JAM-A IS HIGHLY EXPRESSED ON HUMAN HEMATOPOIETIC REPOPULATING CELLS AND ASSOCIATES WITH THE KEY HEMATOPOIETIC CHEMOKINE RECEPTOR CXCR4

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Key words: JAM-A; CXCL12; CXCR4; vascular niche; hematopoietic stem/progenitor cells; NSG transplants.

ABSTRACT

Hematopoietic stem/progenitor cells (HSPCs) reside in specialized bone marrow microenvironmental niches, with vascular elements (endothelial/mesenchymal stromal cells) and CXCR4-CXCL12 interactions playing particularly important roles for HSPC entry, retention and maintenance. The functional effects of CXCL12 are dependent on its local concentration and rely on complex HSPC-niche interactions. Two Junctional Adhesion Molecule family proteins, JAM-B and JAM-C, are reported to mediate HSPC-stromal cell interactions, which in turn regulate CXCL12 production by mesenchymal stromal cells (MSCs). Here, we demonstrate that another JAM family member, JAM-A, is most highly expressed on human hematopoietic stem cells with *in vivo* repopulating activity. JAM-A blockade, silencing and overexpression show that JAM-A contributes significantly to the adhesion of human HSPCs to IL-1β activated human bone marrow sinusoidal endothelium. Further studies highlight a novel association of JAM-A with CXCR4, with these molecules moving to the leading edge of the cell upon presentation with CXCL12. Therefore, we hypothesize that JAM family members differentially regulate CXCR4 function and CXCL12 secretion in the bone marrow niche.

INTRODUCTION

The mammalian Junctional Adhesion Molecule (JAM) family is composed of eight members, three classical closely related proteins JAM-A, JAM-B and JAM-C, and five related non-classical members, JAM-4, JAML, endothelial cell-selective adhesion molecule-1 (ESAM-1), the coxsackie virus and adenovirus receptor (CAR), and CAR-like membrane protein (CLMP) [1-8]. These molecules are type 1 integral transmembrane proteins belonging to the Immunoglobulin Superfamily (IgSf), and contain two extracellular Ig-like domains, a single transmembrane domain and cytoplasmic domains of variable length [1-8]. The non-classical members diverge from the classical members in the structure of their cytoplasmic domains, with the latter possessing a short intracellular region of 40-50 amino acids incorporating a single C-terminal intracellular class II PDS95/Dig/ZO-1 (PDZ)-binding domain, and the former possessing longer intracellular domains, which (with the exception of JAML and CLMP) contain a C-terminal class I PDZ-binding domain [1-8].

Although JAM family proteins are expressed on diverse cell types and have important functions in angiogenesis, vascular permeability, tumor progression and leukocyte trafficking [1-8], recent studies demonstrate the expression of specific JAM family members on hematopoietic precursors and/or their putative microenvironmental niche elements, where they potentially play defined roles in regulating hematopoiesis. In the mouse, *Jam-a, Jam-b, Jam-c, Jam-4* and *Esam-1* are expressed by hematopoietic progenitor cells. Sugano *et al.* [9] reported the high expression of Jam-a on murine hematopoietic stem/progenitor cells (HSPC) during embryonic (aorta-gonad-mesonephros $CD34^+kit^+$ cells) and fetal development, on LSK ($Lin^-Sca\text{-}1^+kit^+$) cells from murine fetal liver, on murine bone marrow LSK cells with *in vivo* repopulating activity and on approximately 25% of enriched murine bone marrow $CD34^-$LSK or $CD150^+CD48^-CD244^-$ HSPC subsets. Jam-a is also found on *in vitro* clonogenic murine multipotent and a proportion of committed hematopoietic progenitor cells [9]. Transcriptional profiling and gene knock-out studies have identified *Jam-b* and *Jam-c* transcripts in enriched putative murine HSCs [10,11]. Of note, *Jam-b* transcripts are low in, but restricted to, murine $CD34^-$LSK HSPCs, even though *Jam-b* deficient mice do not demonstrate overt hematological abnormalities at least during embryonic development and in young adults [11]. Praetor *et al.* [12] further demonstrated that Jam-c is also highly expressed by the murine bone marrow Lin^- LSK enriched long and short term *in vivo* repopulating HSPC subsets, with reduced expression on more committed common myeloid progenitor (CMP), common lymphoid progenitor (CLP),

granulocyte-macrophage progenitor (GMP) and megarkaryocyte-erythroid progenitor (MEP) subsets. It is also highly expressed by $Lin^{-}Sca\text{-}1^{+}AA4.1^{+}$ HSPCs from murine fetal liver [12]. Although less than one third of *Jam-c*-deficient mice are viable beyond weaning, the surviving mice demonstrate increased bone marrow cellularity, resulting from increases in myeloid progenitor cells and granulocytes [12]. This increased myelopoiesis was not evident in *Jam-c*$^{-/-}$ murine fetal liver [12], indicating that *Jam-c* is highly expressed on murine HSPCs and modulates murine bone marrow myelopoiesis. Further studies by Arcangeli *et al.* [13] revealed that Jam-c antibody blockade on murine bone marrow LSK HSPCs partially reduced their homing to and 12 week hematological reconstitution of bone marrow post-irradiation, a process involving the interaction of Jam-c on murine HSCs with Jam-b on murine mesenchymal stromal cells [13].

With respect to non-classical JAM family members, transcriptome analyses have detected *Esam-1* expression in $Thy1.1(CD90)^{lo}Flk2^{-}LSK$, $Thy1.1^{lo}Flk2^{+}LSK$ and $Thy1.1^{-}Flk2^{+}LSK$ murine adult bone marrow HSPC subsets enriched for long-term HSC, short-term HSC and multipotent progenitor (MPP) subsets respectively [14]. Isolation of murine HSCs with Esam-1 improves HSC purity when used in place of such markers as c-kit, and Esam-1 deficient mice have more HSCs than their wild type counterparts [15]. The *Esam-1* transcript is highly expressed and a durable HSC marker throughout murine fetal and adult life [16]. Most $Esam\text{-}1^{hi}$ murine HSCs were found located near bone marrow perivascular niches post 5FU treatment, and *Esam-1* deficient mice displayed a prolonged lack of hematopoietic reconstitution post 5-FU treatment [17]. It has been suggested that Esam-1 expression is a major indicator of murine HSC activation, with the $Esam\text{-}1^{hi}$ HSC subset maintaining its stemness even after entering the cell cycle [17]. JAM4 is another non-classical JAM molecule expressed on both murine $CD34^{-}$ and $CD34^{+}$ KSL bone marrow HSPCs, although Jam-4 deficient mice do not display overt hematological abnormalities [18].

Although important in murine hematopoiesis, fewer studies have been conducted on the JAM family during human hematopoiesis. In the human, JAM-A, JAM-C and ESAM-1 are expressed by $CD34^{+}$ or $CD133^{+}$ HSPCs from cord blood or mobilised peripheral blood [15,19-21] with JAM-C expression being detected on human $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ HSPC and more immature $Lin^{-}CD41^{-}CD38^{-}CD34^{+}CD45RA^{-}CD90^{+}$ HSC and ESAM-1 on the human $Lin^{-}CD34^{+}CD38^{-}CD90^{+}$ HSCs [15,19]. Certain JAM family members may also potentially play important roles on HSPC microenvironmental niche cells. For example, *JAM-B* and *JAM-C* are found in human bone marrow

$CD146^+$ mesenchymal stromal cells and recombinant human (rh) JAM-C can partially inhibit human $CD34^+$ HSPC adhesion to MSCs, but not to osteoblasts or human umbilical vein endothelial cells (HUVEC) [13]. Since CXCL12-CXCR4 interactions play a key role in the retention of HSC in the bone marrow HSC niche [22], and since *Jam-b* deficient mice show increased CXCL12 secretion in the bone marrow [23], the interaction of JAM-C on HSPCs with JAM-B on MSCs may modulate CXCL12 production by MSCs and hence enhance their quiescence and retention within the bone marrow compartment [23].

In this manuscript, we have sought to examine the expression and function of JAM-A on normal human umbilical cord blood (UCB) HSPC subsets. Our studies demonstrate that *in vivo* repopulating human HSC express high levels of JAM-A, that JAM-A principally mediates adhesion of human HSPCs to bone marrow endothelial cells and that JAM-A associates with the CXCL12 receptor CXCR4 on human UCB HSPCs.

MATERIALS AND METHODS

Detailed Materials and Methods are presented in Supplementary data.

Human cells and *in vivo* studies

Human umbilical cord blood (UCB) was collected from the John Radcliffe Hospital, Oxford, UK and used with informed, written pre-consent and ethical approval from the South Central Oxford C and Berkshire Ethical Committees and approval of the NHSBT R&D committee. Mononuclear cells (MNCs; density <1.077g/ml) were CD34 or CD133 enriched using direct microbead kits (Miltenyi Biotec) and used fresh or cryopreserved until use [24-26]. Where indicated before or after thawing, $CD34^+$ or $CD133^+$ cells were cultured in StemSpan or StemSpan ACF medium (Stem Cell Technologies) with 100 ng/ml rh Flt-3L, IL-6 and SCF, and 20 ng/ml rh TPO (all from R&D Systems) overnight prior to assay [24,25]. Human BMEC-60 [27,28] and bone marrow mesenchymal stem/stromal cells were cultured and characterized as described in Supplementary Figures S1, S2 and S3 and in [20,27-30]. Protocols for these and details of hematopoietic cell lines and *in vivo* studies [31] are described in Supplementary Materials and Methods.

Antibodies and flow cytometry

Flow cytometric monoclonal antibodies and protocols are described in the Supplementary Materials and Methods and Supplementary Table S1 [24,25,31-36].

CXCL12 staining of and production by BMEC-60 cells

CXCL12 staining of BMEC-60 cells, and ELISAs [28,29] to quantitate CXCL12 produced by BMEC-60 and bone marrow stromal cells are detailed in Supplementary Materials and Methods.

Adhesion and migration assays

Adhesion assays of HL-60, Jurkat and UCB $CD34^+$ cells to BMEC-60 cells, the migration of UCB $CD34^+$ cells across BMEC-60 cells, and JAM-A antibody blockade, over-expression or RNAi knock-down studies are described in Supplementary Materials and Methods and Supplementary Table S2 and are based on previous studies [24,25,37,38].

Co-immunoprecipitation-Western blotting studies and immunofluorescence co-localisation and proximity ligation assays.

For Western blotting, total protein lysates (45-50 μg) were probed with specific antibodies as described in Supplementary Materials and Methods. Co-immunoprecipitation combined with Western blotting of HEK293T cells over-expressing human JAM-A-FLAG and human CXCR4-HA were used to demonstrate that JAM-A interacts with CXCR4. The co-localization of CXCR4 with JAM-A at the leading edge of the cell after CXCL12 stimulation was demonstrated using immunofluorescence microscopy [24] and the Duolink™ in situ Proximity Ligation Assay (PLA). Details for each method are described in the Supplementary Materials and Methods.

RESULTS

JAM-A is highly expressed on *in vivo* repopulating human cord blood hematopoietic stem cells

To study the surface expression of JAM-A on human UCB HSPCs, an anti-JAM-A monoclonal antibody (mAb), M.Ab.F11, was used to probe both freshly isolated mononuclear cells (MNCs) as well as immunomagnetically enriched $CD133^+$ and $CD34^+$ cells (purity: 87.5±2.4% $CD133^+$ and 95.4±2.3% $CD34^+$). In the initial experiments, the latter had been cryopreserved, thawed and cultured in serum free Stemspan medium with SCF, IL-6, TPO and Flt-3L for 24 hours prior to analysis. These subsets were used because the $CD133^+$ fraction of human UCB contains all *in vivo* repopulating HSC as measured in surrogate models of hematopoiesis, and the $CD34^+$ fraction contains most HSC, multipotent lymphoid myeloid progenitors (MMP) and GMP cell subsets [39-43], while the $CD34^+CD133^-$ subset also contains MEP [43]. In the freshly isolated MNCs, 100±0% $CD133^+$ cells and 90.4±6.5% $CD34^+$ cells co-expressed JAM-A and these comprised 0.2±0.1% and 0.6±0.4% of the MNCs respectively. Of the purified cells, all $CD133^+$ cells (100±0%; M.F.I. 5017±378) and 96.9±1.6% (M.F.I. 1381±196) of $CD34^+$ cells were JAM-A^+ (Figures 1A and 1B; n=3-6 independent experiments). Differential expression of JAM-A was analyzed on the purified $CD34^+$ cells, with the bulk of these cells demonstrating on average higher JAM-A positivity (P2; 1220.0±187.7 M.F.I.) than the more highly CD34 positive cells (P4; 695.0±78.0 M.F.I.) and the $CD34^{lo}$ cells (P3; 270.3±47.2 M.F.I.; n=3; Figure 1C). Using multiparameter flow cytometric analyses of the human UCB $CD34^+$ cells, we next examined JAM-A expression on phenotypically defined $CD34^+$ HSPC subsets. For these experiments,

we compared freshly isolated cells with cells that had been cryopreserved and then thawed and either not cultured or cultured for 20-24h with the cytokines, SCF, TPO, Flt-3L and IL-6. The viable HSPC subsets examined were defined phenotypically based on recent studies [31-36] as hematopoietic stem cells (HSC): $Lin^-CD38^-CD34^+CD45RA^-CD90^+CD49f^+$; multipotent progenitor cells (MPP): $Lin^-CD38^-CD34^+CD45RA^-CD90^-$; common lymphoid progenitor cells (CLP): $Lin^-CD38^-CD34^+CD45RA^+CD90^-$; common myeloid progenitor cells (CMP): $Lin^-CD38^+CD34^+CD45RA^-CD123^+$; granulocyte-macrophage progenitor cells (GMP): $Lin^-CD38^+CD34^+CD45RA^+CD123^+$; and megakaryocyte-erythroid progenitor cells (MEP): $Lin^-CD38^+CD34^+CD45RA^-CD123^-$ (Figure 2A). Between 93 to 100% of cells in these HSPC subsets expressed JAM-A (Figures 2B, 2C and 2D). Stimulation of the freshly isolated $CD34^+$ HSPCs with cytokines enhanced JAM-A expression most significantly in the HSC subset (M.F.I. of 2184±181 versus 3952±321 respectively, n=5; $p<0.001$; Figure 2E). This equated to a 1.85±0.20 fold enhancement in JAM-A expression with cytokines for HSC. A similar enhancement in JAM-A expression on thawed HSC was observed after 20-24h in cytokines (M.F.I. 2366±90 versus 4087±198 respectively or 1.73±0.04 fold increase, n=3; $p<0.001$; Figure 2F) although all HSPC subsets tested showed enhanced JAM-A expression after cytokine stimulation of the thawed $CD34^+$ cells (2F). Given the benefits of upregulated JAM-A expression upon cytokine stimulation and unless otherwise specified, the UCB HSPCs were cultured with cytokines SCF, TPO, Flt-3L and IL-6 for 20-24h prior to analysis and are therefore referred to as cultured $CD34^+$ cells.

To confirm that the repopulating HSC were present in the most positive JAM-A fraction, cultured UCB $CD34^+$ cells were sorted into JAM-A high, intermediate, and low expressing subfractions, representing 13.2±1.15, 76.9±1.2 and 5.6±1.2% of $CD34^+$ cells respectively (Figure 3A) and their ability to engraft NSG mice was assessed. To compare the rates of engraftment in the sorted subfractions, NSG mice injected with comparable numbers of cells ($5x10^4$) from each subfraction were assessed whenever possible (Figure 3B). However, the number of $CD34^+$/JAM-A^{High} cells inoculated ranged from 1.5-$5x10^4$ cells (Figure 3C). Human $CD45^+$ cells were first detected in peripheral blood (PB) aspirates of recipients of $CD34^+$/JAM-A^{High} cells at week 4 and the levels of engraftment steadily increased thereafter, irrespective of whether lower cell numbers were injected. $CD34^+$/JAM-A^{Int} cells ($5x10^4$) took another week to engraft and PB human $CD45^+$ levels did not exceed 4.2%, observed at week 7. Human cells could not be detected in mice inoculated with $CD34^+$/JAM-A^{Low} cells until week 4-5 and levels remained very low in the PB throughout (Figure 3B).

In order to compare the extent of NSG engraftment, mice were inoculated with a range of JAM-A sorted subfractions (Figure 3C). At week 15, the highest levels of bone marrow engraftment were obtained using $CD34^+/JAM\text{-}A^{High}$ cells (2-73% $CD45^+$ with 1.5-5x10^4 cells injected, Figure 3C). These levels were significantly higher than those obtained using 2x10^4-10^5 $CD34^+/JAM\text{-}A^{Int}$ cells (0-19.2% $CD45^+$) and using 1.5-5.6x10^4 $CD34^+/JAM\text{-}A^{Low}$ cells (0-0.6% $CD45^+$, p=0.008). JAM-A expression was retained on the cells recovered from murine bone marrow (Figure 3C). Engrafted cells also expressed human CD19 (0.39-28% in $JAM\text{-}A^{Int}$ and 5-65% in $JAM\text{-}A^{High}$ recipients). Modest levels of $CD34^+$ cells were detected in engrafted recipients (0.2-1.7% in the $JAM\text{-}A^{Int}$ fraction and 0.2-16% in the $JAM\text{-}A^{High}$ fraction), with similar levels of $CD33^+$ cells (0.4-3% in the $JAM\text{-}A^{Int}$ fraction and 2-14% in the $JAM\text{-}A^{High}$ fraction) detected. Cells ($JAM\text{-}A^{Int}/JAM\text{-}A^{High}$) recovered from murine bone marrow from 8 samples were transplanted into secondary animals to assess self-renewal ability. Cells were not re-sorted prior to inoculation into secondary recipients but they received equivalent numbers of human cells as their primary counterparts. Engraftment levels in secondary recipients were at least equivalent to those observed in the primary recipients and higher in several animals (Figure 3D), demonstrating self-renewal capacity of $JAM\text{-}A^+$ cells. Median engraftment in secondary recipients of $JAM\text{-}A^{Int}$ cells was 12.1% $CD45^+$ (range 3-30%), while in corresponding primary animals it was 10.3 % (range 5-19.3%, p=0.17). Likewise, engraftment was higher in secondary recipients of $JAM\text{-}A^{High}$ cells (median 30%, range 11.3-78%) compared with primary animals (median 26.5, range 5.9-72.6%, p=0.15). Expression of JAM-A, CD19, CD33 and CD34 on bone marrow cells recovered from the secondary recipients was similar to that of primary NSG mice (p≥0.72).

JAM-A is expressed on human bone marrow endothelial, but not mesenchymal stem/stromal cells

The expression of JAM-A was next examined on representative cultured human bone marrow niche cells (viz. the mesenchymal stromal cells: BMSCs that support LTC-IC and bone marrow MSCs containing tri-lineage cells, and the bone marrow sinusoidal endothelial cell line BMEC-60 before and after IL-1β activation). Of these, only BMEC-60 cells expressed JAM-A (Supplementary Figure S4A). Although there was a slight decrease in JAM-A expression on BMEC-60 cells after IL-1β activation, this did not reach statistical significance for either cell surface (p=0.584) or total JAM-A expression (p =0.124; Supplementary Figure S4B). Of note, BMEC-60 cells remained LFA-1 (αLβ2 integrin;

CD11a/CD18) negative, became CD62E (E-selectin) positive, and demonstrated enhanced CD54 (ICAM-1) expression and decreased CD51/CD61 ($\alpha v\beta 3$ integrin) cell surface expression after IL-1β activation (Supplementary Figure S3), characteristics typical of bone marrow sinusoidal endothelium.

JAM-A promotes CD34$^+$ human UCB cell adhesion to bone marrow endothelial cells

The HL-60 hematopoietic progenitor cell line, like UCB CD34$^+$ cells and BMEC-60, expresses JAM-A (Supplementary Figure S5A) and was used as a model for subsequent UCB CD34$^+$ cell functional analyses *in vitro*.

First, JAM-A was examined for its role in mediating adhesion of HL-60 cells to BMEC-60 using siRNA knockdown and an anti-JAM-A blocking antibody. JAM-A knockdown, when optimised in HL-60 cells, was shown to be most effective with JAM-A specific siRNAs 150 and 151 at 24 hours post-nucleofection when compared to a non-silencing (NS) control siRNA (Supplementary Figure S6A and S6B). Significantly more untreated HL-60 cells (30.3±2.0%) adhered to IL-1β activated BMEC-60 cells than to control BSA (1.9±0.8%; $p<0.01$, Figure 4A left). Anti-JAM-A antibody blockade and JAM-A siRNA knock-down reduced HL-60 cell adhesion to IL-1β activated BMEC-60 cells by 50.3±15.1% ($p<0.05$) and 41.5±15.0% ($p<0.05$) respectively (Figure 4A middle and right). Similarly, as shown in Figure 4B (left), 33.7±3.2% cultured UCB CD34$^+$ cells adhered to IL-1β stimulated BMEC-60 cells ($p<0.01$ compared to BSA). JAM-A siRNA knock down in these UCB CD34$^+$ cells resulted in a 71.3±10.4% decrease in cell surface JAM-A expression (based on M.F.I. in NS control vs the JAM-A siRNA nucleofected cells 24 hours after nucleofection; $p<0.05$, Supplementary Figure S6C). This JAM-A knock-down in UCB CD34$^+$ cells reduced their cell adhesion to IL-1β-pre-treated BMEC-60 cells by 43.0±11.1% ($p<0.01$ for JAM-A siRNA vs. NS siRNA treated cells, Figure 4B, right). Addition of JAM-A blocking antibody also reduced UCB CD34$^+$ cell adhesion to IL-1β-pre-treated BMEC-60 cells (by 28.5±8.4%, $p<0.01$; Figure 4B, middle).

To further confirm these effects on adhesion, JAM-A was overexpressed in the JAM-A-null Jurkat cell line and the adhesion of JAM-A expressing or non-expressing Jurkat cells to IL-1β stimulated BMEC-60 cells examined. After transduction, JAM-A was highly expressed in 70.6±8.6% of the stably transduced Jurkat cells compared to 0.4±0.2% and 0.3±0.1% in Jurkat cells which were not transduced or transduced with empty lentiviral vector respectively (respective M.F.I.s

for JAM-A cell surface expression by flow cytometry of 12006±2063, 76±8 and 65±6). Figure 4C shows that although Jurkat JAM-A–null cells could adhere to IL-1β stimulated BMEC-60 cells, their adhesion increased significantly by 59.5±19.1% after transduction with JAM-A ($p<0.05$).

JAM-A antibody blockade and knock down with specific siRNAs were next used to assess if JAM-A functioned in UCB $CD34^+$ cell migration across BMEC-60 towards CXCL12. JAM-A antibody blockade reduced $CD34^+$ cell trans-endothelial (across IL-1β stimulated BMEC-60) migration towards CXCL12 from 23.1±2.6% to 20.7±1.6% but this was not statistically significant ($p=0.210$, n=6; Supplementary Figure S7A). JAM-A siRNA 150 +151 knockdown also reduced UCB $CD34^+$ cell transendothelial (IL-1β stimulated BMEC-60) migration towards CXCL12 from 28.5±2.7% to 19.3±2.2%, without reaching statistical significance ($p=0.076$, n=4; Supplementary Figure S7B). Taken together, our studies suggest that a primary function of JAM-A on UCB $CD34^+$ cells is to contribute to cell adhesion to bone marrow endothelial cells.

The role of CXCL12 in promoting adhesion

BMEC-60 cells constitutively express low levels of CXCL12 (Supplementary Figure S4C), which is also produced, albeit much more highly, by bone marrow MSCs in the perivascular niche [28], (reviewed in [44]). We confirmed the levels in the BMEC-60 cell lysate and in BMEC-60 and bone marrow MSC secretome after 24 and 48 hours of *in vitro* culture and showed that bone marrow MSCs produced substantially more CXCL12 than BMEC-60 cells (Supplementary Table S3). HL-60 cells (Supplementary Figure S8), a proportion of freshly isolated UCB $CD34^+$ cells and the majority of UCB $CD34^+$ cells after overnight culture in Flt-3 ligand, TPO, SCF and IL-6 (Supplementary Figure S4D) express cell surface CXCR4, the cognate receptor for CXCL12. Notably, in three independent experiments, the M.F.I. for anti-CXCR4 flow cytometric staining of UCB $CD34^+$ cells increased from 60.0±17.8 before culture to 175.0±21.0 ($p<0.05$) after overnight culture when most cells co-expressed CXCR4 and JAM-A. Since the CXCR-4 transduced HPC line, KG1a, shows enhanced adhesion to activated BMEC-60 cells which have been coated with CXCL12 [38], we pre-coated IL-1β activated BMEC-60 cells with CXCL12 and observed that HL-60 cell adhesion to these BMEC-60 cells significantly increased (in these experiments from 28.6±2.3% to 40.2±6.2%; $p<0.05$; Figure 4D; left). Pre-treating HL-60 cells briefly (10 minutes at 37°C) with CXCL12 also enhanced HL-60 adhesion to

IL-1β activated BMEC-60 cells (from 28.6±2.3% to 48.8±4.5%; $p<0.01$; Figure 4D; left), although this was not further enhanced when adhesion was to IL-1β activated BMEC-60 cells pre-coated with CXCL12 (Figure 4D; left). When JAM-A was knocked down in HL-60 cells with siRNAs 150 and 151 for 24 hours prior to a brief 10 minute exposure of the HL-60 cells to CXCL12, their adhesion to IL-1β activated BMEC-60 cells was impaired (45.6±2.5% of NS siRNA vs 20.7±3.8% of JAM-A siRNA, $p<0.01$; Figure 4D; middle). Less striking results were observed for UCB $CD34^+$ cells. We found increased adhesion of cultured UCB $CD34^+$ cells (after a brief exposure to exogenous CXCL12 vs no CXCL12) to IL-1β activated BMEC-60 cells but this did not reach statistical significance ($p= 0.063$). Subset analyses of the HSPCs adhering to IL-1β activated BMEC-60 cells, without or with CXCL12 pre-coating, revealed a very small increase in the percentage of HSC, CMP, GMP and MEP adhering in the presence of CXCL12 (Supplementary Figure S9). Knock-down of JAM-A reduced adhesion of cultured UCB $CD34^+$ cells to BMEC-60 cells (significantly in the absence of CXCL12; $p<0.05$) and from 50.2±10.0% adhesion with NS siRNA to 26.4±4.8% with JAM-A siRNAs 150+151 in the presence of CXCL12 ($p=0.09$; Figure 4D; right).

JAM-A interacts with CXCR4 and modulates CXCR4 signaling on human UCB $CD34^+$ cells

As JAM-A and UCB $CD34^+$ cells adhere to IL-1β activated BMEC-60 cells and as CXCL12 and CXCR4 can promote HSPC retention in the bone marrow [22], we examined whether CXCR4 might interact with JAM-A. First, we examined if JAM-A could interact with CXCR4. Here, we transiently co-expressed HA-tagged CXCR4 with FLAG-tagged JAM-A in HEK293T cells, lysed the transfected cells with Brij-35 or Triton-X100 detergents and then immunoprecipitated CXCR4 with an anti-HA-antibody. This was followed by Western blotting the whole cell lysates or immunoprecipitated proteins with anti-JAM-A, anti-FLAG and anti-HA antibodies. Mock transfected HEK293T cells were used as the negative control. Notably, Figure 5 demonstrates that JAM-A-FLAG co-precipitated with CXCR4-HA when co-transfected into HEK293T cells, indicating the ability of these molecules to closely interact with each other.

Secondly and given that CXCR4 interacts with JAM-A in HEK293T cell transfectants in the absence of CXCL12 stimulation, we assessed if presentation of CXCL12 to HL-60 and cultured UCB $CD34^+$ cells would promote the re-localization of CXCR4 and JAM-A to the leading edge of the cell.

For this we used both polarized microscopic co-localization and proximity ligation assays (PLA). Due to limited supplies of UCB $CD34^+$ cells, assays were first optimized using HL-60 cells. As shown in Figure 6A, JAM-A moved and co-localized with CXCR4 to the leading edge of the HL-60 cells within the first 5-10 minutes post-CXCL12 exposure (**p<0.01; *p<0.05). When UCB $CD34^+$ cells were tested 10 minutes after their exposure to CXCL12, JAM-A was also found to co-localise with CXCR4 at the leading edge of the UCB $CD34^+$ cells in both immuno-fluorescence microscopy and PLA assays (Figure 6B). In these experiments, the number of human UCB $CD34^+$ cells demonstrating these PLA polarized interactions in the presence of CXCL12 ranged from 42 to 48% (**p<0.01; Figure 6B). The enhanced co-localization of the CXCR4 co-receptor CD164 [24] with both CXCR4 and JAM-A (**p<0.01) was also observed at this 10 minute time point, whereas ICAM-3 co-localization with JAM-A to the leading edge of the cell was not significantly different in the presence or absence of CXCL12.

Studies were further designed to determine whether JAM-A modulates CXCR4 signaling via the AKT, ERK1/2 or PKCζ pathways after a brief 30 to 60 second CXCL12 stimulation of HSPCs. As sufficient UCB $CD34^+$ cells were difficult to source and as we and others have confirmed that hematopoietic cells respond to CXCL12 by phosphorylating AKT and PKCζ [24] and ERK1/2 [24,45], the effects of JAM-A knockdown on these three kinases were first examined in HL-60 cells. Without JAM-A knock-down (NS), CXCL12 presentation to HL-60 cells led to AKT and ERK1/2 phosphorylation (***p<0.001 and *p<0.05/***p<0.001 respectively), but, while PKCζ signaling was also slightly increased, this was not statistically significant (p=0.13; Figure 7A and B; n=3 independent experiments). With JAM-A knockdown (*p<0.05; **p<0.01), increased phosphorylation of pAKT, pERK1 and pERK2 was also observed after CXCL12 stimulation compared to no CXCL12 stimulation (***p<0.001;*p<0.05). The levels of phosphorylated AKT decreased significantly when the JAM-A knockdown was compared with the NS control after CXCL12 stimulation (*p<0.05; Figure 7A and B). Although JAM-A knockdown also reduced pERK1/2 and pPKCζ levels, these were not statistically significant at this time point (p=0.184, p=0.243 and p=0.501 respectively; Figure 7A and B). In a confirmatory Western blot experiment with cultured UCB $CD34^+$ cells, knockdown of JAM-A in the presence of CXCL12 substantially reduced AKT and ERK1/2 phosphorylation (Figure 7C).

DISCUSSION

This is the first comprehensive report of the differential expression of the JAM-A protein on phenotypically defined human UCB HSPC subsets. We initially examined JAM-A protein expression on the bulk multipotent HSPC subsets defined as being $CD133^+$ or $CD34^+$ since the former contains all *in vivo* $CD34^+$ and $CD34^-$ repopulating HSC (as measured in surrogate models of hematopoiesis), as well as lymphomyeloid and GMP progenitor cell subsets but not MEP [41-43,46] while the $CD34^+$ subset contains the majority of HSCs, as well as MPP, lymphomyeloid, GMP and MEP subsets and also more lineage restricted $CD19^+$ B lymphoid and $CD7^+$ T lymphoid progenitor cells [31-36]. In an extension of earlier transcriptome studies from our laboratory [20] and that of Rossi *et al.* [21], which first identified JAM-A gene expression in human $CD133^+$ and $CD34^+$ cells respectively, and of the subsequent detection of JAM-A protein on human UCB and bone marrow $CD34^+$ cells [37], we observed that all the human UCB $CD133^+$ and the majority of human UCB $CD34^+$ HSPCs, whether freshly isolated or isolated and cultured overnight in Flt-3L, TPO, SCF and IL-6, expressed cell surface JAM-A protein. Within the $CD34^+$ cell subset, the highest JAM-A expression was found on the majority of intermediate to high $CD34^+$ cells.

This observation led us to examine the expression of JAM-A on the more primitive HSPC subsets in more detail. First, we demonstrated that the majority of phenotypically defined HSC, as well as their MMP, CLP, CMP, MEP and GMP progeny expressed JAM-A on their cell surface. Flow cytometric comparisons of each subset revealed that JAM-A expression could be enhanced with overnight culture in the cytokines SCF, TPO, Flt-3L and IL-6. When the cultured $CD34^+$ cell subset was sorted into the JAM-A^{High}, JAM-A^{Int} and JAM-A^{Low} expressing fractions and transplanted into NSG mice, PB $CD45^+$ cells derived from the JAM-A^{Low} and JAM-A^{Int} cells were detected at only low levels from week 4-5. In contrast, engraftment was detected in mice inoculated with JAM-A^{High} cells earlier than the JAM-A^{Int} cells and continued to increase with time. Notably bone marrow engraftment was significantly higher in recipients of JAM-A^{High} cells at termination, even when 2-6.7 fold fewer cells were injected. In addition, the levels of human lymphoid and myeloid cells detected were also higher in mice inoculated with JAM-A^{High} cells. Serial transplantation studies demonstrated that both JAM-A^{Int} and JAM-A^{High} cells were capable of self-renewal. However and notably, engraftment was higher in recipients of JAM-A^{High} cells, despite inoculating up to 10 fold fewer cells than administered to JAM-A^{Int} recipients. The engraftment levels achieved are comparable to those reported by Wiekmeijer *et al.*

[47] who injected 1-2x10^6 $CD45^+$ cells from primary NSG mice that had been originally transplanted with 5x10^4 or 1.5x10^5 $CD34^+$ cells, following an overnight period in culture, similar to the ones used in the present study. These data confirm our phenotypic observations that the highly expressing JAM-A^+ subset contains human *in vivo* repopulating HSPCs. Our results differ to some extent from those described for Jam-a expression on murine bone marrow HSPCs, where most murine bone marrow LSK HSPCs and around 25% of $CD34^-$LSK or $CD150^+CD48^-CD244^-$ HSPCs express Jam-a [9], while virtually all primitive HSPCs in human UCB express JAM-A. However, in both murine bone marrow [9] and human UCB, *in vivo* repopulating HSPCs assayed in murine models both express JAM-A.

Hematopoiesis is dependent on interactions of HSPCs with their microenvironmental niches, and recent studies suggest that most murine HSCs reside in a perivascular location, thus endothelial cells and MSCs are key niche components (reviewed in [44]). The human bone marrow sinusoidal endothelial cell line BMEC-60 (but not bone marrow MSCs) expresses JAM-A, which is known to act as a homophilic adhesion molecule (reviewed in [7]). Therefore we assessed the roles of JAM-A in hematopoietic cell adhesion to and transmigration across BMEC-60 cells. Based on the premises that i) bone marrow sinusoidal endothelium exists in an activated state [38], ii) activation of BMEC-60 cells with IL-1β is not associated with a significant change in their JAM-A protein expression, iii) *Cxcl12* deletion from murine bone marrow endothelial cells reduces HSC repopulating ability (reviewed in [44]), and iv) CXCL12 coating of BMEC-60 cells enhances adhesion of the CXCR4-transduced KG1a hematopoietic progenitor cell line to BMEC-60 cells [38], we also tested HSPC adhesion to IL-1β activated BMEC-60 cells with or without CXCL12 coating. We demonstrated that overexpression of JAM-A in JAM-A-null Jurkat cells enhanced their adhesion to IL-1β activated BMEC-60 cells. Both JAM-A expressing HL-60 cells and UCB $CD34^+$ cells adhered to IL-1β activated BMEC-60 cells and both the anti-JAM-A blocking antibody and JAM-A siRNA knockdown in HL-60 and UCB $CD34^+$ cells significantly reduced this adhesion to these IL-1β activated BMEC-60 cells. Coating IL-1β activated BMEC-60 cells with CXCL12 further enhanced this adhesion, as did a brief exposure of HL-60 or UCB $CD34^+$ cells to CXCL12, although this was significant only for HL-60 cells. A comparison of the effects of JAM-A blockade or knockdown on the migratory versus adhesive properties of HSPCs indicated that JAM-A functions preferentially in adhesion of these cells to BMEC-60 cells. Using HEK293T cells co-transfected with tagged human JAM-A and CXCR4 and co-immunoprecipitation-Western blotting

techniques, we were able to demonstrate that these molecules interact with one another. Furthermore, both co-localization and PLA assays indicated that a brief exposure of HSPCs to CXCL12 promoted the movement of CXCR4 with JAM-A to the leading edge of the cell, while JAM-A knockdown reduced CXCL12 mediated signaling via CXCR4, most significantly and most rapidly via the Akt pathway.

The chemokine, CXCL12, and its receptor, CXCR4, are critical for HSPC retention in bone marrow niches [22]. Earlier research has also demonstrated that CXCL12 concentrations can differentially affect HSPC function; for example high concentrations of CXCL12 (100-500ng/ml) are reported to inhibit cell cycling of human HSPCs [48-50], while low concentrations of CXCL12 (0.05–0.5 ng/ml) promote cell cycling [51,52]. In this context, it is of interest that, in the mouse, Jam-c loss or gain of function studies indicate a role for Jam-c in enhancing CXCL12 production by murine lymph node fibroblastic reticular cells [7,53], whereas Jam-b negatively regulates CXCL12 production by murine bone marrow MSCs [23]. Furthermore, it has been reported that Jam-c on murine HSPCs interacts with Jam-b on MSCs [23] and that this interaction may regulate CXCL12 secretion [7,23]. It is now also well recognised that the functions of the CXCR4 chemokine receptor can be modulated by co-receptors, co-associating molecules and small molecules. Our laboratory has, for example, shown that endolyn (CD164) can modulate CXCR4 signaling on UCB HSPCs in response to CXCL12 presentation [24]. Others have demonstrated that syndecan-4 co-associates with CXCR4 on lymphocytes and macrophages and facilitates CXCL12 binding to these cells, although this has not been demonstrated on HSPCs [54]. Further studies have reported that CXCL12 stimulation can upregulate genes involved in adhesion and homing in human umbilical cord blood (UCB) $CD34^+$ cells [21,55]. Although the JAM family of molecules has not previously been shown to interact with CXCR4, our studies indicate that such an interaction can occur between JAM-A and CXCR4, and that CXCL12 presented briefly to human UCB $CD34^+$ HSPCs can promote the movement of JAM-A with CXCR4 in cis towards the leading edge of the cell. JAM-A also has the capacity to enhance CXCR4 mediated signaling on these cells. Thus, we might speculate that, while JAM-B and JAM-C can modulate CXCL12 production by bone marrow MSCs and adhesion of HSPCs to bone marrow MSCs, JAM-A has a different role and instead co-associates with and regulates the function of CXCR4 on HSPCs as well as the adhesion of HSPCs to bone marrow endothelial cells, thereby enhancing retention of HSPCs in specialized bone marrow niches.

CONCLUSIONS

Thus, in conclusion, our studies and those of others suggest that members of the JAM family contribute to the maintenance of HSPCs within the hematopoietic stem cell niche, and do so in part by regulating CXCL12 production and CXCR4 function.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have no conflict of interests or any disclosures to declare.

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FIGURE LEGENDS

Figure 1. JAM-A is expressed on purified CD133$^+$ and CD34$^+$ human cord blood cells.

CD133 and CD34 MACS purified human UCB cells were cultured for 20-24 hours in SCF, TPO, Flt-3L and IL-6 prior to flow cytometric analyses. (A) Representative dotplots of CD133 MACS purified cells labeled with relevant PE- or APC- isotype controls (left) or with JAM-A-PE (M.Ab.F11) and CD133-APC (293C3) (right). (B) Representative dotplots showing dual-colour analysis of isotype controls (left) or CD34 and JAM-A expression (right) on MACS purified human UCB CD34$^+$ cells. The percentage of cells in each quadrant is indicated to the right of Figures (A) and (B) as the mean ± S.E.M for n= 3 independent experiments. DAPI was used to exclude non-viable cells. (C) Cells expressing different levels of CD34 were gated as shown (left; P2 to P4) and the relative levels of JAM-A-PE fluorescence in each group (right) were determined. The M.F.I.s for JAM-A fluorescence were for the isotype control 43.2±6.5, for the majority of CD34$^+$ cells 1220.0±187.7 (P2, red), for CD34-dim cells 270.3±47.2 (P3, blue) and for CD34-high cells 695.0±78.0 (P4, green).

Figure 2. JAM-A expression on human cord blood CD34$^+$ subsets.

(A) Representative flow cytometric gating strategy of MACS purified human UCB CD34$^+$ cells. Single, live and lineage negative (Lin-) cells (top pseudocolor blots) were discriminated into two subsets based on their expression of CD34 and CD38 (contour plots). The CD34$^+$CD38$^-$ population was further distinguished based on CD90 and CD45RA staining to distinguish HSC, MMP and CLP. In addition, HSC were characterized for CD49f expression. The CD34$^+$CD38$^+$ population was segregated into the progenitor subsets GMP, CMP and MEP based on their expression of CD123 and CD45RA. (B) shows typical histograms of JAM-A expression on freshly isolated or thawed UCB CD34$^+$ cells without culture (blue) or after 20-24h culture in the cytokines SCF, TPO, Flt-3L and IL-6 (red) versus the FMO control (black). The influence of cytokine culture on the frequency of JAM-A positive cells (C,E) and the expression levels of JAM-A on single cells (D,F) were determined for each HSPC subset on freshly isolated (C, D) and thawed CD34$^+$ cells (E,F). Values are means± S.E.M. for n_{fresh}=5 donors, n_{thawed}=3 donors, with a starting purity of 92.4±4.0% CD34$^+$ cells. Statistical analysis was performed in GraphPad Prism using matched 2-way ANOVA and Sidak's multiple comparisons test considering $p<0.05$ as statistically significant.

Figure 3. JAM-A is highly expressed on *in vivo* NSG transplantable human cord blood CD34$^+$ cells.

(A) Sorting strategy for JAM-A subsets. Cryopreserved, thawed and cultured human UCB CD34 enriched cells were stained with antibodies against CD34 and JAM-A and with 7ADD. Viable CD34$^+$ cells were selected and sorted based on expression of JAM-A (left density plot). The JAM-A^{High} and JAM-A^{Low} gates represented the highest 12-14% and lowest 5-7% of the JAM-A expressing cells, respectively, with the JAM-A^{Int} gate representing the remaining cells. (B) Peripheral blood reconstitution of NSG mice as measured by CD45$^+$ human leukocytes in peripheral blood. Data represent average levels of human cells detected at weekly intervals for 14 weeks in PB samples of at least 5 NSG mice from each JAM-A sorted subpopulation. Mice received 1.5-5x10^4 CD34$^+$JAM-A^{High}, 5x10^4 CD34$^+$/JAM-A^{Int} or 5x10^4 CD34$^+$/JAM-A^{Low} cells. The CD34$^+$/JAM-A^{High} cells engrafted more quickly and to a higher extent than the JAM-A^{Int} and JAM-A^{Low} populations (p=0.0002, ANOVA with Tukey's post hoc test). Data shown as mean ±S.E.M. (C) Bone marrow reconstitution of NSG mice at week 15 post-transplantation of the following dose ranges; 1.5-5x10^4 CD34$^+$/JAM-A^{high} cells, 2x10^4-10^5 CD34$^+$/JAM-A^{Int} cells and 1.5-5.6x10^4 CD34$^+$/JAM-A^{low} cells. Graphs depict levels of human CD45$^+$ (top panels) or JAM-A+ (bottom panels) cells in the bone marrow removed from NSG mice inoculated with sorted JAM-A subpopulations. (D) Cells were harvested from the bone marrow of engrafted primary recipients and equivalent numbers of CD45$^+$ cells were inoculated into corresponding serial recipients (1x10^5 in JAM-A^{Int} and 1-4x10^4 in JAM-A^{High} recipients respectively) with bone marrow analyses at week 15. Each sample is represented by a specific symbol.

Figure 4. JAM-A modulates cell adhesion to bone marrow endothelial niche cells.

(A) Left: HL-60 adhering to BSA (negative control) or IL-1β-activated BMEC-60 for 1 hour (n=3). Center: HL-60 incubated with anti-JAM-A antibody (10 μg/ml, AF1103) or Ig control before adhering to IL-1β activated BMEC-60. 29.2±3.9% of cells in Ig control adhered to BMEC-60 and this was normalized to 100% (n=3). Right: HL-60 nucleofected with JAM-A 150+151 siRNAs (n=3) or non-silencing siRNA (NS; n=6) were harvested at 24 hours for adhesion to IL-1β-activated BMEC-60. NS control (34.8±3.2% cells adhering) was normalized to 100%. (B) Similar adhesion assays performed using purified UCB CD34$^+$ cells (n=4-6). (C) Left: JAM-A-null Jurkat adhering to IL-1β-activated

BMEC-60 or BSA. Right: similar adhesion using Jurkat transduced with empty lentiviral vector (LV-Empty vector) or JAM-A (LV-JAM-A). LV-Empty vector transduced Jurkat were normalized to 100% (n= 5). (D) Left: BMEC-60 activated with IL-1β were coated without (black histograms) or with (grey histograms) 100ng/ml CXCL12. HL-60 without or with a brief 10 min exposure to CXCL12 (100ng/ml; x-axis) adhesion to the BMEC-60 for 1 hour at 37°C. Middle and right: HL-60 or cultured UCB $CD34^+$ cells respectively after NS siRNA knockdown (black histograms) or JAM-A 150+151 siRNAs (grey histograms) and without (no CXCL12) or with (CXCL12) a brief 10 min exposure to CXCL12 (100ng/ml) adhering to IL-1β activated BMEC-60 (n= 3). Values are means ± S.E.M. for n=3-6 independent experiments. Using unpaired two tailed Student's t-test, $p < 0.05$ (*) or < 0.01 (**) are considered significant.

Figure 5. JAM-A interacts with CXCR4.

(A) Lysates of sham versus CXCR4-HA and JAM-A-FLAG transfected HEK293T cells were subjected to gel electrophoresis and then immunoblotted with a) mouse anti-HA antibody followed by IRDye 800CW anti-mouse Ig, b) mouse anti-JAM-A antibody followed by biotinylated anti-mouse IgG Veriblot secondary antibody and IRDye 800CW streptavidin, c) mouse anti-FLAG M2 antibody followed IRDye 800CW anti-mouse Ig and d) mouse anti-α-tubulin (clone B-5-1-2) followed by secondary IRDye 800CW anti-mouse Ig**.** (B) Brij-35 lysates were incubated with μ-MACS anti-HA microbeads and bound protein eluted according to the manufacturer's instructions prior to gel electrophoresis and immunoblotting with a) anti-HA antibody followed by IRDye 680CW anti-mouse Ig, and b) anti-FLAG antibody followed by IRDye 800CW anti-mouse Ig. Similar results were obtained with Triton-x100 lysates (not shown). Molecular weight markers are shown on the left hand side of each gel. The densiometric signals for the specific bands are indicated at the top of each lane.

Figure 6. JAM-A co-localizes at the leading edge of the cell with CXCR4 when presented with CXCL12.

HL-60 or cultured human UCB $CD34^+$ cells were presented with CXCL12 (200ng/ml) on fibronectin from 0 to 30 minutes and at each time point cells were stained with JAM-A (M.Ab.F11 or 36-1700 from Invitrogen), CXCR4 (Ab2074), CD164 (N6B6) and ICAM-3 (ICAM-3.3) in different combinations.

(A) For HL-60 cells (a) shows the co-localisation of pairs of molecules without or after CXCL12 presentation for 10 minutes at x80 magnification; (b) shows immunofluorescence images resulting from the PLA assay with the red dots in the images showing interactions of pairs of molecules without or after CXCL12 presentation for 10 minutes at x80 magnification; and (c) shows the quantitative data over time for the PLA assay. (B) For cultured human UCB $CD34^+$ cells, (a) shows the co-localisation of pairs of molecules without or after CXCL12 presentation for 10 minutes at x80 magnification; (b) shows immunofluorescence images resulting from the PLA assay with the red dots in the images showing interactions of pairs of molecules without or after CXCL12 presentation for 10 minutes at x80 magnification; and (c) shows the quantitative data over time for the PLA assay. Values are means ± S.E.M. of 3 independent experiments where 100 cells per experiment were counted. The statistical analysis was done using unpaired two tailed Student's t-test with < 0.05 (*) or < 0.01 (**) considered statistically compared to 0 minutes.

Figure 7. JAM-A knockdown alters CXCR4 mediated signaling in human HL-60 cells and UCB $CD34^+$ HSPCs.

HL-60 or cultured human UCB $CD34^+$ cells transfected with NS or JAM-A 150+151 siRNAs. After 24 hours, cells were treated without (-) or presented with (+) CXCL12 (100nM) for 30 seconds and lysed. Total protein lysates (50 μg) were electrophoresed on 12-15% (w/v) NuPAGE Novex Bis-Tris Gels before blots were probed with mouse anti-human JAM-A (43/JAM-1), rabbit anti-human phospho-p44/42 MAPK/phospho-ERK1/2 (Thr202/Tyr204); #9101), rabbit-anti human p44/42 MAPK/ERK1/2 (#9102), rabbit anti-human phospho-PKC-ζ (sc-12894-R), rabbit anti-human PKC-ζ primary antibody (sc-216), rabbit anti-human phospho-Akt (Ser473; #9271) or rabbit anti-human Akt primary antibody (#9272). Mouse anti-human α-tubulin (B-5-1-2) or GAPDH (2D4A7) served as the loading controls. The blots were developed with IRDye® 800CW goat anti-rabbit or 680CW goat anti-rabbit or goat anti-mouse secondary antibodies prior to signal detection in the ODYSSEY CLx Infrared Imaging System. (A) shows representative Western blots for HL-60 cells, and (B) the mean±S.E.M. of their densitometry plots for n=3 independent experiments (black histograms: no CXCL12 treatment; grey histograms: CXCL12 treated cells). The minimal area of signal was gated, the density/mm^2 determined and the signal density normalised to the loading control protein in each lane. The statistical analysis was done using unpaired two tailed Student's t-test with < 0.05 (*), < 0.01 (**) and

p<0.001 (**) considered statistically significant. (C) shows a single confirmatory experiment with Western blots for UCB $CD34^+$ cells and the molecules described in Figure 7A. The densiometric signals for the specific bands are indicated at the top of each lane.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Phenotype and trilineage potential of human BM MSCs.

(A) Bone marrow mesenchymal stem/stromal cells (BM MSCs) were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the Figure. Flow cytometry plots show representative histograms for two batches of BM MSCs stained positive for the MSC markers CD90, CD105, CD73, CD146 and CD166 (blue and orange) versus the isotype controls (red). (B) Representative images of BM MSC differentiated into osteogenic, adipogenic and chondrogenic lineages versus non-induced controls demonstrate their tri-lineage differentiation potential. BM MSCs were seeded at $2x10^4$ cells/cm^2 and incubated with differentiation media (Lonza Biologicals) for 2-3 weeks. Samples were then stained using Alizarin Red S for osteogenesis, Oil Red O for adipogenesis, and Alcian Blue for chondrogenesis.

Supplementary Figure S2. Phenotype of BMEC-60 and BMSC.

(A) BMEC-60 cells were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the Figure. Flow cytometry plots show representative histograms displaying median fluorescence intensity (M.F.I.) for each marker±S.E.M. above an arbitrary gate set for the matched isotype control (n=3 independent tests). (B) BMSCs were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the figure. Flow cytometry plots show representative histograms displaying M.F.I.±S.E.M. (n=3 independent batches).

Supplementary Figure S3. Phenotype of BMEC-60 cells before and after IL-1β stimulation.

Representative flow cytometry histograms of BMEC-60 stained with the (A) APC-CD62E (68-5H11; E-selectin), (B) APC-CD54 (HA58; ICAM-1), (C) FITC-CD29 (β1-integrin), (D) FITC-CD18 (6.7; β2-integrin), (E) APC-CD11a (HI11; αL-integrin) and (F) APC-CD51/CD61 (23C6; αVβ3 integrin) antibodies (red) and their isotype controls (grey). M.F.I.± S.E.M. and the % positive± S.E.M. above the gate set for the isotype controls are shown for the primary antibody stain in the top right corner of the plot. n=3 independent experiments.

Supplementary Figure S4. BMEC-60 and UCB $CD34^+$ cells express JAM-A.

(A) Representative flow cytometry histograms of (left) BMEC-60, (middle) BMSC and (right) BM MSC stained with PE-JAM-A (M.Ab.F11; grey) or isotype controls (white). M.F.I.± S.E.M. and % positive± S.E.M for JAM-A are on each plot (n=3). (B) Left: Representative histograms of BMEC-60 stained with PE-JAM-A (M.Ab.F11; grey) and isotype controls (white). M.F.I.± S.E.M. and % positive± S.E.M. for JAM-A are on each plot. Middle: JAM-A expression in BMEC-60 cells by Western blotting with densitometry plots normalised to GAPDH. Relative values of BMEC-60 controls (no IL-1β) were then normalized to 1. n=3. (C) BMEC-60 stained with isotype control (bottom) or CXCL12 (Clone 79018, top) followed by Alexa Fluor®488 goat anti-mouse IgG antibody (A-11001) and nuclei counterstained with DAPI (blue). Images represent one from n=3 experiments. Magnification x60. (D) UCB $CD34^+$ cells before or after overnight culture in serum free Stem Span medium with SCF, TPO, Flt3-L and IL-6 were stained with the APC-CXCR4 mAb (Clone12G5). Top: Representative dotplots of the isotype controls and JAM-A versus CXCR4 cell surface staining before (left, middle) and after overnight culture (right) are shown. Lower: The left histogram shows the M.F.I. and mean (bar) of 3 independent batches of $CD34^+$ cells ($p<0.05$), the respective middle and right FACS histograms show that while a proportion of cells were $CXCR4^+$ before culture, this increased so that essentially all cells expressed CXCR4 after overnight culture with cytokines. Statistical analysis with Student's t-test and values <0.05 are considered significant.

Supplementary Figure S5. Expression of JAM-A on cell lines.

Representative flow cytometry histograms of cell lines, including (A-C) acute myeloid leukemic cell lines (HL-60, KG-1 and TF-1 cells) and (D-H) and acute lymphoid leukemic cell lines (Jurkat, CEM and MOLT-4 cells of T-ALL, and NAML-1 and Reh cells of B-ALL), stained with the PE-JAM-A antibody (M.Ab.F11; red) and its isotype control (grey). Of these only Jurkat cells were negative for JAM-A. Values are mean M.F.I.± S.E.M. and the mean % positive± S.E.M for N= 3 independent experiments above the gate set for the isotype controls.

Supplementary Figure S6. JAM-A siRNAs knockdown in HL-60 and $CD34^+$ cells.

(A-B) HL-60 cells were nucleofected with JAM-A siRNA (150, 151 or a combination of 150+151 at 100nM in total) or the non-silencing control (NS). The cells were harvested from 24 to 72 hours and

tested for knockdown of cell surface JAM-A by flow cytometry. (A) Representative FACS histograms of non-silencing (NS) and JAM-A siRNA treated HL-60 cells 24 hours post-transfection stained with the JAM-A-PE antibody (red) and isotype control (grey). M.F.I.± S.E.M. for n=4-8 independent experiments shown. (B) Cell surface JAM-A expression of HL-60 cells 24, 48 or 72 hours after they had been nucleofected with JAM-A siRNAs (150, 151 or a combination of 150+151 at 100nM in total) or the non-silencing control (NS). Values are M.F.I ± S.E.M. for n=3-9 independent experiments normalised to NS siRNA treated HL-60 cells at each time point. n=6 for NS, n=5 for 150 and 151 and n=4 for 150+151 at 24 hour; n=3 for all groups at 48 hour; n=4 for all groups at 72 hours. (C) Representative histograms of non-silencing (NS) and JAM-A siRNA 150+151 treated UCB $CD34^+$ cells 24 hours post-transfection stained with the JAM-A-PE antibody (grey) and isotype control (white). M.F.I.± S.E.M. for n=4-8 independent experiments shown. Statistical analysis with Student's t-test comparing NS to each JAM-A siRNA knockdown and values *p< 0.05, **p<0.01 and ***p<0.001 are considered significant.

Supplementary Figure S7. UCB $CD34^+$ cell chemotaxis towards CXCL12 across BMEC-60 cells after JAM-A blockade or silencing.

The absolute percentage of UCB $CD34^+$ cells migrating across IL-1β stimulated BMEC-60 cells towards CXCL12 in a transwell assay. Cultured UCB $CD34^+$ cells (A) were pre-incubated with JAM-A antibody (anti-JAM-A, 10 μg/ml, AF1103, R&D systems) or control goat IgG for 30 minutes at 37°C or (B) were nucleofected with JAM-A siRNAs 150+151 or non-silencing control siRNA (NS) as described 24 hours prior to the transwell migration assay. Values are means ± S.E.M. for n=6 and n=4 independent experiments respectively.

Supplementary Figure S8: Expression of CXCR4 and CD164 on cell lines.

Representative flow cytometry histograms of acute leukaemic cell lines, including acute myeloid leukaemia cell lines: (A) HL-60, (B) KG-1 and (C) TF-1 cells, and T lymphoid leukaemia cell line (D) Jurkat cells, stained with CXCR4-APC (12G5 clone, red, top plots) and CD164-PE antibodies (N6B6 clone, red, bottom plots) and their isotype controls (grey). KG-1 and TF-1 were used as negative

controls for CXCR4 staining. Values are M.F.I.± S.E.M. and the % positive± S.E.M for CXCR4 or CD164 stained cells in n=3 independent experiments.

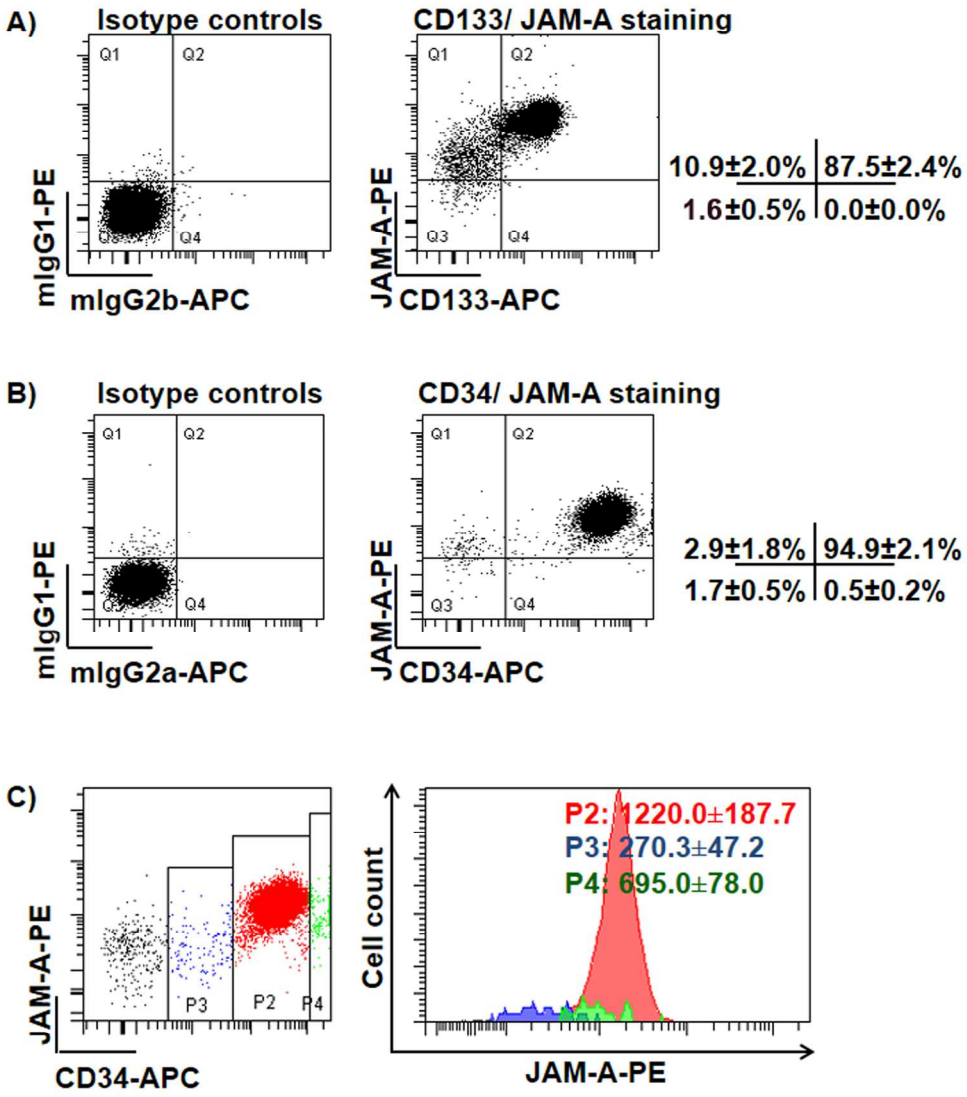
Supplementary Figure S9. Subset analysis of human UCB CD34$^+$ cells adhering to BMEC-60 cells.

UCB CD34$^+$ cells labeled with CFSE were cultured in StemSpan ACF medium supplemented with SCF, Flt-3L, IL-6 and TPO for 20-24 hours and allowed to adhere for 1 hour at 37^{o}C to IL-1β activated BMEC-60 without (control) or with CXCL12 pre-coating (CXCL-12). Adherent cells were analyzed for HSPC subsets using multi-colour flow cytometry with quantification using Countbright beads and acquisition on an LSRII flow cytometer. The gating strategy for identifying HSC, CMP, GMP and MEP that have adhered to BMEC-60 cells is presented in Figure 2. HSC and progenitor subsets were determined by gating on single viable CSFE$^+$ cells and then for HSC (Lin$^-$ CD38$^-$CD34$^+$CD45RA$^-$ CD90^{hi+}), CMP (Lin$^-$ CD38$^+$CD34$^+$CD45RA$^-$CD123$^+$), GMP (Lin$^-$ CD38$^+$CD34$^+$CD45RA$^+$CD123$^+$) and MEP (Lin$^-$ CD38$^+$CD34$^+$CD45RA$^-$CD123$^-$). The Figure shows the adhesion of the different HSC, CMP, GMP and MEP subsets as a % of viable CSFE$^+$CD34$^+$ cells adhering to BMEC-60 (mean±S.E.M.; n= 3 replicates).

Graphical Abstract. Junctional adhesion molecule JAM-A is highly expressed on human hematopoietic repopulating cells and associates with the key hematopoietic chemokine receptor CXCR4.

JAM-A^{High} CD34$^+$ UCB cells contain *in vivo* repopulating cells as demonstrated in primary and secondary transplanted NSG recipients. 2. Adhesion of UCB CD34$^+$ cells to the bone marrow endothelial cell line BMEC-60 is partially inhibited with anti-JAM-A blocking antibody or after JAM-A knock-down. 3. JAM-A co-immunoprecipitates with CXCR4 when both are co-transfected into HEK293T cells. JAM-A moves to the leading edge of the UCB CD34$^+$ cell with CXCR4 when cells are exposed to CXCL12. 4. We hypothesize that, while JAM-B and JAM-C can modulate CXCL12 production by bone marrow mesenchymal progenitor/stromal cells (MSCs) and adhesion of HSPCs to bone marrow MSCs, JAM-A has a different role and instead co-associates with and regulates the function of CXCR4 on HSPCs as well as the adhesion of HSPCs to bone marrow endothelial cells, thereby enhancing retention of HSPCs in specialized bone marrow vascular niches.

Figure 1. Chang et al. 2015 TOP



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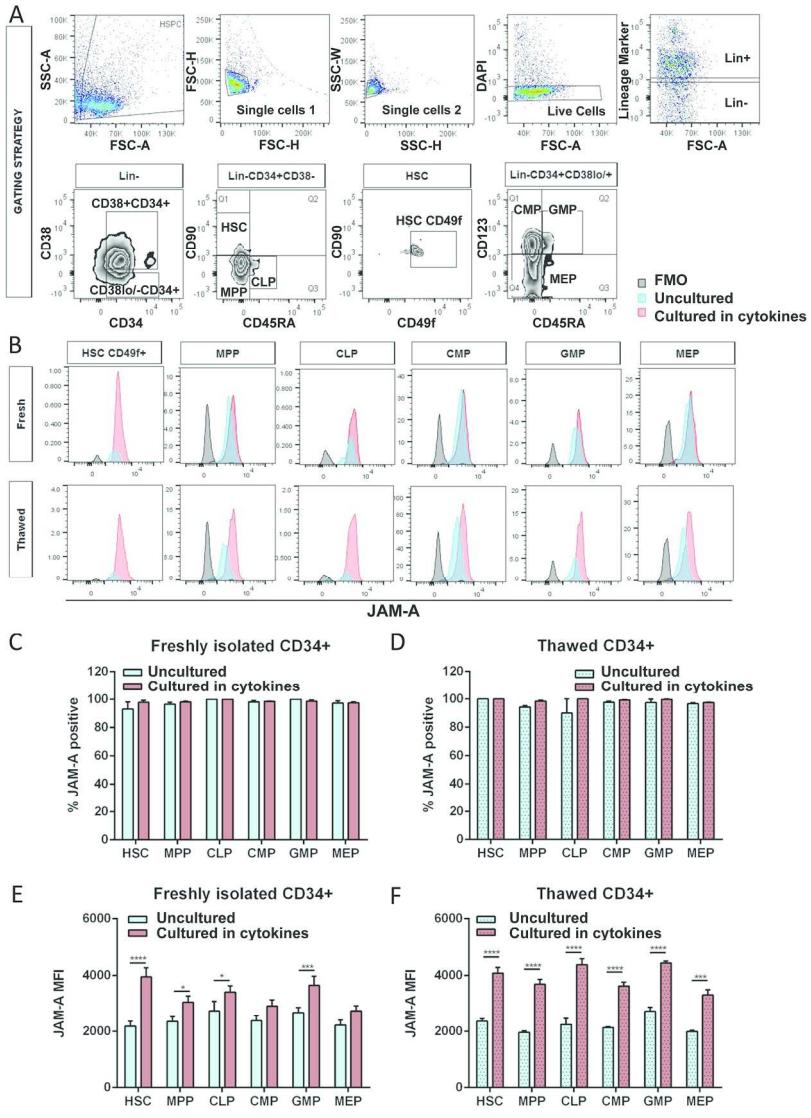

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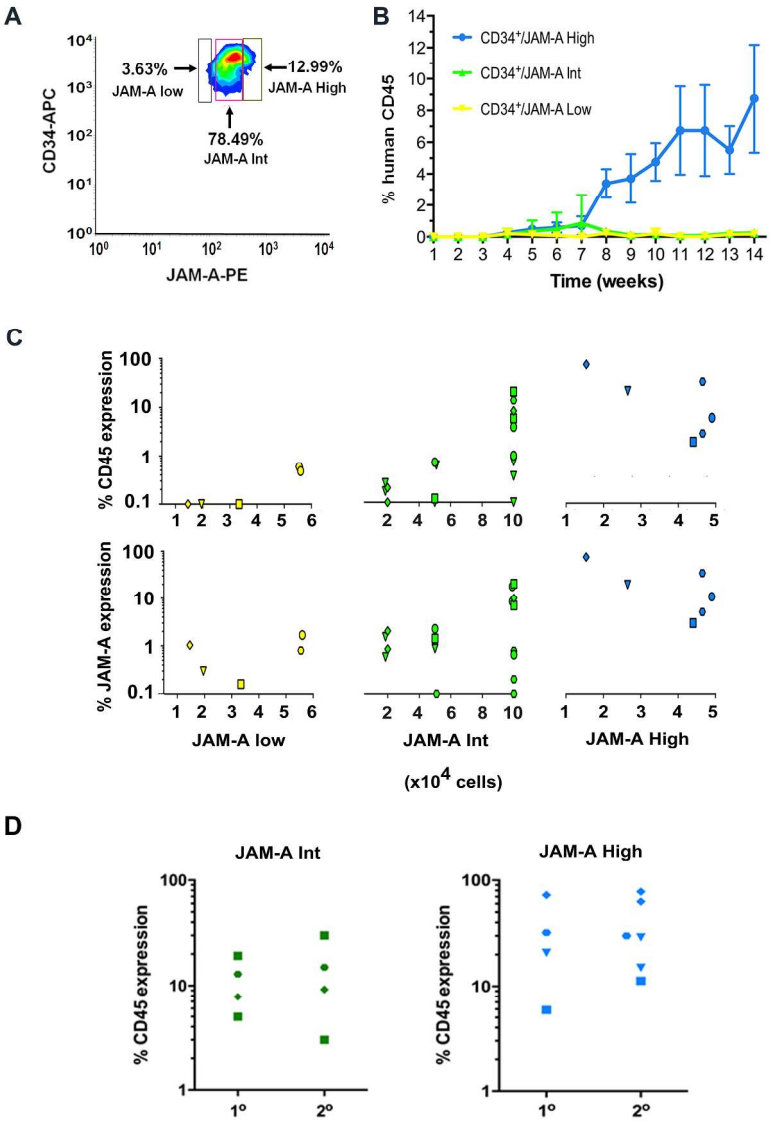

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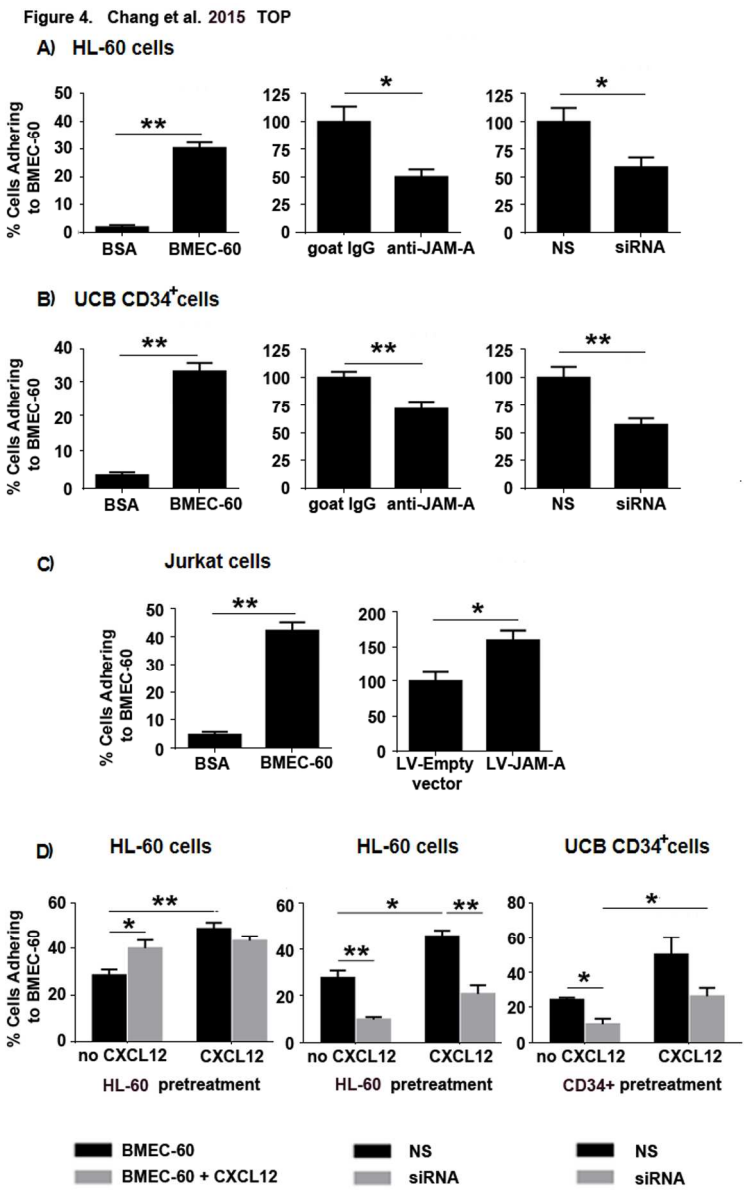

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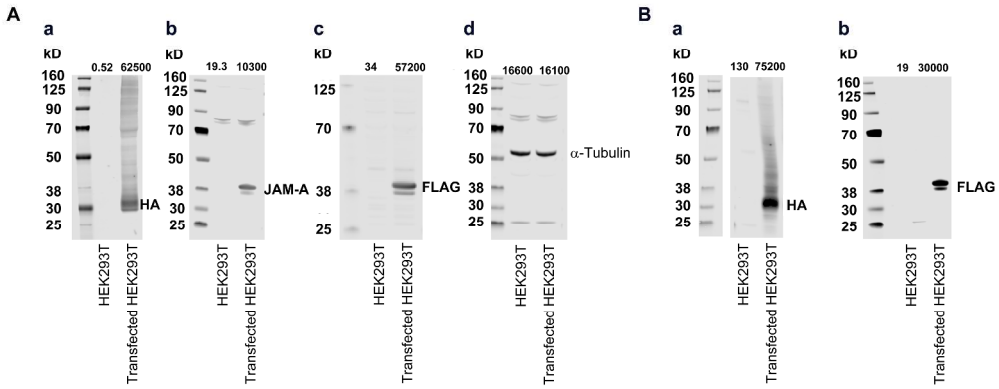

Figure 6A Chang et al. 2015 TOP

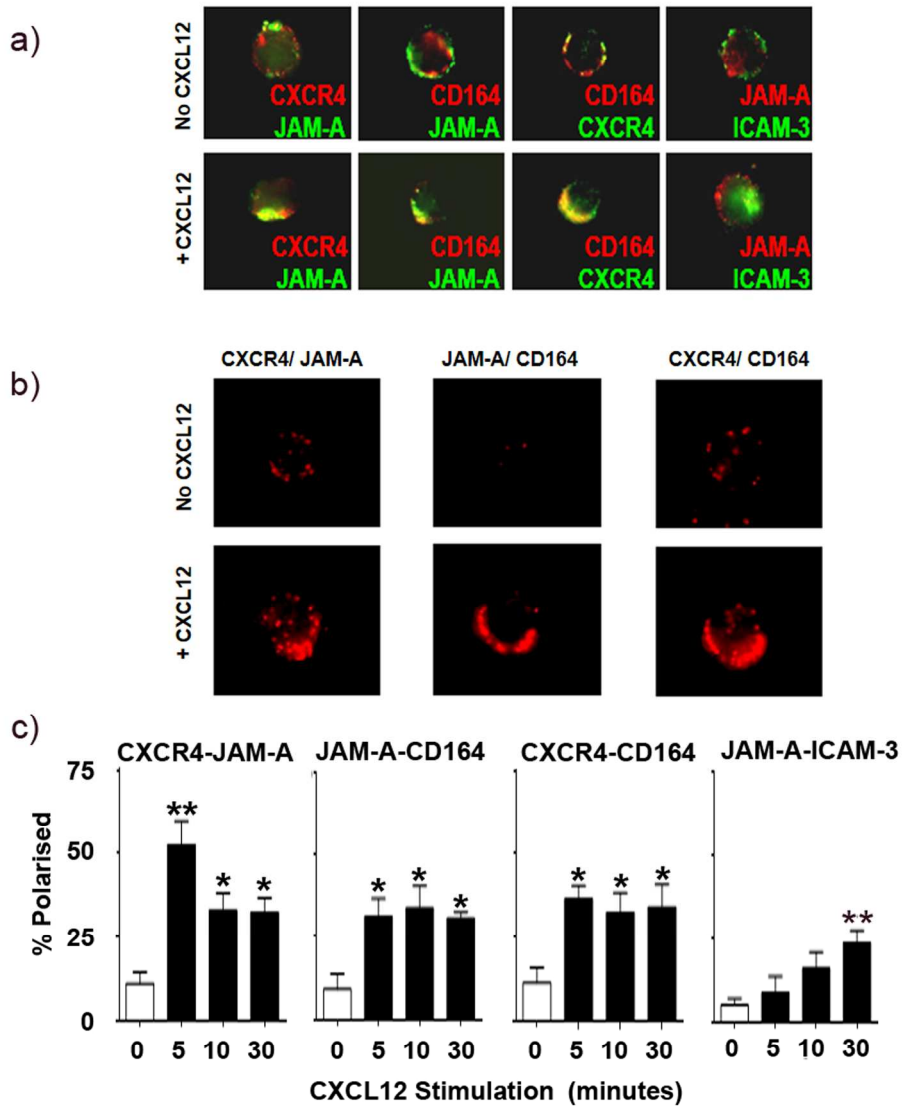

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Figure 6B Chang et al. 2015 TOP

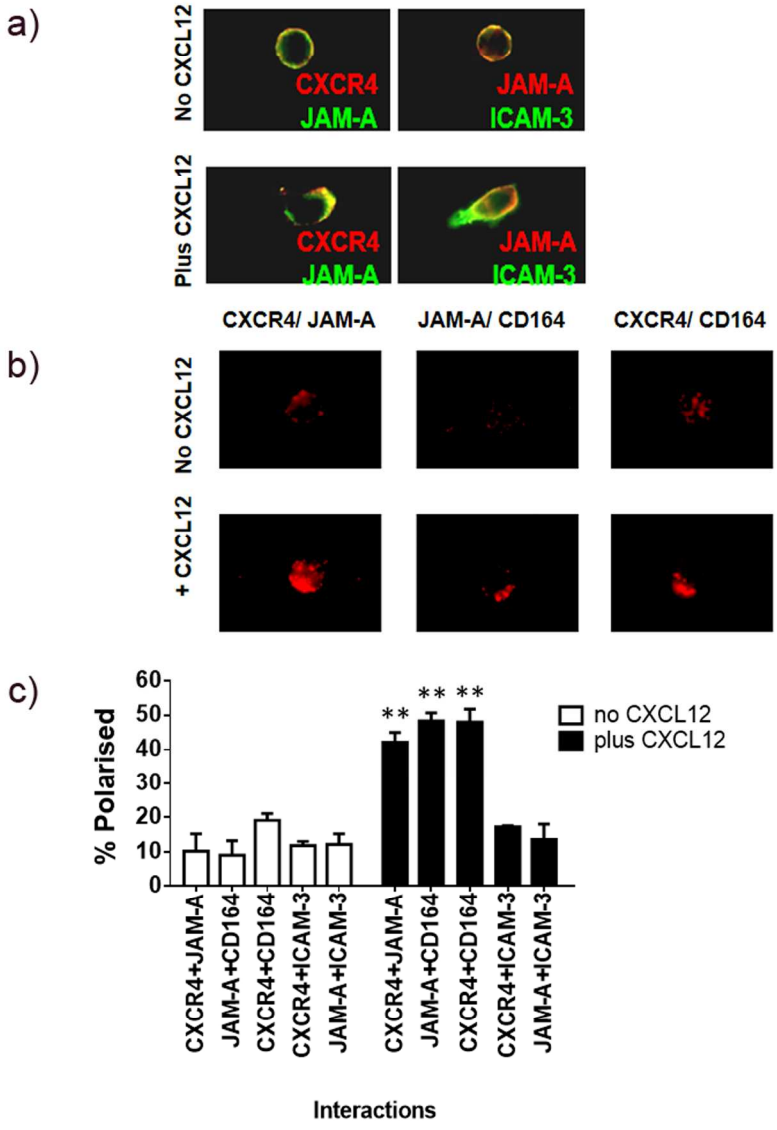

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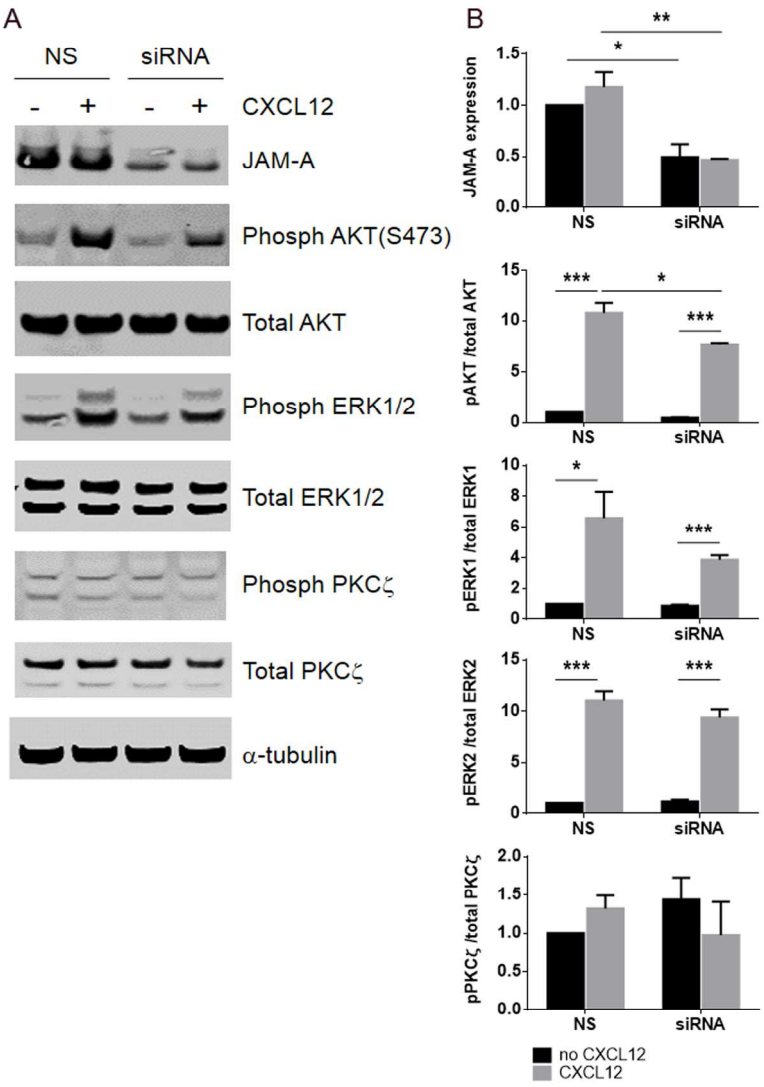
Figure 7 A and B. Chang et al. 2015 TOP



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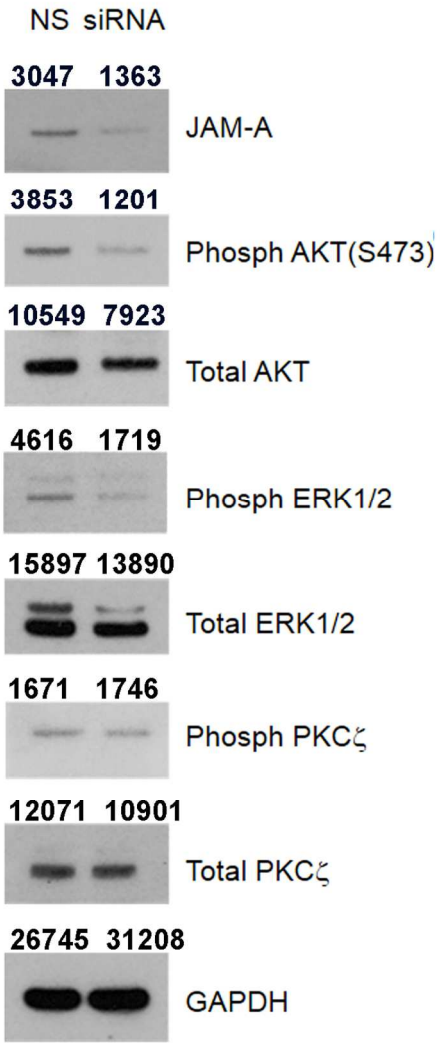

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SUPPLEMENTARY MATERIALS AND METHODS

JAM-A IS HIGHLY EXPRESSED ON HUMAN HEMATOPOIETIC REPOPULATING CELLS AND ASSOCIATES WITH THE KEY HEMATOPOIETIC CHEMOKINE RECEPTOR CXCR4

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Cells and cell lines

The human bone marrow endothelial cell line, BMEC-60 were kindly supplied by Professor CE van der Schoot, Sanquin, Amsterdam, The Netherlands [1], and cultured in complete EGM-2 medium (Lonza Biologics, Workingham, UK) [2]. Human bone marrow (BM) mesenchymal stromal cells (BMSCs with LTC-IC supportive ability; Lonza Biologics) were cultured in Myelocult H5100 medium with 10^{-6} M hydrocortisone sodium succinate (Stem Cell Technologies, Vancouver, Canada). Plastic adherent human trilineage BM mesenchymal stem/stromal cells (BM MSCs; Lonza Biologics) were cultured and characterized (Supplementary Figure S1) as described [2-5]. BMEC-60 and BMSCs were phenotyped as shown in Supplementary Figures S2 and S3 using mAbs described in Supplementary Table S1. HL60 and KG1 cells were cultured in IMDM (Invitrogen Ltd., Paisley, Scotland) with 20% (v/v) fetal calf serum (FCS), Jurkat, CEM, MOLT-4, Reh and NALM-1 cells in RPMI-1640 with 10-20% (v/v) FCS, HEK 293T cells in DMEM containing 10% (v/v) FCS (all media from PAA Laboratories GmbH, Pasching, Austria). The TF-1 cell line was cultured in RPMI-1640 medium with 10% (v/v) FCS (both from PAA Laboratories GmbH) and 2 ng/ml GM-CSF (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany).

Antibodies and flow cytometry

Flow cytometric monoclonal antibodies are shown in Supplementary Table S1. UCB mononuclear or MACS enriched $CD34^+$ or $CD133^+$ HSPCs were stained with CD34-APC or CD133-APC and anti-JAM-A-PE and followed methods previously described [6,7].

Multicolour flow cytometry characterization of $CD34^+$ human umbilical cord blood derived HSPC was performed as described [8-12] or according to Notta *et al.* [13]. For these studies, $CD34^+$ cells were isolated by density gradient centrifugation followed by $CD34^+$ MACS separation no more than 24 hours after UCB collection. Cells were either stained (fresh) immediately for phenotypic multiparameter characterization by FACS or cryopreserved. Some fresh or previously cryopreserved cells were cultured at 20,000 cells/100μl in 96 well round bottom plates containing Stem Span ACF supplemented with SCF, FLT-3L, IL-6 (all at 100ng/ml) and TPO (20ng/ml) for 20-24 hours at 37°C, 5% CO_2, 95% humidity. JAM-A expression levels were determined on uncultured and cytokine cultured fresh and thawed $CD34^+$ HSPC subsets from the same donor by employing the FACS staining protocol described below. The following antibodies (Supplementary Table S1) were used: CD34 AF700 (581), CD45RA APC-H7 (HI100), CD38 PE-TxR (HIT2), CD90 PE (5E10), CD123 PerCP-Cy5.5 (6H6), CD49f PE-Cy7 (GoH3), CD321 (JAM-A) FITC (OV-5B8), and a panel of lineage markers conjugated to PE-Cy5: CD2 (RPA-2.10), CD3 (HIT3a), CD4 (RPA-T4), CD8a (RPA-T8), CD7 (CD7-6B7), CD10 (HI10a), CD11b (ICRF44), CD14 (61D3), CD19 (HIB19), CD20 (2H7), CD235ab (HIR2) and CD56 (B159). Briefly, cells were resuspended in human FcR blocking reagent diluted in MACS buffer (both from Miltenyi Biotec GmbH) and incubated for 10 min, at 4°C. The cells were then incubated with a mixture of fluorescently labeled antibodies diluted in MACS buffer for 20 min on ice. Cells were washed once, resuspended in MACS buffer and acquired immediately on a LSRII flow cytometer (BD Biosciences). DAPI was added at 100ng/ml directly before acquisition to distinguish live and dead cells. Data were analysed on FlowJo (TreeStar Inc., Ashland, OR, USA). HSPC stem and progenitor subsets were determined by gating on single viable, Lin^- cells, and defined as HSC: $Lin^-CD38^-CD34^+CD45RA^-CD90^+CD49f^+$; MPP: $Lin^-CD38^-CD34^+$ $CD45RA^-CD90^-$; CLP: Lin^-CD38^- $CD34^+$ $CD45RA^+CD90^-$; CMP: $Lin^-CD38^+CD34^+CD45RA^-CD123^+$; GMP: $Lin^-CD38^+CD34^+$ $CD45RA^+CD123^+$; and MEP: $Lin^-CD38^+CD34^+CD45RA^-CD123^-$ [13].

CXCL12 staining of and production by BMEC-60 cells

BMEC-60 cells were cultured in EGM-2 medium (Lonza Biologics) containing 10 ng/ml IL-1β (Invitrogen Ltd.) for 4 hours before staining with anti-CXCL12 (79018, R&D Systems, Abingdon, UK) and Alexa Fluor®488 goat anti-mouse IgG antibody (A-11001, Invitrogen Ltd.) and DAPI nucleic acid dye (Sigma-Aldrich Ltd., St Louis, MISS, USA). The labeled cells were examined under an Eclipse TE600 microscope (Nikon UK Ltd., London, UK). BMEC-60 cells seeded in triplicate into 12 well plates at $2x10^5$ cells/well were either treated or not treated with 10ng/ml IL1β in complete EGM-2 medium for 4 hours, washed with PBS and 500µl EBM-2 medium (Lonza Biologics) containing 0.5% (v/v) FCS (PAA Laboratories GmbH) for a further 24 or 48 hours. BM MSC from 3 individual donors were seeded into 24 well plates at $2x10^4$ cells /well, cultured overnight in MSCGM (Lonza Biologics), before media exchange for 250µl α-MEM (Gibco BRL/Invitrogen Ltd.) containing 0.5% (v/v) FCS (PAA Laboratories GmbH) and culture for a further 24 or 48 hours. The conditioned media were collected, centrifuged at 300g for 5 minutes, and the supernatant collected and immediately stored at -80°C. The BMEC-60 cells were also lysed in 100µl RIPA buffer for 10 minutes [2,4], centrifuged at 14,000rpm at 4°C and the supernatant collected. One hundred µl of supernatant without dilution or of lysate (diluted 1:5 in diluent) was added to the ELISA plate to detect CXCL12 (R&D Systems) and the standard ELISA protocol followed [2].

Overexpressing JAM-A in Jurkat cells

The JAM-A cDNA clone in the pENTR221 vector was purchased from I.M.A.G.E. clone (Clone #100066761, EU831732; Geneservice (http://www.lifesciences.sourcebioscience.com) as indicated below, with the JAM-A coding sequence at bps 23 to 919.

JAM-A sequence (EU831732)

GTACAAAAAAGCAGGCTCCACCATGGGGACAAAGGCGCAAGTCGAGAGGAAACTGTTGTGCCTC
TTCATATTGGCGATCCTGTTGTGCTCCCTGGCATTGGGCAGTGTTACAGTGCACTCTTCTGAACC
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GGACTTCGAGTAAGAAGGTGATTTACAGCCAGCCTAGTGCCCGAAGTGAAGGAGAATTCAAACA
GACCTCGTCATTCCTGGTGAAGCTTGACCCAGCTTTCTTGTAC

Plasmids derived from individual bacterial transfected clones were purified using the Midi PureLink™ HiPure Plasmid Filter Purification Kit (Invitrogen Ltd.) according to the manufacturer's instructions, digested with Bam HI and Xho I and purified by gel electrophoresis. JAM-A was then PCR amplified from the appropriate purified DNA fragment using the forward primer (5'-CGGGATCCGCCACCA TGGGGACAAAGGCGCAAGTCG-3') which included two random nucleotides (5'-CG-3'), a *Bam* HI restriction site (5'-GGATCC-3') and a Kozak sequence (5'-GCCACC-3') (309-311), and the reverse primer (5'-CGCTCGAGCTACACCAGGAATGA CGAGGTCTG-3') which included two random nucleotides (5'-CG-3'), an *Xho* I restriction site (5'-CTCGAG-3') and a stop codon (5'-CTA-3') and *PfuT*urbo® DNA polymerase (Stratagene Ltd., Cambridge, UK). The reaction was performed using a 2-step PCR programme (heating to 95°C for 2 minutes, followed by 10 cycles of 95°C for 45 seconds, 56°C for 45 seconds and 72°C for 1.5 minutes, another 25 cycles of 95°C for 45 seconds, 62°C for 45 seconds and 72°C for 1.5 minutes, and a final elongation at 72°C for 10 minutes) using a T3000 PCR ThermoCycler (Biometra GmbH, Göttingen, Germany). In order to ligate the JAM-A PCR amplicons into the TOPO TA cloning® vector (Invitrogen Ltd.), a poly-A tail was added by incubating 15 µl of PCR product with 5mM dATP (Promega, Southampton, UK), 5 units of Immolase™ DNA polymerase (Gentaur GmbH, Aachen, Germany) and 5mM $MgCl_2$ in 1x ImmoBuffer that was diluted from 10x buffer (160 mM $(NH_4)_2SO_4$, 1 M Tris-HCl pH 8.3, and 0.1% (v/v) Tween-20, supplied with the kit; Gentaur GmbH) at 70°C for 45 minutes using a T3000 PCR ThermoCycler PCR (Biometra GmbH). The PCR product was purified with a Qiagen kit and the DNA concentration determined using a Thermo Scientific NanoDrop™ Spectrophotometer (Thermo Scientific Inc., Lutterworth, UK). After purification, the JAM-A DNA was ligated into pre-digested pCR®II-TOPO® vector (Invitrogen Ltd.) using the DNA ligation kit from TaKaRa Bio Inc. (Mountain View, CA, USA) and transformed into One shot® TOP10 (Invitrogen Ltd.) or JM109 (Stratagene Ltd.) competent bacteria according the manufacturer's instructions. The resulting *Bam* HI and *Xho* I digested JAM-A cDNAs were confirmed by sequencing (Source BioScience UK Ltd., Nottingham, UK) using M13 forward and reverse primers. The full-length JAM-A cDNA was cleaved from pCR®II-TOPO® vector using a combination of *Bam* HI

and *Xho* I restriction enzymes and ligated into the pre-digested lentiviral genome vector pLNT/SffvMCS, kindly provided by Professor Adrian Thrasher, Institute of Child Heath, London, UK. The pLNT/SffvJAM-A- lentiviral vector, along with packaging vector pΔ8.91 and envelope vector pVSV-G (both from Professor Adrian Thrasher) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen Ltd.) to produce lentiviral vector particles at 48 hours as described [2,14]. These particles were used to transduce Jurkat cells at an estimated M.O.I. of 10. The transduced Jurkat cells were next tested 48 hours later for cell surface expression of JAM-A by flow cytometry and used in the adhesion assays.

Adhesion assays

For adhesion to BMEC-60 cells, $5x10^4$ BMEC-60 cells were cultured in black 96-well flat bottomed plates (Becton Dickinson, Abingdon, UK) with EGM-2 medium (Lonza Biologics) and activated with 10 ng/ml IL-1β (Invitrogen Ltd.) for 4 hours, washed in EBM-2 medium (Lonza Biologics) containing 0.5% (w/v) BSA (Sigma-Aldrich Ltd.) and $2x10^5$ BCECF-AM labeled [24] UCB $CD34^+$ cells, HL-60 cells, Jurkat cells or JAM-A transduced Jurkat cells (see above) added at 37°C for 1 hour. Absorbance was read before and after washing the cells in PBS using a VICTOR™ X5 multilabel plate reader at a 488nm excitation wavelength (Perkin Elmer Inc., Waltham, PA, USA) and the percentage of adhering cells calculated as follows:

$$\text{Relative adhesion percentage} = \frac{\text{Measured adhering cell absorbance}}{\text{Input cell absorbance}} \times 100\%$$

In some experiments, HL-60 and UCB $CD34^+$ cells were stimulated with 100ng/ml CXCL12 (SDF-1α; PeproTech Ltd., London, UK) for 10 minutes at 37°C before adhesion. For the studies on JAM-A blockade, cells were pre-treated with 10μg/ml goat anti-human JAM-A antibody (AF1103) or normal goat IgG (both from AB-108-C, R&D Systems) for 30 minutes at 37°C prior to assessing adhesion. Similarly, for siRNA knockdown studies, cells subjected to siRNA knockdown (see below) were also assessed for adhesion to BMEC-60 cells. To examine the effects of CXCL12 coating of BMEC-60 cells on HSPC adhesion, $0.75\text{-}2x10^5$ of UCB $CD34^+$ or HL-60 cells/ml were allowed to adhere as above to IL-1β activated BMEC-60 cells after they had been coated without or with 100 ng/ml CXCL12 at 37°C for 10 minutes [15]. To check the adhesion of $CD34^+$ HSPC subsets, $CD34^+$ MACS purified

cells ($1x10^6$ cells/ml) were labeled with 5μM CFSE (Invitrogen Ltd.) in PBS (without $Ca^{2+/}Mg^{2+}$) for 20 minutes at 37°C, washed and cultured in StemSpan ACF medium (Stem Cell Technologies, Vancouver, Canada) supplemented with SCF, Flt-3L, IL-6 (at 100ng/ml each) and TPO (20ng/ml) at $1x10^6$ cells/ml in a 24 well plate overnight at 37°C, 5% CO_2. All cytokines were purchased from R&D Systems. The next day cells were harvested and the total nucleated cell count was determined using Countbright beads (Invitrogen Ltd.) and acquisition on an LSRII flow cytometer (BD Biosciences, Abingdon, UK). CFSE labeled $CD34^+$ cells ($0.75x10^5$ per well) were allowed to adhere for 1 hour at 37°C to IL-1β activated BMEC-60 cells without or with CXCL12 pre-coating as above, before removal of non-adherent cells by gently pipetting up and down 3 times. Adherent cells were then removed by vigorous washing of the BMEC-60 layer with PBS and analyzed for HSPC subsets using multi-colour flow cytometry with quantification using Countbright beads and acquisition on an LSRII flow cytometer (BD Biosciences). Staining and analysis was performed as described above under Antibodies and Flow Cytometry. HSC and progenitor subsets were determined by gating on single viable HSC ($Lin^-CFSE^+CD38^-CD34^+CD45RA^-CD90^+$), CMP ($Lin^-CFSE^+CD38^+CD34^+CD45RA^-CD123^+$), GMP ($Lin^-CFSE^+CD38^+CD34^+CD45RA^+CD123^+$) and MEP ($Lin^-CFSE^+CD38^+CD34^+CD45RA^-CD123^-$) [8,9].

Migration assays

BMEC-60 cells ($2x10^4$) were cultured overnight on 3μm transwell inserts (BD Biosciences) in EGM-2 medium (Lonza Biologics). Non-adherent BMEC-60 cells were removed and the adherent cells activated with 10 ng/ml of rh Interleukin-1β (IL-1β; Invitrogen Ltd.) in EGM-2 medium at 37°C for four hours. The activated BMEC-60 cells were washed twice with the basal EBM-2 medium (Lonza Biologicals) containing 0.5% (w/v) BSA (Sigma-Aldrich Ltd.). Hematopoietic cells ($1-2x10^5$) were then applied to BMEC-60 coated inserts and allowed to migrate towards 100 ng/ml CXCL12 (SDF-1α; PeproTech Ltd.) in the lower chamber for 5 hours at 37°C. Migrating cells were harvested from the lower chamber and from the bottom of the insert and the % migrated cells determined by flow cytometry as described [6].

siRNA nucleofection

An Inventoried Silencer® Select Pre-designed JAM-A siRNA set was synthesised by and purchased from Ambion® (Invitrogen Ltd.), and contained one non-silencing control and two different siRNA

fragments targeting the 3'UTR of human JAM-A mRNA (Supplementary Table S2). To optimise the working concentration of siRNAs using the nucleofection system, a fluorescence-labeled siRNA (Tye563 Fluorescence-labeled siRNA duplex; OriGene Technologies Inc., Rockville, MD, USA) was tested in HL-60 cells at different concentrations prior to JAM-A siRNAs being used. In brief, $2x10^6$ HL-60 cells in 100 μl of the Nucleofector® Solution (Lonza Biologics) and 20nM, 100nM or 200nM of the Tye563 Fluorescent-labeled siRNA duplex were nucleofected using the Amaxa Cell line nucleofector Kit V (Lonza Biologics) and program T-019 (Amaxa Nucleofector® I Device) following the manufacturer's instructions. Between 94 to 96% of cells were nucleofected with the TexRed labeled siRNA at concentrations of 100 nM and 200 nM when measured by flow cytometry 24 hours post nucleofection. HL-60 cells were then nucleofected with 100nM of the siRNAs in Supplementary Table S2B using program T-019 and, after knockdown of JAM-A was assessed at 24, 48 and 72 hours, it was found to be significant with siRNAs 150 and 151 separately, and t with the combined 150+151 siRNAs at 24 hours post-nucleofection (Supplementary Figure S6). For UCB $CD34^+$ cells, $1x10^6$ cells/ml were cultured in StemSpan medium (Stem Cell Technologies) with 100 ng/ml of Flt-3 ligand, IL-6 and SCF and 20 ng/ml of TPO (all from R&D Systems) at 37°C in 5% CO_2 in air overnight before $1x10^6$ cells were resuspended in 100 μl of the Nucleofector® Solution from the Amaxa Human $CD34^+$ cell Nucleofector Kit (Lonza Biologics) with 100 nM of the siRNAs. Program U-008 (Amaxa NucleofectorR® I Device) was used for nucleofection according to the manufacturer's instructions. In the final experiments, UCB $CD34^+$ or HL-60 cells were nucleofected with 100 nM siRNAs {a combination of 150 (5'- GCCUAGUGCCCGAAGUGAAtt -3') and 151 (5'- CCAUCCAAGCCUA CAGUUAtt-3')} or the non-silencing (NS) control using program U-008 (Amaxa NucleofectorR® I Device) according to the manufacturer's instructions [6]. Cells were analysed for changes to cell surface JAM-A expression by flow cytometry or Western blotting at the indicated time points.

Co-immunoprecipitation-Western blotting to assess JAM-A and CXCR4 co-association

cPMV2/3 vectors encoding human JAM-A-FLAG and human CXCR4-HA were obtained from Sino Biological Inc., Beijing, China. The plasmids were transfected together into HEK293T cells using Lipofectamine 2000 over a 5 hour period at 37°C and according to the manufacturer's instructions. The transfection solution was diluted in α-MEM containing 10% (v/v) FCS and the cells cultured for a further 48 hours at 37°C. For Western blotting and co-immunoprecipitation studies, either Brij-35

(10mM Tris-HCl pH7.5, 1mM $CaCl_2$, 1mM $MgCl_2$, 150mM NaCl and 1% (v/v) Brij-35) or Triton-X100 (50mM Tris-HCl pH8, 150mM NaCl and 1% (v/v) Triton-X100) lysis buffers containing protease inhibitors (see below) were used. For co-immunoprecipitation, HEK293T cell lysates were centrifuged and the supernatant incubated with μ-MACS anti-HA microbeads (Miltenyi Biotec GmbH) and bound protein eluted according to the manufacturer's instructions. Western blots were performed on the lysates before or after immunoprecipitation. Lysates of sham or JAM-A-FLAG and CXCR4-HA co-transfected HEK293T cells were heated at 100°C for 5 min. The lysates before or after immunoprecipitation were electrophoresed on 4-12% NuPAGE® Novex Bis-Tris minigels (Invitrogen Ltd.) before the separated proteins were transferred from the gel to nitrocellulose membranes. These membranes were incubated with the Odyssey blocking buffer (LI-COR Biotechnology, Cambridge, UK) and mouse anti-human JAM-A (clone 43/JAM-1; BD Biosciences), mouse anti-FLAG tag (clone M2; Sigma-Aldrich Ltd.), mouse anti-HA tag (clone HA.C5, Abcam) or where appropriate mouse anti-α-tubulin (clone B-5-1-2; Sigma-Aldrich Ltd.) primary antibodies added overnight at 4°C. For JAM-A antibody detection, the reaction was developed with biotinylated anti-mouse Ig VeriBlot secondary antibody (Abcam) and then IRDye 800CW streptavidin (Li-COR Biotechnology), while, for HA-tag antibody detection, the IRDye 800CW or 680CW goat anti-mouse Ig (Li-COR Biotechnology) was used. For the detection of the anti-FLAG or anti-α-tubulin antibodies, the IRDye 800CW goat anti-mouse Ig (Li-COR Biotechnology) was used. The bound complex was detected using the ODYSSEY® CLx Infrared Imaging System (Li-COR Biotechnology). The images were analyzed using the Odyssey Application Software, version 1.2 (Li-COR Biotechnology) to obtain the integrated intensities.

Western blotting

The total protein from 0.5-2x10^6 cells was extracted using 50 μl of lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 5 mM EDTA, 50 mM NaF, containing 5 mM DTT, 0.1 M PMSF, 100 mM Na_3VO_4 and proteinase inhibitors cocktails (AEBSF–[4-(2-aminoethyl)benzene sulphonyl fluoride hydrochloride], aprotinin, bestatin hydrochloride, E-64–[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], leupeptin hemisulfate salt and pepstatin A ; Sigma-Aldrich Ltd.) on ice for 30 minutes, followed by centrifuging at 14000 r.p.m. at 4°C for 30 minutes and storage at -80°C or for protein concentration analysis using the advanced protein concentration assay reagent from Cytoskeleton Inc. (Denver, CO, USA) as described by the manufacturer. Total protein lysates (50 μg)

were electrophoresed on 12-15% (w/v) NuPAGE® Novex Bis-Tris Gels (Invitrogen Ltd.), then transferred to PVDF or NC membranes, which were blocked using Odyssey blocking solution (LI-COR Biotechnology) or with 5% (w/v) skim milk in PBS containing 0.1% (v/v) Tween-20 (both from Sigma-Aldrich Ltd.) for 1 hour. The blots were probed with mouse anti-human JAM-A (43/JAM-1; BD Biosciences), rabbit anti-human phospho-p44/42 MAPK/phosphor-ERK1/2 (Thr202/Tyr204) (#9101, Cell Signaling Technology, Danvers, MA, USA), rabbit-anti human p44/42 MAPK/ERK1/2 (#9102, Cell Signaling Technology), rabbit anti-human phospho-PKC-ζ (sc-12894-R, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-human PKC-ζ primary antibody (sc-216, Santa Cruz Biotechnology), rabbit anti-human phospho-Akt (Ser473; #9271, Cell Signaling Technology), or rabbit anti-human Akt primary antibody (#9272, Cell Signaling Technology). Mouse anti-human α-tubulin ((B-5-1-2, Sigma-Aldrich Ltd.) or GAPDH (2D4A7, Abcam) served as the loading controls. The blots were developed with IRDye 800CW goat anti-rabbit or 680CW goat anti-rabbit or goat anti-mouse secondary antibodies prior to signal detection in ODYSSEY CLx Infrared Imaging System (LI-COR Biotechnology). For CXCL12 (100 nM) stimulation prior to protein extraction, the method by Katagiri *et al.* [16] was followed. Quantitation of the blotted proteins was carried out using Quantity One 4.6.3 basic software (Bio-Rad Laboratories Inc.). The minimal area of signal was gated, the density/mm^2 determined and the signal density normalised to the loading control protein in each lane.

Immunofluorescence co-localisation analysis

Eight-well glass culture slides (BD Falcon™; Becton Dickinson) were coated with 10 μg/ml fibronectin (Sigma-Aldrich Ltd.) in HBSS at 4°C overnight, before air drying and the addition of 200 ng/ml CXCL12 (PeproTech Ltd.) in HBSS at 37°C for 30 minutes [6]. Cells (1-2x10^5 cells per well) in 100 μl basic media with 0.5 (w/v) BSA (Sigma-Aldrich Ltd.) were applied to the CXCL12 coated slides for the indicated times at 37°C, washed and fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich Ltd.) for 15 minutes at room temperature, and then blocked with 4% (w/v) BSA in HBSS for 30 minutes at 4°C. Cells were stained with JAM-A monoclonal (mAb; CD321) or polyclonal antibodies (BD552147, BD Biosciences or #36-1700, Invitrogen Ltd. respectively), CXCR4 polyclonal antibody (Ab2074, Abcam, Cambridge, UK), CD164 mAb (N6B6,BD Biosciences) and ICAM-3 mAb (ICAM-3.3, R&D Systems) or their isotype or Ig controls with 2% (w/v) BSA in HBSS at 4°C overnight. The cells were then gently washed with 4% (w/v) BSA in HBSS three times before Alex Fluor® 488 or Alex Fluor® 555 goat anti-

mouse or rabbit secondary antibodies (Invitrogen Ltd.) were applied to the cells at 4°C for 30 minutes. Finally, the cells were washed with 4% BSA (w/v) in HBSS and stained DAPI (Sigma-Aldrich Ltd.) prior to examination under an Eclipse TE600 microscope (Nikon UK Ltd.). Approximately 100 cells per condition were analyzed.

Duolink™ in situ PLA Proximity Ligation Assays

Cells (1-2x10^5 cells) in their basal media containing 0.5% (w/v) BSA (Sigma-Aldrich Ltd.) were applied to CXCL12 (200ng/ml) precoated slides [6] described above for the times indicated at 37°C before being fixed as above. The fixed cells were then blocked with the Duolink® blocking solution for 30 minutes at 37°C, before addition of the primary mAbs or polyclonal antibodies described above in Duolink® antibody diluent at 4°C overnight and washed with the Duolink® Wash buffer three times at room temperature (all Duolink® reagents from Cambridge Bioscience Ltd., Cambridge, UK). Cells were next labeled with secondary species specific antibodies conjugated with unique short DNA fragments as the anti-mouse PLA probe MINUS and anti-rabbit PLA probe PLUS at 37°C for 1 hour. Interacting MINUS and PLUS probes in close proximity were joined by enzymatic ligation and PCR amplified as described by the manufacturer. TaxRed DNA dye was then applied to visualise the amplified and interacting DNA fragments under an Eclipse TE600 microscope (Nikon UK Ltd.). At least 100 cells per condition were counted per assay.

***In vivo* studies**

NOD/LtSz-scid IL-2R γ (c)-null (NSG) mice were bred and maintained at the University of Bristol Animal Service Unit and studies carried out using procedures licensed by the UK Home Office. Thawed human UCB $CD34^+$ cells (n= 5 batches) were cultured overnight in StemSpan medium (Stem Cell Technologies) with cytokines as above, prior to labeling with APC-CD34, PE-anti-JAM-A antibody (M.Ab.F11) and 7AAD. Cells were sorted using a Becton Dickinson Influx cell sorter and BD Sortware version 1 (BD Biosciences), on the basis of fluorescence intensity after gating on 7AAD (Sigma-Aldrich) negative cells. $CD34^+$ cells were selected on the basis of JAM-A positivity into $CD34^+$JAM-A^{Low}, $CD34^+$JAM-A^{Int} and $CD34^+$JAM-A^{High}. Between 1.5-10x10^4 cells from each fraction in IMDM plus 5% (w/v) human serum albumin (HAS) were injected into the lateral tail veins of 6-8 week old unconditioned NSG mice (n=2-6 per group) as described [17]. Animals were monitored weekly for 14

weeks for the presence of human peripheral blood $CD45^+$ cells, and maintained for 15 weeks before analysis of the bone marrow using antibodies against human CD45, CD7, CD19, CD33 and JAM-A. Cells harvested from bone marrow of some engrafted mice were used for serial transplantation experiments. For comparison with primary transplants, equal numbers of human $CD45^+$ cells were inoculated into serial recipients. These cells were not enriched for any particular phenotype before evaluation in sequential xenografts. Animals were maintained and analyzed as described for the primary transplants.

Statistics

Data are presented as the mean±S.E.M. Statistical analyses used unpaired two tailed Student's t-test or 2-way analysis of variance (ANOVA) with Tukey post hoc testing or Sidak's multiple comparison test and were performed using GraphPad Prism version 3.0 or 6.03 (Graphpad Software Inc., San Diego, CA, USA). P values < 0.05 were considered statistically significant.

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SUPPLEMENTARY TABLES

Supplementary Table S1. Antibodies used in flow cytometric analyses.

Antibody (Clone)	Conjugate	Isotype	Company	Catalog number
CD7 (M-T701)	APC	mouse IgG1	BD Pharmingen™	BD561604
CD7 (M-T701)	FITC	mouse IgG1	BD Biosciences	BD332773
CD11a (HI111)	APC	mouse IgG1	BD Pharmingen™	BD559875
CD18 (6.7)	FITC	mouse IgG1	BD Pharmingen™	BD555923
CD19 (4G7)	PE	mouse IgG1	BD Biosciences	BD345777
CD29 (MAR4)	PE	mouse IgG1	BD Pharmingen™	BD555443
CD29 (4B4LDC9LDH8)	FITC	mouse IgG1	Beckman Coulter	6603109
CD31 (WM59)	PE	mouse IgG1	BD Pharmingen™	BD555446
CD33 (AC104.3E3)	APC	mouse IgG1	Miltenyi Biotec GmbH	130-091-731
CD34 (AC136)	APC	mouse IgG2a	Miltenyi Biotec GmbH	130-090-954
CD34 (8G12)	PerCP	mouse IgG1	BD Pharmingen™	BD345803
CD34 (581)	AF700	mouse IgG1	BD Pharmingen™	BD561440
CD38 (HIT2)	FITC	mouse IgG1	BD Pharmingen™	BD555459
CD38 (HIT2)	PE-TxR	mouse IgG1	ThermoFisher Scientific	MHCD3817
CD44 (515)	PE	mouse IgG1	BD Pharmingen™	BD550989
CD45 (2D1)	PerCP	mouse IgG1	BD Pharmingen™	BD345809
CD45 (H130)	FITC	mouse IgG1	BD Pharmingen™	BD555482
CD45RA (HI100)	APC-H7	mouse IgG2b	BD Pharmingen™	BD560674
CD49f (GOH3)	PerCP-Cy5.5	rat IgG2a	Biolegend	313618
CD49f (GOH3)	Pe-Cy7	rat IgG2a	Biolegend	313622
CD54 (HA58)	APC	mouse IgG1	BD Pharmingen™	BD559771
CD51/CD61 (23C6)	N/C	mouse IgG1	BD Pharmingen™	BD555504
CD62E (68-5H11)	APC	mouse IgG1	BD Pharmingen™	BD551144
CD73 (AD2)	PE	mouse IgG1	BD Pharmingen™	BD550257
CD90 (5E10)	FITC	mouse IgG1	BD Pharmingen™	BD555595
CD90 (5E10)	PE	mouse IgG1	Biolegend	328110
CD105 (166707)	FITC	mouse IgG1	R&D Systems	FAB10971F
CD123 (7G3)	PE-Cy7	mouse IgG2a	BD Pharmingen™	BD560826
CD123 (6H6)	PE-Cy7	mouse IgG1	Biolegend	306010
CD123 (6H6)	PerCP-Cy5.5	mouse IgG1	Biolegend	306016
CD133 (293C3)	APC	mouse IgG2b	Miltenyi Biotec GmbH	130-090-854
CD146 (541-10B2)	APC	mouse IgG1	Miltenyi Biotec GmbH	130-092-849
CD164 (N6B6)	PE	mouse IgG2a	BD Pharmingen™	BD551298
CD164 (N6B6)	FITC	mouse IgG2a	BD Pharmingen™	BD551297
CD166 (105902)	PE	mouse IgG1	R&D Systems	FAB6561P
CD184 (CXCR4;12G5)	APC	mouse IgG2a	BD Pharmingen™	BD555976
JAM-A (M.Ab.F11)	PE	mouse IgG1	BD Pharmingen™	BD552556
JAM-A (M.Ab.F11)	N/C	mouse IgG1	BD Pharmingen™	BD552147
JAM-A (OV-5B8)	FITC	mouse IgG1	Biolegend	353504
Human HSC lineage cocktail 1 containing:				
CD2 (RPA-2.10)	APC	mouse IgG1	eBioscience Inc.	22-7776
CD3 (OKT3)	APC	mouse IgG2a	eBioscience Inc.	22-7776
CD7 (M-T701)	APC	mouse IgG1	BD Biosciences	BD561604
CD10 (HI10a)	APC	mouse IgG1	BD Pharmingen™	BD332777
CD11b (ICRF44)	APC	mouse IgG1	BD Pharmingen™	BD561015
CD14 (61D3)	APC	mouse IgG1	eBioscience Inc.	22-7776
CD16 (CB16)	APC	mouse IgG1	eBioscience Inc.	22-7776
CD19 (HIB19)	APC	mouse IgG1	eBioscience Inc.	22-7776
CD56 (CB56)	APC	mouse IgG1	eBioscience Inc.	22-7776
CD235ab (HIR2)	APC	mouse IgG2b	eBioscience Inc.	22-7776

Human HSC lineage cocktail 2 containing:				
CD2 (RPA-2.10)	PE-Cy5	mouse IgG1	Biolegend	300210
CD3 (HIT3a)	PE-Cy5	mouse IgG2a	Bolegend	300310
CD4 (RPA-T4)	PE-Cy5	mouse IgG1	Biolegend	300510
CD7 (CD7-6B7)	PE-Cy5	mouse IgG2a	Biolegend	343110
CD8a (RPA-T8)	PE-Cy5	mouse IgG1	Biolegend	301010
CD10 (HI10a)	PE-Cy5	mouse IgG1	Biolegend	312206
CD11b (ICRF44)	PE-Cy5	mouse IgG1	Biolegend	301308
CD14 (61D3)	PE-Cy5	mouse IgG1	eBioscience Inc.	15-0149-42
CD19 (HIB19)	PE-Cy5	mouse IgG1	Biolegend	302210
CD20 (2H7)	PE-Cy5	mouse IgG2b	Biolegend	302308
CD56 (B159)	PE-Cy5	mouse IgG1	BD Pharmingen™	555517
CD235ab (HIR2)	PE-Cy5	mouse IgG2b	Biolegend	306606

HSC: Hematopoietic stem cell. N/C: non-conjugated. FITC: fluorescein isothiocyanate, APC: allophycocyanin, PE: phycoerythrin, PerCP: peridinin-chlorophyll-protein complex.

Supplementary Table S2. JAM-A Knock-down using siRNAs

A) JAM-A siRNAs

Description	siRNA ID	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
Non-Silencing control (NS)	N.A.	N.A.	N.A.
JAM-A siRNA-150	s27150	GCCUAGUGCCCGAAG UGAAtt	UUCACUUCGGGCACUAG GCtg
JAM-A siRNA-151	s27151	CCAUCCAAGCCUACA GUUAtt	UAACUGUAGGCUUGGAU GGag
Tye563 Fluorescent-labelled siRNA duplex	SR30002	N.A.	N.A.

N.A.: Not available.

B) Decrease in Cell Surface Expression of JAM-A on HL-60 cells 24 h after 100nM siRNA Nucleofection

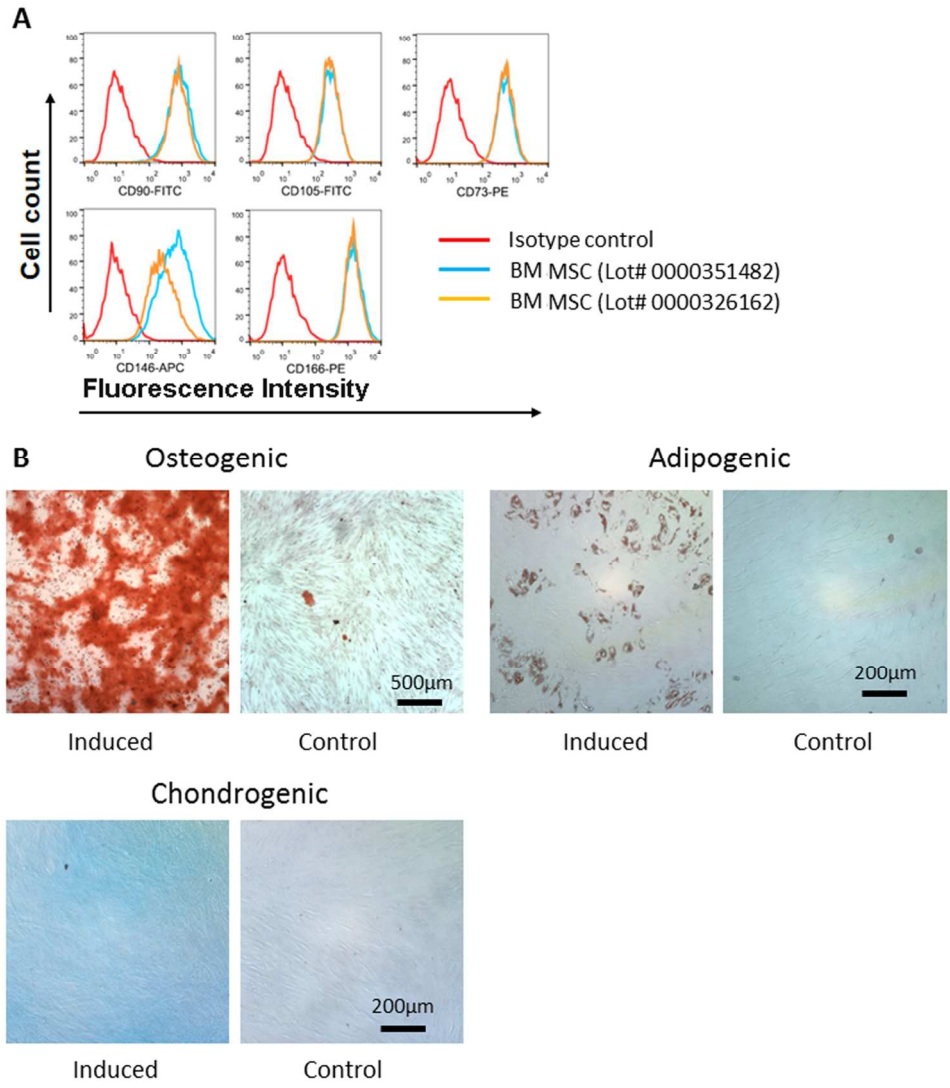
Description	M.F.I. for JAM-A Cell Surface Expression on HL-60 cells
NS	846±127
JAM-A siRNA-150	438±131
JAM-A siRNA-151	627±154
JAM-A siRNA-150+151	287±30

Supplementary Table S3. CXCL12 production by BMEC-60 and BM MSCs

	CXCL12 concentration after 24 hours culture (pg/ml)	CXCL12 concentration after 48 hours culture (pg/ml)
BMEC-60 lysate (untreated 1:5 dilution)	3.3±2.2*	4.2±3.6*
BMEC-60 lysate (IL1β pre-treated 1:5 dilution)	5.0±2.5*	4.1±3.0*
BMEC-60 secretome	ND	ND
BMEC-60 secretome (IL1β pre-treated)	ND	1.7±0.8
BM MSC secretome	70.0±11.8	336.7±32.1

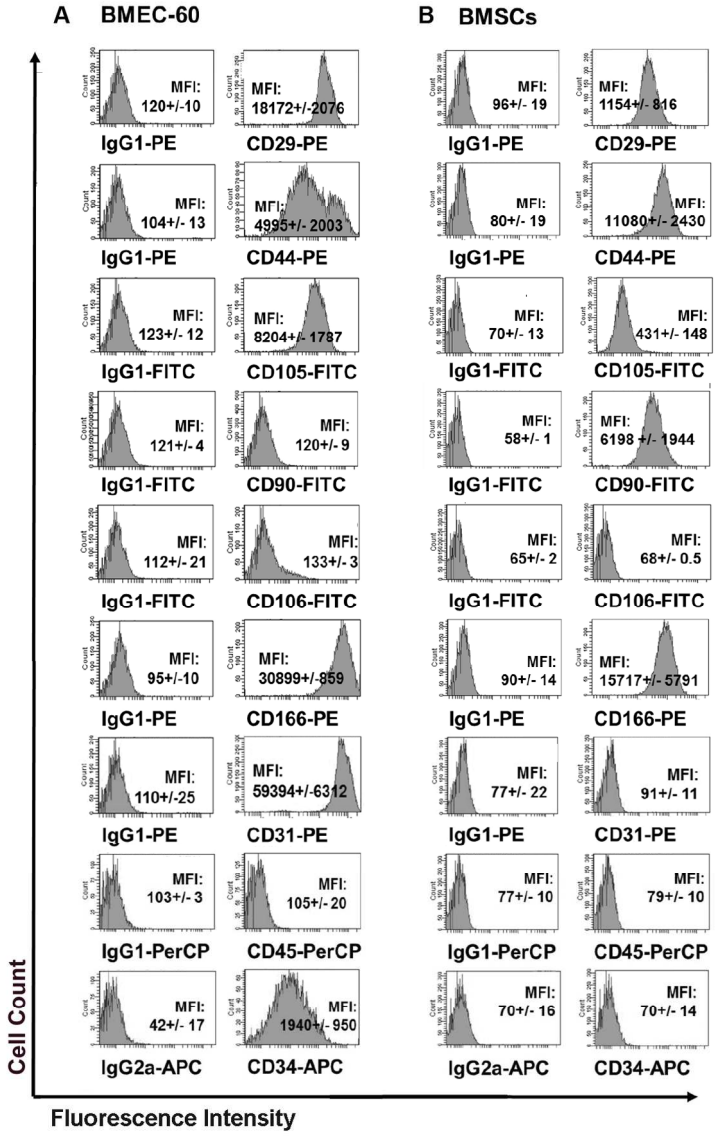
ND = below level of detection in ELISA; * assayed at a 1:5 dilution of lysate. Mean±S.E.M. of n=3.

Supplementary Figure S1. Chang et al. 2015 TOP



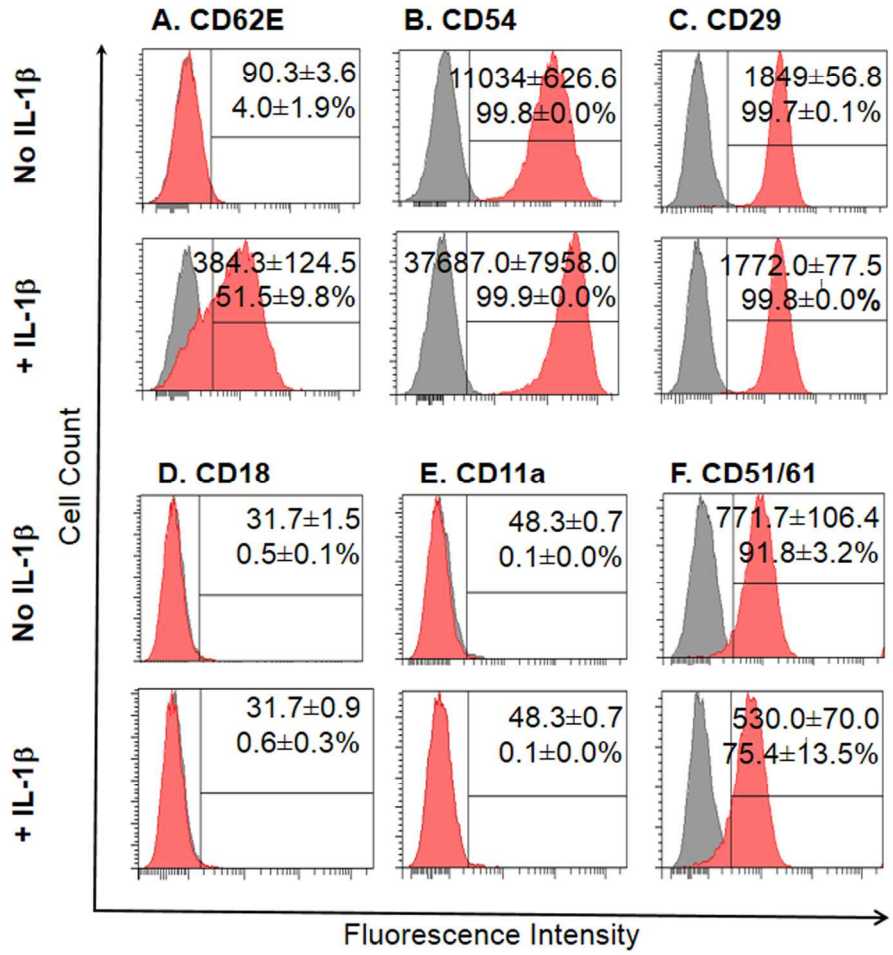
134x154mm (300 x 300 DPI)

Supplementary Figure S2. Chang et al. 2015 TOP



204x327mm (300 x 300 DPI)

Supplementary Figure S3. Chang et al. 2015 TOP



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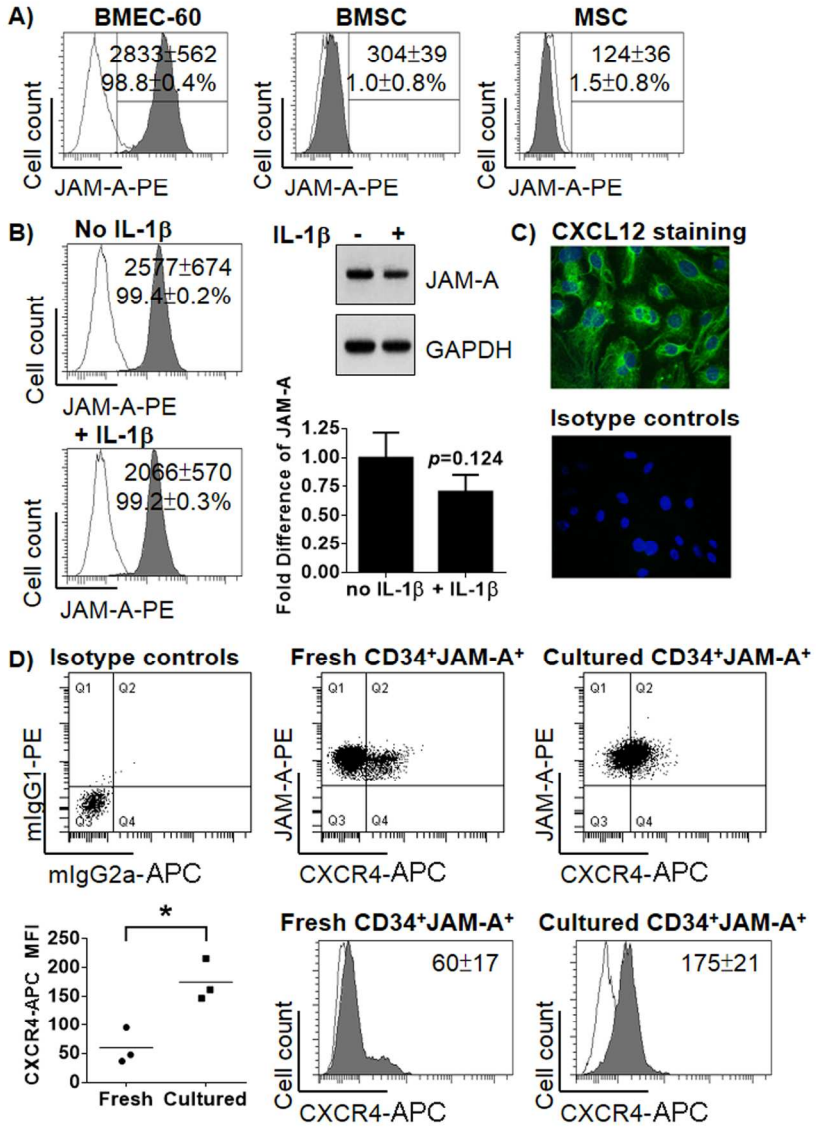
Supplementary Figure S4. Chang et al. 2015 TOP



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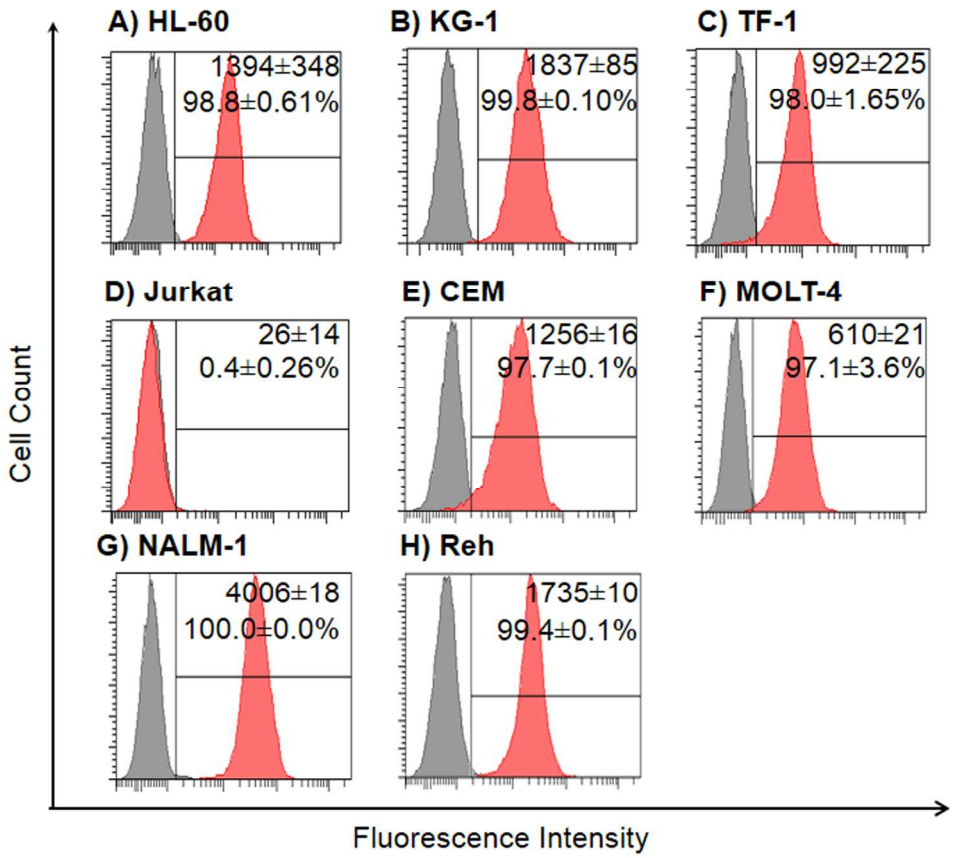
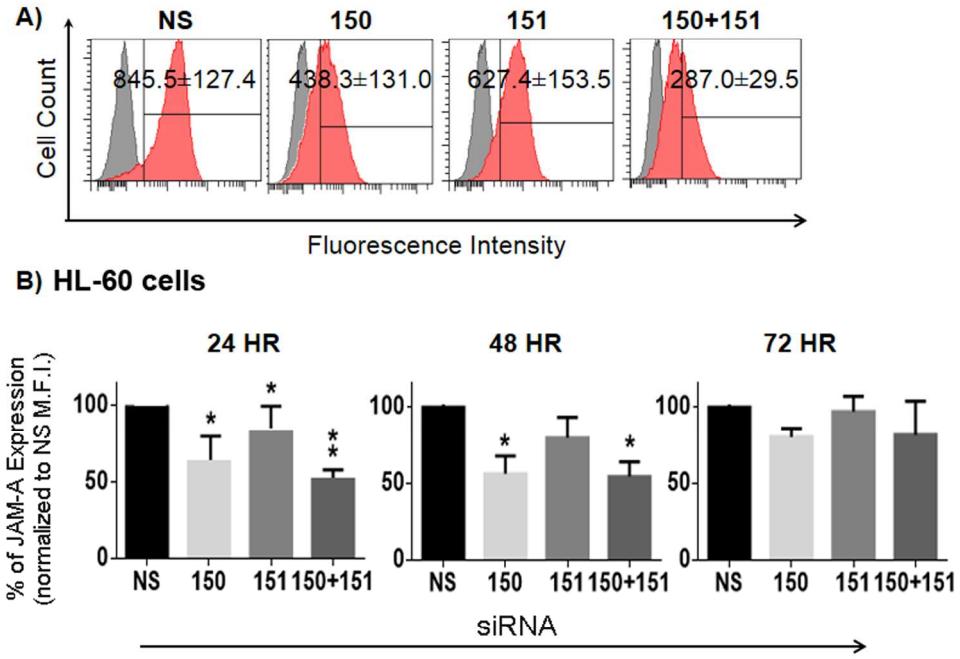
Supplementary Figure S5. Chang et al. 2015 TOP



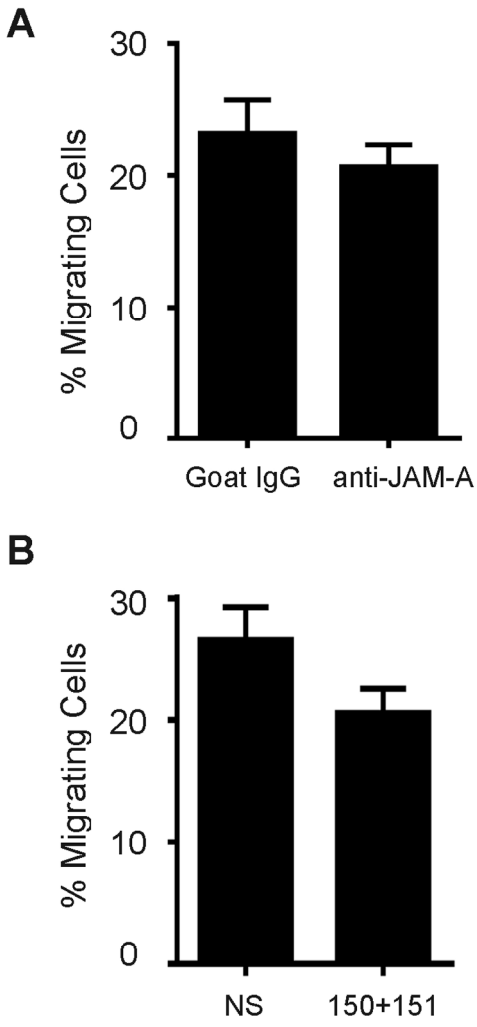
190x189mm (300 x 300 DPI)

Supplementary Figure S6. Chang et al. 2015 TOP



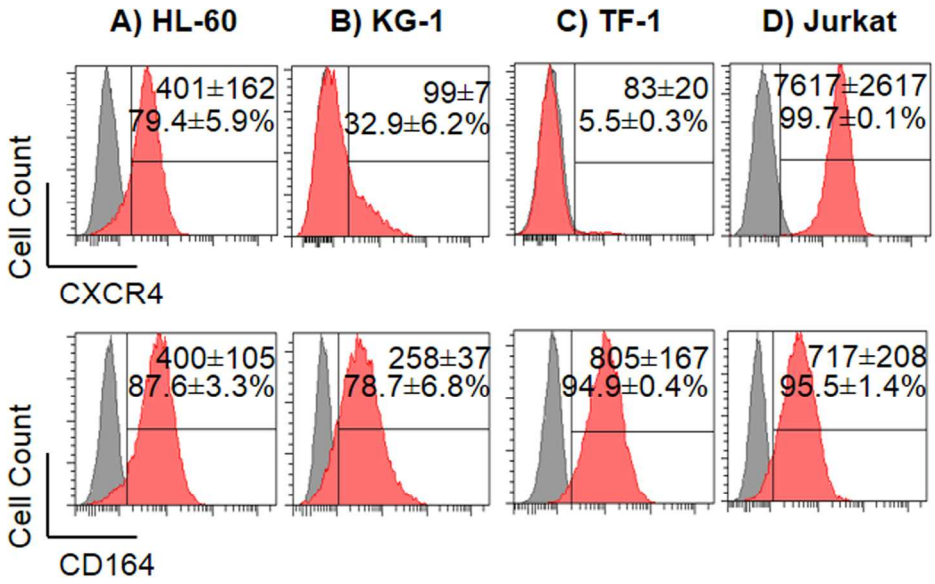
218x234mm (300 x 300 DPI)

Supplementary Figure S7. Chang et al. 2015 TOP



142x163mm (600 x 600 DPI)

Supplementary Figure S8. Chang et al. 2015 TOP



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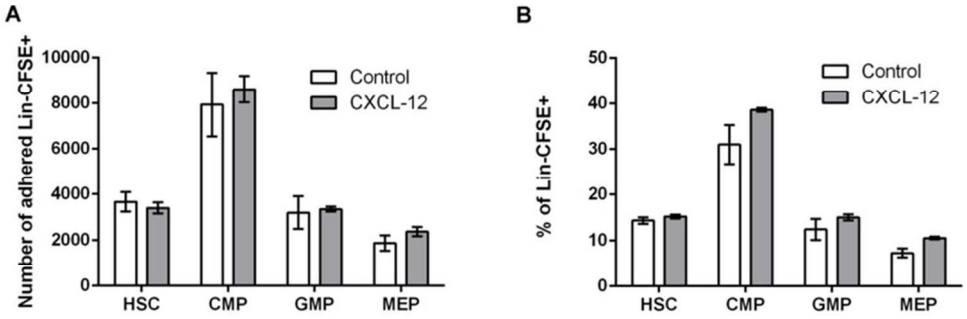
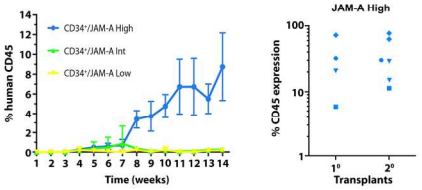
Supplementary Figure S9 Chang et al. 2015 TOP



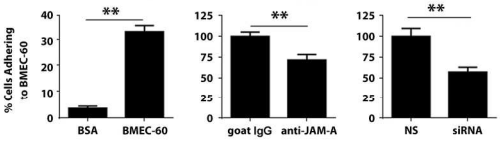
72x31mm (300 x 300 DPI)

Chang et al. 2015. Graphical Abstract

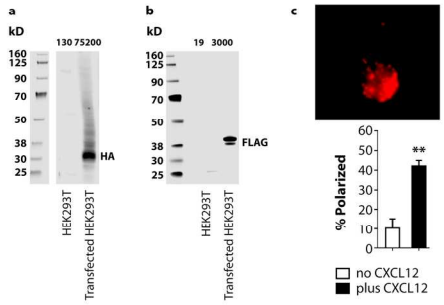
1. JAM-A is highly expressed on human *in vivo* repopulating cells



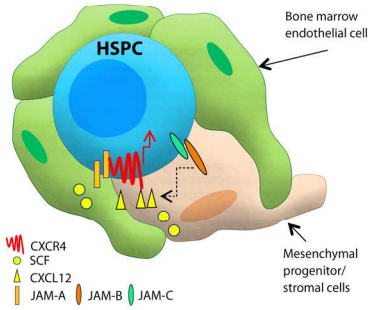
2. JAM-A mediates adhesion to bone marrow endothelium



3. JAM-A co-localizes with CXCR4 and moves with CXCR4 to the leading edge in response to CXCL12



4. Model of JAM-A, B, and C interactions in the bone marrow vascular niche



190x142mm (300 x 300 DPI)