UNIVERSITÉ DE LAUSANNE – FACULTÉ DE BIOLOGIE ET DE MÉDECINE

INSTITUT UNIVERSITAIRE DE PATHOLOGIE

Functional Analysis of Different Haematopoietic Stem Cell Subsets

THÈSE

préparée sous la direction du Professeur Ivan Stamenkovic
avec la collaboration du Professeur Andreas Trumpp de l'EPF Lausanne

et présentée à la Faculté de biologie et de médecine de l'Université de Lausanne
pour l'obtention du grade de

DOCTEUR EN MÉDECINE

par

Raghav CHAWLA

Médecin diplômé de la Confédération Suisse
Originaire de Windisch

Lausanne 2009

RÉSUMÉ (IN FRENCH)

Elucider les bases moléculaires et cellulaires du fonctionnement des cellules souches s'avère crucial dans la compréhension de l'organisation cellulaire au sein des tissus et des organes ainsi que pour le développement de nouvelles stratégies thérapeutiques en médecine régénérative et en oncologie. Les cellules souches adultes les mieux connues sont celles responsables de l'hématopoïèse, les cellules souches hématopoïétiques (CSH). Durant ces dernières années, la recherche a porté une attention particulière à l'isolation prospective de CSH dérivées de la moelle osseuse de souris en utilisant des marqueurs de surface cellulaire ainsi que des propriétés fonctionnelles alléguées. Par la suite, la capacité fonctionnelle des CSH a été vérifiée classiquement par leur transplantation intraveineuse dans des souris réceptrices conditionnées et par l'analyse de leur aptitude à reconstituer le système hématopoïétique à long terme. Des études récentes suggèrent que la transplantation des cellules directement dans la moelle osseuse pourrait non seulement aboutir à une prise de greffe plus rapide et plus efficace, mais pourrait même aider à l'identification de cellules qui ont certes des propriétés intrinsèques de CSH, mais qui n'ont pas la capacité de trouver leur niche au sein de la moelle osseuse et ont donc échoué dans les analyses classiques de reconstitution. Dans cette étude, nous comparons à deux niveaux la fonction de différents sous-groupes de cellules souches de la moelle osseuse, définis par leur phénotype de surface cellulaire. Premièrement, nous étudions leur capacité à reconstituer des souris létalement irradiées après injection soit intraveineuse soit intrafémorale. Deuxièmement, par analyse cytométrique de flux à 8 couleurs, nous comparons leur activité relative de « side population » (SP) par exclusion du colorant fluorescent Hoechst 33342. Nos résultats préliminaires renforcent en effet l'idée que la transplantation intrafémorale aboutit à une greffe plus rapide et plus efficace. Par contre, en utilisant cette approche, nous n'arrivons pas à identifier des cellules capables de prendre greffe spécifiquement quand elles sont injectées en intrafémorale. Finalement, bien qu'une confirmation *in vivo* soit encore nécessaire, nous suggérons sur la base de nos analyses cytométriques de flux, que les cellules SP Sca1$^{\text{très élevé}}$ CD48$^{\text{très bas}}$ sont très enrichies en CSH. Ceci permettrait l'isolation *ex vivo* de CSH de la moelle osseuse de souris par une stratégie à la fois nouvelle et simple.

SUMMARY

Elucidating the molecular and cellular bases of stem cell function is crucial for the understanding of cellular organisation within tissues and organs as well as for the development of new therapeutic strategies in regenerative medicine and oncology. The best-known adult stem cells are those responsible for haematopoiesis, the haematopoietic stem cells (HSCs). In recent years, much effort has been put into the prospective isolation of mouse bone marrow (BM)-derived HSCs using cell-surface markers and alleged functional properties. Upon isolation, the functional capacity of putative HSCs has been classically assessed by intravenous transplantation into conditioned recipient mice and analysis of their ability to reconstitute the haematopoietic system at long-term. It has recently been suggested that transplanting the cells directly into the BM might not only result in more rapid and more effective engraftment, but even help to identify cells that have intrinsic HSC properties but lack the ability to home to their BM niche and have thus failed to succeed in classical reconstitution assays. In this study, we compare the function of different BM cell subsets, as defined by their cell surface phenotype, on two levels. Firstly, we assess their ability to reconstitute lethally irradiated mice, when injected either intravenously or intrafemorally. Secondly, using 8-colour flow cytometric analysis, we compare their relative side population (SP) activity by exclusion of the fluorescent dye Hoechst 33342. Our preliminary results indeed reinforce the idea that intrafemoral transplantation results in faster and more effective engraftment, however, using this approach, we are unable to identify cells that are capable of engrafting specifically when injected intrafemorally. Finally, although *in vivo* confirmation is still required, we propose, based on the results of our flow cytometric analyses, that SP Sca1$^{very\ high}$ CD48$^{very\ low}$ cells should be highly enriched for HSCs. This would allow for a simple new strategy for the isolation of mouse BM HSCs *ex vivo*.

TABLE OF CONTENTS

PRELIMINARY REMARKS

Having been accepted to the Swiss MD-PhD programme in May 2006, I graduated from the University of Lausanne (UNIL) medical school six months later and started my MD-PhD thesis in the laboratory of Prof. Andreas Trumpp at the Swiss Institute for Experimental Cancer Research (ISREC) and the Swiss Federal Institute of Technology Lausanne (EPFL) in March 2007.

In January 2008, Prof. Trumpp announced his appointment as the head of the Division of Cell Biology at the German Cancer Research Centre (DKFZ) in Heidelberg and, related to this, the upcoming move of his laboratory from Lausanne to Heidelberg. With the backing of the Swiss MD-PhD programme, the Swiss Academy for Medical Sciences as well as of my funding agency, the Swiss National Science Foundation, I consequently decided to change my host laboratory and research project. Since March 2008, I have thus been working as a PhD student in the laboratory of Dr. Claus Azzalin under the supervision of Prof. Ulrike Kutay at the Swiss Federal Institute of Technology Zurich.

As the Zurich MD-PhD programme, in contrast to the one in Lausanne, requires its students to apply for an MD degree in addition to and separately from the PhD degree, I decided to write up my MD thesis on the basis of the results I had obtained from my experimental work in the laboratory of Prof. Trumpp at ISREC / EPFL. Prof. Ivan Stamenkovic of UNIL's Institute of Pathology kindly agreed to serve as my official thesis director for this purpose. Furthermore, this endeavour carries the approval of Prof. Amalio Telenti and Prof. Adriano Aguzzi, presidents of the Lausanne and Zurich MD-PhD programme committees, respectively, as well as of Prof. Stephanie Clarke, director of the doctoral school of UNIL's Faculty of Biology and Medicine.

ACKNOWLEDGEMENTS

I would like to thank all members of the Trumpp laboratory for having created a very enthusiastic and motivating environment within the laboratory. Special thanks goes to my supervisor, Prof. Trumpp, for having given me the unique opportunity to work in his lab, for his outstanding support and for his always very constructive and stimulating comments during scientific discussions. Separate mention and acknowledgement also goes to all the following (current and former) lab members for invaluable support: Sandra Offner, Dr. Elisa Laurenti, Dr. Gabriela Oser, Dr. Marieke Essers, Irène Baccelli, Dr. William Blanco, Christelle Dubey and Dr. Mark Murphy.

The submission of this thesis was only made possible through the support of Prof. Stamenkovic, who readily agreed to serve as my official thesis supervisor. I would like to thank Prof. Stamenkovic for always having been available and for having conveyed a lot of enthusiasm and positive thinking throughout recent years. In addition, the submission process was substantially accelerated thanks to the generosity of my current PhD supervisor, Dr. Azzalin, who allowed me to work part-time in the laboratory while I was writing up my MD thesis.

A huge thanks goes to Joanna Roberts for all the hours she spent with me in front of the FACS Vantage DiVa. She was a great sport and made flow cytometry fun even when it didn't work... Furthermore, I thank Dr. Anne Wilson for sharing antibodies and for critical reading of this manuscript, the entire ISREC animal facility staff for caring for my mice, and Tree Star Incorporation for granting me a free temporary FlowJo software license. I also acknowledge everyone (besides the people already mentioned) who showed support during my move from Lausanne to Zurich, notably, Prof. Kutay, Prof. Aguzzi, Prof. Pierre Gönczy and Prof. Clarke.

Finally, I would like to thank the Swiss MD-PhD programme and the Swiss National Science Foundation for their comprehensive (including financial) support and, last but not least, my family, including my wife-to-be, Fabienne Luisier, my parents, Prof. Rakesh and Veena Chawla, my sister, Dr. Radhika Schütz-Chawla, as well as my brother-in-law, Tobias Schütz, for their never-ending love and support.

INTRODUCTION

Stem Cells and Architecture of Self-Renewing Tissues

Stem cells, traditionally subdivided into two categories, embryonic and adult stem cells,[1] hold great promise for the development of new therapeutic strategies in regenerative medicine and oncology.[1 2] Embryonic stem cells are pluripotent cells that derive from the inner cell mass of the blastocyst and are capable of generating all differentiated cell types of the body, including adult stem cells.[1] The latter, in contrast, have restricted pluripotency and usually differentiate only into cell types specific for the organ or tissue they reside in, although some degrees of plasticity seem to exist.[1]

Adult stem cells are *per se* critical for life-long host tissue maintenance and repair after injury.[1] Although they have been described in most (and even low cell-turnover) tissues, they remain best characterised in the "classical" self-renewing tissues, namely the haematopoietic system, the small intestine and the epidermis.[3]

Cells within these tissues are organised in a hierarchical manner (Figure 1).[3] Stem cells, on the uppermost level, are rare pluripotent cells, which are long-lived and capable of self-renewal. Their interaction with the surrounding microenvironment, the so-called stem cell niche, controls their homeostasis and determines their fate (quiescence, self-renewal, or differentiation). While thought to remain quiescent under homeostatic conditions, stem cells are capable of extensive proliferation upon activation. They may divide either symmetrically, giving rise to two stem cells or to two more differentiated cells, or asymmetrically, generating one of either. When their offspring undergoes a commitment to differentiate, it first enters the so-called transit-amplifying phase, during which the cells rapidly proliferate.[3] Upon exhaustion of their proliferative potential, these multipotent cells withdraw from their self-renewal cycle and execute a terminal differentiation programme. Differentiated cells generally have specific characteristic functions, a short life span, and they cannot self-renew.[3]

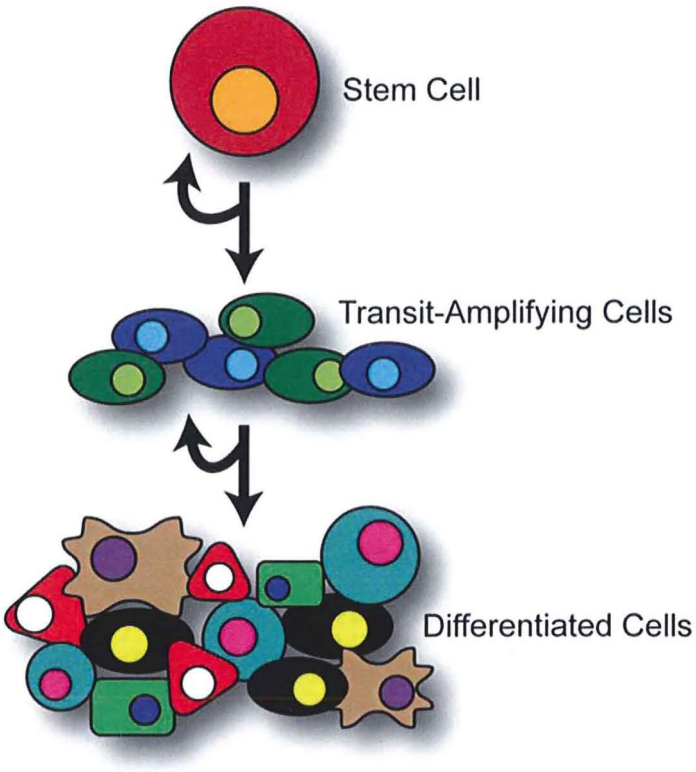

Figure 1. Model illustrating the hypothetical hierarchical organisation of cells in self-renewing tissues. See text for further details.

HSCs and Long-Term Repopulating Assays

The most frequently investigated stem cells are those responsible for replenishing the haematopoietic system, the haematopoietic stem cells (HSCs).[4] In fact, most of the conceptual paradigms and experimental strategies that are pertinent to stem cells in general were first defined in this system.[5] HSCs are cells that have the potential to self-renew and differentiate into all mature blood cell lineages. They are located in the bone marrow (BM) during homeostasis, but can be mobilised to the spleen and liver after BM injury.[6 7] In the BM of an adult mouse, they represent on the order of 1 in 10^4 to 10^5 cells.[8]

HSC activity within the BM has been extensively studied ever since 1956, when it was first shown that lethally irradiated recipients could be durably reconstituted by transplantation of total BM from healthy mice.[9] These early experiments not only suggested that transplanted BM contains cells that are able to clonally differentiate into multiple lineages while self-renewing, but they also formed the basis for what has turned out to establish itself as the gold standard assay to prove enrichment for HSC activity. Although the functional capacity of putative HSCs can also be assessed using *in vitro* assays (such as long-term culture-initiating cell or cobblestone area-forming cell assays) or other *in vivo* assays (such as

spleen colony assays),[4] it is the long-term repopulating (LTR) assays that have become the defining element, the *sine qua non*, of HSC activity. They are based on the capacity of transplanted HSCs to act as long-term reconstituting cells by durably repopulating the entire haematopoietic system in conditioned recipients.[4] The strictest version of LTR assays is known as serial transplantation and requires that HSC-containing donor BM can be re-transplanted from the primary recipient into secondary (and even tertiary) recipients while retaining both self-renewal and multilineage differentiation capacity.[10]

LTR assays have been classically performed using intravenous (IV) injection of putative HSCs, entailing the necessity for them to circulate through blood, recognise and extravasate through BM vasculature and to migrate to a supportive microenvironment, before reconstitution can occur. This awareness recently led to the suggestion that direct injection of the cells into the BM cavity, for example, by intrafemoral (IF) injection, might not only accelerate engraftment but might even help to identify cells that have intrinsic repopulating activities but are unable to home to the BM.[11 12]

Although technically challenging, several groups recently succeeded at performing LTR assays even at the single-cell level, achieving long-term haematopoietic reconstitution of lethally irradiated mice by transplantation of a single, previously purified, BM HSC, thus formally proving the existence of *bona fide* somatic adult stem cells.[13-19]

Enrichment for HSC Activity using Cell Surface Markers

Over the past few decades, considerable efforts have been made in both human and mouse to determine exactly which cell types constitute the HSC compartment. Most recent approaches to purify putative HSCs, or, more precisely, to enrich for putative HSC activity, have used fluorescence-activated cell sorting (FACS) and are based on the level of expression of a combination of cell surface markers.

The highest single-cell long-term reconstitution efficiency (47%) in mice has so far been reported for BM cells with the following *combined* antigenic marker phenotype:[16 20] 1. **Lin$^-$**, absence (or low abundance) of cell-surface markers normally present on lineage-committed haematopoietic cells; 2. **Sca1$^+$**, high-level surface expression of stem-cell

antigen-1; 3. **cKit$^+$**, high-level surface expression of c-Kit receptor; 4. **CD48$^-$**, low-level surface expression of SLAM receptor CD48; 5. **CD150$^+$**, high-level surface expression of the signal activating molecule (SLAM) receptor CD150.

Simultaneously, it has been reported that all long-term reconstitution activity of lineage-depleted adult mouse BM is retained within the Lin$^-$ Sca1$^+$ cKit$^+$ (**LSK**) fraction that shows no or low expression of the antiadhesive sialomucin CD34 (**CD34$^-$**), although the single-cell reconstitution efficiency of cells with this phenotype is lower (22%) than that for the phenotype described above.[13 20]

Recent experimental evidence, involving an assessment of the degree of HSC quiescence by bromodeoxyuridine (BrdU) label-retaining assays in addition to cell surface staining, now indeed suggests that the LSK CD48$^-$ CD150$^+$ CD34$^-$ fraction of BM cells represents the most dormant HSC population (Wilson and Trumpp, unpublished data). Moreover, the observation that CD34$^-$ cells are only found in the LSK CD48$^-$ CD150$^+$ subset, provokes the idea that the acquisition of CD34 surface expression in LSK CD48$^-$ CD150$^+$ BM cells may be one of the determining events in the transition from dormant to actively self-renewing adult HSCs.[20] On the basis of these and other observations, LSK CD48$^-$ CD150$^+$ BM cells have been hypothetically subdivided into dormant (CD34$^-$) and activated (CD34$^+$) HSCs.[20-22] Furthermore, based on the expression of these antigenic markers, a hierarchy of BM stem and transit amplifying cell subsets has been proposed, starting with LSK CD48$^-$ CD150$^+$ CD34$^-$ cells and leading, via the sequential acquisition of CD34 and CD48, followed by the loss of CD150, to LSK CD48$^+$ CD150$^-$ CD34$^+$ cells.[20]

Enrichment for HSC Activity using the Side Population Phenotype

An alternative approach to enrich for putative HSC activity also uses flow cytometry, but is based on alleged physiologic properties of HSCs such as an elevated activity of aldehyde dehydrogenase or an increased ability to efflux the fluorescent DNA-binding dye Hoechst 33342.[23-26] This latter feature is essentially due to an increased activity in HSCs of the ATP-binding-cassette transporter ABCG2, a multidrug resistance (MDR)-related protein, also known as breast cancer resistance protein (BCRP).[27] Display of Hoechst 33342 fluorescence simultaneously at two different emission wavelengths thus allows for

identification of a cell fraction known as the side population (SP), which accounts for 0.07% to 0.10% of mouse BM cells and represents a 2000-fold enrichment for long-term reconstituting cells.[24]

The SP technique has not only proven useful for the prospective isolation of HSCs, but has, in fact, been a big boost to the stem cell field in general, as it has been used to purify stem cell-like subsets from different organisms, tissues, and developmental states.[28-31] Moreover, dye exclusion has been invaluable even for cancer research, as it has allowed for cancer stem cell enrichment from various types of cancers.[32]

More recently, the SP phenotype has been suggested to be a marker of quiescence,[33 34] and innumerable studies have not only addressed the relationship between SP cells and HSCs isolated on the basis of cell surface markers, but have actually used a combination of both strategies to efficiently enrich for HSC activity.[17 26 33-36]

Design and Aim of Project

The aim of this project is to compare functional properties of different HSC subsets. More specifically, we would first like to confirm that mouse LSK $CD48^-$ $CD150^+$ $CD34^-$ BM cells, sorted by fluorescence-activated cell sorting (FACS), are capable of long-term reconstitution (as assessed by long-term analysis of peripheral donor granulocyte frequencies), no matter whether injected IV or IF into lethally irradiated mice. Secondly, we would like to test whether LSK $CD48^-$ $CD150^+$ $CD34^+$ BM cells contain sub-populations which are capable of long-term reconstitution when injected IF (and not when injected IV), the hypothesis being that the presence of CD34 surface expression may hamper homing of HSCs to BM but not necessarily interfere with their intrinsic ability to engraft and reconstitute. Furthermore, using 8-colour flow cytometric analysis, we will directly compare the phenotype of HSCs, based on their expression of the above-mentioned antigenic markers, with their SP-forming capacity.

MATERIALS & METHODS

Mice

All mice were housed under specific pathogen-free (SPF) conditions in individually ventilated cages at the ISREC core animal facility. Animal procedures were performed in accordance with the Swiss federal veterinary office (authorisation number: 1728). For the LTR assays, female C57BL/6J (CD45.2) mice (Harlan Europe) aged 12-16 weeks were used as recipients as well as for preparation of rescue BM, whereas male SJL-Ptprca Pep3b/BoyJ (CD45.1) mice (Charles River Laboratories) aged around 20 weeks were used as donors. For the comparison of HSC surface markers with the side population phenotype, male or female C57BL/6J mice aged 13 weeks were used.

Preparation of Bone Marrow Single Cell Suspension

To retrieve BM, the long bones of hind- and forelegs (and, in the case of donor BM preparation, the vertebral column) were crushed in Roswell Park Memorial Institute-1640 medium with GlutaMAX (RPMI) (Invitrogen) supplemented with 2% foetal calf serum (FCS) (Invitrogen) using a pestle and mortar. Supernatants were filtered through a 35µm nylon mesh (BD Biosciences) prior to further use in order to prevent clumps.

Lineage Depletion of Total Bone Marrow Cells

For preparation of Lin^- BM, cells were first stained (see below) with a cocktail of biotin-conjugated mouse antibodies against lineage markers (CD4, CD8, B220, Ter119, CD11b, and Gr1) (homemade hybridomas) in phosphate buffered saline (PBS) (Sigma) supplemented with 2% FCS and in the presence of 24G2 (see below). After washing with RPMI / 2% FCS, the labelled cells were removed with the help of Dynabeads coated with a polyclonal sheep anti-rat IgG antibody (Invitrogen) according to the manufacturer's instructions (Dynal mouse CD4 negative isolation kit). A bead-to-cell ratio of 4:1 to 5:1 was used, assuming a target frequency of 90%. The supernatant was collected and washed with RPMI / 2% FCS prior to further use.

Hoechst 33342 Staining prior to Flow Cytometry

BM cells were centrifuged and re-suspended at 1 million cells per mL in Dulbecco's modified Eagle's medium with GlutaMAX and 4.5g/L D-glucose (DMEM) (Invitrogen) supplemented with 2% FCS and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) (Invitrogen), prewarmed to 37°C. Hoechst 33342 (Hoechst) (Invitrogen) was added at a concentration of 4µg/mL, and, in addition, verapamil (Sigma-Aldrich) was added to the negative control at 50µM.[24] The cells were incubated at 37°C for 90 minutes, and agitated every 20-30 minutes during incubation. Subsequently, cells were centrifuged and re-suspended in ice-cold Hank's balanced salt solution without Mg^{2+}, Ca^{2+} and phenol red (HBSS) (Invitrogen) supplemented with 2% FCS and 10mM HEPES. Optionally, cells were additionally stained with antibodies as described below, with the exception that cold HBSS (supplemented as above) was used instead of PBS / 2% FCS. In any case, from now on, cells were kept on ice and protected from light until FACS analysis.

Antibody Staining prior to Flow Cytometry

BM cells were centrifuged and re-suspended at 30 million cells per mL of PBS / 2% FCS in the presence of 24G2 hybridoma supernatant containing a monoclonal anti-mouse FcR antibody, thus preventing FcR-mediated binding of the other antibodies. The following antibody-fluorochrome conjugates were used for staining: PECy7-conjugated anti-lineage marker antibodies, Sca1-Alexa700, cKit-APC-Alexa750, CD48-PE, CD34-FITC (all from eBioscience), and CD150-PECy5 (BioLegend). Unstained and single-stained samples were prepared in parallel as compensation controls. Staining was performed by incubating the cells on ice with the appropriate antibodies for 30 minutes. Labelled cells were washed with PBS / 2% FCS, re-suspended in appropriate volumes of the same buffer and stored on ice and in the dark until analysis.

Flow Cytometry

Multicolour analysis in the presence of Hoechst 33342 was performed on a FACS Vantage flow cytometer (BD Biosciences) equipped with 350nm (UV), 488nm and 647nm lasers. Propidium iodide (PI) (Sigma-Aldrich) was added to all samples containing Hoechst 33342,

enabling subsequent identification and exclusion of dead cells by appropriate gating on the Hoechst plot during analysis, as PI^+ cells give an extraordinary high signal on the Hoechst red axis. All cell suspensions were filtered through a 35µm nylon mesh immediately before analysis. Compensation settings were adjusted with the help of unstained and single-stained samples. All post-acquisition analysis of FACS data (including post-acquisition adjustment of compensation settings) was performed using FlowJo software (Tree Star Incorporation).

Long-Term Repopulating (LTR) Assays

Recipient mice were injected intra-peritoneally with 100µg of PK136 (anti-CD161c) antibody (grown and purified in our laboratory) 48h prior to injection of donor BM to minimise host-versus-graft disease.[37] They were lethally irradiated (1x1000rad) 24h later in order to create favourable conditions for HSC homing and engraftment.[38-40] For the first 7 days after total body irradiation (TBI), the drinking water was supplemented with co-trimoxazole (Roche) and paracetamol/acetaminophen (Bristol-Myers Squibb / UPSA). For the subsequent 14 days, the water was supplemented with co-trimoxazole only. During this time, water bottles were changed twice a week.

A Lin^- donor BM single cell suspension (from 2-4 mice per experiment) was prepared and antibody-stained as described above. LSK $CD48^-$ $CD150^+$ and either $CD34^+$ or $CD34^-$ as well as LSK $CD48^+$ $CD150^-$ $CD34^+$ cells (the latter for a negative control that was only done during the first experiment)[20] were sorted with a FACS Vantage and collected in homemade sterile medium consisting of StemPRO-34 serum-free medium (Invitrogen) supplemented with StemPRO-34 nutrient (Invitrogen), mouse stem cell factor, thrombopoietin, Flt-3 ligand, IL-11 (all from R&D Systems), penicillin/streptomycin, L-glutamine (both from Invitrogen) and ciprofloxacin (Bayer).[41-43] After cell counting and centrifugation, appropriate numbers of cells were re-suspended in either 300 or 25µL of non-supplemented PBS, for IV and IF injections, respectively.

Whereas IV injections were performed as usual via one of the tail veins, IF injections were done as follows. 2h prior to injection, mice were injected subcutaneously with the potent opioid analgesic buprenorphine (ESSEX Chemie AG). The mice were anaesthesised using

a diluted mix of ketamine (Dr. E. Graeub AG) and xylazine (Bayer). All following procedures were performed on a heating pad in order to keep the mouse warm. To prevent them from drying, the corneas of the eyes were covered with the ophthalmic gel Lacryvisc (Alcon). With the (previously shaved) knee bent, a hole was made into the distal femur using an 18G needle. The needle was removed and replaced with a 26G needle fixed to a syringe containing the cells to be injected. Upon injection, the needle was removed, and the wound disinfected with liquid Betadine (Mundipharma) and gently rubbed with the ointment Cicatrex (Pfizer). The mouse cages were left on heating pads for at least 12h post-injection to favour recovery.

24-48h later, rescue BM was prepared by putatively depleting total BM (prepared as above) of HSCs using the rat anti-mouse anti-Sca1-PECy5 antibody and the Dynabead system described above with a bead-to-cell ratio of 4:1 to 5:1 and assuming a target frequency of 10%. 1 million cells were injected IV as rescue BM. The following modifications of this protocol were undertaken during the second experiment (see below). Prior to HSC depletion, total BM was first depleted of T cells using complement-mediated cytotoxicity; after an initial 30-minute incubation step on ice with AT83 supernatant (homemade hybridoma), which contains rabbit anti-mouse anti-Thy1.2 antibodies, rabbit complement (Saxon Europe Ltd.) and DNAse (Roche) were added and the cells were incubated for an additional 30 minutes at 37°C. HSC depletion was performed with beads assuming a target frequency of 50%, and only 400'000 cells were injected.

The mice were hereafter regularly checked for survival, and the extent of reconstitution was examined by biweekly analysis of peripheral white blood cell (WBC) and granulocyte chimerism between weeks 4 and 12 post-transplantation. Briefly, 5-8 drops of blood were collected in tubes containing PBS and heparin (Sigma) by tail-vein bleeding. The cell suspension was underlayed with Histopaque (Sigma) and centrifuged. Peripheral WBCs were harvested from the interface, washed and stained (as described above) with the following antibody conjugates: CD45.1-FITC, CD45.2-PECy5.5, and Gr1-PE (all from eBioscience). The labelled cells were analysed (similar to above) on a FACS Scan flow cytometer, and post-acquisition analysis of the FACS data was again performed using FlowJo software.

RESULTS

Long-Term Reconstitution Ability of Different HSC Subsets

To examine the long-term reconstitution ability of LSK CD48$^-$ CD150$^+$ BM cells that are either CD34$^+$ or CD34$^-$, long-term LTR assays were performed in two separate experiments. CD45.1 donor cells were sorted according to their antigenic marker profile (see below for an illustration of the gating strategy) and injected either IV or IF into previously lethally irradiated CD45.2 recipient mice (Table 1). In order to prevent short- and midterm death, all mice were injected with HSC-depleted CD45.2 rescue BM 24-48 hours after HSC injection.

	1st experiment		2nd experiment	
LSK CD48$^-$ CD150$^+$ CD34$^-$	15 cells	5 mice	15 cells	7 mice
LSK CD48$^-$ CD150$^+$ CD34$^+$	15 cells	5 mice	45 cells	5 mice
LSK CD48$^+$ CD150$^-$ CD34$^+$ (Negative Control)	15 cells	3 mice	-	-

Table 1. Overview of cell and mouse numbers used for both reconstitution experiments. The same number of mice was injected with the same number of cells both IV and IF! See text for further details.

Despite the administration of rescue BM, a number of deaths occurred within the first 15 days after transplantation (Table 2). The analyses below are thus based on the remaining (surviving) mice only. To our surprise, despite the documented absence of long-term HSC activity within the LSK CD48$^+$ CD150$^-$ CD34$^+$ subgroup, no deaths were observed among the control-injected mice, neither at short- nor at long-term (Table 2), suggesting, taking into account the absence of engraftment (see below), either inefficient total body irradiation or inefficient HSC depletion of the rescue BM.

The long-term reconstitution ability of the transplanted cells was assessed by measuring the frequencies of peripheral donor (CD45.1) granulocytes biweekly from week 4 to at least 12 weeks post-transplantation. Peripheral granulocyte chimerism has been shown to reflect much more accurately HSC chimerism than the overall donor contribution in the blood.[44] Figure 2 illustrates an example of such an analysis.

	1st experiment		2nd experiment	
	IV	IF	IV	IF
LSK $CD48^-$ $CD150^+$ $CD34^-$	1/5 (20%)	0/5 (0%)	1/7 (14%)	1/7 (14%)
LSK $CD48^-$ $CD150^+$ $CD34^+$	0/5 (0%)	2/5 (40%)	0/5 (0%)	3/5 (60%)
LSK $CD48^+$ $CD150^-$ $CD34^+$ (Negative Control)	0/3 (0%)	0/3 (0%)	-	-

Table 2. Summary of deaths that occurred after HSC injection in each experimental group. Percentages express relative numbers, taking into account the total number of mice used in each experimental group (see Table 1).

In the first experiment, as expected, no chimerism was observed in the LSK $CD48^+$ $CD150^-$ $CD34^+$ (negative control) cell-engrafted mice (data not shown), suggesting that these cells are indeed unable to engraft. In fact, only mice transplanted with LSK $CD48^-$ $CD150^+$ $CD34^-$ cells showed chimerism, namely, 2 (50%) of the 4 surviving IV-injected and 3 (60%) of the 5 surviving IF-injected mice. The average frequency of $CD45.1^+$ granulocytes rose faster and was more elevated at all times in the IF-injected subgroup (Figure 3), suggesting that intra-BM transplantation of HSCs results in more rapid and more efficient engraftment. The difference, however, between the IV- and IF-injected subgroups was minimal at an analysis undertaken at 27 weeks post-transplantation, mainly because one mouse in the IF-injected subgroup displayed diminishing chimerism over time (Figure 3). No evidence of engraftment could be shown for the LSK $CD48^-$ $CD150^+$ $CD34^+$ cells, neither, as expected, for those injected IV, nor, however, for those injected IF.

Taking into account the rather high variability and error bars within the first experiment (Figure 3) and in order to confirm these preliminary findings, a second experiment was undertaken. In order to try to decrease variability, the number of mice transplanted with LSK $CD48^-$ $CD150^+$ $CD34^-$ cells was increased from 5 to 7, and, in order to test whether transplantation of an increased number of LSK $CD48^-$ $CD150^+$ $CD34^+$ cells would result in engraftment at least when injected IF, 45 instead of 15 of these cells were injected (Table 1). No control transplantations were performed during this second experiment, but 2 mice each were injected or not with rescue BM after total body irradiation. As only those that received rescue BM survived, total body irradiation was most likely efficient while HSC depletion within the rescue BM was probably not absolute (data not shown).

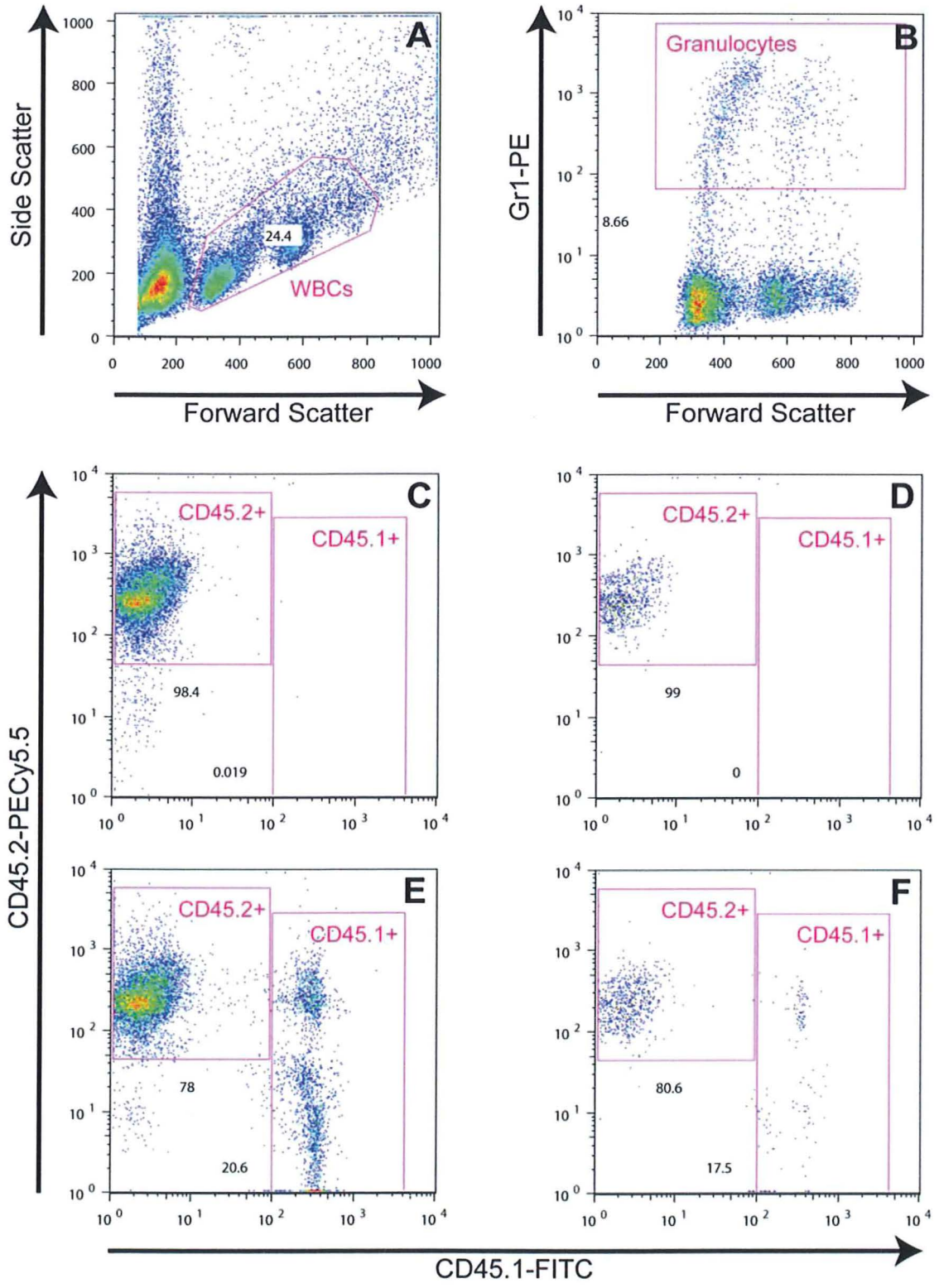

Figure 2. FACS plots illustrating the analysis of peripheral granulocyte chimerism. **(A)** Total events and the WBC gate are displayed. **(B)** WBCs and the granulocyte gate are displayed. **(C-F)** CD45.1^{+} and CD45.2^{+} gates are shown for a mouse displaying no chimerism **(C, D)** and for a mouse displaying chimerism **(E, F)**. Events displayed on the left side **(C, E)** are pre-gated on WBCs, those displayed on the right side **(D, F)** are pre-gated on granulocytes, thus reflecting peripheral granulocyte chimerism. The antibody for CD45.2 is known to partially cross-react with CD45.1.

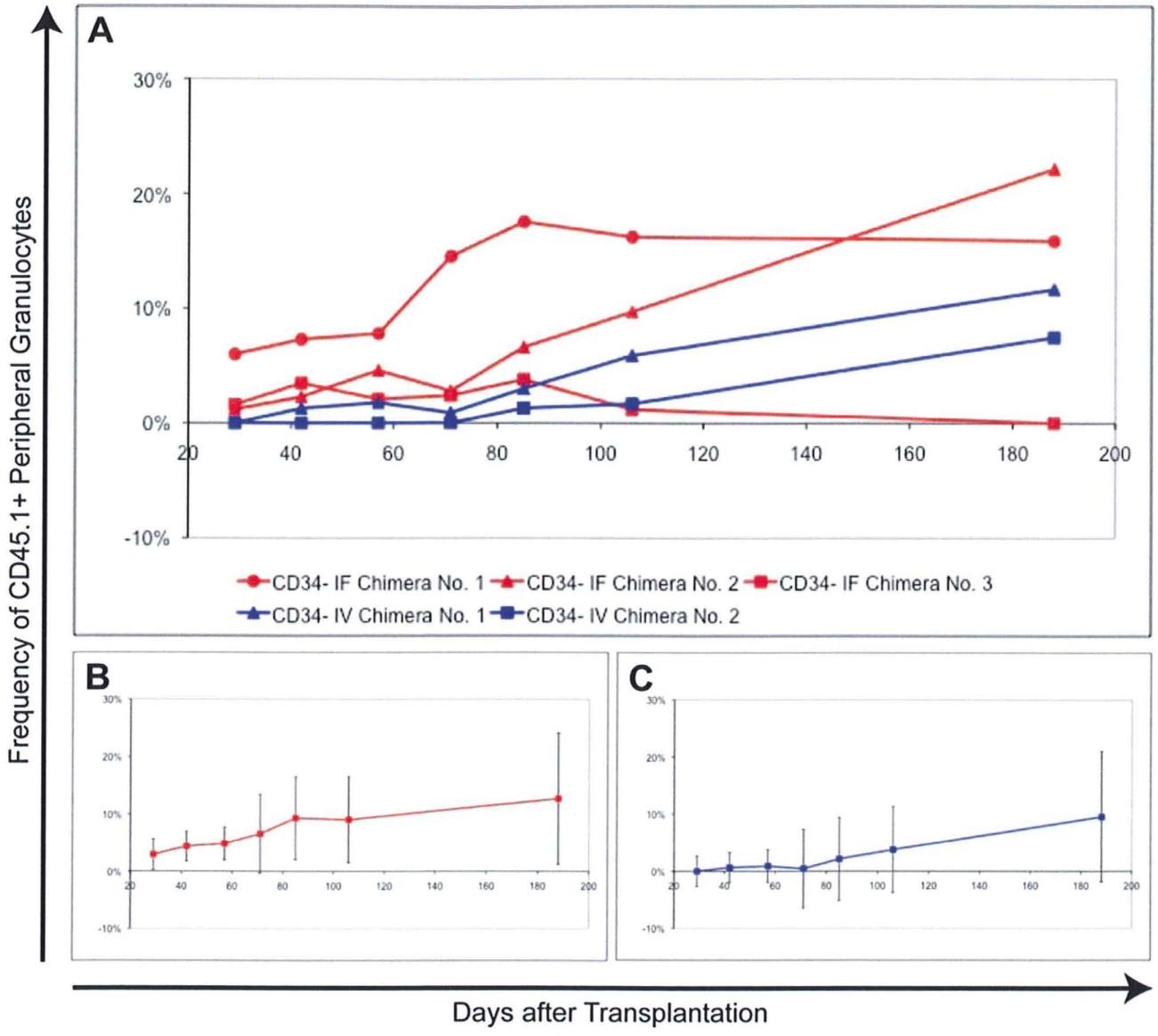

Figure 3. Granulocyte chimerism in the first LTR assay. **(A)** Frequency of CD45.1$^+$ peripheral granulocytes is displayed for individual chimeric IF-injected (red) and IV-injected mice (blue). **(B-C)** Average frequencies for chimeric IF-injected **(B**; N=3**)** and IV-injected mice **(C**; N=2**)**.

Unfortunately, the second experiment did not show the anticipated results: Not only were the results in most cases even more variable and the error bars thus even bigger, but most mice showed diminishing chimerism over time, and, although high CD45.1$^+$ peripheral granulocyte frequencies were observed (especially for one individual) in the subgroup of mice injected IV with LSK CD48$^-$ CD150$^+$ CD34$^-$ cells (chimerism in 5 (80%) of 6 mice), they were substantially lower for the mice injected IF with the same cells (chimerism in 3 (50%) of 6 mice) (Figure 4). Finally, to our surprise and inconsistent with what has been published previously, IV injection of LSK CD48$^-$ CD150$^+$ CD34$^+$ cells resulted in significant chimerism (chimerism in 3 (60%) of 5 mice), whereas no engraftment was observed in the mice injected IF (Figure 4).

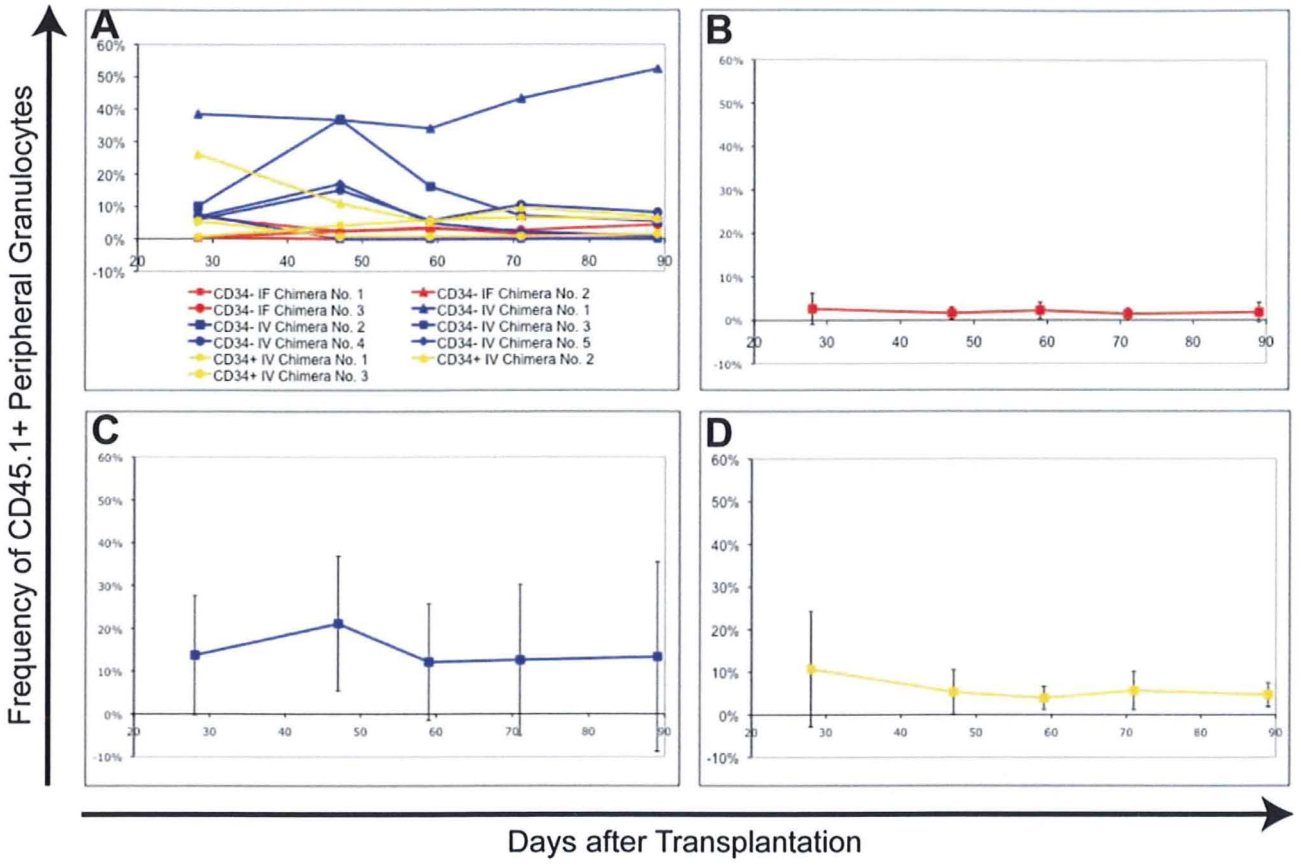

Figure 4. Granulocyte chimerism in the second LTR assay. **(A)** Frequency of CD45.1$^+$ peripheral granulocytes is displayed for individual chimeric mice injected IF (red) and IV (blue) with LSK CD48$^-$ CD150$^+$ CD34$^-$ cells and for those injected IV with LSK CD48$^-$ CD150$^+$ CD34$^+$ cells (yellow). **(B-D)** Average frequencies for chimeric mice injected IF with LSK CD48$^-$ CD150$^+$ CD34$^-$ **(B**; N=3**)**, IV with LSK CD48$^-$ CD150$^+$ CD34$^-$ **(C**; N=5**)** and IV with LSK CD48$^-$ CD150$^+$ CD34$^+$ cells **(D**; N=3**)**.

Side Population Phenotype of Different HSC Subsets

To assess the SP phenotype of different BM cell and, in particular, of putative HSC subsets, 8-colour FACS analyses were performed using Hoechst 33342 and antibody staining in parallel. The results presented below, with exception of the verapamil control, were collected during the same experiment, which was representative of numerous experiments done with slightly differing experimental conditions.

The same antigenic markers as described above were used to define different BM subsets. The gating strategy is illustrated in Figure 5. Lineage-depleted cells, pre-gated on single PI-negative cells, were gated on LSK and divided into further subsets, according to the surface expression of CD48, CD150 and CD34.

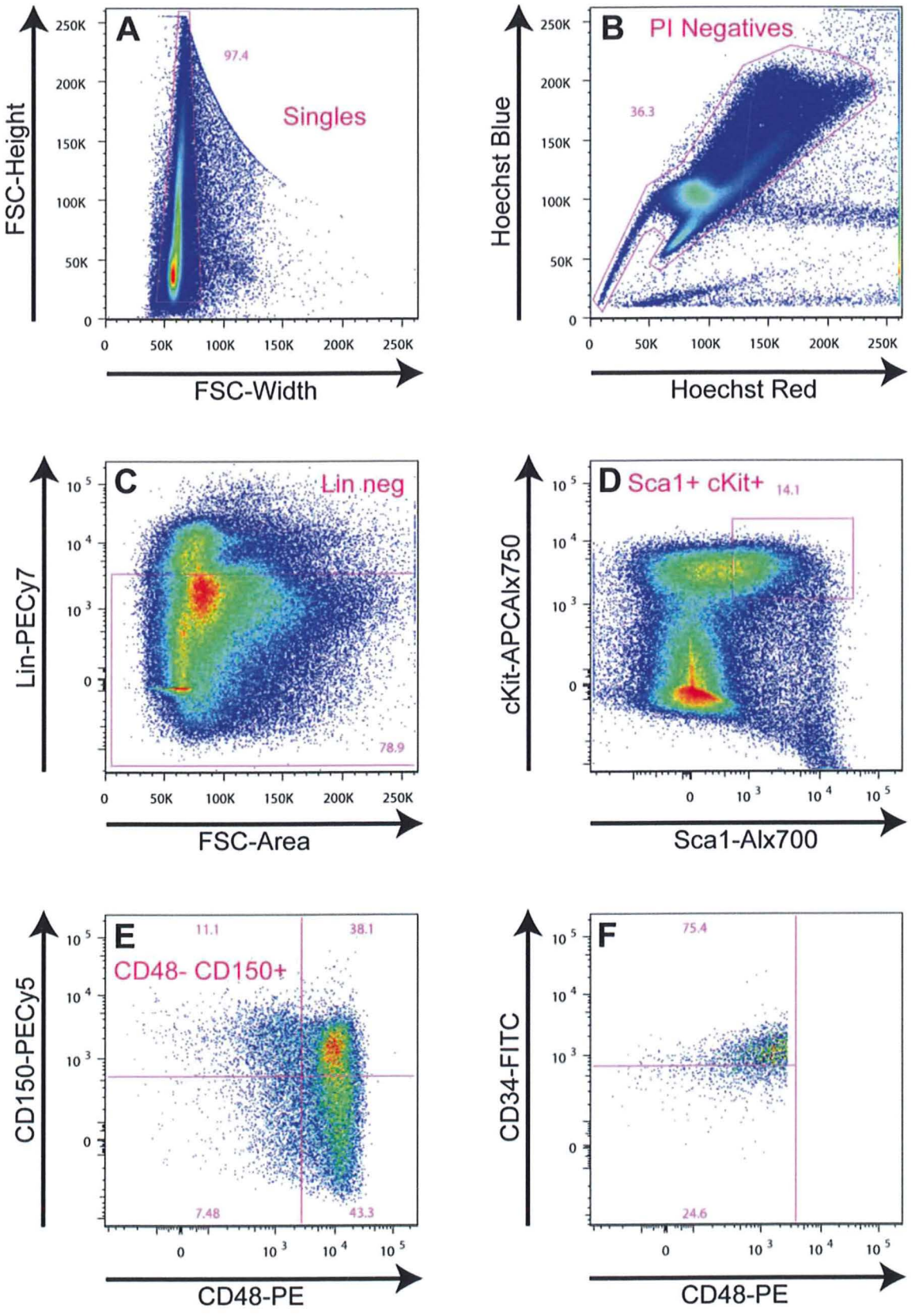

Figure 5. Gating strategy of lineage-depleted BM cells stained with Hoechst 33342 and antibodies against lineage markers, Sca1, cKit, CD48, CD150 and CD34. **(A-F)** Each plot displays only events defined by the gate annotated in the preceding plot. Thus, in **F**, only single PI^- Lin^- $Sca1^+$ $cKit^+$ $CD48^-$ $CD150^+$ cells are displayed. FSC, forward scatter; Alx, Alexa.

SP activity was assessed by displaying Hoechst fluorescent events (previously gated on single PI-negative cells) simultaneously at two different emission wavelengths (Hoechst red versus blue), as previously reported.[24] Figure 6 illustrates the SP and its inhibition upon addition of verapamil, a potent inhibitor of ABC transporters, during incubation with Hoechst 33342.[24 45]

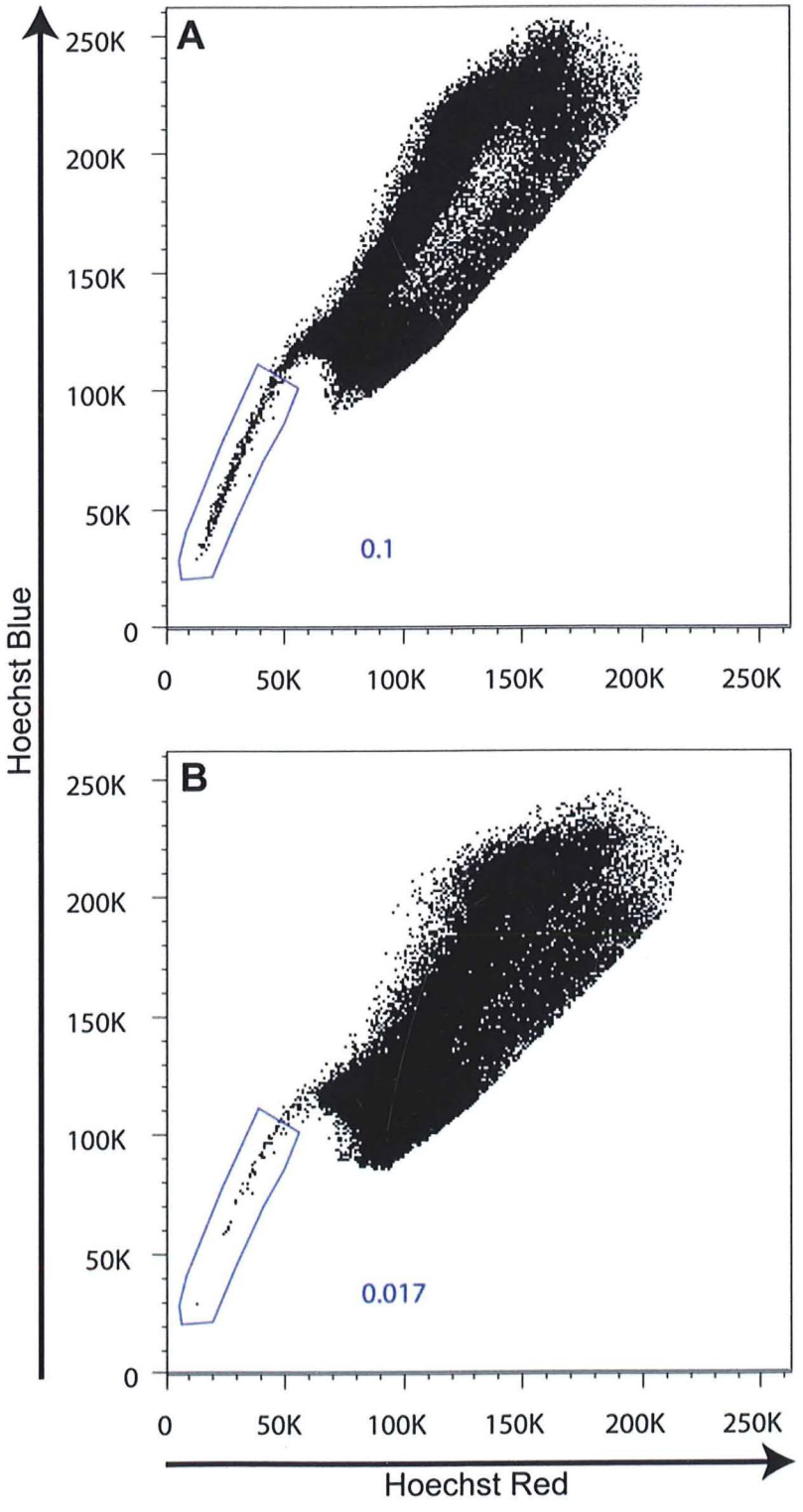

Figure 6. FACS dot plots showing an SP and its suppression upon concomitant treatment with verapamil. Non-lineage-depleted BM cells were incubated for 90 minutes at 37°C either with Hoechst 33342 alone **(A)** or with Hoechst 33342 and verapamil **(B)**. Cells are pre-gated on single PI-negative cells. 300'000 events are displayed per plot.

To examine whether the cells within the tip of the SP contain a higher proportion of cells with a putatively dormant phenotype than the total SP, an "SP tip" gate was defined in addition to the total SP gate (Figure 7).[17]

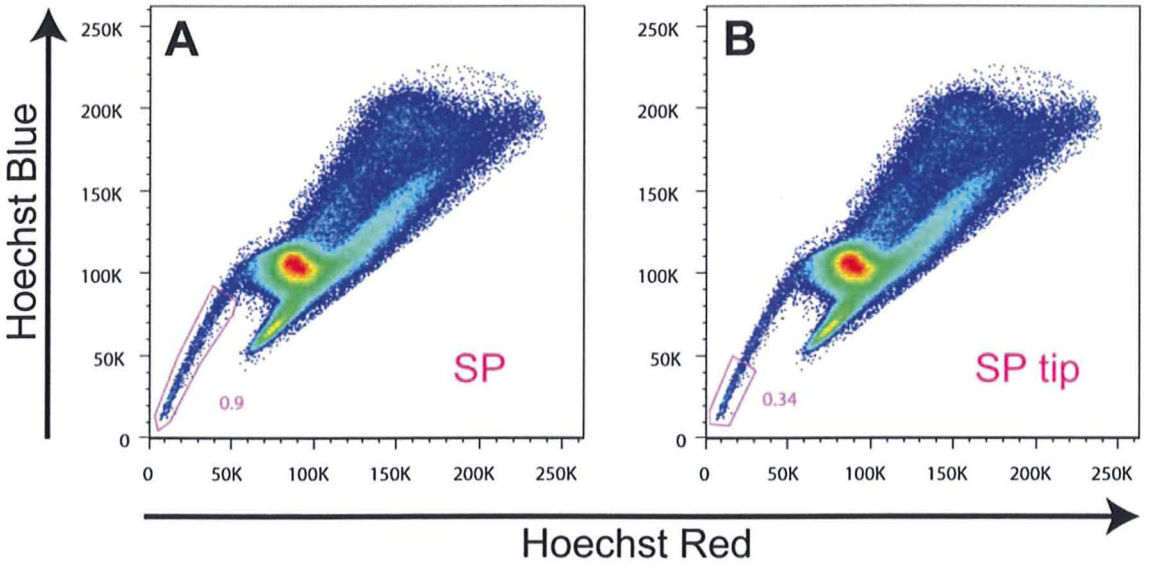

Figure 7. Definition of "SP" **(A)** and "SP tip" **(B)** gates.

Next, to examine SP and SP tip profiles of different BM cell subsets, the latter, as defined in Figure 5, were plotted on Hoechst red versus blue plots (Figure 8). Quantitative analysis of these profiles (Figure 9) shows similar findings for both the SP and SP tip gates, although the proportions are approximately 10x smaller for the latter. The following discussion will therefore concentrate on the SP gate only. Within LSK cells, only $CD48^-$ cells seem to have a significant proportion within the SP (Figures 8C, 8E & 9). This proportion is particularly impressive for the $CD48^-$ $CD150^+$ fraction (14.6%) but is also surprisingly high for the $CD48^-$ $CD150^-$ fraction (6.4%). If the SP profile is really to reflect long-term reconstitution ability, this finding would suggest that the latter is not limited to the $CD48^-$ $CD150^+$ fraction but also concerns $CD48^-$ $CD150^-$ cells and that CD150 might not be such a distinctive marker in terms of reconstitution ability as suggested by the authors who first reported it as an HSC antigenic marker.[16] Interestingly, recent findings indeed confirm this impression by showing that the LSK SP $CD150^-$ compartment contains a substantial fraction of cells capable of long-term reconstitution. The identification of HSCs on the basis of SLAM markers was thus suggested to neglect an important fraction of long-term HSCs.[36]

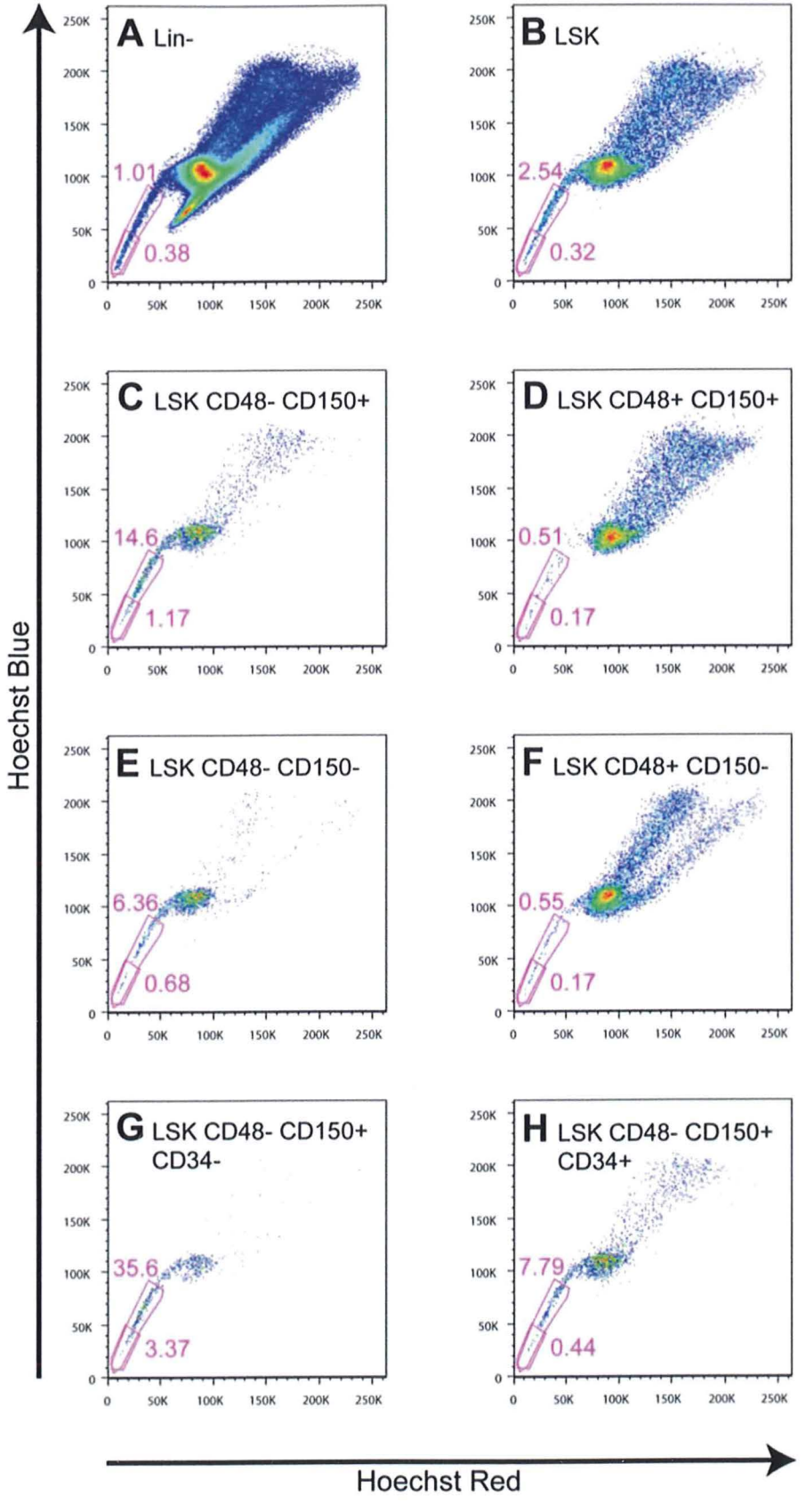

Figure 8. Hoechst plots showing SP and SP tip profiles of different BM cell subsets as defined in Figure 5. All events are pre-gated on single PI-negative cells. The events in **G** and **H** are gated on those shown in **C**.

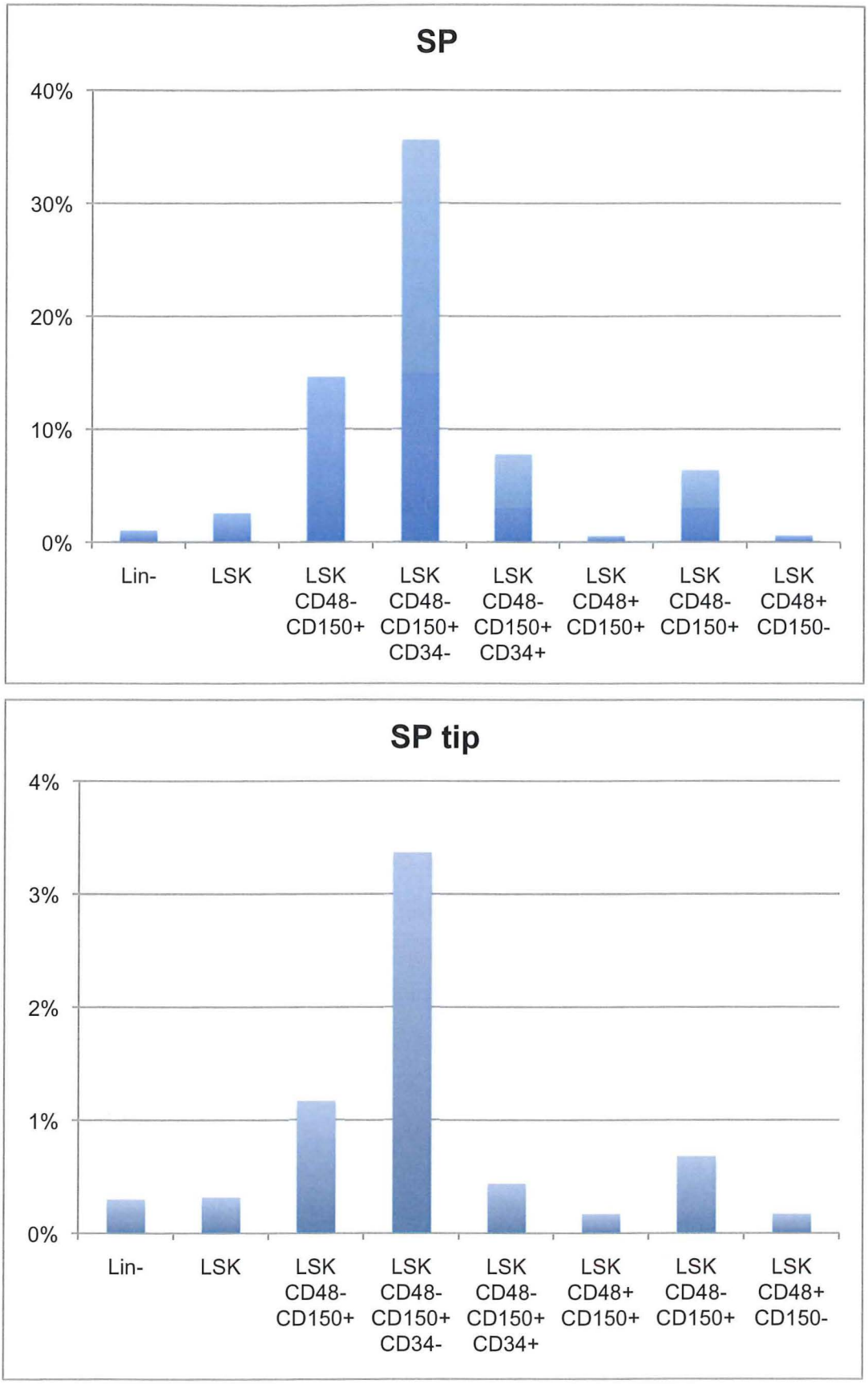

Figure 9. Fractions of various BM cell subsets within SP and SP tip gates with pre-gating on single PI-negative cells (data based on Figure 8).

Finally, within the LSK $CD48^-$ $CD150^+$ subset, $CD34^-$ cells constitute a much higher proportion of SP cells than $CD34^+$ cells (35.6% vs. 7.8%; see Figures 8G, 8H & 9), suggesting a substantial enrichment for LSK $CD48^-$ $CD150^+$ $CD34^-$, the supposedly most dormant HSCs, within the SP. As already hinted above, however, no additional enrichment was found within the cells responsible for formation of the SP tip gate.

Subsequently, we analysed the data the other way round, by examining the antigenic marker profile of SP cells. BM cells were pre-gated on single PI-negative cells, as in Figure 5, and then further gated on SP cells. These were then plotted using the same gating cascade as presented above, allowing us to calculate the fraction of SP cells belonging to the different BM cell subsets (Figures 10 & 11). Due to very low numbers, the corresponding analyses for SP tip cells was not very informative (data not shown).

In comparison with total single PI^- cells, relatively high fractions of SP cells were found to be located within the Lin^- (88.5%), the LSK (31.4%) and the LSK $CD48^-$ $CD150^+$ subsets (20.2%), especially within the $CD34^-$ fraction of the latter (12.1% versus 8.1% for the $CD34^+$ fraction) (Figure 11), implying a significant enrichment for SP cells within these subsets.

It has been suggested that SP LSK (also known as "SParKLS") cells may represent a subset of cells very highly enriched for long-term HSCs.[26 45] Figure 10G indeed somewhat favours such an hypothesis by showing that a majority (64.2%) of SP LSK cells are simultaneously $CD48^-$ and $CD150^+$. Within the SP LSK cells, approximately 50% are $CD34^-$ (data not shown).

The profiles in Figure 10, however, also suggest that there is some overlap between the SP phenotype and expression of some of the antigenic HSC markers, especially those responsible for defining the LSK subset. As such, after the exclusion of doublets and dead cells, SP gating of lineage-depleted cells may allow for a significant enrichment for HSCs without the need to use, for example, lineage markers, cKit or perhaps even CD150 (the validity of which as an HSC marker has already been questioned above, independently of SP gating).

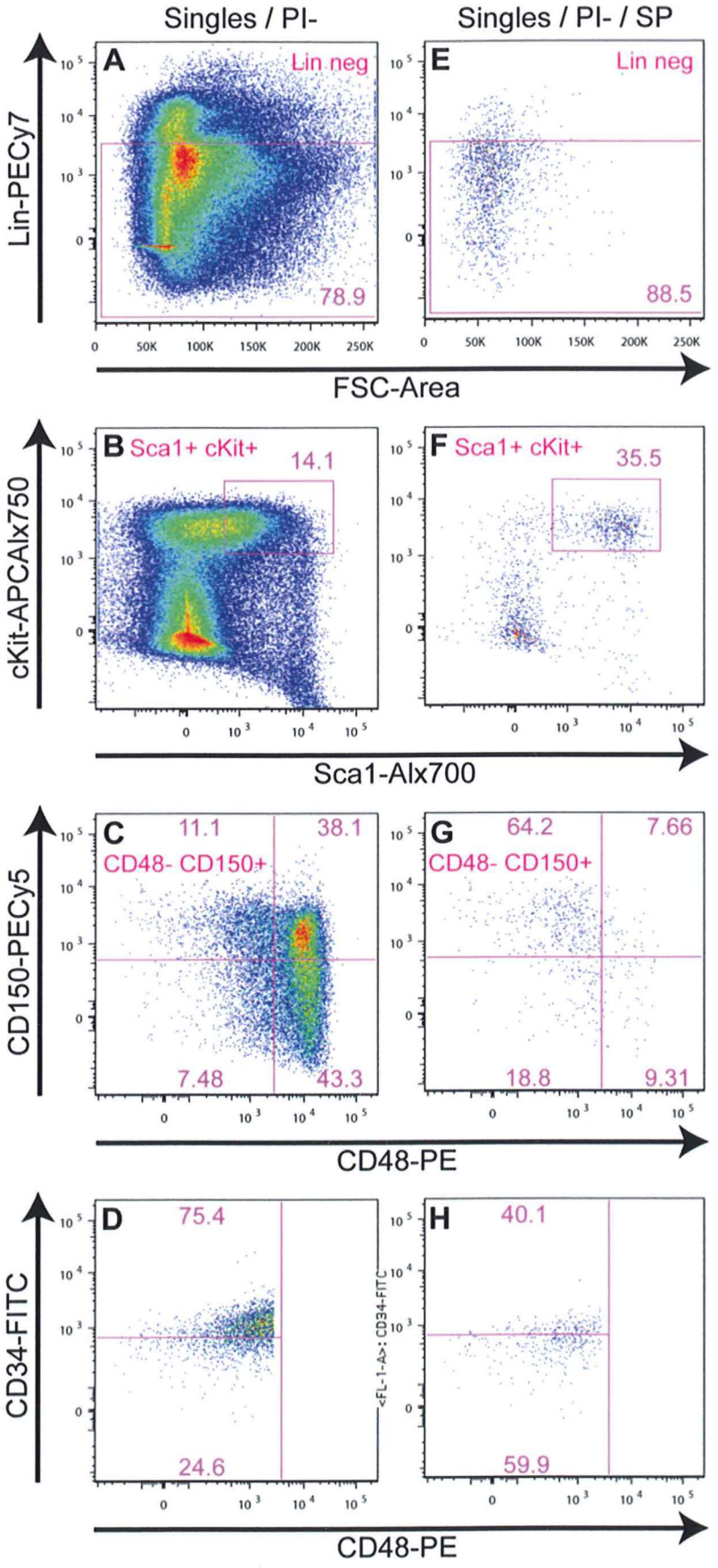

Figure 10. HSC antigenic marker profiles of SP cells. Cells were pre-gated either on single PI-negative cells only (left side, **A-D**) or, additionally, on SP (right side, **E-H**). Plots A-D are identical to plots C-F in Figure 5. The same gating strategy was used as presented in Figure 5, with each plot displaying only events defined by the gate annotated in the plot immediately above. FSC, forward scatter; Alx, Alexa.

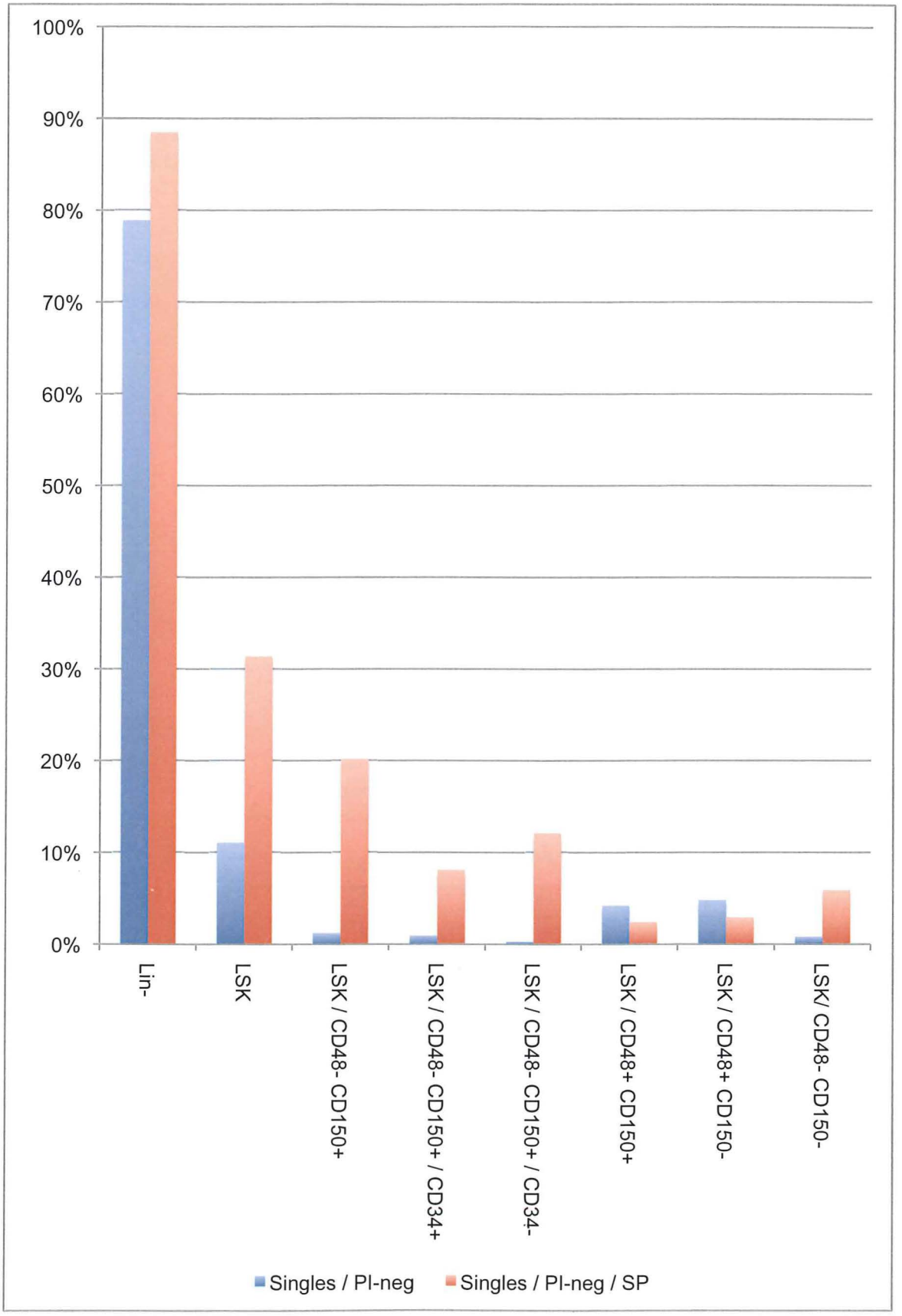

Figure 11. Frequencies of SP cells belonging to various BM cell subsets. Cells were pre-gated either on single PI-negative cells only (blue) or, additionally, on SP (red).

Indeed, if SP-forming cells are more stringently gated according to Sca1 and CD48 expression than done above, in that only *very* high Sca1-expressing and *very* low CD48-expressing cells are selected (Figure 12), this new subset of BM cells (singles / PI^- / SP / $Sca1^{very\ high}$ / $CD48^{very\ low}$) is extremely enriched for Lin^- cells (99%), $cKit^+$ cells (93%) and even CD150 (86%) (Figure 13). Within this subset, 63% are $CD34^-$ (Figure 13).

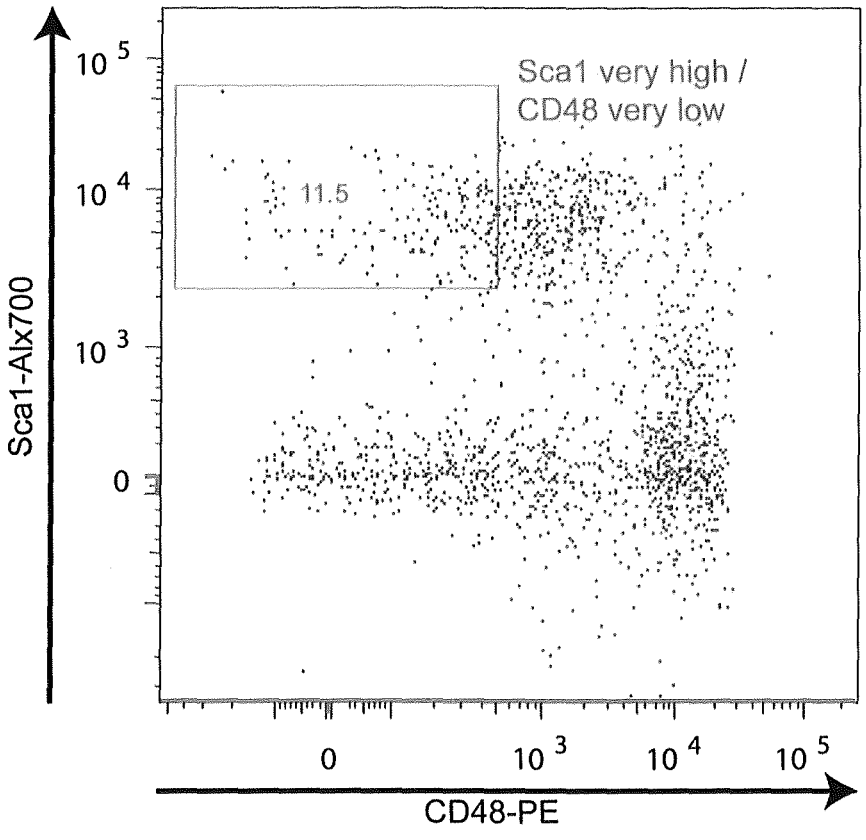

Figure 12. The definition of the $Sca1^{very\ high}$ $CD48^{very\ low}$ gate. Displayed events are pre-gated on singles, PI^- and SP. Alx, Alexa.

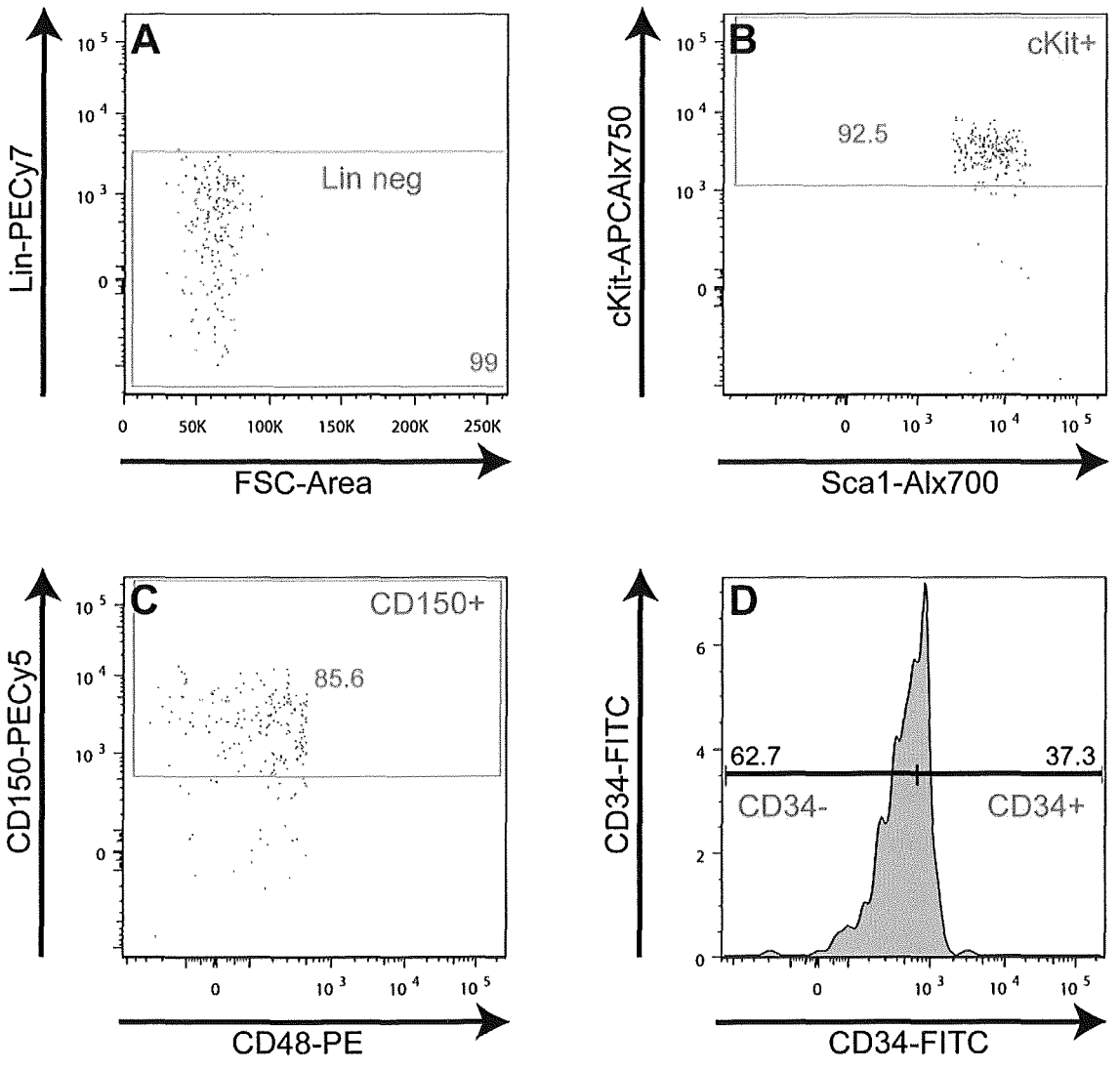

Figure 13. SP Sca1$^{very\ high}$ CD48$^{very\ low}$ cells are highly enriched for Lin$^-$ **(A)**, cKit$^+$ **(B)** and CD150$^+$ cells **(C)** and significantly enriched for CD34$^-$ cells **(D)**. All events displayed are pre-gated on singles, PI$^-$, SP, Sca1$^{very\ high}$ and CD48$^{very\ low}$ cells. FSC, forward scatter; Alx, Alexa.

DISCUSSION

The first successful enrichment for HSC activity from total mouse BM was reported in 1988, having been achieved by sorting cells previously stained with fluorescence-coupled antibodies against surface markers.[46] In the last 20 years, this pioneering work was refined by a number of laboratories, and, in the meantime, the strategies to enrich for putative HSC activity are plentiful, each laboratory having its favourite one(s).[8 14 19 47] Although the different approaches result in significantly overlapping populations, there are also some differences, and, therefore, this wealth of strategies can be problematic when attempting to compare different publications involving the isolation of HSCs.

As such, the choice of surface markers we used for the presented research also reflects, to some extent, the history within our laboratory, and, although they are well-established mouse HSC markers, it should be kept in mind that they are not the only ones. Other commonly used mouse HSC markers include Thy1.1[48] (which is strain-specific) and Flk-2[49], the absence or low expression of which excludes not only fully differentiated cells but also more committed progenitors, just as is said to be the case for the Lin markers and CD34. Furthermore, Tie-2[33] and endoglin[50] are other examples of markers expressed at high levels on the surface of HSCs, and, finally, CD244[16] is another SLAM family member that seems to be absent on HSCs and can be used for negative selection, similar to CD48.

Long-Term Reconstitution Ability of Different HSC Subsets

As outlined above, one of the aims of this study was to test whether functional HSCs are indeed $CD34^-$ or whether this is only a requirement for homing of HSCs to their niche after IV transplantation, which is typically used in the LTR assay. We therefore attempted to transplant putative HSCs directly into their physiological niche by IF injection. This transplantation technique was recently used to identify a new class of human HSCs, capable of more rapid multi-lineage and long-term reconstitution in the severe combined immunodeficiency (SCID) repopulating (SRC) xenotransplant assay than control HSCs injected IV.[11]

We used sorted LSK CD48$^-$ CD150$^+$ CD34$^-$ cells as a positive (HSC) control for both IV and IF injections and LSK CD48$^+$ CD150$^-$ CD34$^+$ as a negative control. LSK CD48$^-$ CD150$^+$ CD34$^+$ represented the actual "test" cells: We wanted to examine their ability to engraft, as assessed by long-term analysis of peripheral granulocyte chimerism, when injected IF, in contrast to when injected IV.

Both controls from the first experiment presented above (Figure 3) seem to have worked as anticipated: The negative control-injected cells were unable to engraft, although the recipient mice survived even at long-term, which may be explained by insufficient HSC depletion of the rescue BM (see above). The LSK CD48$^-$ CD150$^+$ CD34$^-$ (positive HSC control) cell-injected mice engrafted both IV and IF, and, moreover, IF transplantation resulted in more rapid and more effective engraftment than IV transplantation, consistent with what has been reported for the human SRC assay.[11] LSK CD48$^-$ CD150$^+$ CD34$^+$ cells, however, were unable to engraft whether IF nor IV, thus providing no evidence for the validity of our hypothesis that CD34 surface expression specifically interferes with homing of putative HSCs to the BM.

Regrettably, as illustrated above (Figure 4), the second experiment did not provide any conclusive results. The higher frequency of donor granulocytes within the positive control IV-injected subgroup as compared to the first experiment may be explained by a slightly delayed administration of more efficiently HSC-depleted rescue BM (see materials & methods). The pattern of results, however, is rather puzzling and leaves us with the unanswered question as to how well the staining and/or sorting of the donor cells actually worked during this experiment.

Given the low numbers and therefore only preliminary results of the first and the non-conclusive results of the second experiment, it is, at this point, impossible to definitely confirm or reject our initial hypothesis. In order to obtain more conclusive results, more LTR assays would have to be performed. However, in neither of the experiments were IF-injected LSK CD48$^+$ CD150$^-$ CD34$^+$ cells able to engraft, and, therefore, we think it is unlikely that they would do so in an additional experiment using the same conditions.

Regarding the deaths reported in Table 2, it is worth noticing that, summarising the two experiments, 24% of IF-injected mice died within the first 15 days, in contrast to only 8% of

the IV-injected ones, suggesting that injecting mice IF is more traumatic. This may be due a combination of the trauma itself, of trauma-related haemorrhage, of administration of morphinic analgesia before and of the general anaesthesia used during the procedure.

It should be mentioned that CD34 expression on mouse HSCs has been discussed in a rather controversial manner in recent years. Not only is there compelling evidence that, in contrast to the dormant ones, activated HSCs are $CD34^+$ (see above), but, moreover, CD34 surface expression also seems to undergo changes during development. HSCs within the mouse foetus and till at least 5 weeks of age have been reported to be exclusively $CD34^+$, and only from 7 weeks of age has long-term HSC activity been reported within the $CD34^-$ fraction of BM cells.[51-53] This situation, however, as it occurs in unborn and young mice, seems reversed in the adult mouse – from 10 weeks of age onwards, the majority of HSCs are $CD34^-$.[53] Finally, it has even been suggested that the observed exclusivity of long-term HSC activity within the LSK $CD34^-$ subset is an artefact of the lineage-negative enrichment protocol used prior to cell sorting.[54] In fact, some limited HSC activity was indeed previously documented within the Lin^- fraction of cells,[55] and more recent evidence even explicitly suggests that lineage depletion may inadvertently discard important populations of $CD34^+$ HSCs.[56] At the same time, however, sorting even just the number of cells required for the experiments presented in this study justifies an enrichment protocol, as the frequency of the studied cell phenotypes would be much too low and the sorting procedure would take by far too much time in its absence.

The last comment on the LTR assays concerns the follow-up studies of the recipient mice after transplantation. We are totally aware that analysis of peripheral granulocyte chimerism is clearly not sufficient to prove multi-lineage and long-term engraftment ability of the transplanted cells, but that a more detailed analysis of donor-derived haematopoietic contribution within the recipient mice (by analysis of the stem and progenitor cell pool as well as of multiple lineages) and even serial transplantations would be necessary to achieve this goal. Given our preliminary and partially contradicting results, however, we believe that further long-term and costly analysis at this point was not going to lead to any more conclusive results.

Side Population Phenotype of Different HSC Subsets

As mentioned above, some widely used approaches to enrich for HSC activity rely on alleged functional properties of HSCs such as the capacity to exclude the fluorescent dye Hoechst 33342 and to form an SP on a Hoechst red versus blue bivariate FACS plot. Given the confirmed correlation between long-term HSC activity and SP activity, one could even consider using the latter as a preliminary functional test for HSCs. Therefore, we set out to perform 8-colour FACS analyses, comparing the various BM cell subsets described above with their ability to form an SP and thereby also attempting to examine the overlap between different currently used methods to isolate putative HSCs.

As discussed above, this approach indeed allowed us to validate some of the markers commonly used to isolate HSCs, but, simultaneously, it also led us to question others. Moreover, it prompted us to propose a new HSC isolation strategy (SP / Sca1$^{very\ high}$ / CD48$^{very\ low}$) that might lead to similar or even better reconstitution efficiencies than the ones achieved so far, while enabling the use of substantially less antibodies and thus allowing HSC isolation by less complicated FACS experiments. This result being only very preliminary, however, this hypothesis obviously needs confirmation by assaying reconstitution activity *in vivo*.

Furthermore, within the LSK CD48$^-$ CD150$^+$ fraction, there seems to be a significantly higher correlation between the SP and CD34$^-$ cells than between the SP and CD34$^+$ cells. Considering the idea that ABC transporters are thought to protect normal and cancer stem cells by preventing intracellular accumulation of toxic compounds,[57] it seems plausible that, the more undifferentiated and dormant a stem cell is, the more protection it might need and the more active its transporters might be. As such, the above correlation is indeed consistent with the hypothesis that CD34$^-$ cells are more primitive and/or dormant than CD34$^+$ cells. Interestingly, it has been reported that this correlation is even more significant if mice aged below 10 weeks are used.[26] This observation may be due to differential CD34 expression during development (see above), but may also be related to the fact that the SP phenotype seems to vary quite dramatically between BM cell samples of mice of different ages.[58 59]

At this point, it should be reminded that the SP technique does have some caveats. Firstly, although there are undeniably significant correlations, in terms of HSC surface marker expression, SP cells seem to be very heterogeneous. For example, only 30% of SP cells belong to the LSK subset (Figure 11). This heterogeneity could be partly explained by the fact that the SP phenotype does most likely not just depend on dye efflux but also on uptake, the kinetics of which is probably entirely unrelated to the ability of SP cells to act as stem cells.[60]

A major problem of the Hoechst 33342 dye is its significant toxicity.[30 61 62] It has even been suggested that the cells that stain poorly (either because they have not imported or because they have exported the toxic dye) display a higher reconstitution capacity because they remain more viable and therefore more functional than the remainder of the cells.[63] Although this is vividly discussed at conferences, to our knowledge, so far, no *in vivo* data clearly addresses and obliterates these concerns. An alternative, therefore, when aiming at transplanting cells isolated on the basis of their SP phenotype, is to use other, less toxic dyes such as the mitochondria-binding dye rhodamine-123.[64]

Furthermore, the requirement of an (expensive) UV laser for SP analysis using Hoechst 33342 increases the urge to look for alternative dyes. In this context, one group recently reported the successful use of DyeCycle Violet (DCV) as a replacement for Hoechst 33342 on instruments with violet lasers.[65]

Finally, as mentioned above, an important issue in stem cell biology (but not only) is the reproducibility of results. The SP technique has indeed shown certain limitations due to the difficulty to produce comparable results in different laboratories – not only is the technique extremely delicate and sensitive to the slightest changes in the experimental conditions, but it also requires rather advanced FACS instrumentation knowhow. Following precisely a well-established protocol is therefore primordial when working with the SP technique.

CONCLUSION & OUTLOOK

In this study, we first analyse the ability of different HSC subsets to reconstitute lethally irradiated mice, when injected either IV or IF. Although the results from our LTR assays using LSK $CD48^{-}$ $CD150^{+}$ $CD34^{-}$ cells support the idea that IF transplantation leads to faster and more effective engraftment than IV transplantation, we are unable to show that LSK $CD48^{-}$ $CD150^{+}$ $CD34^{+}$ cells are capable of engrafting specifically when injected IF. We therefore believe that our initial hypothesis – that CD34 expression on IV injected donor BM cells specifically hampers their homing to the recipient BM – is unlikely to be true. However, given the preliminary nature of our results, definite rejection of our hypothesis would require additional LTR assays.

Secondly, we compare the relative SP activity of different BM cell subsets, as defined by their expression profile of the above antigenic markers. The results of our flow cytometric analyses prompt us to question some of the markers commonly used to isolate mouse BM HSCs *ex vivo* and to propose a simplified new enrichment strategy. We suggest that SP $Sca1^{very\ high}$ $CD48^{very\ low}$ cells might lead to similar or even better reconstitution efficiencies than the ones achieved so far with more complicated purification schemes. It will therefore be interesting to test the *in vivo* functional capacity of these cells using LTR assays.

BIBLIOGRAPHY

1. Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? *Nat Med* 2001;7(4):393-5.

2. Weissman I. Stem cell research: paths to cancer therapies and regenerative medicine. *JAMA* 2005;294(11):1359-66.

3. Fuchs E, Segre JA. Stem cells: a new lease on life. *Cell* 2000;100(1):143-55.

4. Bonnet D. Haematopoietic stem cells. *J Pathol* 2002;197(4):430-40.

5. Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell* 1997;88(3):287-98.

6. Kollet O, Dar A, Lapidot T. The multiple roles of osteoclasts in host defense: bone remodeling and hematopoietic stem cell mobilization. *Annu Rev Immunol* 2007;25:51-69.

7. Shizuru JA, Negrin RS, Weissman IL. Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. *Annu Rev Med* 2005;56:509-38.

8. Ema H, Morita Y, Yamazaki S, Matsubara A, Seita J, Tadokoro Y, et al. Adult mouse hematopoietic stem cells: purification and single-cell assays. *Nat Protoc* 2006;1(6):2979-87.

9. Ford CE, Hamerton JL, Barnes DW, Loutit JF. Cytological identification of radiation-chimaeras. *Nature* 1956;177(4506):452-4.

10. Domen J, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Molecular medicine today* 1999;5(5):201-8.

11. Mazurier F, Doedens M, Gan O, Dick J. Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. *Nat Med* 2003;9(7):959-963.

12. Mazurier F, Doedens M, Gan O, Dick J. Characterization of cord blood hematopoietic stem cells. *Ann N Y Acad Sci* 2003;996:67-71.

13. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 1996;273(5272):242-5.

14. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 2002;297(5590):2256-9.

15. Uchida N, Dykstra B, Lyons KJ, Leung FY, Eaves CJ. Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Experimental Hematology* 2003;31(12):1338-47.

16. Kiel M, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison S. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 2005;121(7):1109-21.

17. Camargo FD, Chambers SM, Drew E, McNagny KM, Goodell MA. Hematopoietic stem cells do not engraft with absolute efficiencies. *Blood* 2006;107(2):501-7.

18. Ema H, Sudo K, Seita J, Matsubara A, Morita Y, Osawa M, et al. Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. *Dev Cell* 2005;8(6):907-14.

19. Dykstra B, Kent D, Bowie M, McCaffrey L, Hamilton M, Lyons K, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* 2007;1(2):218-29.

20. Wilson A, Oser GM, Jaworski M, Blanco-Bose WE, Laurenti E, Adolphe C, et al. Dormant and self-renewing hematopoietic stem cells and their niches. *Ann N Y Acad Sci* 2007;1106:64-75.

21. Sato T, Laver JH, Ogawa M. Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* 1999;94(8):2548-54.

22. Tajima F, Sato T, Laver JH, Ogawa M. CD34 expression by murine hematopoietic stem cells mobilized by granulocyte colony-stimulating factor. *Blood* 2000;96(5):1989-93.

23. Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* 1990;75(10):1947-50.

24. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;183(4):1797-806.

25. Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA* 1999;96(16):9118-23.

26. Pearce DJ, Ridler CM, Simpson C, Bonnet D. Multiparameter analysis of murine bone marrow side population cells. *Blood* 2004;103(7):2541-6.

27. Raaijmakers M. ATP-binding-cassette transporters in hematopoietic stem cells and their utility as therapeutical targets in acute and chronic myeloid leukemia. *Leukemia* 2007;21(10):2094-2102.

28. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997;3(12):1337-45.

29. Nadin BM, Goodell MA, Hirschi KK. Phenotype and hematopoietic potential of side population cells throughout embryonic development. *Blood* 2003;102(7):2436-43.

30. Uchida N, Fujisaki T, Eaves AC, Eaves CJ. Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype. *J Clin Invest* 2001;108(7):1071-7.

31. Challen GA, Little MH. A side order of stem cells: the SP phenotype. *Stem Cells* 2006;24(1):3-12.

32. Wu C, Alman B. Side population cells in human cancers. *Cancer Letters* 2008;268(1):1-9.

33. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004;118(2):149-61.

34. Yoshihara H, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* 2007;1(6):685-97.

35. Matsuzaki Y, Kinjo K, Mulligan RC, Okano H. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* 2004;20(1):87-93.

36. Weksberg D, Chambers S, Boles N, Goodell M. CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood* 2008;111(4):2444-2451.

37. Lee LA, Sergio JJ, Sykes M. Natural killer cells weakly resist engraftment of allogeneic, long-term, multilineage-repopulating hematopoietic stem cells. *Transplantation* 1996;61(1):125-32.

38. Weiss L, Bullorsky E, Ashkenazi YJ, Slavin S. Optimal time interval between myeloablative whole body irradiation and reconstitution with syngeneic bone marrow graft. *Bone Marrow Transplant* 1988;3(3):207-10.

39. Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest* 2000;106(11):1331-9.

40. Lapidot T. How do stem cells find their way home? *Blood* 2005;106(6):1901-1910.

41. Domen J, Weissman IL. Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med* 2000;192(12):1707-18.

42. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. *J Exp Med* 2000;192(9):1281-8.

43. Audet J, Miller CL, Rose-John S, Piret JM, Eaves CJ. Distinct role of gp130 activation in promoting self-renewal divisions by mitogenically stimulated murine hematopoietic stem cells. *Proc Natl Acad Sci USA* 2001;98(4):1757-62.

44. Bhattacharya D. Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. *Journal of Experimental Medicine* 2006;203(1):73-85.

45. Lin KK, Goodell MA. Purification of hematopoietic stem cells using the side population. *Meth Enzymol* 2006;420:255-64.

46. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988;241(4861):58-62.

47. Benveniste P, Cantin C, Hyam D, Iscove N. Hematopoietic stem cells engraft in mice with absolute efficiency. *Nat Immunol* 2003;4(7):708-713.

48. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1994;1(8):661-73.

49. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci USA* 2001;98(25):14541-6.

50. Chen CZ, Li M, de Graaf D, Monti S, Göttgens B, Sanchez MJ, et al. Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells. *Proc Natl Acad Sci USA* 2002;99(24):15468-73.

51. Sánchez MJ, Holmes A, Miles C, Dzierzak E. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* 1996;5(6):513-25.

52. Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* 1997;7(3):335-44.

53. Ito T, Tajima F, Ogawa M. Developmental changes of CD34 expression by murine hematopoietic stem cells. *Experimental Hematology* 2000;28(11):1269-73.

54. Ogawa M. Changing phenotypes of hematopoietic stem cells. *Experimental Hematology* 2002;30(1):3-6.

55. Szilvassy SJ, Cory S. Phenotypic and functional characterization of competitive long-term repopulating hematopoietic stem cells enriched from 5-fluorouracil-treated murine marrow. *Blood* 1993;81(9):2310-20.

56. Ishida A, Zeng H, Ogawa M. Expression of lineage markers by CD34+ hematopoietic stem cells of adult mice. *Experimental Hematology* 2002;30(4):361-5.

57. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer.* 2005;5(4):275-84.

58. Pearce D, Bonnet D. Ageing within the hematopoietic stem cell compartment. *Mech Ageing Dev* 2008.

59. Pearce DJ, Anjos-Afonso F, Ridler CM, Eddaoudi A, Bonnet D. Age-dependent increase in side population distribution within hematopoiesis: implications for our understanding of the mechanism of aging. *Stem Cells* 2007;25(4):828-35.

60. Ibrahim S, Diercks A, Petersen T, Vandenengh G. Kinetic analyses as a critical parameter in defining the side population (SP) phenotype. *Experimental Cell Research* 2007;313(9):1921-1926.

61. Fried J, Doblin J, Takamoto S, Perez A, Hansen H, Clarkson B. Effects of Hoechst 33342 on survival and growth of two tumor cell lines and on hematopoietically normal bone marrow cells. *Cytometry* 1982;3(1):42-7.

62. Van Zant G, Fry CG. Hoechst 33342 staining of mouse bone marrow: effects on colony-forming cells. *Cytometry* 1983;4(1):40-6.

63. Sales-Pardo I, Avendaño A, Barquinero J, Domingo JC, Marin P, Petriz J. The Hoechst low-fluorescent profile of the side population: clonogenicity versus dye retention. *Blood* 2006;108(5):1774; author reply 1774-5.

64. Lo KC, Brugh VM, Parker M, Lamb DJ. Isolation and enrichment of murine spermatogonial stem cells using rhodamine 123 mitochondrial dye. *Biol Reprod* 2005;72(3):767-71.

65. Telford WG, Bradford J, Godfrey W, Robey RW, Bates SE. Side population analysis using a violet-excited cell-permeable DNA binding dye. *Stem Cells* 2007;25(4):1029-36.