

Research Article

Recovery and Biodistribution of *Ex Vivo* Expanded Human Erythroblasts Injected into NOD/SCID/IL2R γ^{null} mice

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Ex vivo expanded erythroblasts (EBs) may serve as advanced transfusion products provided that lodgment occurs in the macrophage-niche of the marrow permitting maturation. EBs expanded from adult and cord blood expressed the receptors (CXCR4, VLA-4, and P-selectin ligand 1) necessary for interaction with macrophages. However, 4-days following transfusion to intact NOD/SCID/IL2R γ^{null} mice, CD235a^{pos} EBs were observed inside CD235a^{neg} splenic cells suggesting that they underwent phagocytosis. When splenectomized and intact NOD/SCID/IL2R γ^{null} mice were transfused using retrovirally labeled human EBs, human cells were visualized by bioluminescence imaging only in splenectomized animals. Four days after injection, human CD235a^{pos} cells were detected in marrow and liver of splenectomized mice but only in spleen of controls. Human CD235a^{pos} erythrocytes in blood remained low in all cases. These studies establish splenectomized NOD/SCID/IL2R γ^{null} mice as a suitable model for tracking and quantification of human EBs *in vivo*.

1. Introduction

Culture conditions capable of generating *ex vivo* human erythroblasts (EBs) in numbers sufficient for transfusion have been established by several investigators (reviewed in [1]) increasing the likelihood that these cells could serve as alternative transfusion products [2]. The preclinical evaluation of these products includes testing the *in vivo* potency of *ex vivo* generated cells in animal models [3]. The development of animal models to assess the potency of human EBs generated *ex vivo* requires a better understanding of the interactions between human EBs and the murine microenvironment and identification of animal manipulations which may favor lodgment of these cells in the marrow.

In vivo, EBs mature in specialized areas of the marrow in close proximity to macrophages which favors both

hemoglobinization, by facilitating iron uptake, and enucleation [4, 5]. Following enucleation, reticulocytes lose their association with the macrophage and egress into the blood stream. EBs interact with the macrophages through the surface adhesion receptors CXCR4 (CD184), P-selectin ligand1 (PSGL1, CD162), and VLA-4 (CD49d, $\alpha 4$ integrin) which recognize CXCL12 (also known as SDF1), P-selectin and VCAM1, respectively, on the surface of the macrophage [4]. Under steady-state conditions, few EBs egress from the marrow and these are usually cleared by macrophages in the spleen [6]. Smears of cultured human EBs often contain macrophages surrounded by clusters of EBs indicating that cultured EBs are capable of interacting with human macrophages derived from blood. However, the adhesion receptor profile of *ex vivo* generated EBs and the efficiency

of the interaction of these cells with murine macrophages are presently unknown.

Neildez-Nguyen et al. have reported that human carboxyfluorescein diacetate succinimidyl ester- (CFSE-) labeled EBs expanded *ex vivo* from CD34^{pos} cells isolated from cord blood differentiate into red blood cells when transfused to NOD/SCID mice [7]. The transfusion protocol included coadministration of exogenous human erythropoietin (EPO) and intraperitoneal administration of packed human red cells to block the reticuloendothelial system (leading to transient functional splenectomy). Initially, human CFSE^{pos} cells were detected in bone marrow, liver, spleen and lung of the transfused animals and, beginning at day 4, also in peripheral blood. However, these experiments have been viewed with skepticism because human CD34^{pos} cells engrafted in immunodeficient mice typically generate robust levels of lymphoid and myeloid cells but barely detectable numbers of erythroid cells [8]. These data have been interpreted as evidence that the murine microenvironment is not permissive for maturation of EBs. Although the molecular structures of human and murine EPO are very similar, it has been suggested that murine EPO may fail to support optimal maturation of human EBs because it does not induce dimerization after binding to the human receptor [9]. This hypothesis was indirectly supported by Nicolini et al who reported that treatment with human EPO after transplantation greatly increases the generation of human erythroid cells when human CD34^{pos} cells are injected into immunodeficient mice [10]. However, the relative contribution of administration of human EPO and packed human red cells to the success of the animal model for human transfusion developed by Neildez-Nguyen et al. [7] has not been clarified as yet.

The aim of our study was to test whether *ex vivo* generated EBs express the adhesion receptor profile necessary to complete their maturation *in vivo* and whether inhibition of the interaction between *ex vivo* generated EBs and splenic macrophages will permit establishment of an *in vivo* model for functional evaluation of *ex vivo* expanded human EBs.

2. Materials and Methods

2.1. Human Subjects. Low volume cord blood units (CB) were obtained from the New York Blood Center (New York, NY, USA). Peripheral blood (PB) was collected from normal adult donors at the transfusion center of “La Sapienza” University (Rome, Italy). Both specimens were collected according to guidelines established by institutional ethical committees and provided as deidentified samples.

2.2. Mice. 12-week-old female NOD/SCID/IL2Ry^{null} mice were purchased from Jackson laboratory and housed in the animal facility of Memorial Sloan-Kettering Cancer Center. All murine studies were performed according to institutional animal care and use committee approved protocols.

2.3. Cell Preparation. Mononuclear cells (MNCs) from CB and AB were obtained by centrifugation over Ficoll-Hypaque

(Amersham Pharmacia Biotec, Uppsala, Sweden). CD34^{pos} cells from CB were separated using the Human Cord Blood CD34 Selection Kit (STEMCELL Technologies Inc, Vancouver, BC, Canada), as described by the manufacturer.

2.4. Expansion of Human Erythroblasts. CD34^{pos} cells and MNC (5×10^4 and 10^6 cells/mL, resp.) were cultured under human erythroid massive amplification (HEMA) conditions in a proliferative phase stimulated with stem cell factor (SCF, 10 ng/mL, R&D System, Minneapolis, MN, USA), erythropoietin (EPO, 5 U/mL, Epogen, Amgen, Thousand Oaks, CA, USA) and interleukin 3 (IL-3, 1 ng/mL, Biosource, San Jose, CA, USA), dexamethasone and estradiol (both at 10^{-6} M, Sigma, St. Louis, MO, USA) up to 10 days and subsequently in a differentiative phase stimulated with EPO (5 U/mL), insulin (10 ng/mL, Calbiochem, La Jolla, CA, USA), and FeSO₄ (10^{-6} M, Sigma) up to 4 days, as previously described [11, 12].

2.5. Colony Forming Assay. The colony forming ability of sorted cells was evaluated in standard semisolid methylcellulose cultures (40%, Fluka Biochemika) stimulated with SCF (10 ng/mL), IL-3 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/mL), granulocyte colony-stimulating factor (G-CSF, 100 ng/mL) and EPO (5 U/mL). The cultures were incubated at 37°C in a fully humidified 5% pCO₂ atmosphere and scored after 2 days for colony forming unit erythroid (CFU-E) and after 8 days for burst forming unit erythroid (BFU-E) and colony forming unit granulocyte-macrophages (CFU-GM) colonies.

2.6. Cell Viability and Phenotypic Analysis. Cell numbers and viability were assessed by microscopic evaluation after trypan blue (Boston Bioproducts, Ashland, MA, USA) staining. Erythroid cells were identified using standard morphological criteria by visual examination of cytocentrifuged cell preparations (Cytospin 3, Shandon, Astmoor, England) stained with May-Grünwald-Giemsa (Fisher Scientific, Pittsburg, PA, USA) using the Axioscope light microscope equipped with a Coolsnap video camera (Zeiss, Oberkochen, Germany). For flow cytometric analyses, cells were suspended in Ca²⁺/Mg²⁺-free phosphate-buffered saline, supplemented with 1% BSA, stained with either phycoerythrin- (PE-) conjugated CD36 (antithrombospondin receptor) [13], -CD184 (CXCR4), -CD162 (PSLG1), -CD49d (VLA-4), or allophycocyanin- (APC-) conjugated CD235a (anti-glycophorin A), pan hematopoietic human- (FITC-) conjugated CD45 or appropriate isotype controls (all from Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). For GFP determinations, autofluorescence of untreated cells was analysed as negative control. Fluorescence was analyzed with the FACS Canto (Becton Dickinson Biosciences) equipped with three air-cooled and solid-state lasers (488 nm, 633 nm and 407 nm). Dead cells were excluded by propidium iodide (PI, 5 µg/mL, Sigma) staining.

2.7. Fluorescence Labelling of Human EBs. CFSE Labeling. EBs obtained at day 11 of HEMA cultures were stained with

CFSE (10 μ M, Invitrogen, Carlsbad, CA, USA) as described [14] and cultured for 24 additional hrs under HEMA conditions. *Retrovirally mediated labeling.* CB CD34^{pos} cells were cultured for 3 days in X-VIVO 10 media (Lonza Walkersville, Walkersville, MD, USA) containing thrombopoietin (TPO, 100 ng/mL), SCF (100 ng/mL), Flt3 Ligand (Flt3L, 100 ng/mL), IL-3 (20 ng/mL), and the PG-13 retroviral producer supernatant [15, 16] which contains the membrane anchored form of the Gaussia luciferase enzyme (extGLuc) [16] was added twice 24 hrs apart. The transfected CD34^{pos} cells were then cultured under HEMA conditions for 6 additional days. An aliquot of nontransduced CD34^{pos} cells from the same isolation was cultured in parallel under HEMA condition as control.

2.8. Transfusion Protocol. Two protocols were investigated. In the first experiment (See Supplementary Figure 1 in Supplementary Material available online at doi: 10.4061/2011/673752), 2 NOD/SCID/IL2R γ ^{null} mice were injected via the tail vein with 25×10^6 CFSE-labeled EBs. Twenty-four hrs prior to injection, mice were bled (500 μ L) to increase endogenous EPO levels. In the second experiment (Supplementary Figure 2), intact and splenectomized NOD/SCID/IL2R γ ^{null} mice (4 mice per group) were used. NOD/SCID/IL2R γ ^{null} mice were anaesthetized with isoflurane (Baxter, Deerfield, USA) and splenectomized after double ligation of the splenic artery and vein [17]. The muscle, peritoneum, and skin were closed in separate layers using sterile 5–0 absorbable suture. Intact and splenectomized mice were transfused via the tail vein with 25×10^6 retrovirally labeled EBs together with human EPO (20 U/mouse). Twenty four hours earlier, mice had been bled (500 μ L). In both experiments, mice receiving culture media only served as negative controls and all the mice were sacrificed 4–5 days following transfusion for further analyses.

2.9. Immunocytochemistry. Smears of single cell suspensions from femur, spleen, and liver and cultured EBs prepared by cytocentrifugation (Shandon, Astmoor, England) were fixed with paraformaldehyde (3.7%, Electron Microscopy Science/Hatfield, PA, USA) for 30 min and rinsed 2 times with double distilled water. Smears were incubated with an antihuman glycophorin A antibody (CD235a, Abcam, Cambridge, MA, USA) and the immunoreaction detected with the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) on slides counterstained with hematoxylin-eosin, as described by the manufacturer. Aliquots of injected EBs and smears from media-treated mice were used as positive and negative controls, respectively. Histological observations were carried out using a ZEISS AXIOSKOPE light microscope (Jena, Germany) equipped with a Coolsnap Videocamera. Enumeration of human CD235a^{pos} cells was performed by two separate investigators in a blinded fashion.

2.10. Bioluminescence Imaging. Bioluminescence was detected using a Xenogen IVIS Imaging System (Xenogen) as previously described [18]. Imaging was performed 24–48 hrs

following intravenous injection of coelenterazine (250 μ g) (Nanolight Technology) via the tail vein. Dorsal and ventral images of the animals were acquired with acquisition times in the range of 1 to 3 min. Field of view of 15, 20, or 25 cm with low, medium, or high binning in an open filter was utilized to maximize signal intensity and sensitivity. We obtained acquisition of image datasets and measurements of signal intensity through region of interest (ROI) analysis using Living Image software (Xenogen). Normalized images are displayed on each dataset according to color intensity.

2.11. Statistical Analyses. Results are expressed as mean (\pm SD) of at least three replicate experiments unless stated otherwise. Statistical analysis was performed by Anova using the computer software Origin 5.0 for Windows (Microcal Software, Inc., Northampton, MA, USA).

3. Results

3.1. Cord Blood MNC Generate Greater Numbers of Less Mature Erythroid Cells Than Adult Blood MNC under HEMA Conditions. Our group has previously identified a culture strategy for massive production of EBs *ex vivo* from both CB and AB MNC defined as human erythroid massive amplification (HEMA) culture [11]. Under these culture conditions, in the experiments included in this paper, AB MNC generated EBs with an FI = 15 while the number of EBs generated by CB MNC was 3-times greater (FI = 40 Figure 1).

By CD36/CD235a profiling, EBs generated under HEMA conditions may be separated into 4 classes of progressively more mature cells represented by CD36^{high}/CD235a^{neg} (class I, gate R1) and CD36^{high}CD235a^{low} (class II, gate R2) cells, which contain colony-forming unit-erythroid (CFU-E) and pro-EBs, and CD36^{high}CD235a^{high} (class III, gate R3) and CD36^{low}CD235a^{high} cells (class IV, gate R4), which contain basophilic-polychromatic and orthochromatic EBs, respectively (Supplemental Figure 3). BFU-E- and CFU-GM-derived colonies are not detectable in cells generated in HEMA culture from day 8 on (data not shown). At day 10 of HEMA culture, a significant proportion of EBs generated in cultures of AB and CB MNC are represented by immature class I and class II EBs (38 and 78%, resp.) (Figure 1 and Table 1). Exposure of Day 10 EBs derived from both AB and CB to EPO alone for 4 days increased the proportion of mature class III and IV EBs up to 52–70%. CB-derived EBs contained a lower fraction of mature cells than AB-derived EBs both in HEMA culture and when cultured with EPO alone (Figure 1 and Table 1).

Macrophage islands surrounded by 6–9 EBs were commonly detected in smears of both AB and CB derived EBs beginning at day 7 and up to day 10 of HEMA culture (Figure 1(c)). The frequency of these islands on the smears was correlated with the frequency of nonerythroid cells which had survived in culture (data not shown). The random nature the macrophages in HEMA culture prevented quantification of this phenomenon. Further studies in which cultured EBs will be exposed to monocytes purified from

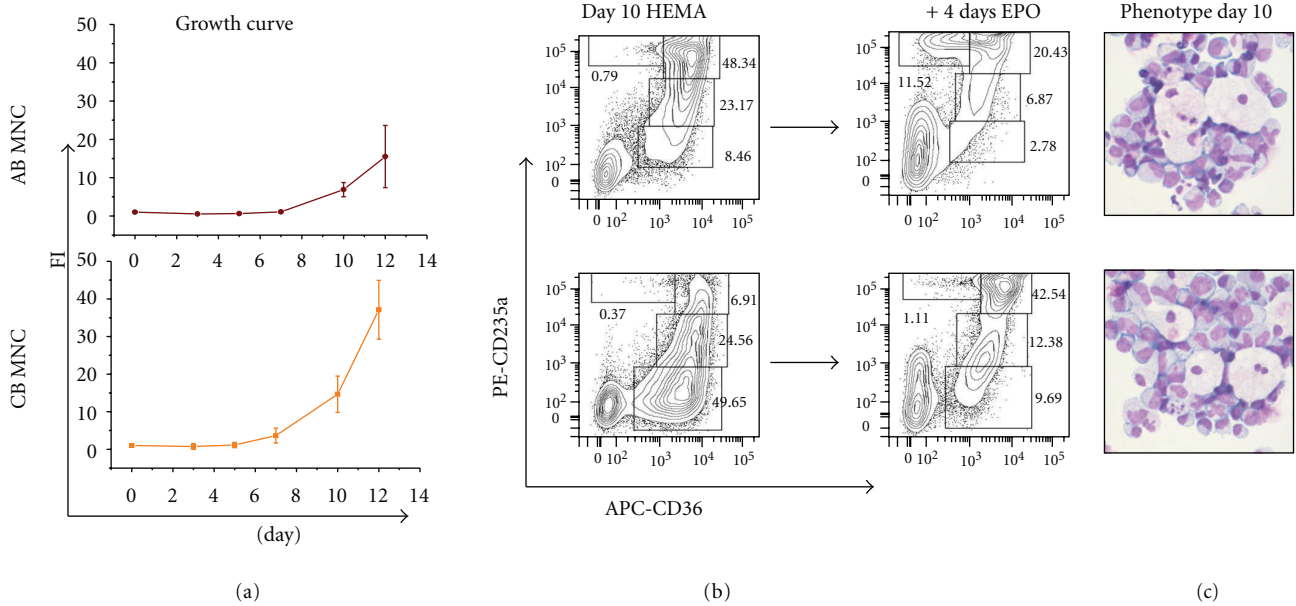


FIGURE 1: MNC from AB and CB generate great numbers of EBs under HEMA conditions. (a) Cell number (as Fold Increase, FI, with respect to day 0), (b) maturation profile (cytofluorimetric analysis on the basis of the expression of CD36 and CD235a), and (c) representative morphology (by May-Grunwald staining) of EBs generated in HEMA culture seeded with MNC from either CB or AB. The ability of CB and AB EBs obtained at day 10 to proceed along the maturation pathway after 4 days of culture in the presence of EPO only is also compared (b). The flow cytometric profile used to define EBs maturation is presented in Supplemental Figure 3. The flow charts are representative of those obtained in at least three independent experiments with MNC from different CB or AB donors. The numbers in the quadrants present the frequency of EBs in the gates R1 to R4. Frequencies obtained in multiple experiments are presented as mean (\pm SD) in Table 1.

TABLE 1: Frequency of EBs divided according to their maturation profile at day 10 of HEMA culture seeded with AB and CB MNC and after 4 days of additional culture in EPO alone to induce maturation. The gates used to define the 4 maturation classes used in this study are described in Supplemental Figure 3.

Maturation classes	AB MNC		CB MNC	
	Day 10 (%)*	Day 4 + EPO (%)	Day 10 (%)	Day 4 + EPO (%)
Class I (CD36 ^{high} CD235a ^{neg})	17.0 \pm 6.2	1.6 \pm 0.6	53.8 \pm 8.9	11.9 \pm 4.8
Class II (CD36 ^{high} CD235a ^{low})	21.7 \pm 4.1	9.7 \pm 1.1	21.5 \pm 6.5	10.6 \pm 4.5
Class III (CD36 ^{high} CD235a ^{high})	49.2 \pm 2.4	55.1 \pm 7.4	9.1 \pm 4.6	49.5 \pm 6.4
Class IV (CD36 ^{low} CD235a ^{high})	1.2 \pm 1.1	15.8 \pm 4.4	0.2 \pm 0.6	1.7 \pm 1.1

* Percentages were calculated with respect to the total cell population present in culture (EBs and non EBs).

human blood, marrow, and spleen are required to define the interaction between these cell populations *in vitro*.

3.2. Dynamic Pattern of Adhesion Receptor Profiling during the Maturation of AB- and CB-Derived EBs in Culture. The expression patterns of CXCR4, PSLG1, and VLA-4 on EBs obtained from AB and CB at day 10 of HEMA and induced to mature for 4 days in cultures containing EPO alone is presented in Figures 2, 3, and Supplemental Figure 4.

Expression of CXCR4 was barely detectable either in AB- or CB-derived EBs at day 10 of culture, irrespective of the stage of maturation (Figures 2 and 3). Exposure of these cells to EPO alone rapidly (within 1 day) activated CXCR4 expression first on immature cells (Class 1, R1) and then by day 4 also on cells expressing a more mature phenotype (Class 2 for AB EBs and Class 2 + 3 for CB EBs). The most mature Class 4 EBs (both from AB and CB) never expressed

CXCR4 (Figures 2 and 3). The dynamic pattern of CXCR4 expression is visualized by the direct comparison of CD184 (CXCR4) and CD235a (glycophorin A) expression as AB and CB EBs matured in EPO alone culture presented in Supplemental Figure 4. Although similar patterns of CXCR4 expression were observed in EBs derived from AB and CB, a greater percentage (30–80% versus 15–40%) of CB-derived EBs expressed CXCR4 than AB EBs and the mean fluorescence intensity (MFI) was also greater for CB (range 1000–2500 versus 500–1000).

At day 10, both AB and CB derived EBs expressed high levels of PSLG1 regardless of their maturation stage (80–90% PSLG1^{pos} cells with MFI in the range of 1000–4000) (Figures 2 and 3). The expression of PSLG1 was conserved when EBs were induced to mature with EPO alone for 4 days (Figures 2 and 3) although direct comparison of PSLG1 expression against CD235a indicated an association

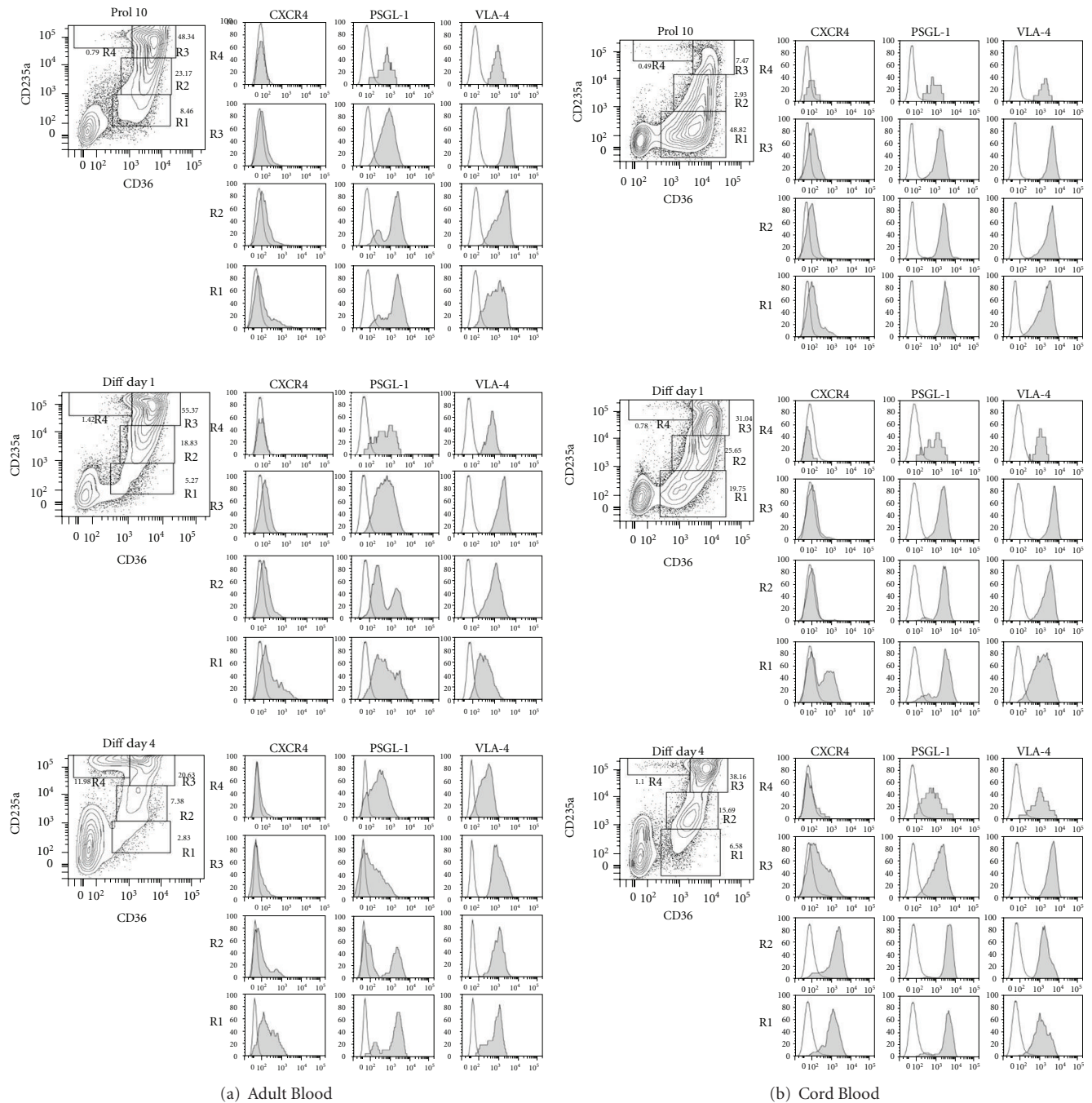


FIGURE 2: Adhesion receptor profiling during *in vitro* maturation of EBs expanded from AB (a) and CB (b). Three color flow cytometry analysis for CD36 and CD235a in combination with either CXCR4 (CD184), PSGL-1 (CD162), or VLA-4 (CD49d) of EBs obtained at day 10 of HEMA culture from AB and CB MNC (Prol 10) and at day 1 (Diff Day 1) and 4 (Diff Day 4) of maturation with EPO alone, as indicated. EBs were divided into 4 maturation classes defined by the levels of CD36 and CD235a expression identified by the gates R1 to R4 and corresponding to Class 1 to 4 (see Supplemental Figure 3 for further details).

between reduced expression of this receptor and maturation which was particularly evident in the case of AB-derived EBs (Supplemental Figure 4).

A great percentage (60–100%) of day 10 EBs expressed robust levels of VLA-4 (MFI in the range of 1000–3000 for AB EBs and 2000–6000 for CB EBs) regardless of their maturation status. The frequency of VLA4^{POS} EBs remained

high when the cells were induced to mature with EPO for 4 days, although in the case of AB EBs the levels of VLA4 expression per cell decreased with time in culture when stimulated with TPO alone (Figures 2 and 4). The apparently high maturation-independent pattern of VLA4 expression in AB and CB EB was also observed by direct analyses of VLA4/CD235a expression patterns (Supplemental Figure 4).

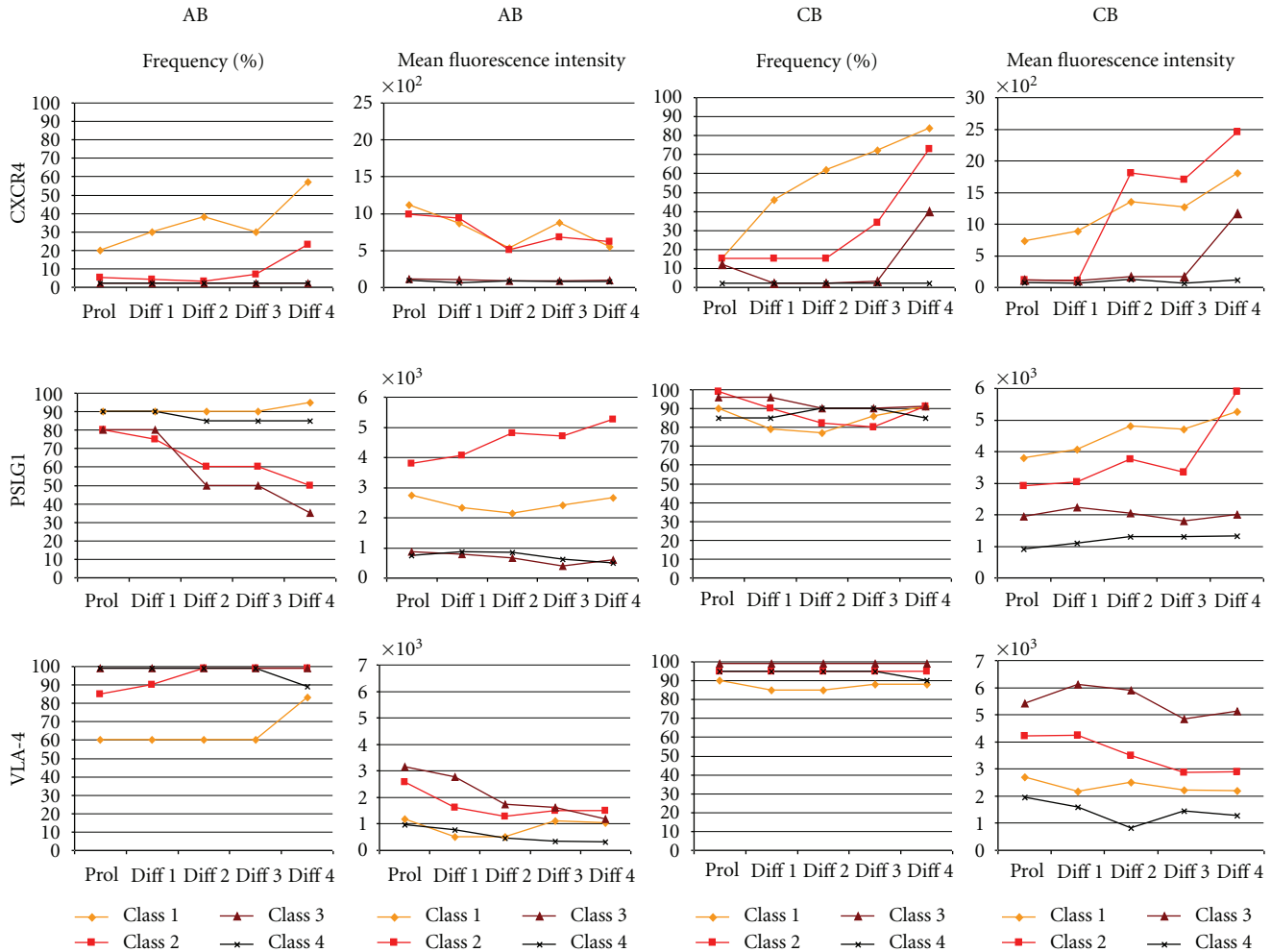


FIGURE 3: Time course of the frequency and MFI of CXCR4, PSL1, and VLA4 expression during *in vitro* maturation of EBs expanded from adult and cord blood, as indicated. Values observed with EBs at different stages of maturation are color coded: Class 1 (gate R1): light orange, Class 2 (gate R2): red, Class 3 (gate R3): brown, and Class 4 (gate R4): black (see also Supplemental Figure 1). The results correspond to the flow charts presented in Figure 2.

In conclusion day 10 EBs express low levels of CXCR4 but robust levels of PSLG1 and VLA4. Induction of these cells to mature by exposure to EPO alone rapidly activates CXCR4 expression but has modest or no effect on the expression of PSLG1 and VLA4, with the exception of a reduction in the number of PSLG1^{POS} class 2 and class 3 EBs observed in culture of AB-derived cells.

3.3. Cell Fate of Ex Vivo Expanded CB EBs in NOD/SCID/IL2Ry^{null} Mice. The greater levels of adhesion receptor expression of CB EBs suggested that these cells might be more able than AB EBs to establish the cellular interactions necessary for maturation *in vivo* and therefore more suitable for use in animal models to analyze cell fate following transfusion. We chose the NOD/SCID/IL2Ry^{null} mouse as the animal model for these experiments because it has been shown to readily support engraftment of human CD34^{POS} hematopoietic stem cells and represents a superior, long-lived model suitable for studies employing xenotransplantation strategies [20, 21].

In preliminary experiments, the fate of murine RFP-labeled EBs derived from liver and transfused into syngeneic thalassemic mice was determined. Murine RFP-labeled EBs ($30\text{--}150 \times 10^6$) transfused in unbled thalassemic mice generated undetectable or barely detectable levels of red cells. By contrast, when mice were bled $150\text{--}600 \mu\text{L}$ 24 hrs before transfusion, the same numbers of murine RFP-labeled EBs generated as many as $5 \times 10^9\text{--}5 \times 10^{10}$ red cells (A.R. Migliaccio and P. Frenette, unpublished observations). These results indicate that bleeding facilitates *in vivo* maturation of transfused murine EBs, probably by increasing endogenous EPO levels. On the basis of these data we decided to bleed animals 24 hr prior to transfusion with human EBs.

In the initial three experiments, CFSE-labeled EBs derived from CD34^{POS} CB cells after 11 days in HEMA culture were transfused via the tail vein (25×10^6 EBs per mouse) into NOD/SCID/IL2Ry^{null} mice that were bled ($500 \mu\text{L}$) the day before (Supplemental Figure 3). Mice were sacrificed at day 4 after transfusion for detection of human CD235a^{POS} EBs and

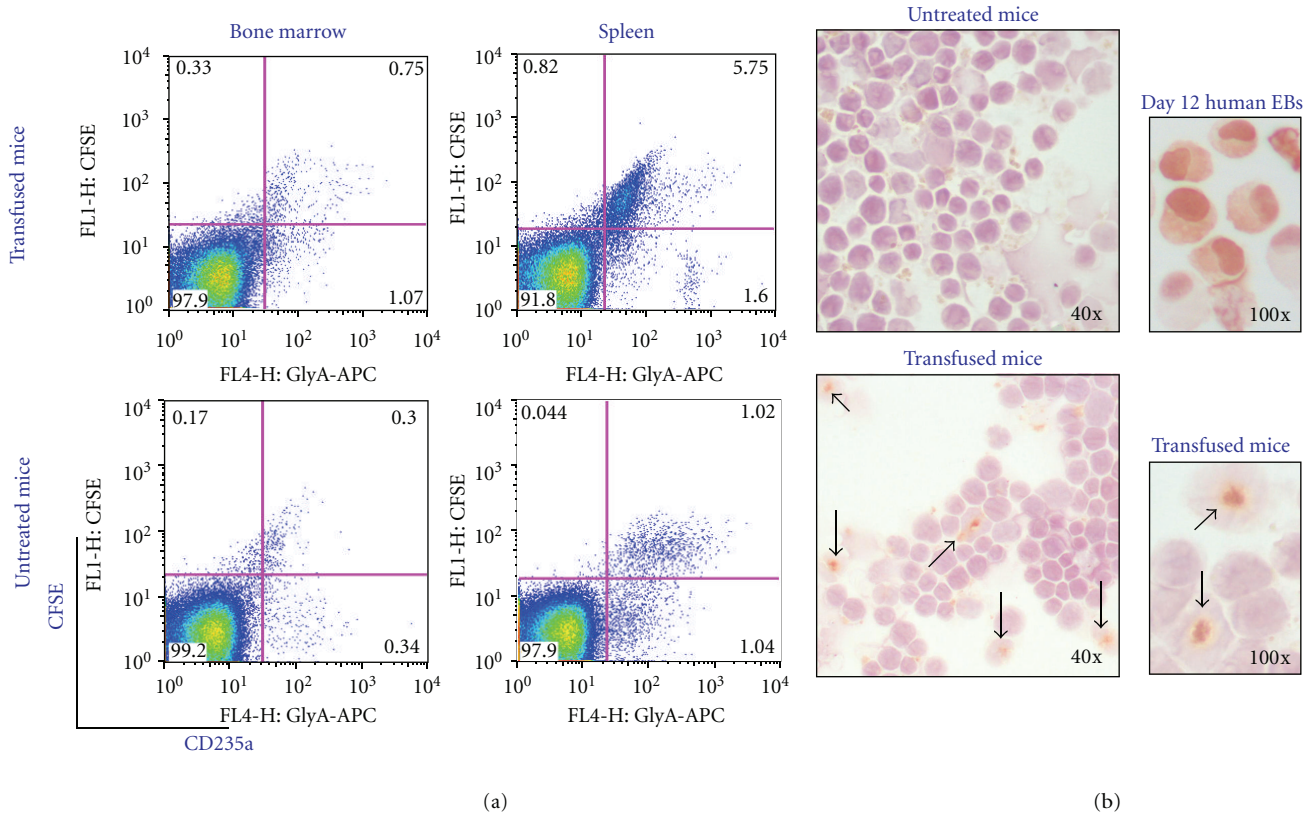


FIGURE 4: At day 4 after transfusion, human EBs are mostly detectable in spleen of NOD/SCID/IL2R γ^{null} mice. (a) Flow cytometric analysis for human CD235a and CFSE expression of bone marrow and spleen cells from transfused and nontransfused (negative control) NOD/SCID/IL2R γ^{null} mice. The results are representative of those obtained with 2 mice per group (see Table 2 for further details) and are representative of those observed in three separate experiments. (b) Immunocytochemistry for human CD235a of cells from the spleen of untransfused (top left panel) and transfused (lower panels) mice sacrificed 4 days after the human EBs transfusion, as indicated. Cells from control mice (top left panel) are negative while cultured human EBs (top right panel) are all positive. In transfused mice, human CD235a^{pos} cells (arrows) represented up to $18.2 \pm 5.6\%$ of total splenic cells. However, they were trapped inside larger CD235a^{neg} cells, probably macrophages of murine origin. Magnifications 40x and 100x, as indicated.

TABLE 2: Frequency and total number of cells expressing the human CD235a in bone marrow and spleen of NOD/SCID/IL2R γ^{null} mice transfused 4 days earlier with CFSE-labeled EBs. Both CFSE-positive and CFSE-negative human CD235a^{pos} cells were detected in the organs of the animals after transfusion, an indication that some of the human EBs had proliferated *in vivo*.

	CSFE ⁺ CD235a ⁺ (%)	CSFE ⁻ CD235a ⁺ (%)	CSFE ⁺ +CSFE ⁻ CD235a ⁺ -control (%)	Total no. of CD235 ⁺ cells/organ*
BM				
Control	0.3	0.34	0	
Transfused mice (2 mice)	0.53–0.75	1.1–1.07	0.99–1.18	$3.0\text{--}3.5 \times 10^6$
Spleen				
Control	1.02	1.04	0	
Transfused mice (2 mice)	3.34–5.75	1.3–1.6	2.57–5.29	$2.6\text{--}5.3 \times 10^6$

The frequency of human CD235a⁺ cells was obtained after subtracting the background signal observed in untreated controls.

*The total number of cells per organ was calculated assuming that a mouse contain 300×10^6 BM and 100×10^6 spleen cells [19].

at day 21 for the presence of human hematopoietic cells of all lineages (CD45^{pos}).

At day 4, FACS analyses revealed the presence of 0.99–1.18% and 2.57–5.29% of CD235a^{pos} cells (both CFSE^{pos} and CFSE^{neg}) in bone marrow and spleen, respectively, of the transfused mice (Figure 4(a) and Table 2). The presence of

CFSE^{neg}CD235a^{pos} cells indicated that some of the human EBs had undergone proliferation *in vivo* during the 4 days after transfusion. Assuming that the total number of cells in bone marrow and spleen of a mouse is 300×10^6 cells and $\sim 10^8$ cells, respectively [19], it is calculated that the bone marrow and spleen of the transfused animals contained

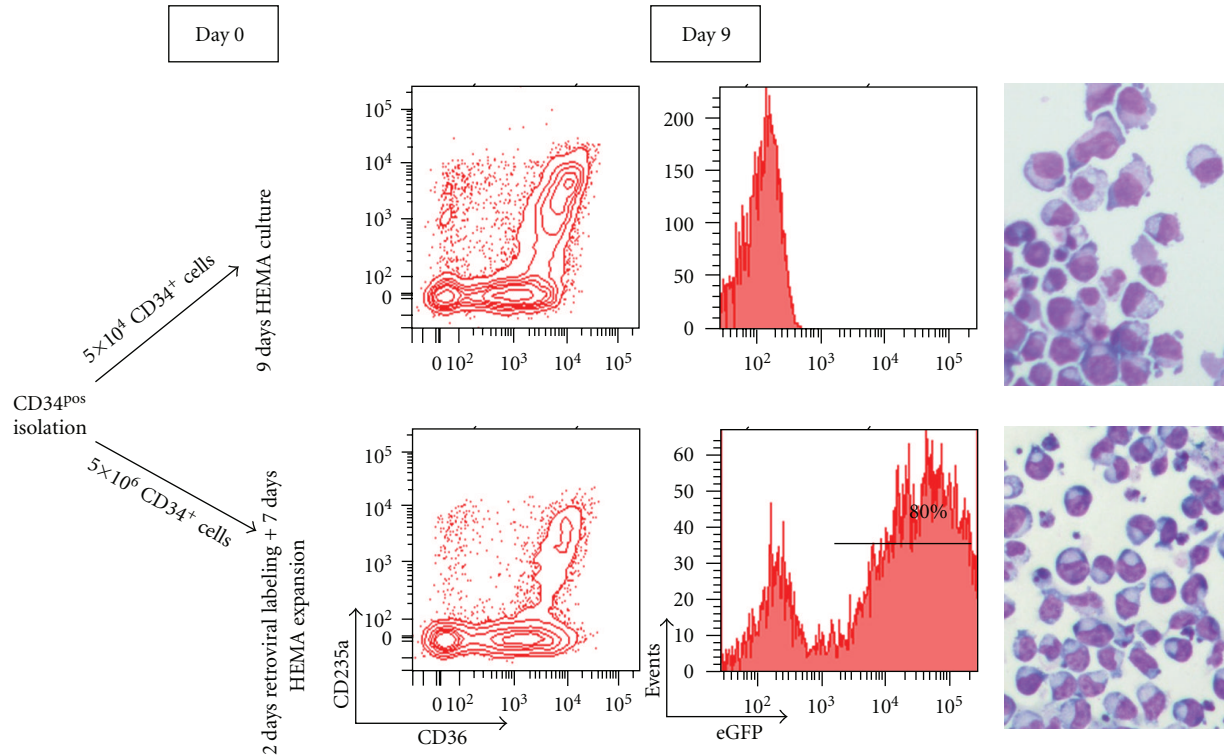


FIGURE 5: *Ex vivo* expanded extGLuc⁺ EBs express high level of eGFP by FACS analyses. Maturation profile and transfection efficiency of nontransfected (as control, top panels) and transfected (bottom panels) EBs at day 9 of HEMA culture identified on the basis of CD36/CD235a and eGFP expression (on the left) and on the basis of morphology (May-Grunwald staining, on the right), magnification 40x. The transfected EBs express great levels of eGFP. The transfection did not alter the morphology of the cells but slightly reduced the expression of the CD235a probably due to interference with the GFP signal. At day 9, in transfected and control cultures the fold increase was 48-fold and 325-fold, respectively, and the viability was 98% in both cases.

$\sim 3.5 \times 10^6$ (1.7% of 3×10^8 cells) and 7×10^6 (6.8% of 10^8 cells) human EBs, respectively (Table 2). Therefore, the majority of human EBs lodged in the spleen. Immunocytochemical analyses for the expression of the human erythroid CD235a marker confirmed the presence of numerous (18% of the total cell population) human CD235a^{pos} cells in the spleen of the transfused animals (Figure 4(b)). However, the CD235a^{pos} cells were trapped inside larger CD235a^{neg} cells, probably macrophages of murine origin. By day 18, very few (0.01–0.06%) human CD235a^{pos} cells were detectable in the blood. By day 21, human CD45^{pos} cells were undetectable in marrow, spleen, and liver of the transfused NOD/SCID/IL2R γ ^{null} mice, confirming the colony forming data, which indicated that CB EBs expanded for 10 days under HEMA conditions no longer contain hematopoietic progenitor cells. These results suggest that human EBs lodged preferentially in the spleen where they were engulfed and probably destroyed by macrophages.

To determine if splenectomy would facilitate lodgment in the marrow, the fate of *ex vivo* expanded EBs transfused to splenectomized and intact NOD/SCID/IL2R γ ^{null} mice was compared. Cell biodistribution was analyzed by retroviral-mediated labeling with the external Gaussia luciferase genes (extGLuc), using bioluminescence imaging, and e green fluorescent protein (eGFP) was used for flow cytometry determinations of CD34^{pos} CB cells which were

then expanded under HEMA conditions (see [16] and Supplemental Figure 2). The extGLuc enzyme is a newly engineered Gaussia luciferase which contains the CD8 transmembrane domain at its carboxy terminus. The presence of this domain allows membrane anchoring and cell surface retention of extGLuc, significantly enhancing the bioluminescent signal [16].

The expansion potential of retrovirally transduced and control CD34^{pos} CB cells is compared in Figure 5. By day 9, 5×10^4 unmanipulated CD34^{pos} CB cells generated 16×10^6 EBs (FI = 325-fold) while 5×10^6 CD34^{pos} CB cells which had been cultured for 3 days under conditions permissive for retroviral transduction (see Supplemental Figure 2) generated 240×10^6 EBs (FI = 48), the great majority of which (80%) expressed robust levels of eGFP (MFI > 5×10^4). The difference in FI between HEMA culture of unmanipulated and retrovirally transduced CD34^{pos} CB cells was not associated with changes in death rates, as evaluated by propidium iodide staining (data not shown) and may be related to the likelihood of underestimations of the low number of cells (5×10^4) used to initiate the cultures of unmanipulated cells. By FACS analysis, the majority of CD36^{pos} EBs derived from retrovirally transduced CD34^{pos} CB cells did not express CD235a. However, by morphological analysis the maturation stage of these cells was comparable to that of EBs derived from un-manipulated CB cells (Figure 5).

We believe that lower expression of CD235a by the retrovirally transduced EBs was a consequence of interference between the fluorescence signal of this antibody and that of eGFP.

Aliquots (25×10^6) of retrovirally transduced EBs were injected via the tail vein together with 20 U of human EPO in splenectomized and control NOD/SCID/IL2R γ^{null} mice which had been bled (500 μL) the day before. Bioluminescent imaging was performed 24 and 48 hrs after transfusion. Twenty-four hrs after transfusion, the extGLuc bioluminescent signal was virtually undetectable, other than at the site of injection in intact mice (Figure 6). In contrast, significant levels of extGLuc signal were observed in legs and skull of splenectomized mice, an indication that human EBs may have homed to the BM. In these mice, significant levels of bioluminescent signal were also detected in the abdomen (Figure 6). These data demonstrate that in the absence of the spleen CB EBs lodge in marrow and liver.

Four days after transfusion, mice were sacrificed and the presence of human CD235a^{pos} cells in bone marrow and liver of the transfused NOD/SCID/IL2R γ^{null} mice was analyzed by FACS. In the case of intact mice, great numbers (~15% of the total cell population) of human CD235a^{pos} cells were detected in spleen while human CD235^{pos} cells in marrow and liver were barely detectable. By contrast, in splenectomized animals, great numbers of human CD235a^{pos} cells were detectable in bone marrow (25%) and liver (7%) (Figure 7). Interestingly these cells did not express CD36, an indication that they had matured *in vivo*. However, red cells in the blood remained low both in intact and splenectomized mice (data not shown).

These results indicate that the removal of the spleen favors homing of human EBs to the bone marrow of NOD/SCID/IL2R γ^{null} mice.

4. Discussion

An important issue for the quality and safety of cellular products lies in the development of a surrogate assay for a cell product that is as close as possible to the final product intended for therapeutic use. Short of a phase I clinical study, the optimal evaluation of human cell products is represented by preclinical studies performed in immunodeficient mouse models. In our experience, such an approach can not only help validation studies and the establishment of a potency assay, but also help guide the development of the production process itself by defining optimal cell purification procedures, culture conditions, and scale-up systems. This study focused on establishing a xenogeneic model to test EBs expanded *ex vivo* under HEMA conditions as potential transfusion products. The availability of such a model may advance other areas of research by providing a single method to compare the functional status of EBs derived from primary hematopoietic stem/progenitor cells with those derived from other stem cells sources such as human embryonic stem cells and induced pluripotent stem cells.

This study demonstrates that the maturation of *ex vivo* generated CB- and AB-derived EBs is associated with a dynamic pattern of adhesion receptor expression. At day

10–11 of HEMA culture, the majority of immature AB- and CB-derived EBs expressed low levels of CXCR4 and high levels of both VLA-4 and P-selectin ligand 1 (Figures 2 and 3). Once induced to mature by exposure to EPO alone, these EBs rapidly (within 24 hrs) activated expression of CXCR4 while retaining expression of VLA-4 and downmodulating expression of PSGL-1. By day 4 of maturation culture, the majority of EBs had progressed to the mature CD235a^{high}CD36^{high} phenotype (Figure 1 and Table 1) and had reduced expression of all the adhesion receptors. Minor ontogenetic differences were observed since CB EBs expressed greater levels of CXCR4, PSGL-1, and VLA4 and activated CXCR4 expression upon EPO exposure more readily than AB EBs. While the adhesion receptor pattern expressed by human erythroblasts maturing *in vivo* is not known, these data are available in mice. Immature murine EBs obtained from both fetal liver and adult bone marrow express robust levels of CXCR4, PSGL-1 and $\alpha 4$ integrins. These cells lose PSGL-1 expression and retain CXCR4 and VLA-4 expression with maturation [22, 23]. These high levels of CXCR4 represent the major difference between the pattern of adhesion receptors expressed by murine cells and those expressed by *ex vivo* expanded human EBs. The low levels of CXCR4 expression in human EBs generated in HEMA and the fact that these cells rapidly, within 24 hrs, acquire CXCR4 expression upon exposure to EPO alone are consistent with reports that CXCR4 expression is suppressed by dexamethasone [24], one of the components of HEMA culture [11]. On the other hand, the low levels of CXCR4 expression observed when human EBs mature (Class 4) is expected since these cells are supposed to downregulate expression of CXCR4, the major receptor involved in cell retention in the marrow [25], as part of the process which allows them to egress from the marrow [6].

A separate set of experiments evaluated the fate of CFSE- or retrovirally-labeled CB-derived human EBs when transfused in NOD/SCID/IL2R γ^{null} mice. Bleeding and/or infusing the animals with human EPO to increase EPO levels greatly increased the detection of human EBs in the animals. By day 4 after transfusion, 1.5–5% of the cells in BM and spleen, respectively, of the animals were positive for human CD235^{pos} by flow cytometry (Figure 4(a)). However, by immunohistochemistry, high numbers (18%) of cells positive for human CD235a by immunohistochemistry were detectable in the spleen (Figure 4(b)). The human CD235a^{pos} cells, however, appeared trapped inside larger CD235a cells suggesting that they had probably undergone phagocytosis by murine macrophages. These results indicated that preferential splenic lodgment and clearance of human EBs may represent a barrier to the use of mouse models for functional evaluation of human transfusion products.

CXCR4 plays a major role in cell lodgment and retention in bone marrow [25] but is dispensable for cell homing in spleen [17, 19]. We hypothesized that failure of human CB EBs to lodge in bone marrow may be determined by unbalanced expression of CXCR4 with respect to the expression of other adhesion receptors, such as PSGL1, VLA-4, and CD36 (CD36 specifically guide cell interaction with the endothelial system [26]). Therefore, removal of

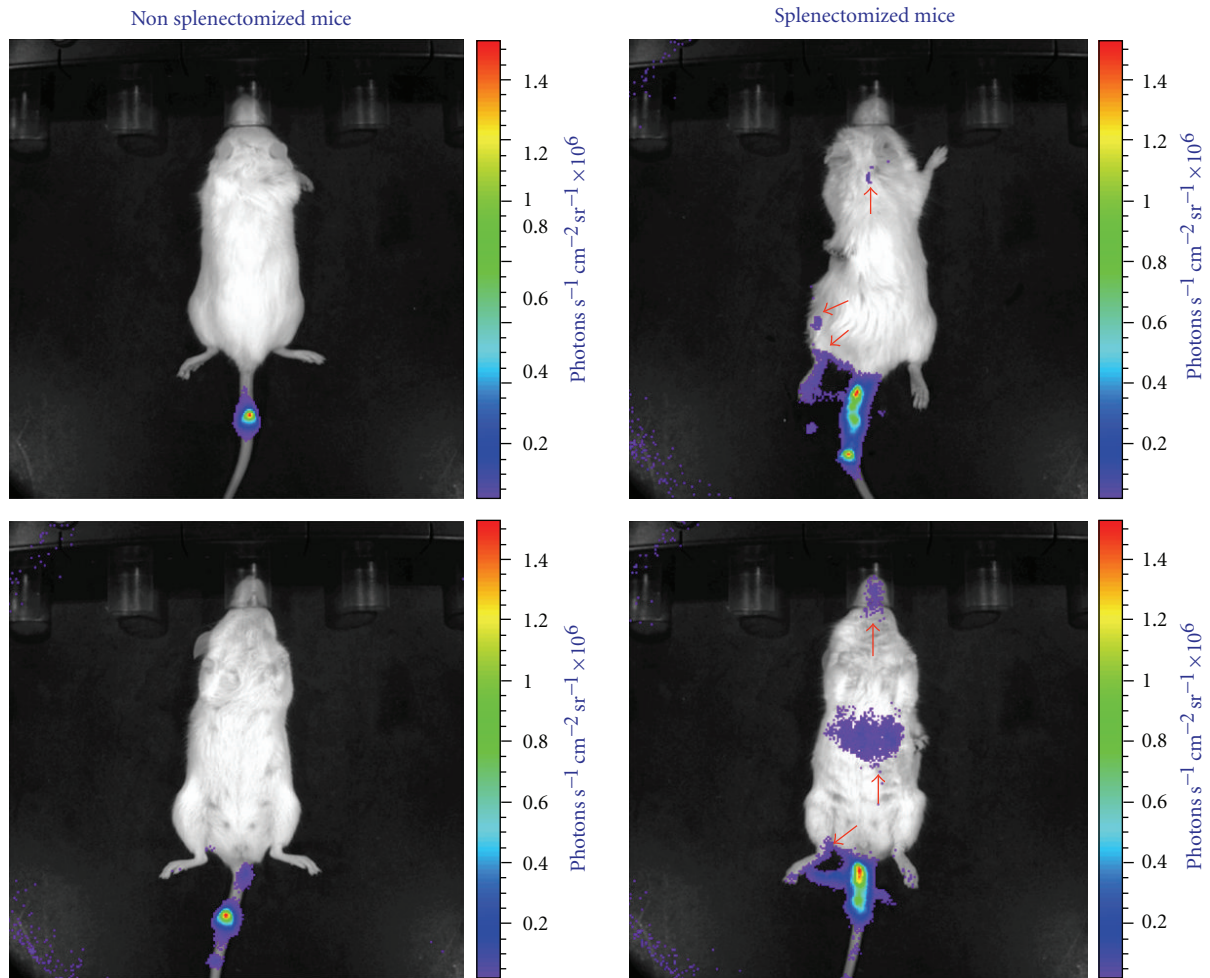


FIGURE 6: Significant level of extGLuc signal is detectable in splenectomized mice 24 hrs after transfusion. Bioluminescent imaging of nonsplenectomized (left panels) and splenectomized (right panels) mice 24 hrs after injection with extGLuc⁺EBs, as indicated. Dorsal and ventral imaging is presented in the top and bottom panels, respectively. In intact mice, the bioluminescent signal was virtually undetectable other than at the site of injection (the tail). In the splenectomized mice, significant signal levels were observed in limbs (lower extremity), skull (indicating marrow localization), and abdomen (indicating liver localization because the spleen had been removed). Arrows indicate the positive signal. Data are representative of those observed with 4 mice per experimental point.

the spleen, by reducing cell interactions mediated by these receptors, may favor lodgment in the marrow of EBs expressing low levels of CXCR4. This hypothesis was tested by comparing lodgment of human EBs in the bone marrow of intact and splenectomized NOD/SCID/IL2R γ^{null} mice. Bioimaging, followed by flow cytometry analyses, indicated that in the absence of the spleen retrovirally-labeled human EBs lodge in greater numbers in the marrow of the transfused animals (Figure 6). These results indicate that removal of the spleen greatly improves the survival of human EBs in mice. However, although signs of *in vivo* maturation were detected (detection in the marrow of cells positive for human CD235a but negative for CD36), the numbers of human erythrocyte detected in the blood of the transfused animals remained low.

Since CXCR4 expression was likely upregulation when human EBs were transfused *in vivo* (the cells were no longer exposed to DXM but exposed to human EPO), it is possible that, in addition to splenectomy, lodgment of human EBs in the marrow of the transfused animals may be improved

by experimental approaches designed to increase CXCR4 expression, such as short pretransfusion exposure to EPO *in vitro* (Figures 2 and 3). Since cycling cells are known to have inferior homing [27, 28], in addition to increasing CXCR4 expression, these pretreatments may also improve homing by reducing the number of proliferating EBs. Indirect proof that CXCR4 over-expression may facilitate homing of human EBs in the marrow was recently obtained. Although treatments with many chromatin modifying agents decreases CXCR4 expression [29] the HDAC inhibitor valproic acid (VPA) is well known for its ability to upregulate CXCR4 expression while downmodulating expression of α -integrins and other adhesion receptors in several cell types [30, 31]. Recently Chaurasia et al. [32] have demonstrated that *ex vivo* expanded EBs obtained from VPA-treated CB CD34^{POS} cells colonize, in addition to the spleen, the bone marrow when transfused into NOD/SCID and NOD/SCID/IL2R γ^{null} mice which had been functionally splenectomized by blocking the reticuloendothelium system with intraperitoneal injection

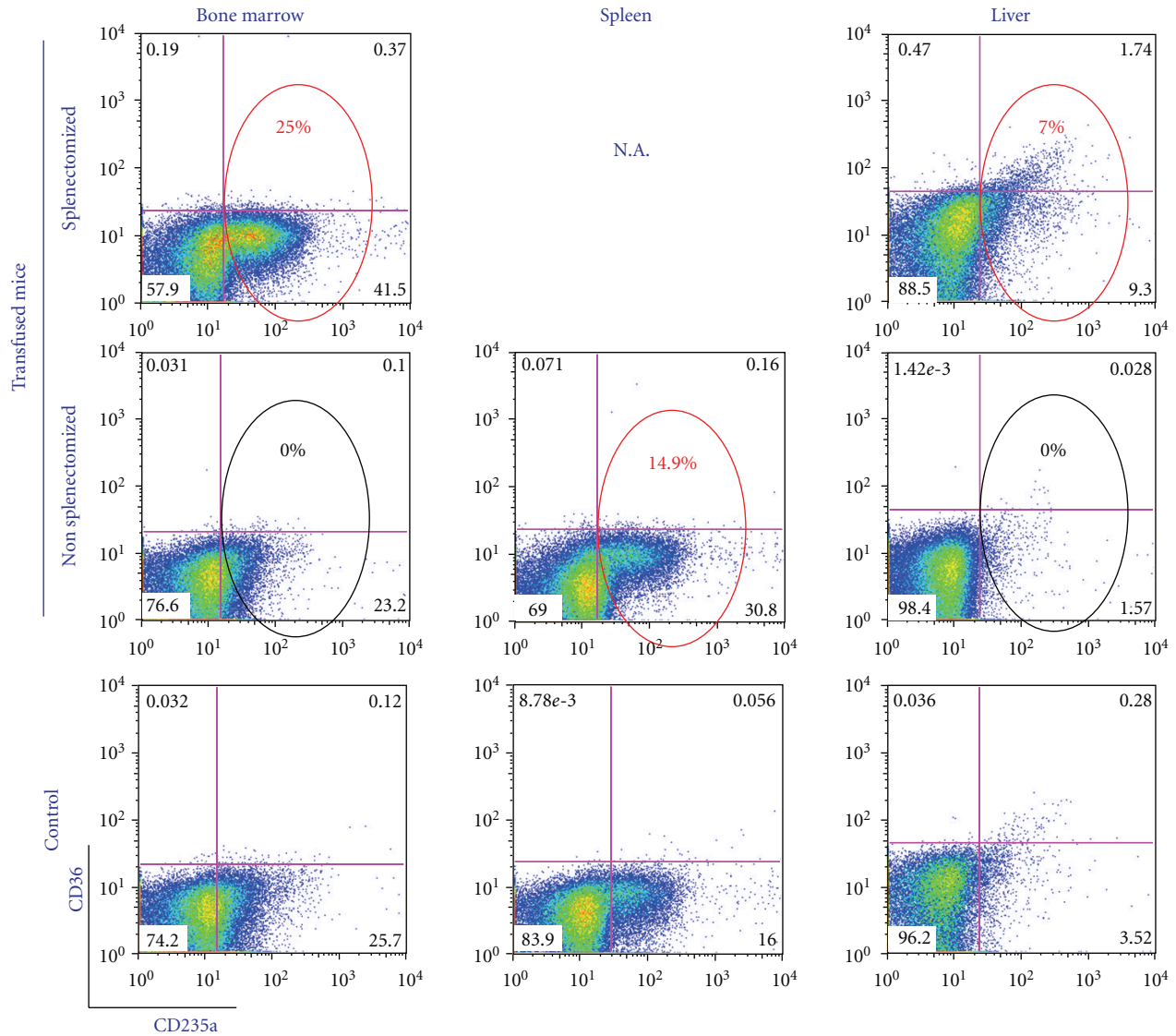


FIGURE 7: By flow cytometry of human CD36/CD235a expression, human CD235a^{pos} cells were detectable in marrow and liver of splenuctomized mice 4 days after transfusion. Flow cytometry analysis of bone marrow (left panels), spleen (middle panels) and liver (right panels) from splenuctomized (top panels), non splenuctomized (middle panels), and nontransfused controls (bottom panels) of representative mice are shown. The small numbers indicate the frequency of the cells detected within the respective quadrant. Values within circles correspond to the percentage of human cells calculated by subtracting the frequency detected in the negative controls in the corresponding bottom panels. The red circles indicate the frequency of human cells. Of note, the majority of the human CD235a positive cells were negative for CD36, an indication that the human EBs had matured *in vivo*. Similar results were obtained by analyzing the cells for eGFP expression. Data are representative of those obtained with 3 mice per experimental point.

of human type O+ red cells, as first reported by Neildez-Nguyen et al. [7]. However, since Chaurasia et al. [32] did not measure the levels of adhesion receptors expressed by VPA-treated EBs, it is not possible to assess the relative contribution of functional splenectomy and/or upregulation of CXCR4 (and/or down regulation of VLA-4 and PSGL-1 expression) to the improved lodgment of these cells in the marrow. Further studies are necessary to clarify this issue in order to establish the optimal pre-treatment (splenuctomy versus functional reticulo-endothelium blockade) of the mouse model for functional evaluation of transfusion products generated by different stem cell sources.

A major difference between this study and the report by Chaurasia et al. [32] is represented by the levels of human erythrocytes detected in the blood of the transfused animals. In our study, human erythrocytes were barely detectable in the blood at any time point after transfusion while in the study of Chaurasia et al. discrete numbers of mature erythrocytes were detectable in the circulation starting from day 7 after transfusion (15% in NOD/SCID mice and 30% in the improved NOD/SCID/IL2R γ^{null} model). However, progenitor cells were no longer detectable among the human EBs expanded under HEMA conditions in this study (CD45^{pos} cells were undetected in the transfused mice)

while representing a significant proportion (7%) of the VPA-treated cells transfused by Chaurasia et al. [32]. It is therefore possible that in this case release of human erythrocytes in the blood was favored by the presence of human macrophages generated by human hematopoietic progenitors in the animal model. Another possibility, however, is that in our experiments, the erythrocytes released in the blood were cleared by the immune system.

Immunological responses have represented a barrier to the development of mouse models for functional assessment of human red cell products [33]. These barriers include cell-mediated and humoral immune responses triggered by antigens specifically expressed on red cells. Since some of the epitopes present on red cell antigens are expressed also by proteins on the membrane of bacteria presents in gut flora [34], red blood cells may trigger immune reactions leading to red cell lyses even in the absence of any prior sensitization. NOD/SCID, and the improved NOD/SCID/IL2R γ ^{null} mice, lack cell mediated and immunoglobulin-mediated humoral responses (these mice do not have B cells). In addition, a great proportion of the immune-response against red blood cell antigens are often initiated in the spleen [35] which was transiently inactivated in [7, 32] and permanently removed in our study. The complement system plays a significant role in the humoral mediated red cell clearance *in vivo*. Since NOD/SCID/IL2R γ ^{null} mice lack C5 [21], they have reduced activation levels of the alternative complement pathway. However, these mice express C3 [21] and may activate the classical complement pathway responsible for lysis of the human CD55 and CD59 deficient red cells observed in paroxysmal nocturnal hemoglobinuria (PNH) [36], preventing the use of NOD/SCID/IL2R γ ^{null} mice for development of animal models for PNH studies. Human CB EBs expanded *ex vivo* under HEMA conditions express CD55 (53–87% positive EBs, MFI = 110–330) and CD49 (54–70%, MFI = 1,900–2500). However, the expression of these antigens greatly decreased when the cells were induced to mature with EPO for 5 days *ex vivo* (CD55: 17–27% EBs, MFI = 30–32; CD59: 28–56%, MFI = 20–76, data from 2 separate cultures) (A.R.Migliaccio and C.Whitsett, unpublished data). It is therefore possible that *ex vivo* expanded human EBs also lost CD55/CD59 expression during their maturation in the marrow of NOD/SCID/IL2R γ ^{null} mice making their progeny susceptible to C3-mediated lysis. Since C3 deficient mice have been recently developed [37], we predict that C3-deficient NOD/SCID/IL2R γ ^{null} mice will represent a better read-out animal model for the presence of human erythrocytes in the blood.

In conclusion, we describe data indicating that splenectomized NOD/SCID/IL2R γ ^{null} mice represent a surrogate *in vivo* model to assess the potency of transfusion products generated *ex vivo* from different stem cell sources.

Disclosure

All the authors have read the paper, concur with its content, and state that its content has not been submitted elsewhere. The authors have no conflict of interest to disclose.

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