



# Kinetics of *in vivo* homing and recruitment into cycle of hematopoietic cells are organ-specific but CD44-independent

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## Summary:

In this study, we investigated the homing and initiation of division of fluorescently labelled adult mouse bone marrow cells after their intravenous injection into lethally irradiated congenic mice. After 2 h, only 3% of the transplanted cells remained in the blood, and ~35% could be retrieved from the marrow, liver and spleen in approximately equal numbers. Subsequently, the proportion of injected cells found in blood, liver and spleen decreased further, but increased slightly (to ~17%) in the marrow. Homing of progenitors followed a similar pattern. At 22 h post transplant, almost half of the injected cells in the blood, liver and spleen had completed a first mitosis; although these did not include progenitors with *in vitro* clonogenic ability. At the same time, >90% of the injected cells recovered from the marrow had not yet divided. Parallel studies with CD44<sup>-/-</sup> mice showed these to contain a numerically and functionally normal stem cell population whose homing and activation in either CD44<sup>+/+</sup> or CD44<sup>-/-</sup> hosts appeared unaltered. These results indicate homing mechanisms that favor more stable retention of transplanted marrow cells in the marrow of the recipient, more rapid activation of some of those cells that home to other sites, and a lack of change in either of these responses when either the transplanted or the recipient cells do not express CD44. *Bone Marrow Transplantation* (2000) 26, 559–566.

**Keywords:** hematopoiesis; homing; CD44; CFSE

Hematopoiesis in the adult takes place primarily within the extravascular spaces of the bone marrow, where the developing hematopoietic cells are in close contact with different types of stromal cells (fibroblastoid cells, adipocytes, smooth muscle-like cells and endothelial cells) and macrophages. Stromal cells are known to secrete a number of extracellular matrix molecules into their immediate environment including several types of collagen, fibronectin, laminin, thrombospondin, hemonectin, tenascin, hyaluronic acid, as well as chondroitin- and heparan sulfate-modified proteins.<sup>1,2</sup> Adjacent hematopoietic cells can then

interact with many of these. Stromal cells also express a variety of adhesion molecules on their surfaces which allow them to interact directly with hematopoietic cells.<sup>2</sup> *In vitro* studies suggest that the strength of the interactions of hematopoietic cells with extracellular matrix proteins depends on transient activation signals stimulated by concomitant interactions of the hematopoietic cells with cytokines like GM-CSF and Steel factor (SF)<sup>3</sup> or certain costimulatory adhesion molecules like the  $\beta$ 1-integrins, CD44 and ICAM-3.<sup>4–6</sup> Such activation signals peak between 15 min and 2 h after ligand binding and then decline, thereby providing a dynamic process of adhesion and detachment which facilitates the ability of hematopoietic cells to move through the type of stromal cell network that extends throughout the bone marrow cavity.

Hematopoietic cells also exit from the bone marrow cavity into the circulation by migrating through the vascular endothelial cell layer. From the circulation, they may enter other tissues, like the spleen, thymus, liver, or distant bone marrow cavities, although the molecular mechanisms involved in this trafficking process are poorly understood. Early studies showed that intravenously injected bone marrow cells disappear rapidly from the circulation of irradiated recipients and very soon after injection begin to accumulate in the marrow and spleen.<sup>7–9</sup> The initial lodgment of at least some types of early hematopoietic progenitors in the spleen is, however, transient, as the percentage of injected colony-forming cells (CFC) recovered from the spleen has been found to decrease substantially between 4 and 24 h after their intravenous injection. On the other hand, in the bone marrow, the number of injected CFC increases between 2.5 and 4 h after injection and then remains relatively constant for the next 24 h.<sup>10</sup>

Recent evidence indicates that VLA-4, its ligands VCAM-1 and the CS1-motif in fibronectin, as well as the P- and E-selectins are involved both in the homing as well as the mobilization of hematopoietic progenitors.<sup>11–13</sup> In addition, a strong case has been made for involvement of the SF receptor, c-kit.<sup>14,15</sup> It has also been shown that antibodies to the cell surface glycoprotein CD44<sup>16</sup> could affect the homing of hematopoietic progenitors in both mice<sup>17</sup> and rats.<sup>18</sup> These latter studies did not, however, exclude a role of the CD44 antibodies used in influencing the recovery process by other mechanisms nor did they establish a unique role of CD44 in the inhibition seen. In addition, non-specific antibody effects on the opsonization of target cells *in vivo* are well known.<sup>19</sup> On the other hand, we and others have demonstrated that antibodies against CD44

can affect the growth, differentiation and adhesion of normal and leukemic hematopoietic progenitors *in vitro* in different ways, depending on the epitope specificity of the anti-CD44 antibodies tested.<sup>6,16,20–22</sup> Thus, it was of interest to further examine the role of CD44 in the homing and engraftment of normal bone marrow cells in a syngeneic setting using other strategies. To address this question we compared various parameters of *in vitro* and *in vivo* hematopoietic stem cell activity and transplant engraftment in normal mice and mice deficient in CD44 expression (CD44<sup>-/-</sup> mice). CD44<sup>-/-</sup> mice are healthy and fertile and have been found to be hematologically normal with normal WBC counts, normal marrow progenitors and normal T and B cell numbers and functions,<sup>23</sup> but do show a decreased IL-2 activation of lymphokine-activated killer cells.<sup>24</sup> However, the number and function of more primitive hematopoietic cells types in adult CD44<sup>-/-</sup> mice and the ability of these mice to serve as transplant recipients has not been previously investigated. The results of the present studies demonstrate interesting differences both in the kinetics of engraftment of different hematopoietic organs and in the rate of recruitment into division of the injected cells found in different organs. However, the absence of CD44 (on either the donor and/or the recipient) was not found to affect the number or *in vivo* homing of any type myeloid progenitor or stem cell.

## Materials and methods

### Animals

C3H/HeJ (Ly-5.2), C57BL/6J (B6) (Ly-5.2) and B6:Pep3b (Pep3b, Ly-5.1) mice were bred and maintained in the animal facility of the British Columbia Cancer Research Center (BCCRC, Vancouver, BC). CD44<sup>-/-</sup> mice 23 originally obtained from Dr TW Mak (Amgen Institute, Ontario Cancer Institute, Toronto, ON, Canada) were further backcrossed in Vancouver on to a B6 background for at least 15 more generations selecting each time for absence of CD44 expression from F<sub>2</sub> progeny using the anti-CD44 IM7 antibody conjugated to fluorescein-isothiocyanate (FITC) from Pharmingen (San Diego, CA, USA). These CD44<sup>-/-</sup> mice were then further crossed with Pep3b mice and CD44<sup>-/-</sup> Ly-5.1 F<sub>2</sub> progeny selected on the basis of an absence of cellular staining for both CD44 (IM7) and Ly5.2 (AL14A2 antibody, also from Pharmingen), and positive staining for Ly5.1 (A20–1.7 antibody, also from Pharmingen). Details of these staining procedures are described below. All animals were kept under micro-isolators and provided with sterilized food and acidified water (pH 3) *ad libitum*.

### Cell suspensions

Single cell suspensions were prepared individually from the marrow contents of the two tibiae and two femurs of each mouse analyzed (unless otherwise indicated) in Hanks' balanced salt solution (HBSS) containing 2% fetal bovine serum (HF/2, StemCell Technologies, Vancouver, BC, Canada). Single cell suspensions were prepared from the

entire spleen, thymus, and a defined portion of the liver by gently pressing organ fragments through a 70  $\mu$ m mesh filter (Falcon, Franklin Lakes, NJ, USA) into HF/2. Red blood cells in a measured volume of peripheral blood, and in aliquots of the spleen and marrow cell suspensions were lysed by resuspension of the pelleted cells in ice-cold ammonium chloride solution (StemCell) and incubation for 10 min on ice. The nucleated cells were then washed once in PBS and twice in HF/2, and viable cell numbers determined using a hemocytometer to count trypan blue-excluding cells (Sigma Chemicals, St Louis, MO, USA). To facilitate final comparisons of the number of cells present in different organs, these values were calculated directly for the spleen and thymus and were extrapolated from the blood, marrow and liver samples assessed. For the latter, it was assumed that the total blood volume of each mouse was 3 ml and that two femurs plus two tibiae constituted 25% of the total marrow.<sup>25</sup> The portion of liver assessed (one or two lobes) was determined by comparing its wet weight to the wet weight of the total liver. It was then assumed that the cellularity of the liver was proportional to its wet weight.

### CFC and long-term culture initiating cell (LTC-IC) assays

CFC numbers were determined by plating suitable aliquots of test cells in methylcellulose medium (Methocult; StemCell) supplemented with 10 ng/ml murine IL-3 (expressed and purified from the murine IL-3 cDNA in the Terry Fox Laboratory), 10 ng/ml human IL-6 (Cangene, Mississauga, Ontario, Canada), 50 ng/ml murine SF (Terry Fox Laboratory), and 3 U/ml human erythropoietin (StemCell). After 12–14 days of incubation at 37°C,<sup>26</sup> erythroid, granulopoietic and multi-lineage colonies, containing  $\leq 30$  cells were scored. For the determination of the LTC-IC content of cell suspensions, a limiting dilution assay was performed as described,<sup>27</sup> using as feeders, previously established LTC adherent layers set up with marrow cells from (C3H  $\times$  B6)F<sub>1</sub> mice and irradiated with 20 Gy when close to confluence ( $\sim 2$  weeks later). LTC-IC frequencies were calculated by the method of maximum likelihood from the proportion of negative wells (wells containing no CFC) measured for each input dilution of cells tested using the L-Calc software package (StemCell).

### Assay for day 12 colony-forming units-spleen (CFU-S<sub>12</sub>)

The method was performed essentially as originally described.<sup>28</sup> In brief, mice were irradiated with 900 cGy of <sup>137</sup>Cs  $\gamma$ -rays at a dose rate of 95 cGy/min and then injected intravenously with  $5 \times 10^4$  cells (200  $\mu$ l) via the tail vein. Twelve days later, the mice were sacrificed, their spleens placed in Tellesniczky's fixative (90 ml of 70% ethanol, 5 ml glacial acetic acid, and 5 ml of 37% formaldehyde) and the number of macroscopic spleen colonies counted at  $\times 2$  magnification.

### Assay for competitive repopulating units (CRU)

This assay has also been described in detail previously.<sup>29,30</sup> In brief, mice of the Ly-5.2 genotype were irradiated with

900 cGy and then injected intravenously with  $10^4$  to  $5 \times 10^4$  bone marrow cells from Ly-5.1<sup>+</sup> donors as well as  $10^5$  normal marrow cells from mice of the same Ly-5.2 genotype as the recipient. Peripheral blood was obtained from the tail vein of the recipients at intervals from 6 weeks to 24 weeks later and assessed for the presence of donor-derived (Ly-5.1<sup>+</sup>) lymphoid and myeloid cells after lysis of the red blood cells with ammonium chloride and staining the nucleated cells for 30 min on ice with either biotinylated anti-GR-1 (clone RB6-8C5), anti-Mac-1 (clone M1/70) and anti-Ly-1 (clone 53-7.3) or anti-B220 (clone RA3-6B2). This was followed by a single wash in HF and a further 30 min incubation with FITC-anti-Ly5.1 (clone A20-1.7) and phycoerythrin (PE)-conjugated streptavidin. The cells were then washed twice in HF with 1  $\mu$ g/ml propidium iodide (PI; Sigma) included in the final wash, and analyzed on a FACSort (Becton Dickinson, San Jose, CA, USA). Animals were considered to be reconstituted (positive) only if their blood contained >1% donor derived (Ly-5.1<sup>+</sup>) myeloid (GR-1<sup>+</sup>/Mac-1<sup>+</sup>) and lymphoid cells (Ly-1<sup>+</sup>/B220<sup>+</sup>) cells. CRU frequencies were derived by the method of maximum likelihood (using L-calc) from the proportions of mice that were negative when these were assessed 12 to 24 weeks post transplant.

#### *Measurement of homing and cell division tracking in vivo*

Single cell suspensions of bone marrow cells to be injected were washed once in HBSS and resuspended in 5 ml HBSS ( $5 \times 10^6$ – $10^7$  cells/ml). To this suspension, 2  $\mu$ l of a 50 mM stock solution of 5- (and 6-) carboxymethylfluorescein diacetate succinimidyl ester (CFSE, C-1157; Molecular Probes, Eugene, OR, USA) in DMSO was added to give a final concentration of 20  $\mu$ M CFSE). The cells were then incubated at 37°C for 10 min. Further uptake of the CFSE was terminated by addition of 5 ml of cold FCS. The suspension was washed twice in cold HF/2 and finally resuspended in HF/2 at a concentration of  $5 \times 10^7$  cells/ml. Recipient mice were irradiated with 900 cGy and injected intravenously with  $\sim 10^7$  ( $7 \times 10^6$  to  $1.5 \times 10^7$ , depending on the experiment) CFSE-labelled cells. Blood, marrow, spleen, liver and thymus cell suspensions obtained from the mice 2 to 22 h later were washed twice in cold HF/2 with 1  $\mu$ g/ml PI in the final wash and analyzed for the presence of CFSE<sup>+</sup> cells on a FACSort equipped with an argon ion laser (Becton Dickinson) set to 488 nm. The photomultiplier for the CFSE detection channel (FL1) was adjusted so that the CFSE-labelled cells cultured in the presence of colcemid peaked at around  $\sim 2 \times 10^3$  on a four decade logarithmic scale. Between  $5 \times 10^4$  and  $2 \times 10^5$  ungated events were collected to obtain a measure of the proportion of CFSE<sup>+</sup> cells. This value was then used to calculate the total number of CFSE<sup>+</sup> cells in the organ from which the sample was prepared by multiplying this value times the total number of viable cells in the organ determined as described above under Cell suspensions. In some experiments, an aliquot of  $10^6$  cells was removed and cultured for the same period in long-term culture (LTC) medium (HCC-5300; StemCell) at 37°C in 95% humidity and 5% CO<sub>2</sub> in the presence of 0.1  $\mu$ g/ml colcemid (Gibco/Life Technologies, Paisley, UK) to prevent cell divisions. These cells were

then analyzed at the same time as the cells obtained from the mice.

## Results

### *Characterization of adult hematopoiesis in CD44<sup>-/-</sup> mice*

Initial characterization of the CD44<sup>-/-</sup> mice that had been fully backcrossed on to a B6 background showed the number of nucleated cells in their bone marrow, spleen and peripheral blood to be the same as in congenic +/+ (B6) littermates (Table 1). Differentials performed on blood and marrow showed these also to be similar (data not shown). The number of CFC and LTC-IC in the marrow, spleen and circulation of CD44<sup>-/-</sup> and +/+ mice were also the same (Table 1), as were the marrow CFU-S<sub>12</sub> and CRU populations (assayed in either CD44<sup>-/-</sup> or +/+ recipients) (Table 2). These results extend previous similar findings for CFC and CFU-S from CD44<sup>-/-</sup> mice,<sup>23</sup> although we failed to confirm any alteration in the size of the splenic CFC population.

### *CD44<sup>-/-</sup> mice are as sensitive to radiation as +/+ mice*

CD44 has been described to play a role in cell survival after apoptotic stimuli<sup>31</sup> and the survival of irradiated mice is dependent on the number of stem cells with rapid reconstituting ability that do not succumb to the apoptosis-inducing effects of the irradiation. However, as can be seen in Figure 1, the survival of CD44<sup>-/-</sup> mice to 850 cGy (a sublethal dose for +/+ mice) and 900 cGy (a lethal dose for +/+ mice) was similar for the two genotypes. Subsequent experiments in which irradiated CD44<sup>-/-</sup> and +/+ mice were used as recipients of CD44<sup>-/-</sup> and +/+ marrow cells for short-term homing measurements also showed no difference in the cytoreductive effects of 900 cGy on the major hematopoietic organs (see below and Figure 2a).

### *Kinetics of in vivo homing of bone marrow cells from CD44<sup>-/-</sup> and +/+ mice*

To look for possible effects of a lack of CD44 on the early homing kinetics of bone marrow cells, various tissues were analyzed from mice sacrificed 2, 4 and 22 h after being injected with CFSE-labelled marrow cells as described in Materials and methods. These experiments included an analysis of all four possible combinations of CD44<sup>-/-</sup> and +/+ donors and recipients. About an hour prior to injection, the mice were lethally irradiated (900 cGy). At each time point post transplant, the total number of cells in each organ was determined and the percentage of CFSE<sup>+</sup> cells present measured by flow cytometry. The results of the cell counts, shown in Figure 2a, confirm that a lack of CD44 expression either on the injected cells or on those of the recipients, or both, does not affect the cytoreductive effects of a lethal dose of radiation. The results of the calculated homing data, shown in Figure 2b indicate unique patterns of organ homing but these were similar in all four types of donor-recipient pairs. On average, the proportion of cells injected that could be recovered from the blood, thymus, marrow, spleen

**Table 1** Number of hematopoietic progenitors in +/+ and CD44<sup>-/-</sup> mice are similar

Cells assayed	CD44 genotype	No. per femur	No. per spleen	No. per ml blood
Nucleated cells	+/+	1.5 ± 0.2 × 10 <sup>7</sup>	1.0 ± 0.2 × 10 <sup>8</sup>	3.1 ± 0.8 × 10 <sup>6</sup>
	-/-	1.9 ± 0.3 × 10 <sup>7</sup>	1.2 ± 0.1 × 10 <sup>8</sup>	3.8 ± 0.9 × 10 <sup>6</sup>
CFC	+/+	1.1 ± 0.2 × 10 <sup>5</sup>	2.0 ± 0.7 × 10 <sup>4</sup>	1.6 ± 0.7 × 10 <sup>2</sup>
	-/-	1.2 ± 0.2 × 10 <sup>5</sup>	3.0 ± 1.0 × 10 <sup>4</sup>	1.7 ± 0.5 × 10 <sup>2</sup>
LTC-IC	+/+	7.4 × 10 <sup>2</sup>	4.9 × 10 <sup>3</sup>	ND
	-/-	9.1 × 10 <sup>2</sup>	8.4 × 10 <sup>3</sup>	ND

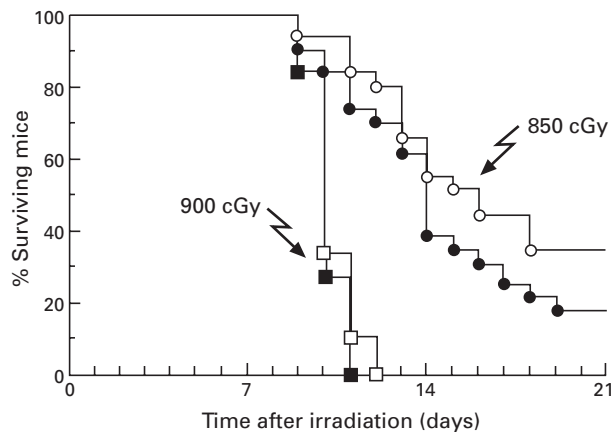
Values shown are the mean ± s.e.m. for determinations made in parallel on up to six (10–15 week old) mice. ND = not done.

**Table 2** Frequencies of CFU-S<sub>12</sub> and CRU in +/+ and CD44<sup>-/-</sup> marrow are similar and are not affected by assessment in CD44<sup>-/-</sup> recipients

CD44 genotype		CFU-S <sub>12</sub> (No. per 5 × 10 <sup>4</sup> cells) <sup>a</sup>	CRU (No. per 10 <sup>5</sup> cells) <sup>b</sup>
Donor	Recipient		
+/+	+/+	10 ± 1 (10)	4.0 (3.4–5.0, n = 39)
-/-	+/+	9 ± 1 (10)	3.9 (3.1–4.9, n = 20)
+/+	-/-	8 ± 1 (7)	4.5 (3.3–6.0, n = 23)
-/-	-/-	11 ± 2 (9)	4.2 (3.1–5.6, n = 22)

<sup>a</sup>Values are the mean ± s.e.m. (number of spleens counted) of results from two independent experiments.

<sup>b</sup>Values are shown with the range defined by ± s.e.m. and the number of mice used to make the determination in brackets (four independent experiments). The latter were assessed 12 to 24 weeks post transplant as described in Materials and methods.

**Figure 1** Survival curves of CD44<sup>-/-</sup> (solid symbols) mice and +/+ mice (B6, open symbols) to 850 (circles) and 900 cGy (squares) whole body irradiation.

and liver declined from approximately 40% to 30% over the first 22 h. A marked decline was seen during this period in the number of CFSE<sup>+</sup> cells detected in the blood, spleen, and liver, whereas the proportion of injected donor cells to be found in the bone marrow actually increased slightly from 12% at 2 h post transplant to 17% after 22 h. Although the numbers of injected marrow cells that could be recovered from the thymus were much lower (maximum of 0.2% of the original cells injected detectable 22 h later),

these cells, like those in the marrow appeared to increase during the first day.

#### Kinetics of in vivo homing of CFC from CD44<sup>-/-</sup> and +/+ mice

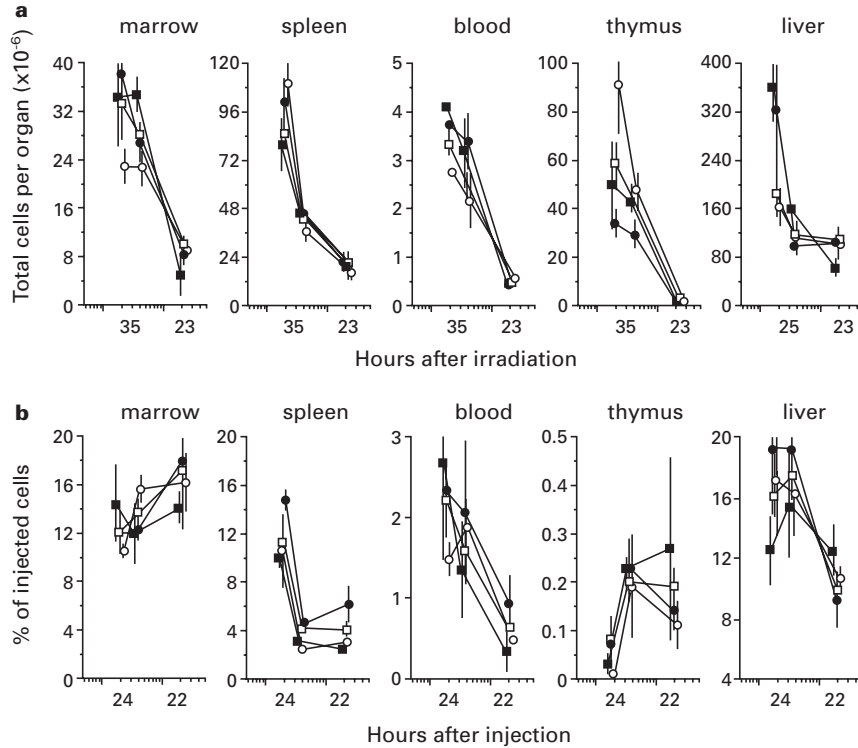
To determine the distribution of more primitive cells in different tissues 22 h after the injection of marrow cells into irradiated mice, we isolated viable CFSE<sup>+</sup> cells from the bone marrow and the spleen using the FACS and then measured their CFC content. As shown in Table 3, approximately 16% of the injected CFC could be recovered 1 day later from the marrow and approximately 4% from the spleen, regardless of the CD44 genotype of the donor or recipient. These findings are similar to those obtained for the total marrow cell population injected.

#### Lack of involvement of CD44 in the homing of CFU-S<sub>12</sub> or CRU

The detection of CFU-S<sub>12</sub> and long-term repopulating cells depends on the ability of these cells to home to the spleen and marrow, respectively. Therefore the finding that both of these populations are present in CD44<sup>-/-</sup> mice in normal frequencies when assayed in +/+ recipients would suggest that a lack of CD44 expression on these primitive hematopoietic cells does not affect their homing integrity. To determine whether CD44 expression on the marrow and spleen cells of the host might influence their function, CD44<sup>-/-</sup> and +/+ CFU-S<sub>12</sub> and CRU were also assayed in irradiated CD44<sup>-/-</sup> recipients. As can be seen in Table 2, CFU-S<sub>12</sub> and CRU frequencies in CD44<sup>-/-</sup> mice were the same as in +/+ mice and none of these values changed when the assays were performed in CD44<sup>-/-</sup> recipients. Since the cellularity of the marrow of CD44<sup>-/-</sup> and +/+ mice is also the same, this means that the total number of stem cells generated in the CD44<sup>-/-</sup> mice is also not affected by their lack of CD44 expression or their production in an environment lacking CD44. In addition, the average number of blood cells produced per CD44<sup>-/-</sup> CRU in +/+ or CD44<sup>-/-</sup> mice was the same as that measured for +/+ CRU (data not shown).

#### Different proliferative activity of transplanted marrow cells found in different sites 1 day later

Since the fluorescence of CFSE-labelled cells exactly halves at each mitosis, CFSE allows the proliferative his-



**Figure 2** Time course analysis of total and donor-derived cells in different organs of lethally (900 cGy) irradiated mice. Panel **a** shows results for total cells in +/+ (B6, open symbols) or CD44<sup>-/-</sup> (solid symbols) mice injected with  $0.7\text{--}1.5 \times 10^7$  CFSE-labelled marrow cells from +/+ (squares) or CD44<sup>-/-</sup> donors (circles). Panel **b** shows the total number of CFSE<sup>+</sup> cells in each organ, expressed as a percentage of the CFSE<sup>+</sup> cells injected. Values are the mean  $\pm$  s.e.m. for results from four to 10 individually analyzed mice from two to six independent experiments. A more detailed description of how these values were calculated is given in the Materials and methods. Note that the follow-up times are shown on a log scale.

**Table 3** Proportion of CFC found in the marrow and spleen 1 day post transplant is unaffected by the CD44 genotype of the donor or host

CD44 genotype		% of injected marrow CFC found in different organs 22 h later	
Donor	Recipient	Marrow	Spleen
+/+	+/+	16 $\pm$ 1 (9)	3.5 $\pm$ 0.4 (8)
-/-	+/+	16 $\pm$ 2 (6)	6.1 $\pm$ 1.5 (6)
+/+	-/-	14 $\pm$ 1 (4)	2.4 $\pm$ 1.3 (4)
-/-	-/-	17 $\pm$ 6 (6)	3.0 $\pm$ 0.4 (4)

Values shown are the mean  $\pm$  s.e.m. derived from measurements of the number of mice shown in brackets in three independent experiments. Values for marrow are based on an assessment of two femurs and two tibias multiplied by 4 on the assumption that two femurs plus two tibias comprise 25% of the total marrow of a mouse.<sup>25</sup> Same experiments as those shown in Figure 3.

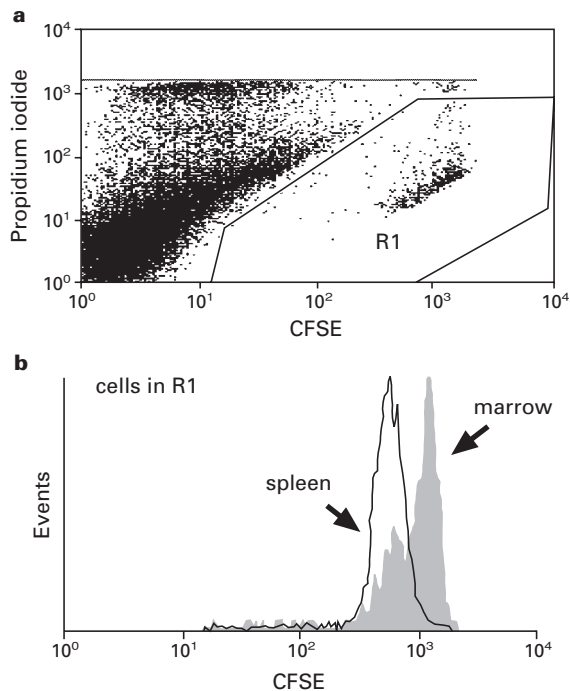
tory of cells dividing asynchronously *in vitro* and *in vivo* to be analyzed.<sup>32,33</sup> The use of CFSE labelling in the present study thus allowed the rates of recruitment into division of cells homing to different organs to be assessed and compared. The results of such analyses are shown in Table 4. It can be seen that during the first 2 h post transplant, <20% of the injected cells had divided, regardless of the site in which they were found. This situation persisted in the marrow for another day and a similar situation pertained in the thymus. However, on average, approximately 40%

**Table 4** Different cell cycle kinetics of transplanted marrow cells homing to the marrow by comparison to other hematopoietic organs

Organ assessed	% of undivided injected cells (in each organ)		
	2 h	4 h	22 h
Blood	81 $\pm$ 5	89 $\pm$ 7	58 $\pm$ 6
Spleen	81 $\pm$ 4	83 $\pm$ 4	57 $\pm$ 10
Liver	86 $\pm$ 2	90 $\pm$ 3	58 $\pm$ 12
Marrow	96 $\pm$ 2	96 $\pm$ 2	88 $\pm$ 4
Thymus	ND	84 $\pm$ 9	70 $\pm$ 9

For each organ, the number of undivided CFSE<sup>+</sup> cells recovered was expressed as a % of the total CFSE<sup>+</sup> cells recovered from the same mouse. Values shown are the mean  $\pm$  s.e.m. of data from six to 10 mice sacrificed at the times indicated. Same experiments as described in Figure 3 and Table 3. Only results for +/+ cells injected into +/+ recipients are shown.

of the CFSE<sup>+</sup> cells present in blood, spleen and liver 1 day post transplant had already completed a first division. Figure 3 shows an extreme example for the spleen and the marrow of one such mouse. Interestingly, in two experiments, separate isolation of the undivided cells from the spleen showed these to contain most of the CFSE<sup>+</sup> CFC present suggesting that the first cells to divide there may be later cell types. Note that the detection of cells that have already divided by day 1 post transplant means that the values shown in Figure 2b would slightly overestimate the proportions of injected cells present in the blood, spleen



**Figure 3** Different post-transplant proliferative histories of injected marrow cells found in the marrow and spleen of congenic recipients 22 h later. Panel **a** shows the gating used (R1) to distinguish viable (PI<sup>-</sup>) CFSE<sup>+</sup> (donor)  $+/+$  marrow cells from other marrow cells in a  $+/+$  recipient. Panel **b** compares the fluorescence histograms of gated populations for the spleen and marrow cells of the same mouse shown in panel **a**. In this example, 82% of the injected (CFSE<sup>+</sup>) cells in the spleen had divided once (decreased their fluorescence by two-fold) whereas in the marrow, >11% of the CFSE<sup>+</sup> cells had divided at the same time point.

and liver at that time. However, this distortion would represent a very small overall change, due to the relatively small numbers of injected cells persisting in these sites 1 day after injection.

## Discussion

In the present study, we demonstrate the utility of CFSE labelling to measure both the distribution and activation kinetics of intravenously transplanted syngeneic marrow cells injected into lethally irradiated recipients. Our results confirm and extend the distribution findings obtained by others using both functional assays of retrieved cells and direct analysis of PKH26-labelled cells after injection of either unfractionated or stem cell-enriched populations, all of which have indicated different homing patterns of syngeneic marrow cells to different organs.<sup>7,9,10,34–36</sup> This includes the observation that the proportion of injected marrow cells retained within the spleen and liver declines two- to three-fold during the first day post transplant, in contrast to the marrow and thymus where a stable and in the case of the marrow, an ultimately higher level of engraftment is achieved much faster (70% of maximum within 2 h post transplant). In addition, we show that, as is seen in the spleen and liver, there is a marked decline of the injected marrow cells in the blood during the first day post transplant, although the precise kinetics of the early decline seen

in each of these sites appear to be different. Because the interval between irradiation and injection of the CFSE-labelled cells was kept the same in the present experiments, it was also possible to demonstrate that the organ-specific homing kinetics noted are not explained by differences in the rate at which endogenous cells in the corresponding organs die and disappear since the latter decreased at a similar and constant rate throughout the first day. Independence of homing from organ cellularity changes was also suggested by the experiments of Hendriks *et al*<sup>34</sup> who showed less than three-fold differences in the homing efficiency of a murine stem cell-enriched (low density WGA<sup>+</sup>) marrow population in lethally irradiated by comparison to unirradiated recipients.

Comparison of the homing efficiency of the total marrow population and the CFC contained within that population 1 day post transplant showed no differences between these two in our experiments (17% vs 16% in the marrow and 4% vs 4% in the spleen). Although Szilvassy *et al*<sup>10</sup> reported slightly higher homing efficiencies of injected CFC than of total cells in a similar analysis of unfractionated marrow transplants, their day 1 CFC homing values (9% to the marrow and 6% to the spleen) are remarkably similar to the homing values that we obtained and that were reported by others.<sup>8,36</sup> Similar results were also obtained by Hendriks *et al*,<sup>34</sup> who injected a highly CFU-S<sub>12</sub>-enriched marrow subpopulation (10% of which homed to the marrow and 5% to the spleen, when the same formula for calculating the total size of the marrow is used). Although these data do not necessarily predict the homing efficiency of long-term repopulating cells from adult marrow, marrow homing values for these cells of 20 to 30% can be extrapolated from the highest reported purities of these cells.<sup>37,38</sup> Interestingly, these numbers agree quite well with the number of all injected marrow cells that we have calculated as being in the marrow and spleen 1 day post transplant.

Our findings also reveal differences in the rate at which some of the cells that home to different organs are stimulated to divide. In most sites, approximately 40% of the cells present could be shown to have completed a first mitosis by the end of the first day post transplant. However, in the marrow, engrafted cells were just beginning to proliferate at this time. These results could reflect a unique difference in the mitogenic environment of the marrow or, alternatively, a difference in the cell populations that home to these two organs in terms of the time they require to be activated. Differences in the stem cell activities of cells that home to the spleen and marrow have also been noted by others. In one case, superior stem cell activity (on a per cell basis) was obtained with cells that had homed to the spleen (as compared to the marrow) 3 h after being injected.<sup>10</sup> In another, the reverse was found to be true but the cells analyzed were recovered 2 days later.<sup>35</sup> Interestingly, in this study, the more primitive cells that preferentially homed to the marrow also still showed a low cycling activity at that time.

The present study confirms that hematopoiesis in CD44<sup>-/-</sup> mice is normal, and that the ability of CD44<sup>-/-</sup> bone marrow cells to initiate long-term marrow cultures, form spleen colonies *in vivo*, and repopulate lethally irradiated mice long term is not detectably compromised.

In addition, it was demonstrated that the homing behavior of CD44<sup>-/-</sup> bone marrow cells and CFC is identical to that of CD44-expressing cells. Although we did not find a reduced number of CFC in the spleens of CD44<sup>-/-</sup> mice as reported by Schmits *et al.*<sup>23</sup> this disparity may have been the result of differences in the methodologies used to detect CFC in the two studies. CD44 is one of the most thoroughly investigated surface adhesion receptors of the last decade. Evidence of a role of CD44 in the normal development of limbs and the mandibular arch,<sup>39,40</sup> in the extravasation of activated T cells,<sup>41</sup> and in the homing of hematopoietic progenitors<sup>17,18</sup> has been reported. Nevertheless, CD44<sup>-/-</sup> mice develop normally, demonstrate normal *in vitro* and *in vivo* T cell responses,<sup>23</sup> and, as further shown here, exhibit no alteration of hematopoietic cell development or post-transplant homing in myeloablated recipients. This suggests that the role of CD44 in the latter processes implicated from antibody studies may be functionally redundant, at least under conditions where CD44 expression is prevented. CD44 is thought to mediate much of its activity by binding hyaluronan (HA) but its role in this regard does not appear to be unique since CD44<sup>-/-</sup> cells can still be mitogenically activated by HA.<sup>24</sup> This is likely due to the activity of one or more of the rapidly growing number of other molecules with HA binding activity. These include several extracellular matrix molecules (aggrecan, brevican, link protein, neurocan, and versican), TSG-6 (TNF-stimulated gene-6),<sup>42</sup> RHAMM (receptor for HA-mediated motility),<sup>43</sup> and LYVE-1 (lymphatic vessel HA receptor 1).<sup>44</sup> However, only CD44 and RHAMM are known to be co-expressed by hematopoietic cells. Interestingly, RHAMM, like CD44, exists in several different isoforms, and has been implicated in HA-mediated motility,<sup>43</sup> as well as in the trafficking of hematopoietic progenitors.<sup>45</sup> Both normal and leukemic B cells can utilize either CD44 or RHAMM to adhere to HA,<sup>46</sup> suggesting that RHAMM and CD44 could have overlapping functions on hematopoietic cells. It will therefore be of interest to investigate the hematologic phenotype of RHAMM<sup>-/-</sup> mice and mice deficient for RHAMM as well as CD44.

In summary, we have shown how the increased resolution that is possible with CFSE-labelling can provide simultaneous homing and cell division history data to reveal organ-specific differences in both of these parameters and their lack of dependence on CD44 expression, either on the injected cells or the cells of the host. These findings do not negate previous evidence of specific anti-CD44 antibody effects on hematopoietic cell homing,<sup>18,21</sup> stroma-dependent hematopoiesis *in vitro*<sup>47</sup> or leukemic hematopoiesis.<sup>20,22</sup> However, they do suggest that the latter are mediated either by antibody-stimulated activation of intracellular signalling pathways, opsonization, or the induction of other indirect effects on hematopoiesis which can be functionally replaced by other molecules.

### Acknowledgements

We would like to thank Jessyca Maltman, Maya Sinclair and Gayle Thornbury for excellent technical assistance, Jo-Ann Woo for typing the manuscript, Dr Tak Mak (Amgen Institute, Ontario

Cancer Institute, Toronto, ON) for kindly providing the original CD44<sup>-/-</sup> breeding pairs, and Cangen and StemCell for generous gifts of reagents. This work was supported by the National Cancer Institute of Canada (NCIC) with funds from the Terry Fox Run and NIH P01 HL55435. CJ Eaves is a Terry Fox Cancer Research Scientist of the NCIC.

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