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-MP12int/20 population (comprising 30% of the BM cells, including all lymphocytes) contained 90% to 95% of the LTRA cells and immature day-28 CAFC (CFAC-28), 75% of the CFU-S-12, and very low numbers of CFU-C. In contrast, the ER-MP12hi20 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-MP12lo 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-MP20int/20. 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 immediately 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.

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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. 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. 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). 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 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). The different protocols included separation on the basis of rhodamine-123 (Rh123) retention, Hoechst 33342 fluorescence, and wheat germ agglutinin (WGA) affinity. This resulted in a 850- to 2,000-fold enrichment for multilineage long-term repopulating stem cells with relatively low numbers of purified CFU-S-12. 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, showing six distinct subpopulations of murine BM cells when used in two-color immunofluorescence. 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. 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-MP12hi20). After intravenous transfer, however, both the ER-MP12hi20 and ER-MP20subpopulations generated B lymphocytes and myeloid cells in addition to T lymphocytes, indicating the presence of pluripotent hematopoietic cells. Although fre-
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...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 (CFAC)-7 through CFAC-35[23] and long-term in vitro repopulating cells, using an α-thalassemic chimeric mouse model as was recently described.[24] 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/cAnCrlJ mice, and female heterozygous α-thalassemic BALB/c (Hba−/−) mice,[24] were bred at the former Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands. C57BL/6-Ly5.1-pep3b (Thy-1.2, Ly-5.1) and (CBA X C57BL/6)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-pep3b 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[23]), whereas ER-MP12 was biotinylated (ER-MP12[6]) using N-hydroxy-succinimidyl-biotin (Sigma) according to standard procedures.[35] Streptavidin-conjugated R-phycocyanin (SAV-PE) and streptavidin-conjugated TRICOLOR (SAV-TRI) were obtained from Caltag Laboratories (San Francisco, CA).

Hybridoma supernatants were used for MoAbs RA3-6B2 (anti-R20),[26] RB6-8C5 (anti-GR-1),[27] and M1/70 (anti-Mac-1).[36] Erythroid lineage specific MoAb TER-119[23] 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-phycocyanin-conjugated mouse-adsorbed goat antirat IgG (H + L) (Gra-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.[25] 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[6] in PBS containing 0.1% (wt/vol) NaN3, washed, and subsequently incubated for 15 minutes with streptavidin-conjugated MACS microbeads (Miltenyi Biotec) in PBS containing 0.1% NaN3 and 5 mmol/L EDTA (Titriplex III; Merck, Darmstadt, Germany). After incubation, the cells were washed in PBS supplemented with 0.1% NaN3, 5 mmol/L EDTA and 1% (wt/vol) bovine serum albumin (BSA; Fraction V; Sigma) and separated using the MACS columns 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[6]) 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 x 10⁶ cells per 50 μL) were incubated with ER-MP12[6], washed in PBS containing 0.1% NaN3 and 0.5% BSA, and subsequently incubated with ER-MP20[6] 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 Gera-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-MP20[6] followed by ER-MP20[6] 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[6] and SAV-PE for further sorting. All incubations were performed for 30 minutes at 0°C. Cells were analyzed using a FACSscan 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 x 10⁶ 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) methicellulose (Methodol 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 x 10⁻³ mol/L sodium selenite (Merk) and 8 x 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 μM 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% CO₂, 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.[214] 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),
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). 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) 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.

**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). Four of the subpopulations expressed one of the two antigens, at either an intermediate or a high level. The ER-MP12*20* and ER-MP12*20* subsets contained 2% and 30% of the nucleated cells, respectively, whereas the ER-MP12*20* and ER-MP12*20* 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*20* population 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*, ER-MP12*20*, and ER-MP12*20* 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*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). All ER-MP12*20* cells appeared to express the TER-119 Ag, while the ER-MP12*20* cells all expressed Gr-1. The majority of the cells in the ER-MP12*20* population, 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 clonal progenitors, respectively.

**CAFC subsets differentially express the ER-MP12/20Ag.** 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. Compared with unseparated BM, the ER-MP12*20*, ER-MP12*20*, and ER-MP12*20* subsets were enriched for CAFC (Fig 3A). The ER-MP12*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*20* subset 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

<table>
<thead>
<tr>
<th>Population*</th>
<th>Frequency in NBMC (%)</th>
<th>Granuloid Early</th>
<th>Late</th>
<th>Erythroid Early</th>
<th>Late</th>
<th>Lymphoid</th>
<th>Monocytes</th>
<th>Blasts</th>
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<tr>
<td>ER-MP12^20^-</td>
<td>2.1</td>
<td>2.0</td>
<td>0</td>
<td>6.4</td>
<td>0.3</td>
<td>1.7</td>
<td>0.4</td>
<td>10.0</td>
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<tr>
<td>ER-MP12^-20</td>
<td>30.0</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>0</td>
<td>96.2</td>
<td>7.8</td>
<td>20.5</td>
</tr>
<tr>
<td>ER-MP12 20^+</td>
<td>5.0</td>
<td>21.9</td>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>0</td>
<td>76.3</td>
<td>3.2</td>
</tr>
<tr>
<td>ER-MP12^20^med</td>
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<td>30.6</td>
<td>96.8</td>
<td>0</td>
<td>0</td>
<td>5.3</td>
<td>10.2</td>
<td>33.8</td>
</tr>
<tr>
<td>ER-MP12^20^+</td>
<td>9.2</td>
<td>45.5</td>
<td>2.7</td>
<td>28.1</td>
<td>1.8</td>
<td>1.8</td>
<td>10.2</td>
<td>33.8</td>
</tr>
<tr>
<td>ER-MP12 20^-</td>
<td>28.1</td>
<td>0</td>
<td>0.4</td>
<td>63.5</td>
<td>97.8</td>
<td>0.3</td>
<td>0</td>
<td>23.9</td>
</tr>
</tbody>
</table>

* 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 Gramsa 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 polychromatophilic 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-MP12^20^- 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-MP12^20^- 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.
of the microcytic recipients as previously described. Different numbers of unseparated BM cells (Fig 5A) and low-density ER-MP12med20- and ER-MP12med20- 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-MP12med20- subset contained about 25% of the BM CFU-S-12, whereas 75% was found in the ER-MP12med20- 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-MP12med20- and ER-MP12med20- 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-MP12med20- and ER-MP12med20- subsets, the subpopulations were tested for day 7 and day 14 CFU-C in standard methylcellulose cultures (Table 3). The ER-MP12med20- subset was 30-fold enriched when compared with unseparated BM, and contained 67% to 71% of the CFU-C. The ER-MP12med20-, on the other hand, was not enriched and contained 14% to 20% of the CFU-C. The ER-MP12med20- 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. 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 monocye-macrophage lineage (Table 4). Although differences between the recoveries of individual lineages were small, the ER-MP12med20 subset gave rise to an overall higher percentage of colonies containing granulocytes, megakaryocytes, and erythroblasts. After counting the number of lineages per colony, irrespec-
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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-MP12med and ER-MP12high 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-MP12med 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-MP12med 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-MP12med cells, and low-density ER-MP12high 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 x 10⁶, (●) 1 x 10⁶, (▲) 3 x 10⁵ cells per mouse; (B) low-density ER-MP12med: (●) 116,000, (▲) 39,000, (■) 13,000, (□) 4,300 cells; and (C) low-density ER-MP12high: (●) 39,000, (▲) 13,000, (■) 4,300 cells.
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.\(^{29,30}\) We previously reported that the ER-MP12\(^{hi}20^{-}\) 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.\(^{21}\) 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 immediately 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.\(^{31}\) 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.\(^{22-24}\) 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

<table>
<thead>
<tr>
<th>Population</th>
<th>Injected per Mouse</th>
<th>CFU-S-12 per Spleen*</th>
<th>Colonies per 10⁶ Cells (±SD)</th>
<th>Mean (±SEMI)</th>
<th>Enrichment Factor</th>
<th>Recovery of CFU-S-12</th>
<th>Relative Contribution</th>
</tr>
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<tr>
<td>NBMC</td>
<td>5 × 10⁴</td>
<td>6.8 ± 2.9</td>
<td>12.5 ± 6.6</td>
<td>1.5 ± 0.8</td>
<td>1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.2 ± 3.2</td>
<td>18.4 ± 6.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 × 10⁴</td>
<td>6.3 ± 2.5</td>
<td>15.6 ± 6.4</td>
<td>1.5 ± 0.8</td>
<td>1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.1 ± 2.2</td>
<td>15.2 ± 6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 × 10⁴</td>
<td>4.0 ± 1.2</td>
<td>16.0 ± 4.6</td>
<td>1.5 ± 0.8</td>
<td>1</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>ER-MP12(^{med}20^{-})</td>
<td>1,500</td>
<td>3.1 ± 1.4</td>
<td>206.7 ± 91.3</td>
<td>179.6 ± 20.9</td>
<td>11.4 ± 1.4</td>
<td>27.3%</td>
<td>24.9%</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 1.3</td>
<td>206.7 ± 86.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8 ± 1.9</td>
<td>118.5 ± 128.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>2.3 ± 1.6</td>
<td>186.4 ± 128.3</td>
<td>179.6 ± 20.9</td>
<td>11.4 ± 1.4</td>
<td>27.3%</td>
<td>24.9%</td>
</tr>
<tr>
<td>ER-MP12(^{med}20^{-})</td>
<td>2.5 × 10⁴</td>
<td>6.2 ± 2.4</td>
<td>24.8 ± 9.6</td>
<td>48.6 ± 8.9</td>
<td>3.1 ± 0.8</td>
<td>82.5%</td>
<td>75.1%</td>
</tr>
<tr>
<td></td>
<td>2.0 × 10⁴</td>
<td>9.1 ± 2.2</td>
<td>45.5 ± 11.2</td>
<td>48.6 ± 8.9</td>
<td>3.1 ± 0.8</td>
<td>82.5%</td>
<td>75.1%</td>
</tr>
<tr>
<td></td>
<td>11.9 ± 2.2</td>
<td>59.4 ± 11.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.9 ± 2.2</td>
<td>64.5 ± 11.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 Telleyesniczky 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\(^{med}20^{-}\) and ER-MP12\(^{med}20^{-}\) populations were calculated using their average frequencies of 2.4% and 28.8%, respectively.

‡ The relative contribution of CFU-S-12 in the ER-MP12\(^{med}20^{-}\) and ER-MP12\(^{med}20^{-}\) subpopulations was calculated using their respective recoveries.
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,26,48-51 or intracellular dyes like rhodamine-123 and Hoechst 33342.15,20

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-2b, H-2d, H-2k, H-2q', H-21q) 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.34 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-2b) and BALB/c (H-2d) 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 3. Recovery of Day 7 and Day 14 CFU-C in ER-MP20^- BM Cells Separated on the Basis of ER-MP12 Ag Expression

<table>
<thead>
<tr>
<th>Population</th>
<th>Frequency in NBMC</th>
<th>Day 7 CFU-C per 10^6 Cells</th>
<th>Recovery (%)</th>
<th>Day 14 CFU-C per 10^6 Cells</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBMC</td>
<td>100.0</td>
<td>15.2 ± 2.7</td>
<td>100.0</td>
<td>23.6 ± 4.2</td>
<td>100.0</td>
</tr>
<tr>
<td>ER-MP12^20^-</td>
<td>2.3</td>
<td>480.2 ± 85.9</td>
<td>70.7 ± 18.2</td>
<td>676.0 ± 55.5</td>
<td>66.6 ± 13.0</td>
</tr>
<tr>
<td>ER-MP12^-20*</td>
<td>22.5</td>
<td>13.6 ± 2.2</td>
<td>20.0 ± 4.8</td>
<td>14.4 ± 6.0</td>
<td>13.7 ± 6.2</td>
</tr>
<tr>
<td>ER-MP12^-20*</td>
<td>19.5</td>
<td>&lt;0.2</td>
<td>&lt;0.3</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

* 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.

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

<table>
<thead>
<tr>
<th>Population</th>
<th>Day 7 CFU-C</th>
<th>Day 14 CFU-C</th>
<th>% Day 7 CFU-C</th>
<th>% Day 14 CFU-C</th>
<th>Percentage of CFU-C-14 colonies with specific lineage expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBMC</td>
<td>16.8 ± 1.6</td>
<td>125.8 ± 17.2</td>
<td>2.4 ± 0.5</td>
<td>0.2 ± 0.2</td>
<td>Granuloid: 12.0</td>
</tr>
<tr>
<td></td>
<td>17.8 ± 4.8</td>
<td>202.5 ± 24.6</td>
<td>7.3 ± 2.0</td>
<td>0.2 ± 0.1</td>
<td>Mono/maph: 100.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.0 ± 15.9</td>
<td>11.0 ± 2.8</td>
<td>2.0 ± 1.4</td>
<td>Megakaryocytic: 16.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>79.4 ± 13.0</td>
<td>19.2 ± 5.7</td>
<td>1.4 ± 0.7</td>
<td>Erythroid: 14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mast cells: 4.8</td>
</tr>
<tr>
<td>ER-MP12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Granuloid: 12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mono/maph: 100.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Megakaryocytic: 16.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Erythroid: 14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mast cells: 4.8</td>
</tr>
<tr>
<td>ER-MP12^20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Granuloid: 12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mono/maph: 100.0</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Megakaryocytic: 16.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Erythroid: 14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mast cells: 4.8</td>
</tr>
</tbody>
</table>

* Number of day 7 and day 14 CFU-C per 10^6 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-MP12^20, 127; ER-MP12^-20, 83; and ER-MP12, 22.
‡ The number of lineages per CFU-C colony was determined irrespective of the particular lineage type.
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⁻ Sca-1⁺ CD4⁻ (the low-CD4 precursor), that did not have spleen colony-forming capacity upon intravenous (IV) infusion. On intrathymic transfer, however, this precursor took less time to generate CD4⁻ CD8⁻ thymocytes than did Sca-2⁻ Thy-1,1⁻ Sca-1⁺ Lin⁻ BM cells, which are highly enriched for CFU-S-12. This observation suggests that the average CFU-S is more primitive than the thymic and BM⁻ 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. 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, a low expression of Thy-1.1, and a low to negative expression of Thy-1.2. In addition, they were positively identified by the expression of a high level of c-kith and a high level of the major histocompatibility class I Ag H-2K. 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) and Fall-3, 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. 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. 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. 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-MP12² 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⁻ and ER-MP12⁺ BM cells expressed Sca-1⁺ suggesting that ER-MP12 could be used to identify subsets within the Thy-1⁻ Lin⁻ Sca-1⁺ subpopulation, which remains to be tested. Taken together, the present study identifies the ER-MP12

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**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**

<table>
<thead>
<tr>
<th>Population</th>
<th>% of BM</th>
<th>% of BM of Lin⁻ Cells</th>
<th>% pre-T</th>
<th>% pre-B</th>
<th>% CFU-C</th>
<th>% CFU-S-12</th>
<th>% LTRA 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sca-1⁻</td>
<td>5-6</td>
<td>0.2</td>
<td>50-80</td>
<td>ND</td>
<td>10 (68)</td>
<td>~50</td>
<td>~100</td>
</tr>
<tr>
<td>Sca-1</td>
<td>94-95</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>90 (35)</td>
<td>low</td>
<td>0</td>
</tr>
<tr>
<td>Fall-3⁻</td>
<td>15-30</td>
<td>10-25</td>
<td>ND</td>
<td>7</td>
<td>48</td>
<td>65</td>
<td>75</td>
</tr>
<tr>
<td>Fall-3</td>
<td>70-85</td>
<td>ND</td>
<td>ND</td>
<td>93</td>
<td>52</td>
<td>35</td>
<td>~35</td>
</tr>
<tr>
<td>ER-MP12⁻</td>
<td>2-4</td>
<td>1-2</td>
<td>Majority</td>
<td>ND</td>
<td>80-90</td>
<td>25</td>
<td>5-10</td>
</tr>
<tr>
<td>ER-MP12⁻</td>
<td>30-35</td>
<td>2-3</td>
<td>Minority</td>
<td>ND</td>
<td>10-20</td>
<td>75</td>
<td>90-95</td>
</tr>
<tr>
<td>ER-MP12⁻</td>
<td>61-68</td>
<td>1-2</td>
<td>None</td>
<td>ND</td>
<td>1-2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figures on the expression and distribution of Sca-1 are from multiple references. The distribution of Fall-3 is described in Muller-Sieburg, while the ER-MP12 data come from Slierker et al²⁵ and were described in the present study.

Abbreviation: ND, not determined or unknown.

* Determined by intrathymic transfer into sublethally irradiated animals.

1 Determined at 2 weeks in Whitlock-Witte cultures.

2 Determined by intrathymic transfer into sublethally irradiated animals.

3 Distribution of day 7 and day 14 CFU-C stimulated with IL-3; ER-MP12: day 4 through day 14 CFU-C stimulated by IL-3, SF, IL-11 and IL-12.

4 Frequency analysis by CAFC (day 28/35) in vitro, and LTRA (1-5 mo) in vivo.

5 Data refer to the (Thy-1⁻ Lin⁻) Sca-1⁺ and (Thy-1⁻ 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 L.L. Weissman for providing us with breeding pairs of the α-thalassemic and C57BL/6-Ly5.1-pegb mice, respectively, Dr S. Neben of Genetics Institute for the recombinant murine cytokines, and Prof Dr W. van Ewijk for critically revising the manuscript.

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33. Ploemacher RE, Van der Sluij JP, Van Beurden CAJ, Baert MRM, Chan PL: Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen
Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12

JC van der Loo, WA Slieker, D Kieboom and RE Ploemacher