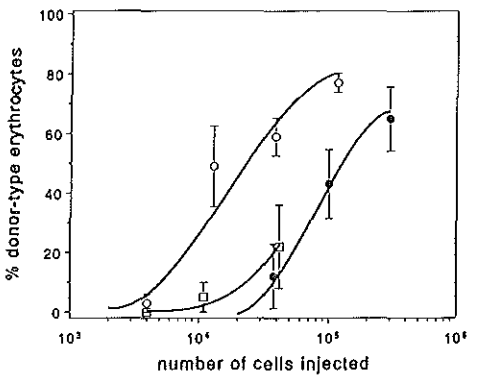

Fig 6. Donor-type red blood cells 52 weeks after transplantation of (●) unseparated BM cells, (○) low-density ER-MP12^{med}20$^-$, or (□) low-density ER-MP12^{hi}20$^-$ BM cells; in 5-Gy-irradiated α-thalassemic mice (six mice per group).

in the size of individual cobblestone areas, that would show an effect on the proliferative capacity of CAFCs, was also not observed. Therefore, replating studies were not performed. In conclusion, these data do not support a functional role of the ER-MP12 Ag in hematopoiesis in vitro.

DISCUSSION

ER-MP12 and ER-MP20, two novel MoAbs that had been raised against an immature BM macrophage precursor line,[28] allowed the identification of six distinct subpopulations of murine BM cells when used in two-color immunofluorescence.[29,31] We previously reported that the ER-MP12^{hi}20$^-$ BM subpopulation, as opposed to the ER-MP12^{med}20$^-$ subset, contained a high frequency of precursor cells with thymus repopulating ability, upon intrathymic injection.[29] On intravenous transfer, however, cells of both subsets gave rise to T, B, and myeloid repopulation.[31] These observations prompted us to define the expression of the ER-MP12 and ER-MP20 antigens among other hematopoietic cells and on hematopoietic stem cell subpopulations. The present study shows that the ER-MP12 Ag was intermediately expressed by LTRA cells, of which 90% to 95% were recovered from the ER-MP12^{med}20$^-$ subpopulation. With differentiation, the ER-MP12 Ag expression increased, reached a maximum in the in vitro clonable progenitors (CFU-C), and disappeared from most lineages during final maturation, with the exception of T and B lymphocytes (Fig 7). Hence, ER-MP12 recognizes a novel Ag on hematopoietic stem cells, which expression is related to the primitiveness of the stem cells within the hematopoietic stem cell hierarchy. The ER-MP20 Ag Ly-6C was expressed on only a small percentage of the more mature in vitro clonable progenitors,[47] but was absent on primitive CAFC, CFU-S-12, and LTRA cells.

ER-MP12 and ER-MP20 enable a separation of the four major hematopoietic lineages in the BM, as was demonstrated by FACScan analysis and differential counting. Granulocytes and monocytes did not express the ER-MP12 Ag, but expressed the ER-MP20 Ag Ly-6C at intermediate and high levels, respectively.[30,47] We previously demonstrated that LTRA cells have a low-affinity for the lectin WGA, while the large majority of CFU-S-12 have a high affinity for WGA, allowing high enrichment factors for both CFU-S-12 and LTRA cells when used for cell sorting.[22-24] The ER-MP12^{med}20$^-$ subpopulation, which contains the LTRA cells and the majority of CFU-S-12, consists for about 90% of lymphocytes that do not bind WGA.[22] Monocytes, granulocytes, and erythroblasts, on the other hand, show variable

Table 2. Distribution of CFU-S-12 on the Basis of ER-MP12 and ER-MP20 Ag Expression

Population	Injected per Mouse	CFU-S-12 per Spleen*	Colonies per 10^5 Cells (±SD)	Mean (±SEM)	Enrichment Factor	Recovery of CFU-S-12†	Relative Contribution‡
NBMC	5×10^4	6.8 ± 2.8	13.5 ± 5.6				
		9.2 ± 3.2	18.4 ± 6.4				
	4×10^4	6.3 ± 2.5	15.8 ± 6.4	15.8 ± 0.8	1	100%	
		6.1 ± 2.2	15.2 ± 5.6				
	2.5×10^4	4.0 ± 1.2	16.0 ± 4.6				
ER-MP12^{hi}20$^-$	1,500	3.1 ± 1.4	206.7 ± 91.3				
		3.1 ± 1.3	206.7 ± 86.0	179.6 ± 20.9	11.4 ± 1.4	27.3%	24.9%
		1.8 ± 1.9	118.5 ± 128.1				
	1,250	2.3 ± 1.6	186.4 ± 128.3				
ER-MP12^{med}20$^-$	2.5×10^4	6.2 ± 2.4	24.8 ± 9.6				
	2.0×10^4	9.1 ± 2.2	45.5 ± 11.2	48.6 ± 8.9	3.1 ± 0.6	82.5%	75.1%
		11.9 ± 2.2	59.4 ± 11.0				
		12.9 ± 2.2	64.5 ± 11.2				

* Sorted or unseparated BM cells (NBMC) were injected into groups of seven to 15 (eight Gy) irradiated recipients. Spleens were taken out and fixed in Tellyesniczky after 12 days. Data represent the mean of 4 to 5 separate experiments (±1 SD) and were corrected for the number of endogenous colonies observed in the control irradiated groups (0.1 colony/spleen).

† The recovery and relative distribution of CFU-S-12 in the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ populations were calculated using their average frequencies of 2.4% and 26.8%, respectively.

‡ The relative contribution of CFU-S-12 in the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations was calculated using their respective recoveries.

Table 3. Recovery of Day 7 and Day 14 CFU-C in ER-MP20$^-$ BM Cells Separated on the Basis of ER-MP12 Ag Expression

Population	Frequency in NBMC*	Day 7 CFU-C per 10^4 Cells†	Recovery (%) Day 7 CFU-C	Day 14 CFU-C per 10^4 Cells	Recovery (%) Day 14 CFU-C
NBMC	100.0	15.2 ± 2.7	100.0	23.6 ± 4.2	100.0
ER-MP12^{hi}20$^-$	2.3	460.2 ± 85.9	70.7 ± 18.2	676.0 ± 55.5	66.6 ± 13.0
ER-MP12^{med}20$^-$	22.5	13.5 ± 2.2	20.0 ± 4.8	14.4 ± 6.0	13.7 ± 6.2
ER-MP12$^-$20$^-$	19.5	<0.2	<0.3	0.8 ± 0.4	0.7 ± 0.3

* Average frequencies in these particular experiments.

† Normal BM cells were separated on the basis of ER-MP12 and ER-MP20 Ag expression. Cultures contained 20% horse serum, 1% BSA, and were stimulated by 10% PWM-MSCM and 2 U/mL Epo. Colony numbers from two experiments, four dishes per group, are given as the mean ± 1 SD.

affinity for WGA, but do not bind ER-MP12, as was shown in the present study. Therefore, if combined with sorting on the basis of WGA affinity, ER-MP12 may enhance and simplify the purification procedure for LTRA cells, which is currently being investigated. This combination would enable a separation of LTRA cells from the large majority of CFU-S-12 and at the same time exclude all mature BM cells, without the use of an additional panel of lineage-specific MoAbs,[19,26,48-51] or intracellular dyes like rhodamine-123 and Hoechst 33342.[18-20]

To investigate whether the ER-MP12 Ag is functionally important for hematopoiesis, BM cells were cultured in the presence of ER-MP12 or an isotype control MoAb, in the CAFC assay. The protocol and concentration of the MoAbs were comparable to that used for the inhibition of hematopoiesis in LTBMC by MoAbs against c-*kit* and Pgp-1/CD44.[52,53] However, no effect of ER-MP12 on the CAFC frequencies was observed. These data indicate that ER-MP12 does not either interfere with hematopoiesis in LTBMC, or does not block a functional epitope on the ER-MP12 Ag. Although not conclusive, these data do not support a functional role of the ER-MP12 Ag in the regulation of stroma-dependent hematopoiesis in vitro.

We previously showed that both the ER-MP12 and ER-MP20 antigens were expressed by mice of different MHC haplotypes (H-2^b, H-2^d, H-2^k, H-2^s, H-2$^{b/q}$) with only slight variations in the distribution of the nucleated cells among the six BM subpopulations,[31] which contrasts with the expression of the Ly-6A/E (Sca-1) and Thy-1 antigens that are haplotype restricted.[54] In the present study, no differences were found in the distribution of CAFC subsets, CFU-S-12, and CFU-C between C57Bl/6-Ly-5.1 (H-2^b) and BALB/c (H-2^d) mice, with respect to ER-MP12 and ER-MP20 Ag expression. Therefore, MoAbs ER-MP12 and ER-MP20 may be applicable for sorting using a wide range of haplotype mice.

Table 4. In Vitro Colony Formation and Lineage Expression After Sorting on the Basis of ER-MP12

	NBMC	ER-MP12^{hi}	ER-MP12^{med}	ER-MP12$^-$
Number and distribution of day 7 and day 14 CFU-C*				
Day 7 CFU-C	16.8 ± 1.6	125.8 ± 17.2	2.4 ± 0.5	0.2 ± 0.1
Day 14 CFU-C	17.8 ± 4.8	202.5 ± 24.6	7.3 ± 2.0	0.2 ± 0.1
% day 7 CFU-C		87.0 ± 15.9	11.0 ± 2.8	2.0 ± 1.4
% day 14 CFU-C		79.4 ± 13.0	19.2 ± 5.7	1.4 ± 0.7
Percentage of CFU-C-14 colonies with specific lineage expression†				
Granuloid	12.0	6.3	22.9	4.5
Mono/maph	100.0	99.2	94.0	95.5
Megakaryocytic	16.9	10.2	26.5	9.1
Erythroid	14.5	10.2	25.3	9.1
Mast cells	4.8	12.6	12.0	0.0
Percentage of colonies with unilineage or multilineage character‡				
1 lineage	73.5	78.7	53.0	81.8
2 lineages	9.6	11.8	26.5	9.1
3 lineages	10.8	6.3	12.1	4.6
4 lineages	6.0	2.4	7.2	4.6
5 lineages	0.0	0.8	1.2	0.0

* Number of day 7 and day 14 CFU-C per 10^4 cells inoculated (mean ± 1 SD). Cultures were stimulated by Epo, IL-3, SF, IL-11, and IL-12. Colonies were counted in six replicate dishes with a maximum of 40 colonies/dish. The relative contribution (mean ± 1 SD) of the different CFU-C subsets was calculated on the total number of CFU-C recovered.

† CFU-C colonies were individually picked at day 14. Colonies were transferred to slides, stained with May-Grünwald/Giemsa and differentiated by microscope. Number of colonies picked: NBMC, 83; ER-MP12^{hi}, 127; ER-MP12^{med}, 83; and ER-MP12$^-$, 22.

‡ The number of lineages per CFU-C colony was determined irrespective of the particular lineage type.

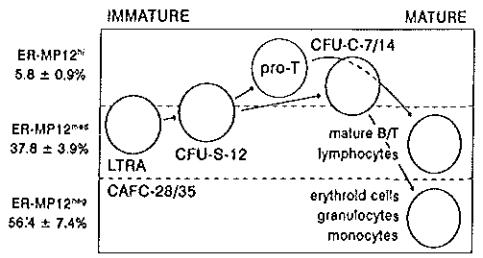

Fig 7. Schematic representation of the ER-MP12 Ag expression during hematopoietic differentiation. Circle areas depict the distribution (%) of several subsets with respect to the different levels of Ag expression as determined in the present study. For clarity, the overlap between different subsets is not taken into account. Frequencies are averaged from four experiments (±1 SEM). Pro-T defines a subset that has the ability to repopulate the thymus 21 days after IV injection into sublethally irradiated animals (data from Slieker et al[31]). The distribution of the LTRA was determined on low-density cells.

Recently, a common lymphoid and dendritic cell precursor population has been identified in the adult mouse thymus by the phenotype Sca-2$^+$ Thy-1.1^{lo} Sca-1$^+$ CD4^{lo} (the low-CD4 precursor), that did not have spleen colony-forming capacity upon intravenous (IV) infusion.[55-57] On intrathymic transfer, however, this precursor took less time to generate CD4$^+$CD8$^+$ thymocytes than did Sca-2$^-$ Thy-1.1^{lo} Sca-1$^+$ Lin$^-$ BM cells,[58] which are highly enriched for CFU-S-12.[59] This observation suggests that the average CFU-S is more primitive than the thymic and BM[60] pre-T cell, which is supported by the recent observation that individual spleen colonies were able to give rise to T and B lymphocytes upon IV and intrathymic transfer.[61] Such a differentiation sequence would be consistent with the observed increase in ER-MP12 Ag expression, as was found in the present study (Fig 7).

Phenotypically, LTRA cells have been identified by a very low expression of the lineage markers B220, TER-119, CD4, and Gr-1,[25,26,62-64] a low expression of Thy-1.1, and a low to negative expression of Thy-1.2.[25,48,49,54,65] In addition, they were positively identified by the expression of a high level of *c-kit*,[26,50-52,66,67] and a high level of the major histocompatibility class 1 Ag H-2K.[25,68] However, all of these markers were indiscriminately expressed by the different subsets of the hematopoietic stem cell compartment. Two antigens that could partly distinguish primitive from the more mature hematopoietic stem cell subsets were identified by MoAbs Sca-1 (Ly-6A/E)[48] and Fall-3,[69] which were expressed by 7% to 10% and 15% to 30% of the BM cells, respectively (Table 5). The Sca-1$^+$ cells contained all LTRA cells and about half of the CFU-S-12, while the Sca-1$^-$ cells contained most of the committed myeloerythroid progenitors.[48,49,59,70,71] Similarly, Fall-3 was expressed by the majority of LTRA cells and by 65% of the CFU-S-12, while the Fall-3$^-$ subset contained 52% of the in vitro clonable (IL-3 responsive) progenitors.[69] In addition, the Fall-3$^-$ subset included 93% of the B cell precursors, as was tested in 2-week Whitlock-Witte cultures. However, Fall-3 did not allow the identification of discretely stained subpopulations, which might have been the reason that some radioprotective and LTRA cells have been reported in the Fall$^-$ subset.[69] The present data show that the distribution of the ER-MP12 Ag partly overlaps with that of Fall-3 and is comparable to the distribution of Sca-1 (Table 5). The expression of the ER-MP12 Ag and Sca-1 differ in that the Lin$^-$ Sca-1$^+$ subset contained only 0.2% of all BM cells including 50% of the CFU-S-12, while the Lin$^-$ ER-MP12^{med} subset comprised 2% to 3% of the BM cells, including 75% of the CFU-S-12. FACScan analysis showed that ER-MP12 was heterogeneously expressed on Sca-1$^+$ BM cells and that 30% of both the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells expressed Sca-1,[31] suggesting that ER-MP12 could be used to identify subsets within the Thy-1^{lo} Lin$^-$ Sca-1$^+$ subpopulation, which remains to be tested.

Taken together, the present study identifies the ER-MP12

Table 5. Comparison of the Distribution of Hematopoietic Stem Cell and Progenitor Cell Subsets With Respect to the Expression of the ER-MP12 Ag, Ly-6A/E (Sca-1), and Fall-3

Population	% of BMC	% of BMC of Lin$^-$ Cells	% pre-T*	% pre-Bf	% CFU-C‡	% CFU-S-12	% LTRA§
Sca-1$^+$	5-6	0.2	50-80‖	ND	10‖ (65)	~50‖	~100‖
Sca-1$^-$	94-95	ND	0‖	ND	90‖ (35)	low‖	0‖
Fall-3$^+$	15-30	10-25	ND	7	48	65	Most, if not all
Fall-3$^-$	70-85	ND	ND	93	52	35	Almost none
ER-MP12^{hi}	2-4	1-2	Majority	ND	80-90	25	5-10
ER-MP12^{med}	30-35	2-3	Minority	ND	10-20	75	90-95
ER-MP12$^-$	61-68	1-2	None	ND	1-2	0	0

Figures on the expression and distribution of Sca-1 are from multiple references.[48,49,54,59-71] The distribution of Fall-3 is described in Müller-Sieburg,[69] while the ER-MP12 data come from Slieker et al[29] and were described in the present study.

Abbreviation: ND, not determined or unknown.

* Determined by intrathymic transfer into sublethally irradiated animals.

† Determined at 2 weeks in Whitlock-Witte cultures.

‡ Sca-1: distribution of day 7 and day 14 CFU-C stimulated with IL-3. Between brackets the distribution after stimulation with IL-3, SF, IL-1 and IL-6. Fall-3: distribution of day 10 CFU-C stimulated by IL-3; ER-MP12: day 4 through day 14 CFU-C stimulated by IL-3, SF, IL-11 and IL-12.

§ Frequency analysis by CAFC (day 28/35) in vitro, and LTRA (>5 mo) in vivo.

‖ Data refer to the (Thy-1^{lo} Lin$^-$) Sca-1$^+$ and (Thy-1^{lo} Lin$^-$) Sca-1$^-$ subsets of BM.

Ag as a novel positive marker on adult mouse BM hematopoietic stem cells. Like the previously defined stem cell antigens Fall-3 and Sca-1, MoAb ER-MP12 can be used for a further dissection of the hematopoietic stem cell compartment and identification of cell surface molecules on functionally different hematopoietic stem cell subsets.

ACKNOWLEDGMENT

We thank Dr C. van den Bos for his assistance with the α-thalassemic mice, Dr T. Kina for MoAb TER-119, Dr R.A. Popp and Dr I.L. Weissman for providing us with breeding pairs of the α-thalassemic and C57BL/6-Ly5.1-pep^{3b} mice, respectively, Dr S. Neben of Genetics Institute for the recombinant murine cytokines, and Prof Dr W. van Ewijk for critically reviewing the manuscript.

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CHAPTER 5

Two phenotypically defined bone marrow subsets with *in vivo* thymus-repopulating ability show different reconstitution profiles upon transfer into deoxyguanosine-treated fetal thymus lobes

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Summary

Using *in vivo* cell transfer studies we recently identified a new cell surface marker, ER-MP12 antigen, on mouse thymus-repopulating progenitor cells in the bone marrow (BM) of the mouse. Thymus-repopulating BM cells express either high (ER-MP12^{hi}) or intermediate (ER-MP12^{med}) levels of ER-MP12 antigen. In addition, they are characterized by a lack of ER-MP20 antigen expression (ER-MP20$^-$). The highest frequency of thymus-repopulating progenitor cells was detected among ER-MP12^{hi}20$^-$ BM cells. Moreover, ER-MP12^{hi}20$^-$ BM cells reconstituted the thymus more rapidly than ER-MP12^{med}20$^-$ BM cells. In the present study we used deoxyguanosine-treated fetal thymus organ cultures (dGuo-FTOC) to determine *in vitro* the thymus-repopulating ability (TRA) of BM cells sorted on the basis of differences in the level of ER-MP12 antigen expression. In agreement with the *in vivo* transfer studies, high frequencies of thymus-repopulating cells were only detected among ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells. However, in contrast to the differentiation *in vivo*, the majority of the *in vitro* progeny of ER-MP12^{hi}20$^-$ BM cells consisted of CD4$^-$CD8$^-$ cells, while ER-MP12^{med}20$^-$ BM cells mainly gave rise to CD4$^-$CD8$^+$ thymocytes. Moreover, a five-fold higher percentage of CD3$^+$ thymocytes was generated in dGuo-FTOC reconstituted with ER-MP12^{med}20$^-$ BM cells. Subsequent *in vivo* intrathymic (i.t.) injection of thymocytes generated from ER-MP12^{hi}20$^-$ BM cells in dGuo-FTOC resulted in the development of mature single positive thymocytes. From this observation we conclude that dGuo-FTOC cannot support the full differentiation of ER-MP12^{hi}20$^-$ BM cells into mature thymocytes. Apparently, additional *in vivo* thymic microenvironments are required to complete their development into mature single positive thymocytes. T cell reconstitution of dGuo-FTOC with ER-MP12^{med}20$^-$ BM cells, on the other hand, may have coincided with the restoration of the *in vitro* thymic microenvironment, possibly by the presence of precursors of dendritic cells within the ER-MP12^{med}20$^-$ subset of BM cells.

Introduction

In adult life, maintenance of intrathymic T cell development requires a continuous influx of very low numbers of BM-derived progenitor cells into the thymus [1]. The subsequent differentiation of these immature cells into functionally competent T lymphocytes is strongly influenced by thymic microenvironments [2], while evidence is now emerging that conversely the integrity of the thymic microenvironments depends on the presence of developing T cells [3-6]. The exact nature of the thymus-repopulating progenitor cells is still not elucidated. Cells belonging to the T lineage are ultimately derived from multipotent hematopoietic stem cells (HSC), as are the cells of all

other hematopoietic lineages [7-10]. Up to now, however, it is not clear whether the thymus is seeded by multipotent HSC or by progenitor cells committed solely to T cell development [11]. This issue has become more controversial as recent studies have shown that even the earliest intrathymic precursor cells are not exclusively committed to T cell differentiation [12-14]. To be able to discriminate between multipotent HSC and more restricted progenitor cells it is necessary to identify cell surface antigens that are specifically or at least differentially expressed by either population. To this purpose, we recently identified a new surface marker, the ER-MP12 antigen, on mouse thymus-repopulating BM cells using *in vivo* transfer assays [15,16]. Thymus-repopulating progenitor cells appeared to express either high (ER-MP12^{hi}) or intermediate (ER-MP12^{med}) levels of ER-MP12 antigen. They were further characterized by a lack of ER-MP20 antigen expression (ER-MP20^{-}). We found that the ER-MP12^{hi}20^{-} BM subset contained the highest frequency of thymus-repopulating progenitor cells and reconstituted the thymus more rapidly than the ER-MP12^{med}20^{-} BM subpopulation, suggesting that the thymus-repopulating progenitor cells expressing high levels of ER-MP12 antigen are more mature than those with an intermediate ER-MP12 antigen expression.

In the present study, we used dGuo-FTOC [17,18] to assess *in vitro* the TRA of BM cells expressing distinct levels of ER-MP12 antigen. As expected, high frequencies of thymus-repopulating cells (identified by the expression of donor-type Thy-1 antigen) were only detected among ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells. However, in contrast to *in vivo* development, the development *in vitro* of ER-MP12^{hi}20^{-} BM cells was mainly limited to the generation of CD4^{-}CD8^{-} (DN) thymocytes, while ER-MP12^{med}20^{-} BM cells mainly gave rise to CD4^{-}CD8^{+} thymocytes. It appeared that this maturation arrest was not an intrinsic effect since subsequent *in vivo* i.t. transfer of thymocytes generated from ER-MP12^{hi}20^{-} BM cells in dGuo-FTOC resulted in the generation of mature CD4 and CD8 single positive (SP) thymocytes. Our data indicate that, in contrast to the *in vivo* thymus, the *in vitro* dGuo-treated fetal thymus, lacks microenvironments required for full maturation of progenitor T cells defined by high levels of ER-MP12 antigen expression.

Material and methods

Mice

Breeding pairs of C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2, Ly-5.1) mice, originally provided by Dr. I.L. Weissman (Stanford University, Stanford, California, USA), and C57BL/Ka BL-1 (Thy-1.1, Ly-5.2) mice were cesarean derived and foster reared. Mice were bred and maintained under clean conventional conditions at the mouse facilities of our department. C57BL/6-Ly-5.1-Pep^{3b} mice aged 4-10

weeks were used as BM donors. C57Bl/ka BL-1 fetal embryos at day 14 of gestation (GD 14) were obtained from timed matings (the time allowed for mating was approximately 15 h). Day 0 of gestation was appointed by the detection of a vaginal plug in mated females.

mAb, reagents, and media

mAb ER-MP12 and ER-MP20 [19], 30H12 (anti-Thy-1.2) [20], H129.19 (anti-CD4) [21], 53-6.72 (anti-CD8) [20], KT3 (anti-CD3) [22], H57.597 (anti-TcR αβ) [23], GL-3 (anti-TcR γδ) [24], RB6-8C5 (anti-Gr-1) [25], F4/80 (anti-F4/80 antigen) [26], and RA3-6B2 (anti-B220) [27] were used either conjugated to FITC or biotin (Sigma Chemical Co., St. Louis, MO, USA) after purification from ascites or hybridoma culture supernatant, or as unpurified hybridoma culture supernatant. In the latter case, FITC-conjugated rabbit anti-rat$(Fab)_2$ fragments (RαRa-FITC) (Cappel, Organon Teknika, Turnhout, Belgium) were used as second stage reagent. In some experiments, 30H12 was used labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer, Mannheim, FRG) according to the manufacturer's procedure. PE conjugated to streptavidin (SAV-PE) was obtained from Caltag Laboratories (San Francisco, CA, USA). CD8 conjugated to RED613™ was purchased from Life Technologies (distributed by GIBCO BRL, Breda, The Netherlands).

RPMI 1640 medium without $NaHCO_3$ (GIBCO BRL) adjusted to pH 7.2 and supplemented with 10% heat-inactivated FCS, penicillin and streptomycin was used during the isolation of fetal thymus lobes from time-mated pregnant female mice. Iscove's modified Dulbecco's medium with L-glutamine and 25 mM Hepes (GIBCO BRL) supplemented with 10% 56 °C heat-inactivated FCS, $NAHCO_3$ (3 g/l), penicillin and streptomycin was used as culture medium for fetal thymus organ cultures (FTOC). To obtain alymphoid fetal thymus lobes deoxyguanosine (dGuo; Sigma Chemical Co.) was added to the culture medium to a final concentration of 1.35 mM just before use.

Preparation of cell suspensions

BM cell suspensions were prepared from femora and tibiae cleaned from muscles and tendons by grinding the bones in a mortar in the presence of Dutton's balanced salt solution (GIBCO BRL) supplemented with 5% FCS (DBSS-FCS) as previously described [15]. Single cell suspensions were obtained by aspiration through a 22-gauge needle into a 2-ml syringe. Debris were removed by filtering the cell suspensions over 100 and 30 μm nylon meshes (Polymon PES, Kabel, Amsterdam).

Cell suspensions of reconstituted fetal thymic lobes were prepared by gently

disruption of the lobes in the presence of PBS supplemented with 0.5% BSA and 20 mM NaN_3 (PBS-BSA-NaN_3) using a miniature Potter homogenizer.

In vitro reconstitution and organ culture of fetal thymus lobes

The TRA of unseparated and sorted BM cells was assessed by *in vitro* transfer into dGuo-treated fetal thymus lobes. This *in vitro* reconstitution assay was originally developed by Jenkinson et al. [17]. Throughout the assay, incubations were carried out at 37 °C in a humidified incubator containing 8% CO_2 in air. Fetal thymus lobes at GD 14 were placed on top of preboiled Nucleopore polycarbonate filters (pore size 0.8 μm; Sterilin Ltd., Teddington, UK) resting on strips of gelatin foam (Upjohn, MO, USA) soaked in culture medium containing 1.35 mM dGuo. After five days of incubation the lobes were extensively washed for at least 2 h in a large volume of dGuo-free culture medium prior to seeding with BM cells. Alymphoid lobes were associated with either unseparated or sorted BM cells in hanging drop cultures [18] by transferring the lobes individually into 20 μl of BM cell suspension in microwells of Terasaki trays which were subsequently inverted. Twenty-four or 48 h later the lobes were placed on filters resting on gelatin foam sponges soaked in culture medium and incubated for 12-14 days. Thymocytes, harvested either from individual or pooled lobes, were counted and analyzed for their surface antigen expression by flow cytometric analysis.

Flow cytometric sorting and analysis

For cell sorting, BM cells were stained simultaneously with ER-MP12 and ER-MP20 as previously described [16,17]. In short, 10^8 BM cells were incubated with biotin-labeled ER-MP12. After 30 min on ice, the cells were washed two times using a 25-fold excess of DBSS-FCS and subsequently incubated with FITC-conjugated ER-MP20 and SAV-PE simultaneously for 30 min in the dark. After two washes with DBSS-FCS, stained cells were resuspended in PBS supplemented with 1% BSA and D-glucose (4.5 g/l, Merck, Amsterdam, The Netherlands) to a final concentration of 3 x 10^6 cells/ml.

Cell sorting was performed using a FACS 440 (Becton Dickinson, Mountain-View, CA, USA) at a sorting speed of 2500-3000 cells per second. Just before sorting, the stained cells were filtered through a 30 μm mesh nylon sieve. Deflected drops were collected in FCS-coated conical polypropylene tubes (Falcon, Becton Dickinson). Isolated cells were washed and resuspended in DBSS-FCS. Viable cells were counted using a Bürker hemocytometer. The purity of the sorted BM subpopulations was assessed using a FACScan flowcytometer (Becton Dickinson).

Phenotypic analysis of thymocytes isolated from reconstituted fetal thymic lobes was carried out in 96 microwell plates (round bottom, Nunc, Denmark) on ice with each incubation step lasting 30 min, followed by extensive washing with PBS-BSA-NaN_3. Thymocytes were stained for either one or two cell surface antigens using biotinylated mAb or mAb conjugated to FITC or FLUOS. When hybridoma culture supernatant was used in two-color immunofluorescence staining, RαRa-FITC was used as a second stage fluorescent reagent. In that case the cells were incubated successively with hybridoma culture supernatant, RαRa-FITC (supplemented with 2% normal mouse serum to avoid non-specific binding), 2% normal rat serum (to block any free anti-rat immunoglobulin-binding sites of the conjugate), biotinylated mAb, and SAV-PE. The percentages of the four major thymocyte subpopulations were calculated from 3 different stainings in which FITC-conjugated anti-Thy-1.2 was combined with either biotinylated anti-CD4, anti-CD8, or both anti-CD4 and anti-CD8 (see Table 2).

The cell surface phenotype of donor-derived thymocytes generated from intrathymically injected "FTOC passed" cells was analyzed using three-color immunofluorescence with FLUOS-conjugated anti-Thy-1.2, PE-conjugated anti-CD4, and CD8 conjugated to RED613™.

Stained cells were analyzed using a FACScan flowcytometer (Becton Dickinson). Erythrocytes and dead cells were excluded from analysis by forward and side scatter gating.

Intrathymic injection

I.t. injection was performed as previously described [15]. Briefly, 5000 "FTOC passed" cells in a volume of 10 µl were injected directly into one of the thymus lobes of 5 Gy irradiated C57Bl/Ka BL-1 recipient mice. Twenty days later, thymocyte suspensions were prepared and their cell surface phenotype determined by flow cytometric analysis.

Results

In vitro TRA of phenotypically defined BM subsets isolated on the basis of differential ER-MP12 and ER-MP20 antigen expression

BM cells labeled simultaneously with ER-MP12 and ER-MP20 can be divided into six subpopulations on the basis of differential ER-MP12 and ER-MP20 antigen expression (Figure 1) [15]. We have previously shown that progenitor cells with *in vivo* TRA are confined to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM subsets [15,16]. In those *in vivo* studies, the highest frequency of thymus-

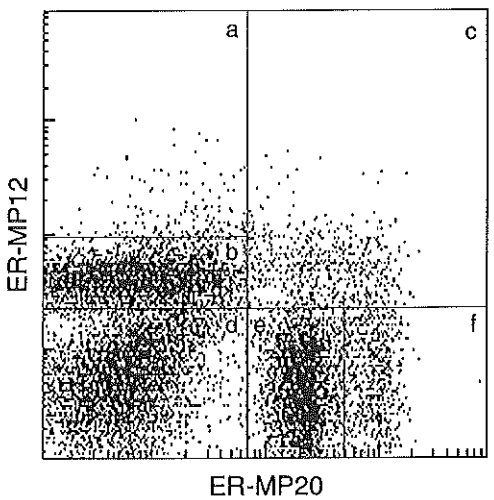

Figure 1. Two-color flow cytometric analysis of ER-MP12 and ER-MP20 antigen expression by total BM cells. Boxes indicate the 6 phenotypically distinct subpopulations: a. ER-MP12^{hi}20^{-} (1.9 ± 0.2%); b. ER-MP12^{med}20^{-} (30.8 ± 2.5%); c. ER-MP12$^{med/hi}$20$^{med/hi}$ (8.7 ± 1.1%); d. ER-MP12^{-}20^{-} (30.2 ± 1.7%); e. ER-MP12^{-}20^{med} (25.9 ± 2.5%); f. ER-MP12^{-}20^{hi} (4.1 ± 0.4%) (mean % ± SEM of 10 experiments).

repopulating activity was detected in the minor subset of ER-MP12^{hi}20^{-} BM cells (1-2% of nucleated BM cells).

In the present study, we questioned whether TRA could be detected in the same subsets of BM cells using a previously described *in vitro* reconstitution assay [17,18]. To this purpose, dGuo-treated fetal thymus lobes were seeded with 5000 BM cells isolated by fluorescence-activated cell sorting (FACS) on the basis of differential ER-MP12 and ER-MP20 antigen expression. After 13 days of organ culture each lobe was analyzed individually for reconstitution with donor-derived (Thy-1.2^{+}) thymocytes (Table 1). No thymocytes could be obtained from unseeded lobes (negative control). Fifteen out of 17 lobes seeded with 5000 fetal thymocytes (GD 14-15) were reconstituted with donor-derived thymocytes (positive control). The highest percentage of reconstituted lobes was found after seeding with ER-MP12^{hi}20^{-} BM cells (11 out of 12 lobes). A high percentage of reconstitution was also seen after seeding with ER-MP12^{med}20^{-} BM cells (14 out of 20 lobes). When unseparated BM cells were used for seeding, only 8 out of 19 lobes were reconstituted with donor-derived thymocytes. In contrast, no donor-derived thymocytes were observed in lobes seeded with ER-MP12^{-}20^{-} BM cells, while only one out of 12 lobes seeded with ER-MP12^{-}20^{med} BM cells and one out of 5 lobes seeded with ER-MP12$^{hi/med}$20$^{hi/med}$ BM cells contained donor-derived thymocytes. Two out of six lobes seeded with ER-MP12^{-}20^{hi} BM cells were reconstituted. Although under the present *in vitro* culture conditions the ER-MP12^{-}20^{hi} BM fraction gave rise to some reconstitution, our data indicate that, comparable with our previously published data using *in vivo* thymus reconstitution assays [15,16], the highest percentage of donor-type reconstitution *in vitro* was observed in lobes seeded with ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells.

Table 1. *In vitro* TRA of sorted BM subpopulations.

cell source	no. reconstituted lobes/no. total lobes[a] (% of positive lobes)		mean % Thy-1.2$^+$ cells[b]
ER-MP12^{hi}20$^-$	11/12	(91.7)	91.9
ER-MP12^{med}20$^-$	14/20	(70.0)	63.5
ER-MP12$^{med/hi}$20$^{med/hi}$	1/5	(20.0)	90.3
ER-MP12$^-$20$^-$	0/10	(0)	–
ER-MP12$^-$20^{med}	1/12	(8.3)	88.5
ER-MP12$^-$20^{hi}	2/6	(33.3)	97.4
unseparated BM	8/19	(42.1)	83.2
fetal thymocytes[c]	15/17	(88.2)	85.9
none	0/15	(0)	–

C57BL/Ka BL-1 (Thy-1.1) dGuo-treated fetal thymus lobes were seeded with 5,000 BM cells of C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2) mice (hanging drop culture time: 24 h). Individual lobes were analyzed for donor-derived (i.e. Thy-1.2$^+$) reconstitution after 13 days of organ culture. a) Lobes were scored as reconstituted if more than 500 events could be obtained in the thymocyte gate during FACscan analysis. b) The mean percentage of Thy-1.2$^+$ thymocytes in reconstituted lobes. c) Thymocytes (GD 14-15) were isolated from BALB/c fetal thymus lobes.

CD4 and CD8 antigen expression by donor-derived thymocytes generated from ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells after in vitro differentiation in dGuo-FTOC

In order to analyze the differentiation status of the donor-derived thymocytes, cells isolated from lobes reconstituted with ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells were stained with anti-Thy-1.2 (donor marker) in combination with either anti-CD4, anti-CD8, or anti-CD4 and anti-CD8 simultaneously. As shown in Table 2, the majority of the cells in lobes seeded with

Table 2. CD4 and CD8 antigen expression by thymocytes generated from ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells after *in vitro* differentiation in dGuo-FTOC.

cell source	exp. no.	cells/lobe (x10^3)	%CD4$^-$8$^-$	%CD4$^+$8$^+$	%CD4$^+$8$^-$	%CD4$^-$8$^+$
ER-MP12^{hi}20$^-$	1	29.7	59.9	1.5	7.3	31.3
	2	54.8	92.5	1.6	3.1	2.8
		(42.3)[a]	(76.2)[b]	(1.5)[b]	(5.2)[b]	(17.1)[b]
ER-MP12^{med}20$^-$	1	18.0	20.8	1.0	3.3	74.9
	2	18.2	16.1	3.3	4.4	76.2
		(18.1)[a]	(18.5)[b]	(2.2)[b]	(3.8)[b]	(75.5)[b]

C57BL/Ka BL-1 (Thy-1.1) dGuo-treated fetal thymus lobes were seeded with 5000 cells of the indicated BM subpopulation isolated from C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2) mice (hanging drop culture: 48 h). Lobes seeded with the same BM subpopulation were pooled and analyzed for reconstitution after 13 days of organ culture. Isolated cells were stained with biotinylated CD4, CD8, or CD4 and CD8 simultaneously (visualized by SAV-PE), followed by anti-Thy-1.2 conjugated to FITC a) mean cell number x 10^3. b) mean percentage of cells.

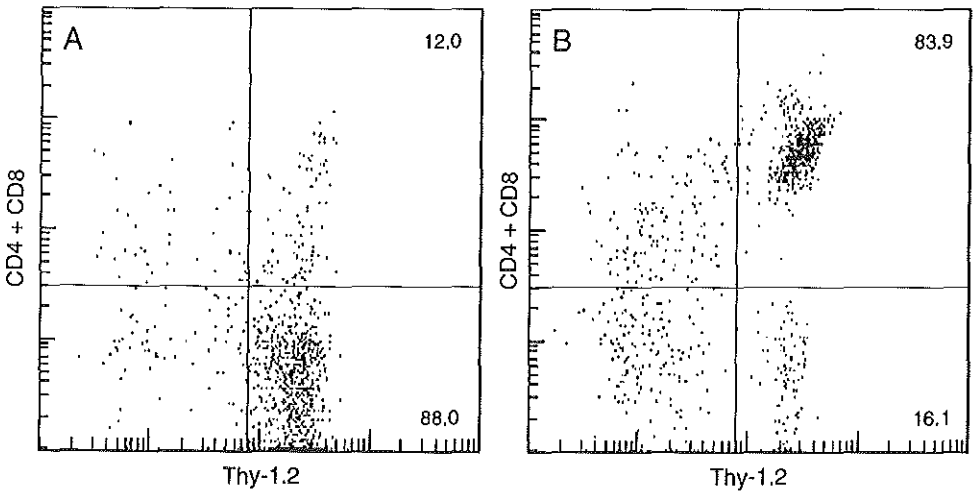

Figure 2. Thy-1.2 (donor-type) expression versus CD4 and CD8 antigen expression by thymocytes isolated from lobes reconstituted with ER-MP12^{hi}20^{-} (A) and ER-MP12^{med}20^{-} (B) BM cells. Analysis was performed 14 days after hanging drop culture. Cells were stained with biotinylated CD4 and CD8 simultaneously (vertical axis) and FITC-conjugated anti-Thy-1.2 (horizontal axis).

ER-MP12^{hi}20^{-} BM cells were DN (mean percentage: 76.2%), while about 17% of the donor-derived thymocytes expressed CD8 only (CD8 SP). In contrast, lobes seeded with ER-MP12^{med}20^{-} BM cells mainly gave rise to CD8 SP thymocytes (75.5%), while approximately 18% expressed neither CD4 nor CD8 antigens. Both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells gave rise to CD4^{+}CD8^{-} (CD4 SP) thymocytes (5.2% and 3.8%, respectively) and CD4^{+}CD8^{+} (DP) thymocytes (~2% with both BM subsets). As shown in Figure 2, the level of CD4 and CD8 antigen expression was high in lobes reconstituted with ER-MP12^{med}20^{-} BM cells (Figure 2). Interestingly, ER-MP12^{hi}20^{-} BM cells gave rise to more (donor-derived) cells per lobe than ER-MP12^{med}20^{-} BM cells (Table 2). These results suggest that, in contrast to the thymus microenvironment *in vivo* [15], the *in vitro* dGuo-treated thymic microenvironment does not promote sufficiently the differentiation of ER-MP12^{hi}20^{-} BM cells into mature thymocytes. Thymocytes derived from ER-MP12^{hi}20^{-} BM cells seem to proliferate to a larger extent than thymocytes derived from ER-MP12^{med}20^{-} BM cells. In addition, T cell development *in vitro* from ER-MP12^{med}20^{-} BM cells is strongly biased towards the generation of CD8 SP thymocytes which are most likely mature, as suggested by their high level of CD4 and CD8 antigen expression.

Analysis of TcR and CD3 antigen expression by thymocytes isolated from dGuo-FTOC reconstituted with ER-MP12^{hi}20^{-} or ER-MP12^{med}20^{-} BM cells

To further analyze the developmental status of thymocytes isolated from dGuo-FTOC reconstituted with either ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells, we studied the surface expression of CD3, TcR αβ, and TcR γδ (Table 3, Figure 3). In this experiment, about 15% of the thymocytes derived from ER-MP12^{med}20^{-} BM cells expressed CD3 antigen, the majority of which belonged to the TcR αβ lineage, while approximately 3% of the CD3^{+} cells expressed TcR γδ. These data demonstrate that mature thymocytes can develop from ER-MP12^{med}20^{-} BM cells in the *in vitro* thymic microenvironment. In contrast, a much lower percentage of CD3^{+} cells (3.4%) was detected in lobes reconstituted with ER-MP12^{hi}20^{-} BM cells. These results again indicate that almost all thymocytes derived from this BM subset are still immature, which is in agreement with their (mainly) DN phenotype (Table 2, Figure 2A).

Table 3. CD3, TcR αβ, and TcR γδ cell surface expression by donor-derived thymocytes isolated from dGuo-FTOC reconstituted with ER-MP12^{hi}20^{-} or ER-MP12^{med}20^{-} BM cells.

cell source	Cells/lobe[a]	%Thy-1.2^{+}	%CD3^{+}	%TcR αβ^{+}	%TcR γδ^{+}
unseparated BM	36.3	91.3	8.1	7.2	2.4
ER-MP12^{hi}20^{-}	43.0	65.1	3.4	2.6	2.6
ER-MP12^{med}20^{-}	39.4	79.1	15.0	14.8	3.2

C57BL/Ka BL-1 (Thy-1.1) dGuo-treated fetal thymus lobes were seeded with 5,000 donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2) mice. Lobes seeded with the same cell source (hanging drop culture: 48 h) were pooled and reconstitution was analyzed after 14 days of organ culture. Isolated cells were stained with either anti-CD3, anti-TcR αβ, anti-TcR γδ followed by anti-Thy-1.2. The percentages of CD3^{+}, TcR αβ^{+}, and TcR γδ^{+} cells were determined among donor-derived (Thy-1.2^{+}) thymocytes. a) Cell number x 10^{3}.

In vivo differentiation potential of cells isolated from dGuo-FTOC reconstituted 12 days earlier with ER-MP12^{hi}20^{-} BM cells

To determine whether the immature thymocytes generated from ER-MP12^{hi}20^{-} BM cells in the *in vitro* thymic microenvironment are able to differentiate further into mature thymocytes, we injected 5000 of these "FTOC-passed" cells intrathymically into sublethally irradiated congenic recipient mice. Twenty days after i.t. injection, the CD4 and CD8 antigen expression by donor-derived thymocytes was analyzed (Figure 4). Clearly, the progeny of ER-MP12^{hi}20^{-} BM cells generated *in vitro* in dGuo-FTOC was able to differentiate into CD4^{+}CD8^{+}, CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes upon *in vivo* i.t. transfer. These results indicate that the progeny of ER-MP12^{hi}20^{-} BM cells generated in dGuo-FTOC is not limited in its differentiation potential.

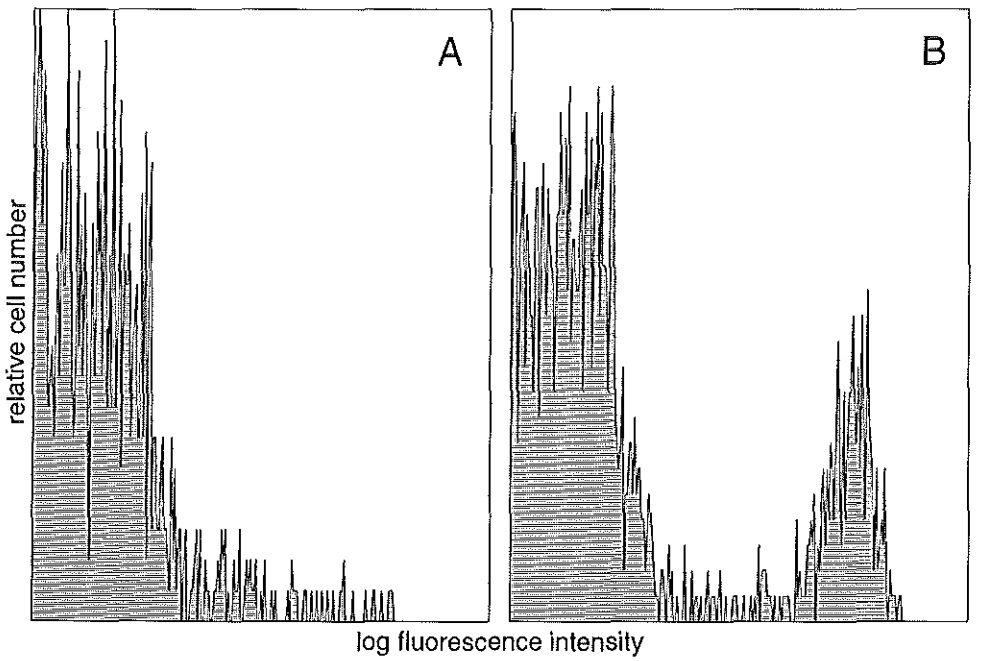

Figure 3. CD3 cell surface expression by donor-derived thymocytes isolated from lobes reconstituted with A) ER-MP12^{hi}20^{-} and B) ER-MP12^{med}20^{-} BM cells. Analysis was performed 14 days after hanging drop culture.

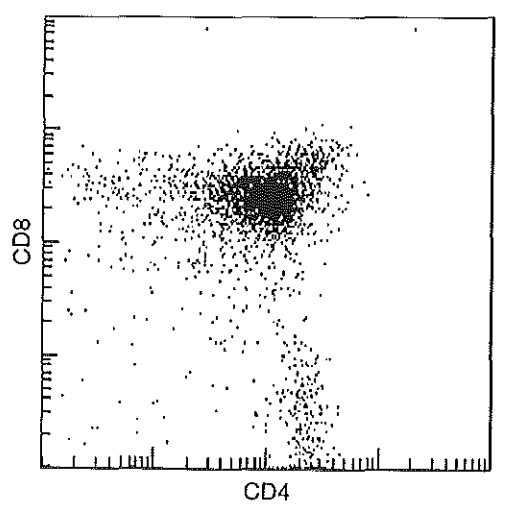

Figure 4. CD4 and CD8 antigen expression by donor-derived thymocytes 20 days after i.t. injection of "FTOC passed" cells. Thymocytes were stained with anti-Thy-1.2, anti-CD4, and anti-CD8 simultaneously using fluorescein-conjugated mAb (see Material and Methods). Life gates were set on Thy-1.2^{+} (donor-derived) thymocytes and the CD4 and CD8 antigen expression of the was subsequently analyzed.

However, the *in vitro* dGuo-treated fetal thymic microenvironment is not sufficient for the differentiation of ER-MP12^{hi}20^{-} BM cells into mature thymocytes. Apparently, the thymus-repopulating cells among ER-MP12^{hi}20^{-} BM cells need additional *in vivo* microenvironments in order to complete their differentiation.

Discussion

Using *in vivo* i.t. and i.v. cell transfer assays we previously reported that thymus-repopulating progenitor cells in mouse BM express either high or intermediate levels of the newly identified cell surface antigen ER-MP12 but lack the expression of the ER-MP20 cell surface antigen [15,16]. In the present study, we investigated the differentiation potential of these BM subsets in dGuo-FTOC [17,18]. Our *in vitro* results demonstrate that, in agreement with the *in vivo* transfer studies, most of the thymus-repopulating cells can be identified among ER-MP20^{-} BM cells expressing either high (ER-MP12^{hi}) or intermediate (ER-MP12^{med}) levels of ER-MP12 antigen. Also in this system, the highest frequency of thymus-repopulating progenitor cells was detected among ER-MP12^{hi}20^{-} BM cells. Analysis of CD4 and CD8 antigen expression of the *in vitro* differentiated cells revealed a clear difference in the *in vitro* differentiation potential of ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells, in that the dGuo-treated fetal thymic microenvironment did not support the differentiation of ER-MP12^{hi}20^{-} BM cells to the same extent as that of ER-MP12^{med}20^{-} BM cells. Whilst the progeny of ER-MP12^{hi}20^{-} BM cells mainly consisted of DN thymocytes, the majority of the thymocytes derived from ER-MP12^{med}20^{-} BM cells were of the CD4^{-}CD8^{+} phenotype. These latter thymocytes are most likely mature, since a higher percentage of CD3^{+} and TcR^{+} thymocytes was found in lobes reconstituted with ER-MP12^{med}20^{-} BM cells than in lobes reconstituted with ER-MP12^{hi}20^{-} BM cells. These data seem to suggest that ER-MP12^{hi}20^{-} BM cells need more time to give rise to mature SP thymocytes than do ER-MP12^{med}20^{-} BM cells. This is most likely not the case, since our *in vivo* cell transfer studies clearly showed that thymus reconstitution by ER-MP12^{hi}20^{-} BM cells was faster compared to that by ER-MP12^{med}20^{-} BM cells [16], suggesting that thymus-repopulating progenitor cells within the ER-MP12^{hi}20^{-} BM subset are more mature than those contained within the subset of ER-MP12^{med}20^{-} BM cells. Indeed, using limiting dilution-type long-term BM cultures [28] we recently demonstrated that the frequency of more mature progenitor cells is higher among ER-MP12^{hi}20^{-} BM cells, whereas conversely the frequency of multipotent HSC is higher in the ER-MP12^{med}20^{-} BM subset [29].

Another phenomenon may explain the observed difference between the *in vitro* and *in vivo* data. In the fetal thymus, not only thymocytes but also BM-derived dendritic cells are sensitive to dGuo-treatment [30]. These cells are an essential component of the thymic microenvironment and are mainly located at the cortico-medullary junction in the adult thymus [2,31,32]. Seeding of dGuo-treated fetal thymus lobes with ER-MP12^{med}20^{-} BM cells may not only have led to T cell differentiation, but also to the reconstruction of the thymic microenvironment by dendritic cell precursors present within this BM subset. On the other hand, ER-MP12^{hi}20^{-} BM cells may be depleted of precursor cells capable of differentiating into dendritic cells. As a result, seeding of dGuo-treated fetal thymus lobes with ER-MP12^{hi}20^{-} BM cells will not result in the restoration of the fetal thymic microenvironment and as a consequence, T cell differentiation will be incomplete. Interestingly, approximately one third of the ER-MP12^{med}20^{-} BM cells are characterized by the expression of low levels of the NLDC-145 antigen, a cell surface determinant found on dendritic cells [33]. In contrast, no NLDC-145 antigen expression could be detected on ER-MP12^{hi}20^{-} BM cells (data not shown). Potential precursors of dendritic cells may thus be contained within the NLDC-145^{dull} subset of ER-MP12^{med}20^{-} BM cells. It is noteworthy that the thymocytes which develop from ER-MP12^{hi}20^{-} cells seem to proliferate to a larger extent than ER-MP12^{med}20^{-} BM cells, as judged by the number of cells isolated from the reconstituted lobes, maybe because they require some interaction with dendritic cells for their further differentiation. In our previously reported *in vivo* transfer studies, thymic dendritic cells are most likely not affected since the recipient mice were treated with low doses of irradiation [15,16] and dendritic cells could develop from host-derived hematopoietic progenitor cells. Therefore we feel that, unlike the *in vitro* dGuo-treated fetal thymic microenvironment, the *in vivo* thymic microenvironment is capable of supporting the differentiation of ER-MP12^{hi}20^{-} BM cells into mature SP thymocytes.

Recently, thymic dendritic cells were found to arise from cells that are phenotypically indistinguishable from the earliest intrathymic precursor cells [13]. As thymus-repopulating BM cells are the predecessors of these intrathymic precursor cells, this would imply that, by definition, these cells must be able to develop into dendritic cells. However, single cell assays are required to determine whether both dendritic cells and thymocytes indeed arise from a shared precursor cell or from distinct subpopulations of precursor cells.

In summary, our *in vitro* data support our previous findings in that most, if not all, thymus-repopulating progenitor cells are detected among BM cells expressing either high or intermediate levels of ER-MP12 antigen. The difference in the *in vitro* differentiation potential between ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells may be explained by the presence of dendritic cell precursors among the latter but not the former BM cells. These precursors of

dendritic cells may have resulted in the restoration of the dendritic component of the dGuo-treated fetal thymic microenvironment, allowing further differentiation of the ER-MP12^{med}20^{-} BM cells towards mature thymocytes. In contrast, the *in vitro* differentiation of ER-MP12^{hi}20^{-} BM cells may have been hampered by the absence of such dendritic cell precursors, resulting in an incomplete thymic microenvironment.

Acknowledgements

We thank Marella de Bruijn for critically reading the manuscript, Tar van Os for preparing the figures and Joop Brandenburg and Els van Bodegom for providing timed pregnant mice.

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CHAPTER 6

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

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European Journal of Immunology 1994;24:2279-2284

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

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.

1 Introduction

Macrophages form a heterogeneous population of cells which play essential roles in a wide variety of biological processes (for reviews see [1, 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 (mAb) using immortalized macrophage precursors as immunogens [3]. Two of these mAb, 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 mAb in two-color flow-cytometric 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.

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Abbreviations: M-CSF: Macrophage colony-stimulating factor **M-CFC:** Macrophage colony-forming cell

Key words: Macrophage / Progenitor / Heterogeneity / Phenotype

2 Materials and methods

2.1 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.

2.2 Antibodies and conjugates

The mAb 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]). Antibodies were applied as hybridoma culture supernatants or as purified mAb conjugated to FITC (fluorescein isothiocyanate, isomer I, Sigma Chemical Co., St. Louis, MO) or biotin (N-hydroxysuccinimidobiotin, Boehringer Mannheim GMBH, Mannheim, FRG) by standard procedures.

R-Phycoerythrin-conjugated streptavidin (SAV-PE; Caltag Laboratories, 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.

2.3 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 (DBSS-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 DBSS-FCS-PS prior to immunofluorescence labeling.

2.4 Immunofluorescence labeling, flow-cytometric analysis and cell sorting

For phenotypic analyses, $1 \times 10^6 - 5 \times 10^6$ 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 20 mM 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 mAb, followed by FITC-labeled mAb 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 mAb followed by FITC-labeled mAb 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 nonproducing 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 mAb (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 exceeded 95%, unless stated otherwise.

2.5 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 (2 mM), penicillin (60 μg/ml), streptomycin (100 μg/ml), β-mercaptoethanol (10^{-4} M) and sodium selenite (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). Per sorted bone marrow subset two different cell concentrations were plated into 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 (≥ 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.

2.6 Morphological analysis

Differential morphological analysis of sorted bone marrow subsets was performed on May-Grünwald-Giemsa stained cytospin preparations. Per subset 500 cells were analyzed.

3 Results

3.1 ER-MP12 and ER-MP20 mAb recognize morphologically distinct bone marrow subsets

Using the anti-macrophage precursor mAb 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$^-$ 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,

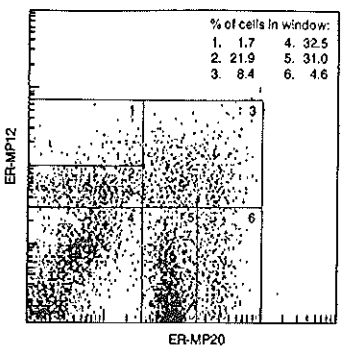

Figure 1. Two-color FACScan analysis of nucleated bone marrow cells labeled with ER-MP12 and ER-MP20 mAb. Percentages are the mean of eight experiments.

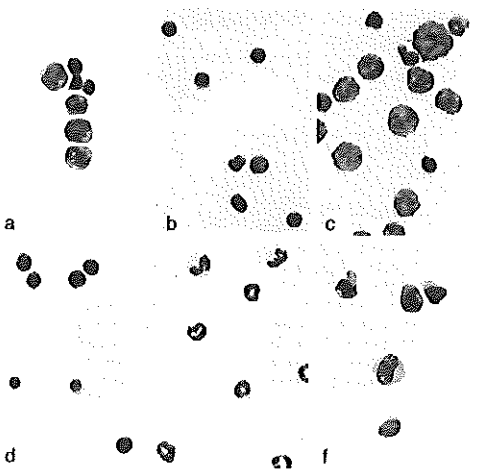

Figure 2. May-Grünwald-Giemsa-stained cytocentrifuge preparations of bone marrow subsets sorted on the basis of ER-MP12 and ER-MP20 expression. (a) ER-MP12^{hi}20^{-} bone marrow subset; (b) ER-MP12^{med}20^{-} subset; (c) ER-MP12^{+}20^{+} subset; (d) ER-MP12^{-}20^{-} subset; (e) ER-MP12^{-}20^{med} subset; (f) ER-MP12^{-}20^{hi} subset. Magnification × 350.

i.e. ER-MP12^{-}20^{-}. In this study we determined the cellular composition of the 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.

3.2 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-

Table 1. Morphological analysis of the bone marrow subsets sorted on the basis of ER-MP12 and ER-MP20 antigen expression[a)]

Bone marrow subset	Myeloid			Erythroid		Lymphoid	Megakaryocytic	Undifferentiated blasts
	Immature progenitors	Band + segmented	Monocytes	Erythroblast	Polychrom + normoblasts			
ER-MP12^{hi}20^{-}	4[b)]	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

a) The sorted bone marrow subsets were spun onto microscopic slides and stained with May-Grünwald-Giemsa. Per subset 500 nucleated cells were examined.

b) 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 underlined.

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[a)]

Bone marrow subset	No. of colonies/10^3 plated cells[b)]	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)[c)]
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[d)]	4 ± 1	13 ± 3	1:59	170 (36/134)

a) 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.

b) 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.

c) **NBMC:** Nucleated bone marrow cells **M-clustFC:** Macrophage cluster-forming cell.

d) **BM:** Bone marrow.

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$^-$20^{hi} bone marrow subset, which consists predominantly of monocytes (cf. Table 1), an average of one out of two plated cells formed a small cluster of usually less than 5 cells, and occasionally a cluster of 15–20 cells. Taking into account the relative sizes of the sorted subsets in bone marrow, these data indicate that macrophage colony-forming cells reside in approximately equal numbers in both the ER-MP12^{hi}20$^-$ 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 Ag expression.

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

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 Ag. 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$^-$20^{hi} cells were Mac-1-positive. This observation was in accordance with the morphological data which showed that this subset almost exclusively contains monocytes and some immature myeloid precursors, which are all Mac-1-positive [10, 12]. Thus, the increase in the proportion of Mac-1 positive cells and level of Ag expression observed from the ER-MP12^{hi}20$^-$ to the ER-MP12$^-$20^{hi} subset supports an increase in maturity of the macrophage precursors present in those subsets.

3.4 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

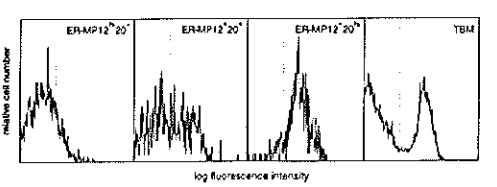

Figure 3. Cell surface expression of Mac-1 Ag by ER-MP12^{hi}20$^-$, ER-MP12$^+$20$^+$, and ER-MP12$^-$20^{hi} nucleated bone marrow cells. Cells were triple-labeled as described in Sect. 2.4 with M1/70 (Mac-1), ER-MP12 and ER-MP20 mAb. Mac-1 expression within the ER-MP12/20 subsets was determined by 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.

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 Ag expression was determined. As a marker for mature macrophages, the expression of the ER-BMDM1 Ag 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 hemopoietic 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, days 5 and 7) which is characteristic of more mature mononuclear phagocytes ([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.

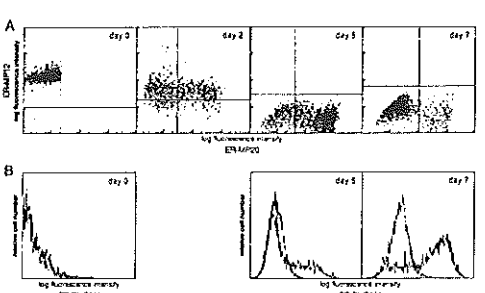

Figure 4. Phenotypic development of ER-MP12^{hi}20^{-} bone marrow cells cultured in the presence of M-CSF. ER-MP12^{hi}20^{-} bone marrow cells were sorted and cultured in the presence of M-CSF-containing conditioned medium. At days 2, 5 and 7 of culture, the cells were collected and the expression 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 (thin lines) are shown in each histogram. Results are from one representative experiment out of two.

4 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 mAb 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 mAb. 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 Ag, which is expressed relatively late during macrophage maturation [10, 11], follows the proposed maturation sequence of the precursor subsets. The Mac-1 Ag 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 phenotypic 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 [13] reported for C57BL/6J mice an M-CFC frequency in bone marrow of approximately twice the frequency of GM-CFC. 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 Ag 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 Ag 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 hemopoietic 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 (J.C.M van der Loo et al., manuscript in preparation). 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 mAb 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 hemopoiesis.

We would like to thank Mr. J. Brandenburg and Ms. E. van Bodegom for animal care, Mr. T. M. van Os for preparation of the figures and Dr. R. E. Ploemacher for his continuous support.

Received June 10, 1994; accepted June 27, 1994.

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CHAPTER 7

General Discussion

The thymus is the central organ for T cell differentiation. Under the influence of its complex microenvironments, hematopoietic progenitor cells of extrathymic origin are induced to proliferate and to differentiate into mature, immunocompetent T cells [1-4]. In postnatal life, this process is maintained by BM-derived cells which under steady state conditions seed the thymus continuously at a very low rate [5,6]. The identity of the progenitor cells which migrate from the BM to the thymus is not elucidated and it still remains to be determined whether the thymus is populated by 1) multipotent HSC, 2) progenitor cells with a lymphoid-restricted developmental potential, or 3) progenitor cells that are committed to differentiate solely along the T cell lineage (i.e pro-thymocytes). The main stumbling-block in the identification of the progenitor cells that actually seed the thymus is the absence of cell surface markers that are specifically expressed by these cells and not by any other stem and progenitor cells in the BM. In all cell separation strategies so far used, TRA has been found to co-purify with a variety of other hematopoietic activities, including RPA, CFU-S d12 activity, *in vitro* colony-forming activity, and multilineage STRA and LTRA [7-11].

In this thesis, we aimed at identifying new cell surface antigens which: 1) allow a positive identification of thymus-repopulating progenitor cells in the BM of the mouse; 2) permit the separation of functionally different classes of thymus-repopulating BM cells; and 3) contribute to the understanding of the progenitor-progeny relationships of the pre-thymic stages of T cell development.

We have focused our attention on two Mab, ER-MP12 and ER-MP20, which previously have been shown to recognize cell surface antigens on subsets of macrophage progenitor cells [12], as well as other immature hematopoietic cells in mouse BM of which the developmental potential was not examined. The observation that fetal thymocytes also express the cell surface antigens recognized by ER-MP12 and ER-MP20 prompted us to investigate whether these Mab could be used for the positive identification of thymus-repopulating progenitor cells in the BM of the mouse. To make optimal use of ER-MP12 and ER-MP20, it was decided to analyze the cell surface expression of the ER-MP12 and ER-MP20 antigens simultaneously using two-color flow cytometry. On the basis of differential ER-MP12 and ER-MP20 antigen expression, BM cells could be divided into six populations of which the *in vivo* TRA was determined following FACS. The experimental work described in **chapters 2 and 3** demonstrate that BM cells with the ability to reconstitute the thymus of irradiated mice express the ER-MP12 antigen but not the ER-MP20 antigen. This finding was confirmed using the *in vitro* thymus reconstitution assay (**chapter 5**). Two phenotypically distinct populations of thymus-repopulating BM cells could be discerned: an ER-MP12^{hi}20^{-} population and an ER-MP12^{med}20^{-} population, comprising 1-2%

and ~30% of total BM cells, respectively. *In vivo* limiting dilution studies revealed that the frequency of thymus-repopulating cells is highest in the small subset of ER-MP12^{hi}20^{-} BM cells. Besides this quantitative difference, a qualitative difference between the two populations was observed; ER-MP12^{hi}20^{-} BM cells were far more efficient in reconstituting the thymus of irradiated mice at three weeks after i.v. transfer than ER-MP12^{med}20^{-} BM cells. When measured one week later, the difference in the level of thymus reconstitution between both populations was no longer observed.

Taken together, we conclude that ER-MP12 detects a new positive marker on BM cells capable of homing to and repopulating the thymus of irradiated mice. Two functionally different populations of thymus-repopulating BM cells can be defined on the basis of differential ER-MP12 antigen expression, i.e. a subset of ER-MP12^{hi}20^{-} BM cells containing "early" thymus-repopulating progenitor cells, and a population of ER-MP12^{med}20^{-} BM cells which encompasses cells that reconstitute the thymus with a delayed kinetics, presumably because they need to undergo additional maturational events within the BM microenvironment before acquiring the capacity to migrate to the thymus.

Using ER-MP12 in combination with ER-MP20, we were not able to identify progenitor cells with a developmental potential committed to the T cell lineage (i.e. pro-thymocytes); in addition to T lymphocytes, both populations of ER-MP12^{+} BM cells gave rise to B lymphocytes and myeloid cells upon i.v. transfer into irradiated recipient mice (**chapter 3**), suggesting that both subsets contained progenitor cells with other developmental potentials and/or multipotent HSC. In an attempt to establish the progenitor-progeny relationship between the two ER-MP12^{+} BM populations, we investigated whether various, functionally defined hematopoietic stem and progenitor cell subsets could be identified by a differential ER-MP12 antigen expression. In **chapter 4** of this thesis, we show that the majority of the most primitive HSC, i.e. LTRC and CAFC-28, are defined by the expression of intermediate levels of ER-MP12 antigen. In contrast, most of the less primitive progenitor cells, i.e. CAFC-5 and *in vitro* clonable progenitor cells (see also ***chapter 6***), are found among BM cells expressing higher levels of ER-MP12 antigen. Separation of BM cells on the basis of differential ER-MP12 antigen expression, however, did not result in a complete segregation of the CFU-S d12 activity with either the ER-MP12^{hi}20^{-} or the ER-MP12^{med}20^{-} subset; although 75% of the CFU-S d12 are characterized by intermediate levels of ER-MP12 antigen expression, about 25% is defined by the expression of high levels of ER-MP12 antigen. Taken together, the results presented in ***chapter 4*** indicate that the ER-MP12 antigen is differentially expressed during the early stages of hematopoiesis (Figure 1). Upon stem cell maturation, the level of ER-MP12 antigen expression increases from intermediate to high. Our

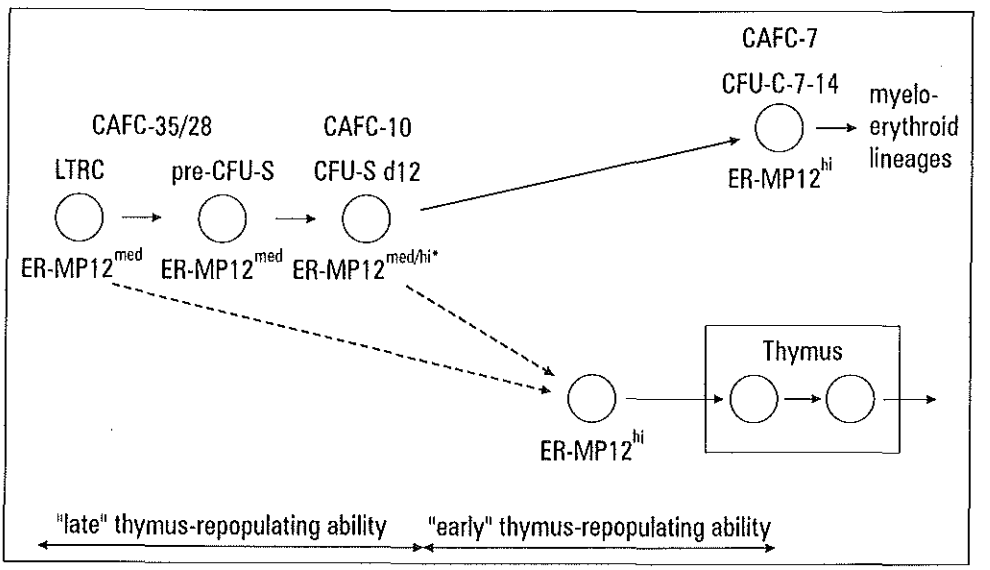

Figure 1. ER-MP12 antigen expression during hematopoietic development. Broken lines: remains to be established. The majority (75%) of CFU-Sd12 are characterized by intermediate levels of ER-MP12 antigen expression, while the remaining 25% express high levels of ER-MP12 antigen.

findings support the hypothesis that "early" thymus reconstitution is a property of less primitive progenitor cells [11-14]. These "early" thymus-repopulating progenitor cells express relatively high levels of ER-MP12 antigen on their cell surface. On the other hand, more primitive HSC, characterized by an intermediate level of ER-MP12 antigen expression, are capable of homing to and repopulating the thymus only after a delay of approximately one week, in which they probably undergo additional maturational events in the BM microenvironment. Although we did not directly establish the lineage relationship between ER-MP12^{med}20^{-} and ER-MP12^{hi}20^{-} BM cells, we hypothesize that the first population must go through a developmental stage at which the level of ER-MP12 antigen expression becomes upregulated before it acquires the capacity to repopulate the thymus of an irradiated mouse (Figure 1).

Although the ER-MP20 antigen is not expressed by thymus-repopulating BM cells, it certainly contributes to their isolation by providing a means of depleting the more mature stages of the myeloid lineages (**chapters 4 and 6**). As the ER-MP12 and ER-MP20 antigens were first identified on macrophage progenitor cells [12], it was important to determine whether distinct subsets of macrophage progenitor cells could be identified on the basis of a differen-

tial expression of the ER-MP12 and ER-MP20 antigens. In **chapter 6** we show that M-CSF-responsive BM cells reside within the ER-MP12^{hi}20^{-}, ER-MP12^{+}20^{+}, and ER-MP12^{-}20^{hi} BM populations. The ER-MP12^{med}20^{-} subset, capable of giving rise to myeloid cells upon i.v. transfer (**chapters 3 and 4**), did not contain progenitor cells capable of responding to M-CSF alone. This result is in good agreement with the data described in **chapter 4**, in that the ER-MP12^{med}20^{-} population contains primitive, yet uncommitted hematopoietic stem and progenitor cells which probably require a broader array of growth factors for growth stimulation. Interestingly, BM cells present in the ER-MP12^{hi}20^{-} population were capable of forming the largest colonies in M-CSF-stimulated BM cultures, whereas ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} BM cells mainly gave rise to large and small clusters, respectively, suggesting that these three populations represent successive stages of macrophage development. Indeed, the progenitor-progeny relationship between these phenotypically defined BM subsets was established by demonstrating that in M-CSF-stimulated cultures, the putative earliest M-CSF-responsive ER-MP12^{hi}20^{-} macrophage progenitor cells successively progressed through the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{hi} stages. Taken together, we hypothesize that lineage restriction, i.e. loss of T cell lineage developmental potential, and commitment to the macrophage lineage coincides with upregulation of ER-MP20 antigen expression.

With regard to the *in vitro* TRA of ER-MP12^{hi}20^{-} BM cells, an unexpected observation was made (**chapter 5**). In contrast to the thymus reconstitution *in vivo* (**chapter 2**), the majority of the Thy-1^{+} progeny generated from ER-MP12^{hi}20^{-} BM cells in dGuo-treated fetal thymus lobes consisted of immature CD4^{-}CD8^{-} (DN) thymocytes, whereas ER-MP12^{med}20^{-} BM cells mainly gave rise to CD4^{-}CD8^{+} thymocytes. Moreover, a much higher proportion of thymocytes derived from ER-MP12^{med}20^{-} BM cells expressed the CD3 antigen and TcR$\alpha\beta$ on their cell surface. These results seem to suggest that ER-MP12^{hi}20^{-} BM cells are less mature than ER-MP12^{med}20^{-} BM cells and require more time to differentiate into mature thymocytes. However, the data presented in **chapters 3 and 4** strongly suggest that the contrary is true; upon i.v. transfer into irradiated mice, ER-MP12^{hi}20^{-} BM cells repopulate the thymus faster than ER-MP12^{med}20^{-} BM cells (**chapter 3**) and analysis of the stem and progenitor cell content of both ER-MP12^{+} populations clearly revealed that ER-MP12^{hi}20^{-} BM cells are less primitive than ER-MP12^{med}20^{-} BM cells (**chapter 4**). We therefore sought for another explanation. It has been shown by others that dGuo is not only toxic to developing thymocytes, but also to thymic dendritic cells and their precursors [15], which are part of the thymic microenvironment. We hypothesized that the observed difference in the *in vitro* thymus reconstitution potential between both subsets of ER-MP12^{+} BM cells may be explained by the

inability of ER-MP12^{hi}20^{-} BM cells to restore the thymic microenvironment. Due to an incomplete microenvironment, ER-MP12^{hi}20^{-} BM cells may not be able to differentiate into mature thymocytes *in vitro*. In contrast, the ER-MP12^{med}20^{-} population may contain progenitor cells that are capable of restoring the thymic microenvironment, allowing the differentiation of ER-MP12^{med}20^{-} BM cells to proceed further than that of ER-MP12^{hi}20^{-} BM cells.

Earlier studies have shown that the majority of thymus-repopulating cells in mouse BM are characterized by the expression of high levels of Sca-1 and c-*kit*, low levels of Thy-1, and very low or undetectable levels of Lin antigens [7-9,11,16-21]. To establish the relationship between Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+}c-*kit*$^{+}$ BM cells and both subsets of ER-MP12^{+} BM cells, it is important to determine whether cells with the Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+}c-*kit*$^{+}$ phenotype can be identified among ER-MP12^{+} BM cells. In ***chapter 3*** we show that ER-MP12^{+} BM cells, in particular those expressing high levels of ER-MP12 antigen, are heterogeneous with respect to the expression of the above mentioned cell surface markers. This phenotypic heterogeneity provides a basis for further enrichment and characterization of the "early" thymus-repopulating progenitor cells that are present among ER-MP12^{hi}20^{-} BM cells. Although we did not test directly whether Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+}c-*kit*$^{+}$ BM cells express the ER-MP12 antigen, the result of the phenotypic analysis presented in chapter 3 suggests that such cells may be found among ER-MP12^{+} BM cells.

The use of ER-MP12 for the enrichment of thymus-repopulating progenitor cells as well as HSC subsets has two important advantages over the mAb that are currently applied for that purpose. *First*, ER-MP12 antigen expression is not restricted to any particular haplotype (**chapter 3**). Moreover, on basis of differential ER-MP12 antigen expression, similar percentages of primitive HSC have been recovered from either C57BL/6-Ly-5.1 or BALB/c mice (**chapter 4**). In contrast, expression of both the Sca-1 antigen and the Thy-1 antigen is not an invariant characteristic of hematopoietic stem and progenitor cells; the Sca-1 antigen is only constitutively expressed within the hematopoietic stem and progenitor cell compartment of Ly-6^{b} haplotype mice [22], while a low level of Thy-1 antigen expression is only a common feature of (virtually all) hematopoietic stem and progenitor cells in Thy-1.1 genotype mice (in Thy-1.2 genotype mice, stem cell activity is found among Thy-1^{lo} as well as Thy-1^{-} BM cells [23]). These markers are therefore not widely applicable for the isolation of hematopoietic stem and progenitor cells from mouse BM. *Second*, the ER-MP12 antigen is differentially expressed within the hematopoietic stem and progenitor cell compartment (Figure 1), whereas the Thy-1 and Sca-1 antigens are not. Sorting on the basis of Thy-1 and Sca-

1 antigen expression therefore results in the co-purification of the various functionally defined classes of hematopoietic stem and progenitor cells. Sorting on the basis of ER-MP12 antigen expression, on the other hand, allows the separation of primitive HSC (including "late" thymus-repopulating cells) from less primitive hematopoietic stem and progenitor cells (which include "early" thymus-repopulating cells) (Figure 1).

The currently available data seem to favor the hypothesis that thymocytopoiesis in postnatal life is maintained by multipotent HSC from BM (see pathway A, Figure 2, Chapter 1) rather than by lymphoid-restricted progenitor cells or pro-thymocytes [24,25]. In an attempt to get more information about the nature of the BM cells that seed the thymus, Wu and co-workers [26] searched for the immediate progeny of such cells in the thymus. A minute population of precursor cells ($CD4^{lo}$ intrathymic precursor population) was identified that resemble the BM-derived multipotent hematopoietic stem and progenitor cells in surface phenotype, except that they also express the Sca-2 antigen [27]. Analysis of the developmental potential of this early intrathymic $CD4^{lo}$ precursor population revealed that it is not restricted to the T cell lineage; upon i.t. transfer, these cells are capable of differentiating into T cells as well as dendritic cells (at very low frequencies) [28], whereas upon i.v. transfer they give rise to both T and B lymphocytes, but not to cells of the myeloid and erythroid lineages [27]. The $CD4^{lo}$ intrathymic precursor cells reconstitute the thymus with a kinetics which is two days delayed when compared to DN thymocytes, but which is two days faster than that of Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells [26]. Similar results were reported with a population of intrathymic precursor cells isolated on the basis of c-*kit* expression, which, upon i.v. transfer was shown to give rise to NK1.1^{+} cells as well [29]. Although the functional potential of these NK1.1^{+} cells was not assessed, it suggests that the early intrathymic precursor cells are capable of differentiating into natural killer (NK) cells. Interestingly, a common precursor for the T cell lineage and NK cell lineage has been identified in the fetal thymus [30]. It should be remarked that cells capable of giving rise to myeloid colonies *in vitro* were detectable in both populations of intrathymic precursor cells [27,29], indicating that these populations are not completely devoid of myeloid progenitor activity. Collectively, these data suggest that the thymus is seeded by uncommitted progenitor cells rather than by lymphoid-restricted progenitor cells or pro-thymocytes. Although the earliest intrathymic progenitor cells are capable of giving rise to T cells, B cells, NK cells, and dendritic cells, they apparently have lost the ability to generate granulocytes, macrophages and erythrocytes following i.v. transfer into irradiated mice. These early intrathymic precursor cells therefore differ from the multipotent Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} BM cells. However, irreversible commitment to the T cell lineage apparently occurs

within the thymus at a developmental stage which is downstream from the $CD4^{lo}$ intrathymic precursor stage.

Following the phenotypic and functional characterization of the earliest intrathymic $CD4^{lo}$ precursor population, Antica et al. [10] searched for their immediate precursors in BM in an attempt to clarify whether restriction in developmental potential takes place before or immediately after thymus seeding. Using Sca-2 antigen expression as one of the selection parameters (Sca-2 is expressed by the earliest intrathymic precursor cells but not by multipotent hematopoietic stem and progenitor cells in BM), they succeeded in isolating a population of BM cells which is characterized by the expression of low levels of Thy-1 antigen and the expression of both Sca-1 and Sca-2 antigens. These BM cells do not express detectable levels of CD4 antigen or any of the other Lin markers. Analysis of the developmental potential of Thy-$1^{lo}Lin^{-}Sca\text{-}1^{+}Sca\text{-}2^{+}$ BM cells revealed that their expansion potential within the thymus is intermediate between that of multipotent Thy-$1^{lo}Lin^{-/lo}Sca\text{-}1^{+}Sca\text{-}2^{-}$ BM cells and intrathymic Thy-$1^{lo}CD4^{lo}Sca\text{-}1^{+}Sca\text{-}2^{+}$ precursor cells. Furthermore, in contrast to the earliest intrathymic precursor cells these Thy-$1^{lo}Lin^{-}Sca\text{-}1^{+}Sca\text{-}2^{+}$ BM cells are capable of differentiating into B cells and myeloid cells upon i.v. transfer into irradiated recipient mice. Compared to Thy-$1^{lo}Lin^{-/lo}Sca\text{-}1^{+}$ BM cells, however, these $Sca\text{-}2^{+}$ BM cells give rise to a reduced number of CFU-S d12, which are also smaller in size. Taken together, these data again support the hypothesis that lineage restriction and T cell commitment take place within the thymus.

Whereas attempts to identify pro-thymocytes in the BM of adult mice have (as yet) been unsuccessful, a subset of precursor cells has recently been identified in fetal blood at day 15.5 of gestation which fully meet the criteria of pro-thymocytes, namely: 1) an efficient thymus-reconstituting potential, 2) no multilineage repopulating activity, and 3) present in a pre-thymic compartment [30,31]. Upon i.t. and i.v. transfer, these fetal blood-derived pro-thymocytes, which are characterized by the expression of intermediate levels of Thy-1 antigen and low levels of c-*kit*, transiently reconstitute the thymus. They however lack *in vivo* and *in vitro* multipotent progenitor activities. Interestingly, a small proportion of these pro-thymocytes has already begun to rearrange their TcR-β genes. A phenotypically identical population was found in fetal blood of athymic mice, suggesting that during embryogenesis T cell lineage commitment may precede thymus colonization. It should be noted, however, that the thymus-reconstituting potential of this particular subset observed in fetal blood of athymic mice was not assessed.

In addition to pro-thymocytes, fetal blood was also shown to contain multilineage LTRC which lack Thy-1 antigen expression and express interme-

diate levels of c-*kit* [30,31]. Thymus reconstitution by this fetal blood Thy-1$^-$c-*kit*$^+$ cells is delayed by five days when compared with fetal blood pro-thymocytes. However, unlike fetal blood pro-thymocytes, these Thy-1$^-$c-*kit*$^+$ cells are capable of long-term reconstitution of the thymus.

It remains to be determined whether pro-thymocytes are confined to fetal development, or whether they also exist at a pre-thymic stage in adult life. In this respect, one should bear in mind that differences between the developmental potential of embryonic and adult HSC have been reported [25]. Therefore, it can not be excluded that pro-thymocytes are generated during fetal life only, and that thymocytopoiesis in postnatal life is maintained by multipotent HSC.

A novel approach to address lineage commitment and differentiation is to analyze the expression patterns of DNA-binding transcription factors which play a critical role in the regulation of gene expression (and thus cellular phenotype) during differentiation [33]. By creating "knockout" mice that carry a mutation in a particular transcription factor gene, it may be possible to identify lineage relationships and early branchpoints during the early stages of lymphocyte development.

Recently, a lymphoid-restricted transcription factor (Ikaros), encoding a family of zinc-finger DNA-binding proteins, has been tested for its role in the regulation of lymphocyte commitment and differentiation [34]. Mice homologous for a germline mutation in the Ikaros gene were shown to lack immature as well as mature T and B cells, and NK cells. In contrast, the myeloid and erythroid lineages were not affected. These results show that the Ikaros transcription factor plays a pivotal role in the development of T, B, and NK cells. However, definite proof demonstrating that Ikaros knockout mice lack progenitor cells with the capacity to generate T cells, B cells, and NK cells has to await cell transfer studies. Although the results may be interpreted as indicative for the existence of a common progenitor cell for the T cell, B cell, and NK cell lineage, it cannot be excluded that absence of functional Ikaros proteins affects unrelated cell lineages that share common regulatory mechanisms.

In conclusion, progenitor cells restricted to differentiate along the lymphoid lineages, as well as pro-thymocytes have not yet been clearly identified in the BM of adult mice. Therefore, the identity of the cells from BM that colonize the thymus throughout life still remains to be established.

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SUMMARY

Summary

The thymus is the major site for T cell development. Throughout postnatal life, thymocytopoiesis is maintained by bone marrow (BM)-derived hematopoietic progenitor cells which populate the thymus continuously at a low rate. The exact nature of these progenitor cells is still not known and it remains to be determined whether the thymus is populated by 1) multipotent hematopoietic stem cells (HSC), 2) progenitor cells with a lymphoid-restricted developmental potential, or 3) precursor cells that are committed to differentiate solely along the T cell lineage (i.e. pro-thymocytes). One approach to identify the pre-thymic stages of T cell development in the mouse is to isolate putative progenitor cell populations on the basis of their cell surface characteristics and to establish lineage relationships by assessing their developmental potential in different *in vivo* and *in vitro* systems. As yet, the only way of identifying BM cells capable of differentiating into mature T cells is by testing their ability to repopulate either the thymus of irradiated mice *in vivo* or lymphoid-depleted fetal thymus lobes *in vitro*. The identification of thymus-repopulating progenitor cells has been hampered by the absence of cell surface antigens that are specifically expressed by these progenitor cells and not by any other stem and progenitor cells in the BM. In all isolation procedures so far used, thymus-repopulating activity has been found to co-purify with a variety of other hematopoietic activities, including the capacity to give rise to B cells, myeloid cells and erythroid cells.

The work described in this thesis aimed at identifying new cell surface markers that contribute to 1) the positive identification of thymus-repopulating progenitor cells in the BM of the mouse, 2) the identification of functionally different classes of thymus-repopulating BM cells, and 3) the understanding of the progenitor-progeny relationships during the pre-thymic stages of T cell development. Two monoclonal antibodies (mAb), ER-MP12 and ER-MP20, which were previously shown to recognize cell surface antigens on progenitor cells in mouse BM, among which those of the macrophage lineage, were studied for their use in the identification of thymus-repopulating progenitor cells. Using ER-MP12 and ER-MP20 simultaneously in two-color flow cytometric analysis, six populations of BM cells could be identified on the basis of a differential expression of the ER-MP12 and ER-MP20 antigens (**chapter 2**). Fluorescence-activated cell sorting (FACS) followed by either direct intrathymic (i.t.) or intravenous (i.v.) transfer of these BM subsets into irradiated recipient mice revealed that thymus-repopulating BM cells were present in two populations that expressed either high (ER-MP12^{hi}) or intermediate (ER-MP12^{med}) levels of ER-MP12 antigen but lacked the expression of ER-MP20 antigen (ER-MP20^{-}) (**chapter 2 and 3**). The highest frequency of thymus-repopulating BM cells was found in the ER-MP12^{hi}20^{-} population (comprising 1-2% of total BM cells). It was concluded that ER-MP12 detects a hitherto unknown cell surface antigen on thymus-repopulating progenitor cells in the BM

of the mouse. The subsets contained functionally different progenitor cells, in that ER-MP12^{hi}20^{-} BM cells were relatively more efficient in repopulating the thymus within three weeks after i.v. transfer (i.e. "early" thymus reconstitution) than ER-MP12^{med}20^{-} BM cells (which were responsible for "late" thymus reconstitution). We hypothesized that the ER-MP12^{med}20^{-} subset contained more primitive HSC or progenitor cells which needed to undergo additional maturational events, presumably in the BM, before they acquired the capacity to home to the thymus, whereas ER-MP12^{hi}20^{-} thymus-repopulating BM cells already had acquired this ability.

Both populations of ER-MP12^{+} BM cells gave rise to B cells and myeloid cells in addition to T cells, upon i.v. transfer into irradiated recipient mice, suggesting that both populations contain progenitor cells with other developmental potentials and/or multipotent HSC. Using different HSC assays, we demonstrated that the ER-MP12 antigen is indeed differentially expressed within the HSC compartment (**chapter 4**). Our findings support the hypothesis that less primitive progenitor cells, expressing relatively high levels of ER-MP12 antigen, are responsible for "early" thymus reconstitution, while more primitive hematopoietic stem and/or progenitor cells, characterized by the expression of intermediate levels of ER-MP12 antigen, need more time to acquire the capacity to home to and repopulate the thymus. We hypothesize that the ER-MP12^{med}20^{-} BM cells must go through a developmental stage at which the level of ER-MP12 antigen expression becomes upregulated before they acquire the capacity to repopulate the thymus.

The thymus reconstitution potential of the six BM populations defined by differential expression of the ER-MP12 and ER-MP20 antigens was also evaluated *in vitro* using deoxyguanosine (dGuo)-treated fetal thymus organ cultures (FTOC) (**chapter 5**). In agreement with the *in vivo* thymus reconstitution studies, thymus-repopulating activity was confined to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets of BM cells. *In vivo* most of the thymocytes generated from ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells expressed both CD4 and CD8 antigens. However, *in vitro* the majority of the thymocytes derived from ER-MP12^{hi}20^{-} BM cells were still in the CD4^{-}CD8^{-} (DN) stage. ER-MP12^{med}20^{-} BM cells, on the other hand, gave *in vitro* rise to mainly CD4^{-}8^{+} thymocytes. In addition, a five-fold higher percentage of CD3^{+} thymocytes was generated in fetal thymus lobes reconstituted with ER-MP12^{med}20^{-} BM cells. These data seem to suggest that ER-MP12^{hi}20^{-} BM cells need more time to differentiate into mature thymocytes than ER-MP12^{med}20^{-} BM cells, which is in sharp contrast with the *in vivo* findings. We hypothesize that the observed difference in the *in vitro* thymus developmental potential between both subsets may be explained by the inability of ER-MP12^{hi}20^{-} BM cells to restore the thymic microenvironment with BM-derived dendritic cells. These cells are part of the thymic microenvironment and are

needed for the final steps in T cell differentiation. It has been demonstrated by others that dGuo treatment also results in the depletion of thymic dendritic cells. The ER-MP12^{med}20^{-} population may contain the required progenitor cells capable of giving rise to dendritic cells, resulting in the restoration of the thymic microenvironment, and allowing the differentiation of ER-MP12^{med}20^{-} BM cells to proceed further than that of ER-MP12^{hi}20^{-} BM cells.

Although the ER-MP20 antigen is not expressed by thymus-repopulating progenitor cells, it contributes to their purification by providing a means of depleting the more mature stages of the myeloid lineages from BM cell suspensions (**chapter 4**). As both ER-MP12 and ER-MP20 antigens were first identified on macrophage progenitor cells, we also determined whether distinct subsets of macrophage progenitor cells could be distinguished in the BM of the mouse according to differences in the levels of ER-MP12 and ER-MP20 antigen expression. On the basis of a differential expression of the ER-MP12 and ER-MP20 antigens, three distinct M-CSF-responsive macrophage precursor subsets could be identified, which represent successive stages along the following maturation pathway: ER-MP12^{hi}20^{-} → ER-MP12^{+}20^{+} → ER-MP12^{-}20^{hi} (**chapter 6**).

Whereas ER-MP12^{med}20^{-} BM cells gave rise to myeloid cells upon i.v. transfer, they were not capable of differentiating into macrophages upon *in vitro* stimulation with M-CSF alone. This finding is in good agreement with the observation that the ER-MP12^{med}20^{-} BM subset is enriched for yet uncommitted, primitive hematopoietic stem and progenitor cells which *in vitro* need more stimuli than M-CSF alone to form mature macrophages. We hypothesize that loss of T cell lineage developmental potential and commitment to the macrophage lineage coincides with the upregulation of ER-MP20 antigen expression.

The use of ER-MP12 for enrichment of thymus-repopulating progenitor cells and HSC subsets has two important advantages over anti-Sca-1 and anti-Thy-1, which are the mAb that are currently widely employed for this purpose. Firstly, ER-MP12 antigen expression is not restricted to any particular haplotype, as is the case of Sca-1 and Thy-1 antigen expression. Secondly, sorting on the basis of ER-MP12 antigen expression permits the separation of "late" thymus-repopulating progenitor cells from "early" thymus-repopulating progenitor cells, whereas sorting on the basis of Thy-1 and Sca-1 antigen expression results in the co-purification of both types of thymus-repopulating progenitor cells. As the ER-MP12^{hi}20^{-} BM population is heterogeneous with respect to the expression of several cell surface antigens, including Thy-1, Sca-1, CD44, c-*kit*, and the B cell differentiation antigen B220 (**chapter 3**), it may be possible to further purify and characterize the "early" thymus-repopulating progenitor cells from this BM population.

The currently available data seem to favor the hypothesis that T cell development in postnatal life is maintained by multipotent progenitor cells rather than by lymphoid-restricted progenitor cells or pro-thymocytes. Multipotent hematopoietic stem and progenitor cells are difficult to detect within the thymus, suggesting that lineage restriction and commitment to the T cell lineage occurs relatively soon after their arrival in the thymus. Pro-thymocytes have recently been detected in mouse fetal blood. However, it cannot be excluded that pro-thymocytes are generated during fetal life only, and that T cell development in postnatal life is maintained by multipotent hematopoietic stem or progenitor cells.

SAMENVATTING

T-celontwikkeling vindt voornamelijk plaats in de thymus. Na de geboorte wordt de T-celontwikkeling in stand gehouden door uit het beenmerg afkomstige hematopoietische voorlopercellen die de thymus onafgebroken in een laag tempo koloniseren. De exacte identiteit van deze voorlopercellen is nog steeds niet opgehelderd en de vraag is of de thymus wordt bevolkt door 1) multipotente hematopoietische stamcellen, 2) voorlopercellen met een lymfoïd-gerestricteerd ontwikkelingsvermogen, of 3) voorlopercellen die gecommitteerd zijn om uitsluitend te differentiëren in de T-celdifferentiatierichting (i.e. prothymocyten). Een manier om de pre-thymaire stadia van de T-celontwikkeling in de muis te identificeren is om vermeende voorloperpopulaties te isoleren op basis van hun celoppervlaktekenmerken en de verbanden tussen de verschillende differentiatielijnen te bepalen. Tot nu toe kan het vermogen van voorlopercellen om te differentiëren tot rijpe T-cellen alleen worden vastgesteld door ze te testen *in vivo* op hun capaciteit om de thymus van bestraalde muizen te repopuleren of *in vitro* op hun vermogen om lymfoïd-gedepleteerde foetale thymuslobben te reconstitueren. De identificatie van thymus-repopulerende voorlopercellen wordt belemmerd door de afwezigheid van celoppervlakteantigenen die uitsluitend tot expressie worden gebracht door deze voorlopercellen. In alle isolatieprocedures die tot dusver zijn gebruikt, viel de thymus-repopulerende activiteit samen met een verscheidenheid aan andere hematopoietische activiteiten, zoals het vermogen om te differentiëren in B-cellen, myeloïde cellen en erytroïde cellen.

Het werk dat beschreven wordt in dit proefschrift had als doel om nieuwe celoppervlaktemarkers te vinden die bijdragen aan 1) de positieve identificatie van thymus-repopulerende voorlopercellen in het beenmerg van de muis, 2) de identificatie van functioneel verschillende klassen van thymus-repopulerende beenmergcellen, en 3) het verkrijgen van inzicht in de voorloper-nakomeling relaties tijdens de pre-thymaire stadia van T-celontwikkeling. Twee monoklonale antistoffen, ER-MP12 en ER-MP20, waarvan eerder was aangetoond dat ze oppervlakteantigenen herkennen op voorlopercellen in het beenmerg van de muis (waaronder onder andere macrofaagvoorlopercellen), zijn onderzocht op hun toepasbaarheid voor de identificatie van thymus-repopulerende voorlopercellen. Door ER-MP12 en ER-MP20 te gebruiken in een twee-kleuren flowcytometrische analyse, konden zes populaties beenmergcellen worden onderscheiden op basis van een differentiële expressie van de ER-MP12 en ER-MP20 antigenen (**hoofdstuk 2**). Fluorescentie-geactiveerde celsortering gevolgd door intrathymaire injectie of intraveneuze transfer van deze beenmergsubpopulaties in bestraalde ontvangermuizen, liet zien dat thymus-repopulerende beenmergcellen aanwezig waren in 2 populaties met ofwel een hoge (ER-MP12^{hi}) of een intermediaire (ER-MP12^{med}) expressie van het ER-MP12 antigeen en geen expressie van het ER-MP20 antigeen (ER-MP20^{-}) (**hoofdstuk 2 en 3**). De hoogste frequentie van thymus-repopulerende beenmergcellen werd

gevonden in de ER-MP12^{hi}20^{-} subpopulatie (bestaande uit 1-2% van alle beenmergcellen). Er werd geconcludeerd dat ER-MP12 een tot nog toe onbekende celoppervlaktemarker herkent op thymus-repopulerende voorlopercellen in het beenmerg van de muis. De subpopulaties bevatten functioneel verschillende voorlopercellen, aangezien de ER-MP12^{hi}20^{-} beenmergcellen relatief efficiënter waren in hun vermogen om de thymus te repopuleren binnen drie weken na intraveneuze transfer (i.e. "vroege" thymusreconstitutie) dan de ER-MP12^{med}20^{-} beenmergcellen (welke verantwoordelijk waren voor een "late" thymusreconstitutie). Een verklaring hiervoor zou kunnen zijn dat de ER-MP12^{med}20^{-} populatie primitievere hematopoietische stamcellen of voorlopercellen bevat, welke additionele maturatiestappen moeten ondergaan, mogelijk in het beenmerg, voordat zij naar de thymus kunnen migreren. ER-MP12^{hi}20^{-} thymus-repopulerende beenmergcellen, daarentegen, zouden deze eigenschap reeds hebben verkregen.

Beide populaties bleken in staat te zijn om naast T-cellen ook B-cellen en myeloïde cellen te kunnen vormen na intraveneuze injectie in bestraalde ontvangermuizen. Dit suggereert dat beide populaties voorlopercellen bevatten met andere ontwikkelingspotenties en/of multipotente hematopoietische stamcellen. Wij hebben met behulp van verschillende hematopoietische stamcelassays aangetoond dat het ER-MP12 antigeen inderdaad differentieel tot expressie wordt gebracht binnen het hematopoietische stamcelcompartiment (**hoofdstuk 4**). Onze bevindingen ondersteunen de hypothese dat minder primitieve voorlopercellen, die het ER-MP12 antigeen relatief hoog tot expressie brengen, verantwoordelijk zijn voor "vroege" thymusreconstitutie. Daarentegen hebben primitievere hematopoietische stamcellen en/of voorlopercellen, welke gekarakteriseerd worden door een intermediaire expressie van het ER-MP12 antigeen, meer tijd nodig om het vermogen krijgen om naar de thymus te migreren. Aangenomen wordt dat ER-MP12^{med}20^{-} beenmergcellen eerst een ontwikkelingsstadium moeten doorlopen waarbij de expressie van het ER-MP12 antigeen wordt opgereguleerd, voordat zij het vermogen krijgen om de thymus te repopuleren.

Het thymus-reconstituerend vermogen van de zes beenmergpopulaties, welke gedefinieerd worden door differentiële ER-MP12 en ER-MP20 antigeenexpressie, is ook onderzocht *in vitro* in deoxyguanosine-behandelde foetale thymusorgaankweken (**hoofdstuk 5**). In overeenstemming met de *in vivo* thymusreconstitutiestudies, was de thymus-repopulerende activiteit beperkt tot de ER-MP12^{hi}20^{-} en ER-MP12^{med}20^{-} populaties van beenmergcellen. *In vivo* brachten de meeste thymocyten die werden gegenereerd uit ER-MP12^{hi}20^{-} en ER-MP12^{med}20^{-} beenmergcellen zowel het CD4 als het CD8 antigeen tot expressie. Echter, *in vitro* bevond de meerderheid van de thymocyten afkomstig van ER-MP12^{hi}20^{-} beenmergcellen zich nog steeds in het CD4^{-}CD8^{-} stadium. ER-MP12^{med}20^{-} beenmergcellen daarentegen, ontwikkelden zich *in vitro*

voornamelijk in $CD4^{-}CD8^{+}$ thymocyten. Daarnaast werd in foetale thymuslobben die gereconstitueerd waren met ER-$MP12^{med}20^{-}$ beenmergcellen een vijfmaal zo hoog percentage $CD3^{+}$ thymocyten gevonden. Deze gegevens lijken te suggereren dat ER-$MP12^{hi}20^{-}$ cellen meer tijd nodig hebben om te differentiëren tot rijpe thymocyten dan ER-$MP12^{med}20^{-}$ cellen, hetgeen in scherp contrast is met de *in vivo* bevindingen. Een verklaring voor het waargenomen verschil in *in vitro* differentiatiecapaciteit tussen beide subsets zou kunnen zijn dat ER-$MP12^{hi}20^{-}$ beenmergcellen niet in staat zijn om zich te ontwikkelen tot dendritische cellen. Deze cellen vormen een deel van de micro-omgeving van de thymus en zijn nodig voor de laatste stappen van T-celontwikkeling. In het verleden is door anderen aangetoond dat deoxyguanosinebehandeling van de foetale thymuslobben ook leidt tot de depletie van de dendritische cellen. De ER-$MP12^{med}20^{-}$ populatie zou voorlopercellen kunnen bevatten die wel in staat zijn om te differentiëren in deze dendritische cellen, met als gevolg dat de micro-omgeving van de thymus wordt hersteld en de multipotente ER-$MP12^{med}20^{-}$ beenmergcellen zo verder kunnen differentiëren dan ER-$MP12^{hi}20^{-}$ beenmergcellen.

Thymus-repopulerende voorlopercellen blijken het ER-MP20 antigeen niet tot expressie te brengen. Toch draagt ER-MP20 bij tot de zuivering van deze voorlopercellen, omdat het kan worden gebruikt voor de depletie van de rijpere myeloïde cellen uit beenmergcelsuspensies (**hoofdstuk 4**). Aangezien zowel het ER-MP12 als het ER-MP20 antigeen in eerste instantie zijn aangetoond op macrofaagvoorlopercellen, is onderzocht in welke van de zes door ER-MP12 en ER-MP20 gedefinieerde beenmergpopulaties macrofaagvoorlopercellen aanwezig waren. Op basis van de differentiële expressie van de ER-MP12 en ER-MP20 antigenen, konden drie verschillende populaties van M-CSF-responsieve macrofaagvoorlopercellen worden onderscheiden in het beenmerg van de muis. Deze populaties representeren opeenvolgende stadia in een lineaire maturatiereeks: ER-$MP12^{hi}20^{-}$ → ER-$MP12^{+}20^{+}$ → ER-$MP12^{-}20^{hi}$ (**hoofdstuk 6**).

Hoewel ER-$MP12^{med}20^{-}$ beenmergcellen in staat waren om te differentieren in myeloïde cellen na intraveneuze injectie in bestraalde muizen, bleken zij niet in staat te zijn om rijpe macrofagen te vormen na *in vitro* stimulatie met alleen M-CSF. Deze bevinding is in overeenstemming met het feit dat de ER-$MP12^{med}20^{-}$ beenmergsubpopulatie verrrijkt is voor nog ongecommitteerde, primitieve hematopoietische stamcellen en voorlopercellen, welke *in vitro* meer stimuli nodig hebben dan M-CSF alleen om in macrofagen te kunnen differentiëren. Onze hypothese is dat opregulatie van de ER-MP20 antigeenexpressie gepaard gaat met "commitment" naar de macrofaagdifferentiatierichting en verlies van het vermogen tot T-celontwikkeling.

De toepassing van ER-MP12 voor de verrijking van thymus-repopulerende voorlopercellen en hematopoietische stamcelsubpopulaties heeft twee belang-

rijke voordelen ten opzichte van anti-Sca-1 en anti-Thy-1, de monoklonale antilichamen die momenteel veelvuldig voor dit doel worden gebruikt. Ten eerste is de ER-MP12 antigeenexpressie niet beperkt tot één bepaald haplotype. Dit is wel het geval voor de Sca-1 en Thy-1 antigenen. Ten tweede, sortering op basis van ER-MP12 antigeenexpressie maakt het mogelijk om "late" thymus-repopulerende voorlopercellen te onderscheiden van "vroege" thymus-repopulerende voorlopercellen, hetgeen op basis van Thy-1 en Sca-1 antigeenexpressie niet mogelijk is. De ER-MP12^{hi}20^{-} populatie is heterogeen met betrekking tot de expressie van onder andere Thy-1, Sca-1, CD44, c-*kit* en de B-celdifferentiatiemarker B220. Deze heterogeniteit kan de basis zijn voor een verdere zuivering en karakterisering van "vroege" thymus-repopulerende voorlopercellen. uit deze populatie beenmergcellen.

De tot nu toe beschikbare gegevens pleiten voor de hypothese dat T-celontwikkeling na de geboorte in stand wordt gehouden door multipotente voorlopercellen en niet door lymfoïd-gerestricteerde voorlopercellen of pro-thymocyten. Echter, multipotente hematopoietische stamcellen zijn niet of nauwelijks aantoonbaar in de thymus. Dit suggereert dat multipotente thymus-repopulerende voorlopercellen vrijwel direkt na hun aankomst in de thymus restrictie en "commitment" naar de T cel differentiatierichting ondergaan.

Onlangs zijn pro-thymocyten aangetoond in het bloed van foetale muizen. Het kan echter niet worden uitgesloten dat pro-thymocyten alleen gedurende de foetale ontwikkeling voorkomen en dat T-celdifferentiatie na de geboorte in stand wordt gehouden door multipotente hematopoietische stamcellen of voorlopercellen.

Abbreviations

Ag	antigen
BSA	bovine serum albumin
BM	bone marrow
CA	cobblestone area(s)
CAFC	cobblestone area-forming cell(s)
CCE	countercurrent centrifugal elutriation
CFC	colony-forming cell(s)
CFU	colony-forming unit
CFU-C	colony-forming unit in culture
CFU-S	colony-forming unit of the spleen
CSF	colony-stimulating factor
d	day
DBSS	Dutton's balanced salt solution
DBSS-FCS	DBSS supplemented with 5% FCS
DBSS-FCS-PS	DBSS supplemented with 5% FCS, 60 μg/ml penicillin and 100 μg/ml streptomycin
dGuo	deoxyguanosine
dGuo-FTOC	deoxyguanosine-treated fetal thymus organ culture
DMEM	Dulbecco's modified Eagle's medium
DN	double negative
DP	double positive
Epo	erythropoietin
ER-MP12^{bio}	biotinylated ER-MP12
ER-MP20^{bio}	biotinylated ER-MP20
ER-MP12^{FITC}	fluoresceinated ER-MP12
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fetal liver
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester
FCS	forward scatter
FLS	forward light scatter
FTOC	fetal thymus organ culture
5-FU	5-fluorouracil
GαRA-PE	goat anti-rat immunoglobulin conjugated to phycoerythrin
GD	day of gestation
h	hour
HPP-CFC	high proliferative potential colony-forming cell(s)
HSC	hematopoietic stem cell(s)
IL	interleukin
i.v.	intravenous
i.t.	intrathymic
Lin	lineage-specific cell surface marker(s)
LTBMC	long-term bone marrow culture(s)
LTRA	long-term repopulating ability

LTRC	long-term repopulating cell(s)
kD	kilodalton
KL	kit ligand
LCM	L cell-conditioned medium
mAb or MoAb	monoclonal antibody
MACS	magnet-activated cell sorting
M-CFC	macrophage colony-forming cell(s)
M-CSF	macrophage colony-stimulating factor
MGF	mast cell growth factor
MHC	major histocompatibility complex
MRA	marrow-repopulating ability
MW	molecular weight
NBM	normal (unseparated) bone marrow
NBMC	normal (unseparated) bone marrow cells
NK	natural killer
PBS	phosphate-buffered saline
PBS-BSA-NaN_3	PBS supplemented with 1% (0.5%) BSA and 20 mM NaN_3
PE	phycoerythrin
PLS	perpendicular light scatter
PWM-MSCM	pokeweed mitogen-stimulated mouse spleen-conditioned medium
RαRa-FITC	rabbit anti-rat$(Fab)_2$ conjugated to FITC
Rh123	rhodamine 123
RPA	radioprotective ability
SAV-PE	streptavidin-conjugated phycoerythrin
SAV-TRI(COLOR)	streptavidin-conjugated TRICOLOR
SCF	stem cell factor
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error of the mean
SSC	side scatter
Sl	Steel
SF	Steel factor
SP	single positive
STRA	short-term repopulating ability
STRC	short-term repopulating cell(s)
TBM	total bone marrow
TcR	T cell receptor
th	thalassemic
TN	triple negative
TRA	thymus-repopulating ability
TC	TRICOLOR
U	unit
W	white spotting
WGA	wheat germ agglutinin

Dankwoord

Uiteindelijk is het er toch van gekomen: mijn boekje is af. Dit zou niet gebeurd zijn zonder het vertrouwen van een drietal mensen die ik op de eerste plaats wil bedanken. Om te beginnen is dat mijn promotor, Prof. (Willem) van Ewijk. Beste Willem, ik denk dat ik zonder jouw geloof in mijn kunnen dit proefschrift nooit zou hebben kunnen schrijven. Jouw enthousiasme voor fundamenteel onderzoek is ongeëvenaard. Jij hebt mij aangestoken met jouw nieuwsgierigheid om het hoe en waarom van de "black box" (de thymus) te doorgronden. Daar zal ik dus mijn hele verdere leven "last" van hebben. Prof. (Rob) Benner en dr. (Herbert) Hooijkaas ben ik zeer erkentelijk voor de ruimte die zij mij hebben geboden om dit proefschrift af te maken. Daarnaast ben ik hen dankbaar voor de mogelijkheid die zij mij hebben gegeven om onder hun leiding de weg in te slaan van de Medische Immunologie.

Degenen die dit boekje hebben doorgebladerd zullen hebben gemerkt dat het beschreven onderzoek het resultaat is van teamwork. Beste Han, onze vraagstellingen lagen inderdaad dicht bij elkaar. Ik zal onze "nachtelijke avonturen" die uiteindelijk hebben geleid tot een aantal gezamenlijke manuscripten niet snel vergeten. Fijn dat je voor mijn promotie de grote oceaan wilt oversteken. Lieve Marella, jij bent voor mij niet alleen een fijne collega, maar ook het zusje dat ik nooit heb gehad. Dit laatste is jou al eens duur komen te staan, toen tijdens de Germinal Centre Conference in Spa iemand per abuis mij complimenteerde met jouw presentatie. Ik ben blij dat jij mijn paranimf bent. Ik hoop volgend jaar jou bij te staan (als een oude, "wijze" en soms vervelende zus) bij het afronden van jouw boekje.

Ik wil Dr. Rob Ploemacher bedanken voor zijn kritische commentaar op ons onderzoek. Beste Rob, ik ben bijzonder blij dat jij als stamcelexpert deel uitmaakt van mijn kleine commissie. Tevens dank ik de overige leden van mijn promotiecommissie voor de tijd die zij hebben gestoken in het lezen van dit proefschrift.

De mensen van mijn "oude" lab wil ik op deze plaats van harte bedanken voor hun collegialiteit en vriendschap. Ik denk nog vaak terug aan onze kampeerweekenden, met name aan het weekend waarin wij onder leiding van onze Zuidafrikaanse gast Prof. Jan Verschoor een tiental kippen op een bedje van gloeiende houtskool diep in de Hollandse klei trachtten te garen. Een aantal mensen van het lab wil ik in het bijzonder bedanken. Beste Pieter, jij staat aan de basis van dit boekje. Ik hoor je kort voor de eerste ER-MP12/ER-MP20 sort nog zeggen: "Wedden dat de pro-thymocyten in deze fractie zitten?" Natuurlijk heb je (bijna) gelijk gekregen. Peter van Soest wil ik bedanken voor zijn technische assistentie bij de eerste intrathymaire injecties. Wat waren we indertijd blij met 5 ingespoten muizen! Jane Voerman bedank ik voor haar hulp in de eerste fase van mijn onderzoek en bij het maken van krankzinnig veel

celsuspensies op een en dezelfde dag. Last but not least wil ik René Brons bedanken voor zijn hulp bij het sorteren op de FACS II en het i.v. spuiten.

I am indebted to Dr. Sophie Ezine for learning me the intrathymic reconstitution assay. I greatly appreciated the exchange of data, mice and reagents between Paris and Rotterdam. I enjoyed our collaboration very much. Unfortunately, our joint venture did not result in a publication, though it has left me with a good friend. I am therefore very pleased to have you in my promotion committee.

De experimenten beschreven in dit boekje zouden niet zijn uitgevoerd zonder de perfecte proefdierverzorging van Joop Brandenburg en Els van Bodegom. Tar van Os wil ik bedanken voor het grafische werk en Tom Vermetten voor alle bestellingen. De secretaresses dank ik voor hun assistentie bij het verwerken van manuscripten, abstracts, posters en correspondentie. Met name wil ik Daniëlle Korpershoek bedanken voor het persklaar maken van dit boekje. Verder bedank ik iedereen die tijdens mijn onderzoek verantwoordelijk is geweest voor schoon glaswerk.

Annemarie, Annemiek en Elvera van de BAL-unit en mijn overige collega's op het diagnostische lab, wil ik bij deze hartelijk bedanken voor hun geduld en bemoedigende woorden. Ik hoop dat niet al te veel mensen hun weddenschap op het gereedkomen van mijn proefschrift hebben verloren.

Mijn ouders en Roel bedank ik voor hun nimmer aflatende steun en goede zorg.

Walentina

The thymus is not only scientifically an interesting organ. For everyone who would like to explore the thymus in a different way, here's a culinary recipe. Enjoy your dinner!

Sweetbreads* with foie gras and truffles

An extravagant little dish, but well worth the expense and trouble, I reckon.

1 small onion stuck with 1 clove
1 bayleaf
1 small carrot
750 g calf's sweetbreads
flour
butter
salt and pepper
a tin of foie gras (100 g)
1 large truffle or more if you can afford it
1 shallot, finely chopped
1 large glass dry white wine
300 ml chicken stock

The day before you prepare this meal, you must put 1 litre water in a saucepan with the onion, bayleaf and carrot and bring to the boil. Pop in the sweetbreads, then turn down the heat and allow to simmer for 30 minutes. Remove from the heat and allow the sweetbreads to cool in the liquid. Once they are cool, carefully peel off the thin skin or membrane which surrounds the sweetbreads. Lay them on a flat surface, such as a teatray, put another teatray on top and load up with weights (tinned food or anything heavy) so that the sweetbreads are pressed. Leave for 3 or 4 hours. Now you have done the boring bit, preparing the dish next day will be fun and it only takes a little while. First, cut the sweetbreads into four portions and dust slightly with flour. Melt some of the butter in a pan and fry the sweetbreads on both sides for about 15 minutes. Season with salt and pepper. They should be slightly golden on the outside and firm and not spongy. Put to one side in a warm place and lay a thin slice of foie gras on each one. Meanwhile, in the same pan fry thin slices of truffle very gently with the shallot. Pour in the wine and bubble furiously for a moment or two. Then add the chicken stock and continue bubbling. Leave this mixture bubbling, but lift out the truffle slices and scatter them over the sweetbreads. Reduce the liquid in the pan to about a half. Whisk in a knob of butter so that the sauce is smooth and shiny. Adjust the seasoning and strain the sauce over the sweetbreads. If necessary, pop the prepared dish under the grill for a second or two, just to make sure that it is really hot.

* *Thymus (Dutch: zwezerik)*

Source: Keith Floyd. Floyd on France. 1987.

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