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Mild heat treatment primes human CD34+ cord blood cells for migration towards SDF-1α and enhances engraftment in an NSG mouse model

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Abstract

Simple efforts are needed to enhance cord blood (CB) transplantation. We hypothesized that short-term exposure of $CD34^+$ CB cells to 39.5°C would enhance their response to SDF-1, by increasing lipid raft aggregation and CXCR4 expression, thus leading to enhanced engraftment. Mild hyperthermia (39.5°C) significantly increased the percent of $CD34^+$ CB that migrated towards SDF-1. This was associated with increased expression of CXCR4 on the cells. Mechanistically, mild heating increased the percent of $CD34^+$ cells with aggregated lipid rafts and enhanced co-localization of CXCR4 within lipid raft domains. By using methyl-β-cyclodextrin (MβCD), an agent that blocks lipid raft aggregation, it was determined that this enhancement in chemotaxis was dependent upon lipid raft aggregation. Co-localization of Rac1, a GTPase crucial for cell migration and adhesion, with CXCR4 to the lipid raft was essential for the effects of heat on chemotaxis, as determined with an inhibitor of Rac1 activation, NSC23766. Application-wise, mild heat treatment significantly increased the percent chimerism as well as homing and engraftment of $CD34^+$ CB cells in sublethally irradiated NSG mice. Mild heating may be a simple and inexpensive means to enhance engraftment following CB transplantation in patients.

Keywords

Hematopoietic stem cells; hematopoietic progenitor cells; SDF-1/CXCL12; chemotaxis; lipid rafts; cord blood transplatation

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Introduction

Cord blood (CB) is a valuable source of hematopoietic stem (HSC) and progenitor cells (HPC) for transplantation to treat patients with malignant and non-malignant disease[1]. One disadvantage of CB is that it contains fewer HSC/HPC than bone marrow (BM) and mobilized peripheral blood. CB transplantation (T) has been improved by use of double CB units, but this has not resulted in shortened time to engraftment[2]. Efforts to enhance engraftment with limited numbers of HSC/HPC in CB could improve efficacy of single unit CBT. One way to accomplish this is to enhance homing of HSC/HPC to the BM microenvironment. The BM stroma secretes the α-chemokine stromal-derived factor-1[3] (SDF-1/CXCL12) which chemoattracts HSC/HPC to the BM[4–6]. HSC/HPC express cell surface CXCR4, a receptor for SDF-1, and SDF-1-CXCR4 signaling may be essential for homing of HSC/HPC to the BM microenvironment[7–9]. This signaling increases adhesion of HSC/HPC to endothelium via activation of the MAPK p42/44 and PI-3K-AKT axis. This increases integrin adhesion to fibronectin and expression of matrix metalloproteinases (MMPs) resulting in increased homing and engraftment[10,11].

Several agents enhance homing of HSC/HPC towards an SDF-1 gradient, including dipeptidylpeptidase 4 (DPP4) inhibitors, prostaglandin-E2 (PGE2), hyaluronic acid, sphingosine-1-phosphate receptor agonist FTY20, UTP, and the complement cascade cleavage fragments anaphylatoxin C3a and C5a amongst other factors[12–20]. These agents allow cells to migrate to lower SDF-1 concentrations. For example, C3a and C5a enhance incorporation of CXCR4 into lipid raft microdomains[19,21]. These are cholesterol- and glycosphingolipid-enriched portions of the plasma membrane that house several proteins required for cytokine signaling, membrane trafficking, and cytoskeleton organization. Normally, CXCR4 is retained within the non-lipid raft portion of the plasma membrane and must be incorporated into lipid rafts for optimal signaling potential[19]. In addition, aggregation of lipid raft microdomains results in concentration or spatial reorganization of several signaling components. This optimizes conditions for activation of signaling pathways, thus modulating signaling intensity[22]. Aggregation of lipid rafts is an important component of SDF-1-CXCR4 signaling, as lipid raft clustering allows for interactions of small GTPases Rac and Rho with their downstream effectors. This ultimately controls membrane ruffling and microtubule stabilization which is essential for cell migration[19,23].

Temperature also influences plasma membrane lipid bilayer and lipid raft organization[24–26]. Physiological temperatures ranging from 33°C (average peripheral body temperature) to 37°C (average core body temperature) alter membrane fluidity which increases as temperature rises, especially within the fever or exercise-induced range of ~38–40°C. At the lower end of the physiologically-relevant temperature spectrum, lipid rafts, which exist as small islands within the membrane, have a height mismatch with non-raft portions of the membrane. This creates line tension and prevents fusion of smaller lipid rafts together[24,26–28]. As temperature increases, membrane fluidity increases, overpowering line tensions, allowing for fusion of lipid rafts[24–26]. This thermal-mediated reorganization of the plasma membrane alters the function of macrophages, natural killer cells, T cells and B cells[25,26,29–32]. Effects of heat on the membrane are short lived, lasting approximately 2 hours[26]. Heat also induces changes in migration patterns of neutrophils, T cells, dendritic

cells, natural killer (NK) cells and macrophages[31–37]. Herein, we examined whether heating $CD34^+$ CB cells to 39.5°C would prime them for optimal migration *in vitro* and homing and engraftment following transplantation in NSG mice. We also evaluated mechanism associated with these effects.

Methods

Mice, cell Line and isolation of $CD34^+$ CB cells

NSG mice (8–10 week old females) were obtained from an on-site breeding core facility at Indiana University School of Medicine. The cytokine-dependent Mo7e cell line[38] was cultured in IMDM with hepes and L-Glutamine (Lonza; Walkersville, MD, USA), 10% FBS (Fisher Scientific; Waltham, MA, USA) and 10ng/mL recombinant human (rh) GM-CSF (R&D Systems; Minneapolis, MN, USA). Mo7e cells express CXCR4 and migrate towards SDF-1[3]. Human CB was obtained from Cord:Use Cord Blood Bank (Orlando, FL, USA). Cells were washed in PBS (Lonza) prior to Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB; Pittsburgh, PA, USA) separation of mononuclear cells. The $CD34^+$ CB cells were then isolated using immunoaffinity selection with MiniMACS paramagnetic beads (Miltenyi Biotec; Auburn, CA, USA) using two sequential columns. The purity of $CD34^+$ CB cells was always above 95%. CB $CD34^+$ cells were acclimated to 37°C overnight in IMDM with 10% FBS and 100ng/mL each of rh-stem cell factor (SCF; R&D Systems), rh-thrombopoietin (TPO; R&D Systems), and rh-fms-related tyrosine kinase 3 (FLT3; Amgen; Thousand Oaks, CA, USA) as the separation process (exposure to cold temperatures and Ficoll separation) alters the surface expression of CXCR4 (as indicated by BD Biosciences). The Indiana University Committee on Use and Care of Animals and the Indiana University Institutional Review Board approved mouse and CB studies.

Antibodies and reagents

PE-conjugated rat anti-human CD184/CXCR4 (clone 1D9, isotype control rat $IgG_{2a,\kappa}$), FITC-conjugated mouse anti-Rac1 (clone 102/Rac1, isotype control mouse $IgG_{2,b}$), APC-conjugated mouse anti-human CD34 (clone 581, isotype mouse $IgG_{1,\kappa}$), PE-conjugated mouse anti-human CD38 (clone HIT2, isotype control mouse $IgG_{1,\kappa}$) and APC-conjugated mouse anti-human CD45 (clone Hl30, isotype mouse $IgG_{1,\kappa}$) were purchased from BD Biosciences (San Diego, CA, USA). Blocking reagents human gamma globulin and mouse gamma globulin were purchased from Jackson ImmunoResearch Laboratories Incorporated (West Grove, PA, USA). BD Cytofix™ fixation buffer was purchased from BD Biosciences. Recombinant human SDF-1α was purchased from R&D Systems. FITC-conjugated Cholera toxin B subunit (CTxB) and methyl-β-cyclodextrin (MβCD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rac1 inhibitor NSC23766 was purchased from BioVision (Milpitas, CA, USA).

Chemotaxis assay

Cells acclimated to 37°C were suspended in pre-warmed IMDM (37°C) with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and either left at 37°C or placed in a water bath at 39.5°C ± 0.2°C for up to 4 hours. Costar® 24-well Transwell® plates with 6.5mm diameter inserts with 5.0μm pores (Corning Incorporated; Corning, NY, USA) were prepared by

placing 650μL of pre-warmed serum-free media (37°C) that contained 0, 12.5, 25, 50, 100 or 200ng/mL rhSDF-1α in the bottom well and allowing plates to acclimate at 37°C for half an hour prior to chemotaxis assay. Cells were suspended at 1×10^5 cells/100μL pre-warmed serum-free media and loaded to the top chamber of the transwell assay. Transwell plates were placed in a 37°C incubator (95% humidity, 5% CO_2) for 4 hours. Percent migration was determined using flow cytometry with background migration (cells that migrated towards media alone; always <4%) subtracted from total migrated cells. To examine the role of lipid rafts, cells maintained at 37 or 39.5°C for 4 hours were incubated for 30 minutes at 37°C in media containing 0, 0.5, 0.75, 1.00, 1.25, 1.50 or 1.75mM MβCD immediately prior to washing and placement in the chemotaxis assay. To examine the role of Rac1, cells maintained at 37 or 39.5°C for 4 hours were incubated for 30 minutes at 37°C in media containing 0, 50, 100, 150, 200, 250 or 300μM NSC23766 prior to washing and placement in the chemotaxis assay.

Flow cytometry and ImageStream analysis

Cells were collected, heated at 39.5°C for up to 6 hours or left at 37°C, washed in PBS, stained with CTxB, anti-human CXCR4 or anti-Rac1, fixed and analyzed on an LSRII flow cytometer (BD Biosciences) using BD CellQuest™ Pro software (version 6.0; BD Biosciences). For quantitative image analysis of GM1, CXCR4, and Rac1 aggregation, fluorescent cell images (40X) were acquired using an ImageStream flow cytometry system (Amnis; Seattle, WA, USA)[26]. Eight thousand images were analyzed using IDEAS software (Amnis). In focus cells were evaluated after gating on live, single, $CD34^+$ cells based on an aspect ratio near one and a low area of the bright field. Bright detail intensity of FITC-CTxB, FITC-Rac1, and APC-CXCR4 staining was used to quantify percent cells with aggregated lipid rafts, Rac1 and/or CXCR4 by calculating the sum of intensity values from the brightest areas within a cell that are morphologically defined as peak fluorescence distributions of three pixel radius or less. A similarity feature determined the amount of overlay between CTxB/CXCR4 and CXCR4/Rac1 staining.

$CD34^+$ CB engrafting studies

Briefly, recipient NSG mice received a single dose of 3.5Gy of total body irradiation (TBI, ^{137}Cs source) followed 24 hours later with an i.v. injection of 80,000 purified CB $CD34^+$ cells[39,40] that had been heated to 39.5°C for 4 hours or left at 37°C. Peripheral blood was collected via tail vein into heparinized microcapillary tubes (Fisher Scientific; Pittsburg, PA, USA) 1, 2, 4, and 6 months post transplantation. Following lysis of red blood cells using lysis buffer (0.155M NH_4Cl, 0.01M $KHCO_3$, 0.1mM EDTA in H_2O; Sigma-Aldrich), cells were washed, blocked with both human and mouse gamma globulin, stained with APC-conjugated anti-human CD45, and fixed prior to flow analysis on a FACSCalibur utilizing BD CellQuest™ Pro software to determine percent human $CD45^+$ cells.

$CD34^+$ CB cell homing assay

Recipient NSG mice received a single dose of 3.5Gy of TBI (^{137}Cs source) followed 24 hours later with an i.v. injection of 500,000 CB CD34+ cells heated to 39.5°C for 4 hours or left at 37°C. BM mononuclear cells from a femur of each recipient mouse was collected 24

hours later, stained with anti-human CD45, and fixed prior to flow analysis on a FACSCalibur to determine percent human $CD45^+$ cells.

Statistical analysis

For chemotaxis assays, data are the mean percent migration +/− SEM of 3–5 wells. For experiments utilizing ImageStream technology or flow cytometry, data for the Mo7e cell line is the mean +/− SEM of 3 tubes. Average CB data is the mean of 3–4 individual CBs +/− SEM. For $CD34^+$ CB homing and engraftment, the average percent human $CD45^+$ cells of 5 individual mice per group was calculated +/− SEM. Student's two-tailed *t* test was used to compare cells kept at 37°C versus 39.5°C, $P < 0.05$ was considered significant.

Results

Heating $CD34^+$ CB cells to 39.5°C enhances chemotaxis towards an SDF-1 gradient

To test the hypothesis that chemotaxis of $CD34^+$ CB cells towards SDF-1 is enhanced by heating the cells to 39.5°C, we performed a transwell assay where $CD34^+$ CB cells were heated for either 1, 2, 3, or 4 hours prior to the assay and then allowed to migrate towards 50ng/mL of rhSDF-1α for 4 hours at 37°C. $CD34^+$ cells that were heated at 39.5°C for 4 hours migrated significantly better towards SDF-1 than cells left at 37°C or heated to 39.5°C for 3 hours or less (Figure 1A). Heated (39.5°C for 4 hours) $CD34^+$ CB cells from 4 different donors migrated better towards SDF-1 than non-heated cells (Figure 1B). This enhancement in migration was also seen for the human factor-dependent Mo7e cell line after cells were heated to 39.5°C for 4 hours (Figure 1B). To determine if this thermal-mediated increase in migration was due to enhanced sensitivity to SDF-1, transwell assays were performed in which $CD34^+$ cells cultured at 37 or 39.5°C for 4 hours prior to the assay were allowed to migrate towards various concentrations of SDF-1 (0–200ng/mL). The maximum percent migration for cells incubated at 37°C (25.2%) required 50ng/mL SDF-1 whereas cells incubated at 39.5°C only required 25ng/mL of SDF-1 to achieve the same percent migration even though the maximum migration when cells were incubated at 39.5°C was still 50ng/mL SDF-1 (38.8%; Figure 1C). Percent migration of $CD34^+$ CB cells towards control media (0ng/mL SDF-1) was comparable between cells incubated at 39.5°C and those kept at 37°C suggesting that heating the cells most likely did not affect the size or other mechanical properties allowing for increased migration due to gravity. Heat-mediated increases in migration of $CD34^+CD38^-$ and $CD34^+CD38^+$ were similar (Figure 1D). These findings demonstrate that heating $CD34^+$ CB cells to 39.5°C primes them to more efficiently migrate towards an SDF-1 gradient.

Heating $CD34^+$ CB cells is associated with increased aggregation and incorporation of CXCR4 Into the lipid raft

Incubating cells at higher temperatures (38–40°C) alters membrane fluidity in several hematopoietic cell lineages (i.e. T and B cells) and increases lipid raft aggregation, thus enhancing the signaling potential of several different signaling pathways[24–26]. However, whether lipid raft reorganization occurs within the $CD34^+$ CB cell population following incubation at a physiologically relevant increase in temperature has not been examined. $CD34^+$ cells from 4 different CB samples were incubated at 37 or 39.5°C for 4 hours, fixed

immediately, stained with Cholera toxin B subunit (CTxB; which binds to the ganglioside GM1 found within lipid rafts) and analyzed by imaging flow cytometry. A diffuse or aggregated lipid raft staining pattern (examples in Figure 2A are from CB#1) was determined on over 8,000 $CD34^+$ CB or Mo7e cells using bright detail intensity feature of IDEAS software. This feature designates which cells have diffuse GM1 staining due to a brighter detail intensity within the membrane region (as determined by a set mask) than those cells with clustered GM1 staining, and calculates percent cells with diffuse versus aggregated lipid raft staining. Heating $CD34^+$ CB or Mo7e cells to 39.5°C for 4 hours increased the percentage of cells with aggregated lipid rafts in each sample tested (~3.3 fold increase, Figure 2B). The heat-mediated increase in lipid raft aggregation was seen following 4 hours of heating and did not increase with longer heat treatment (Supplementary Figure 1A). Upon examining the duration of heats effect it was discovered that the heat-mediated lipid raft aggregation only lasted for 2–3 hours following heat treatment (Supplementary Figure 1B). This increase in lipid raft aggregation at 39.5°C was not due to changes in mean fluorescence intensity (MFI) of GM1 staining as determined by flow cytometry (Supplementary Figure 2) suggesting that the differences seen in GM1 positioning was not due to changes in GM1 expression.

It has been shown that for optimal signaling, CXCR4 must be incorporated into the lipid raft[41–44]. To determine whether thermally-mediated enhancement in lipid raft aggregation was associated with increased clustering of CXCR4 and/or increased co-localization of CXCR4 with GM1, $CD34^+$ cells from 4 different CB samples and Mo7e cells were incubated at 37 or 39.5°C for 4 hours. Both bright detail intensity (to examine clustering of CXCR4) and bright detail similarity (to examine the co-localization of GM1 and CXCR4) features of IDEAS software were utilized on over 8,000 $CD34^+$ CB and Mo7e cells per group. In every sample examined, cells incubated at 39.5°C demonstrated an increased percentage of aggregated CXCR4 than cells incubated at 37°C (~2.7 fold increase, Figure 2C). To determine if this change in bright detail intensity was due to changes in expression levels of CXCR4, we assessed MFI of CXCR4 staining of $CD34^+$ cells on 4 CB samples following incubation at either 37 or 39.5°C. Heating $CD34^+$ cells resulted in a small, but reproducible, increase in surface expression of CXCR4 (average 1.4 ± 0.08 fold increase, Figure 2D). Changes in CXCR4 bright detail intensity was not likely due to changes in CXCR4 levels since there was a more aggregated pattern of CXCR4 staining at the membrane region of $CD34^+$ cells when heated. To examine whether CXCR4 moves into or with lipid rafts, bright detail similarity scores of co-localization of CXCR4 with GM1 on the surface of $CD34^+$ CB cells incubated at 37 or 39.5°C was measured. The bright detail similarity score of $CD34^+$ cells incubated at 37°C was lower (1.8 ± 0.17) than cells incubated at 39.5°C (2.9 ± 0.21, Figure 2E) indicating that CXCR4 was more likely to aggregate with lipid rafts when cells were incubated at the higher temperature. This suggests that heat alone may facilitate CXCR4 incorporation into lipid rafts.

Cholesterol in the plasma membrane is required for the thermal-enhancement of $CD34^+$ cell migration towards SDF-1

To determine if thermal-mediated enhancement in $CD34^+$ CB cell migration towards SDF-1 was due to increased aggregation of lipid rafts, we utilized methyl-beta-cyclodextrin

(MβCD) which depletes cholesterol from the plasma membrane disrupting lipid raft aggregation[45,46]. The dose of MβCD used was determined by transwell assays with a range of MβCD doses (0.5–1.75mM, 30 minutes), with 1.25mM concentration demonstrating minimum inhibition of normal chemotaxis towards SDF-1 (Figure 3A). This dose was chosen for all future experiments. In vehicle controls, heat significantly increased the percent of $CD34^+$ cells with aggregated lipid rafts (Figure 3B) and enhanced migration towards 50ng/mL SDF-1 (Figure 3C). However, when $CD34^+$ cells were treated with MβCD, heat had no effect on lipid raft clustering or cell migration (Figure 3B&C and Supplementary Figure 3) suggesting that lipid raft aggregation plays a role in enhanced migration of $CD34^+$ CB cells towards SDF-1 following cell exposure to 39.5°C. To determine if the inhibition of heat's effects was due to reduced aggregation of CXCR4 within the plasma membrane of heated cells, we examined the location of CXCR4 on the surface of $CD34^+$ CB cells incubated at 37 or 39.5°C for 4 hours that were treated with MβCD or vehicle for the last half an hour of treatment. As already seen in Figure 2C, heat increased the percentage of $CD34^+$ cells with aggregated CXCR4 on the cell surface (Figure 3D). When lipid raft aggregation was inhibited, thermally-enhanced CXCR4 clustering was lost. This suggests that increased CXCR4 aggregation was due to increased lipid raft aggregation, and this contributed to increased $CD34^+$ CB cell migration following cell incubation at 39.5°C.

CXCR4 interacts with Rac1 in the plasma membrane following heat treatment

When Rac1 (a small GTPase crucial for cell migration and adhesion) co-localizes with CXCR4 within lipid rafts, this increases sensitivity of cells to low concentrations of SDF-1 in chemotaxis assays since activated GTP-Rac1 binds more efficiently and preferentially to lipid rafts[19,44,47–50]. To examine whether heat enhances Rac1 and CXCR4 co-localization, $CD34^+$ cells from 4 different CB samples and Mo7e cells were incubated at 37 or 39.5°C for 4 hours, stained for CXCR4 and Rac1, and analyzed. Both bright detail intensity (to examine clustering of Rac1) and similarity (to examine the co-localization of Rac1 and CXCR4) features of IDEAS software were utilized on over 8,000 $CD34^+$ CB and Mo7e cells per group. Cells incubated at 39.5°C demonstrated increased percentage of aggregated Rac1 (~2.5 fold increase, Figure 4A&B). To examine whether reorganization of Rac1 to the plasma membrane was associated with increased co-localization with CXCR4, bright detail similarity score of Rac1/CXCR4 overlapping expression on the surface of $CD34^+$ CB cells incubated at 37 or 39.5°C was measured. The bright similarity score of $CD34^+$ cells incubated at 37°C was lower (1.5 ± 0.15) than cells incubated at 39.5°C (2.6 ± 0.4, Figure 4C) indicating that Rac1 was more likely to co-localize with CXCR4 when cells were incubated at the higher temperature.

Interaction of CXCR4 and Rac1 in the lipid raft promotes Rac activation and results in enhanced sensitivity and responsiveness of $CD34^+$ cells towards a SDF-1 gradient[19]. To examine whether Rac1 activation is essential for thermal-mediated enhancement in $CD34^+$ migration, we utilized an inhibitor (NSC23766) to block Rac1 activation. To determine the optimal dose of NSC23766 to use, transwell assays were performed on cells treated for 30 minutes with various doses of inhibitor (50–300μM) at 37°C (Figure 4D). The NSC23766 dose with minimum disruption of chemotaxis towards 50ng/mL of SDF-1, 200μM, was

assessed to be the optimum dose to determine whether enhanced migration seen at 39.5°C required further Rac1 activation. As already seen in Figure 1, heated CD34$^+$ CB cells demonstrated enhanced migration towards SDF-1 compared to unheated cells (Figure 4E). When Rac1 activation was inhibited there was a significant reduction in effects of heat, suggesting that heat-induced Rac1 activation was required.

Pre-treating donor CD34$^+$ CB cells at 39.5°C for 4 hours prior to transplantation enhances engraftment following transplantation into NSG mice

We wished to examine if heating CD34$^+$ CB cells to 39.5°C would enhance the ability of these cells to engraft following transplantation. NSG mice received 3.5Gy total body irradiation followed one day later with transplantation of 80,000 CD34$^+$ CB cells that had been incubated for 4 hours at 37 or 39.5°C immediately prior to injection. Pre-treating CD34$^+$ cells at 39.5°C significantly enhanced human CD45$^+$ cell recovery following transplantation (1.8, 1.3 and 1.7 fold increase respectively) when compared to recovery of human CD45$^+$ cells in animals transplanted with cells incubated at 37°C (Figure 5A). This suggested that heat may allow CD34$^+$ cells to home better towards the BM microenvironment, leading to better engraftment. To test this, NSG mice received 3.5Gy total body irradiation followed one day later with an i.v. injection of 500,000 CD34$^+$ CB cells that had been incubated for 4 hours at 37 or 39.5°C immediately prior to injection. BM was collected 24 hours later. Heating CD34$^+$ CB cells enhanced the percentage of human CD45$^+$ cells in the BM of NSG mice by ~2.9 fold when compared to BM of mice who received CD34$^+$ CB cells incubated at 37°C (Figure 5B).

Discussion

There are currently three main methods that are used to enhance efficacy of CBT: 1) increasing numbers of cells collected and infused, 2) enhancing homing of cells to the BM microenvironment, and/or 3) increasing numbers of HSC/HPC through *ex vivo* expansion. Here we demonstrate a new method to enhance engraftment of CD34$^+$ CB cells via exposure of these cells to mild heat treatment of 39.5°C for 4 hours. Mildly heating CD34$^+$ CB cells primes them to migrate more efficiently towards an SDF-1 gradient (Figure 1) ultimately engrafting in NSG mice with better efficacy, perhaps by better homing to the BM (Figure 5). By mildly heating cells, lipid raft aggregation increases. This correlates with increased CXCR4 aggregation and co-localization within the plasma membrane lipid rafts (Figure 2). This thermally-mediated increase in CXCR4 aggregation is associated with an increased Rac1 aggregation and co-localization with CXCR4 (Figure 4). Inhibition of either lipid raft aggregation or Rac1 activation blocks effects of heat treatment, indicating that heat-mediated alterations to CD34$^+$ cells migration is mitigated at the membrane level (Figure 3 and 4).

Heat treatment alters fluidity of the plasma membrane[24–26]. Heat-mediated changes in membrane fluidics primes multiple immune cell populations for better antigen presentation (e.g. B cells and macrophages)[25,51] and lowers the threshold of signal (whether it be antigen presentation, cytokine or ‘danger’ signal) required for activation (e.g. NK cells, dendritic cells, macrophages, and T cells)[26,29,32,35] through enhancing lipid raft clustering allowing

for a more rapid, stronger response. These findings, along with those presented herein suggest a physiologically relevant importance of temperature in both mature and immature hematopoietic cells on reorganization/localization of lipids and signaling components within the plasma membrane. This is especially important in the SDF-1-CXCR4 axis as relocation or recruitment of CXCR4 into lipid rafts appears key for optimizing SDF-1 signaling[19,41–44]. Co-localization of CXCR4 with the lipid raft allows the receptor to be in closer contact with signaling components such as Rac1 (enhanced in our model simply by heating the cells alone), which is required for key events to occur in order to get events such as cytoskeleton reorganization[19,23]. Mechanism of heat's effect on priming $CD34^+$ CB cells appears to be through reorganization of key SDF-1 signaling components into the lipid raft, since disrupting lipid raft clustering by extracting cholesterol from the membrane using MβCD blocked heat enhancement of $CD34^+$ cell chemotaxis (Figure 3).

In addition, heat altered the expression of CXCR4 on the surface of $CD34^+$ CB and Mo7e cells. Whether this change in CXCR4 expression was through production of new CXCR4, or incorporation of stored intracellular CXCR4 is not yet elucidated. We hypothesize that changes seen in surface expression of CXCR4 over the short time period examined are probably due to movement of CXCR4 between intracellular and membrane compartments. However, since changes in CXCR4 expression were small, we believe that the most significant mechanism by which heat is enhancing $CD34^+$ cell migration is by altering localization of components within the membrane. This conclusion is supported by the data that there was no difference in chemotaxis between cells incubated at 37 or 39.5°C when treated with MβCD (Figure 3B), and there was no change in Rac1 or GM1 expression in $CD34^+$CB or Mo7e cells by heat as determined by flow cytometry (Supplementary Figure 2).

Conclusions

Previous methods to prime HSC for better engraftment relied on methods that required addition of small molecules (i.e. DPP4 inhibitors, PGE2 and complement fragments C3a and C5a)[12–14,19–21]. Here we propose a novel, inexpensive, and easily performed method to *ex vivo* prime cord blood HSC to home better to the BM simply by heating the cells to 39.5°C for 4 hours. Heat treatment may ultimately be used alone or as an adjuvant with other therapies to enhance efficacy of CBT in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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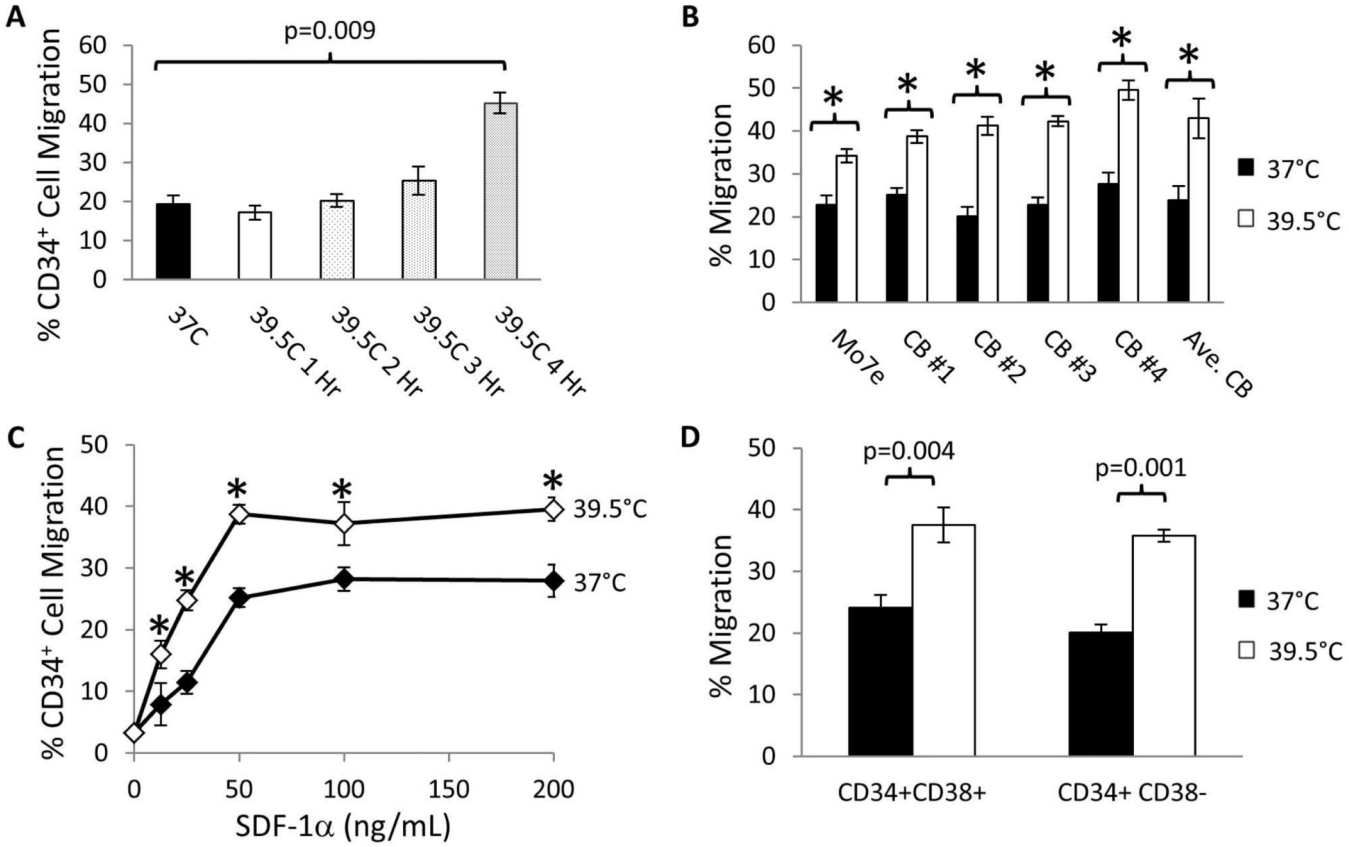
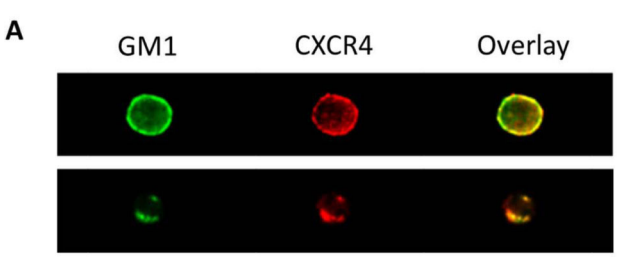
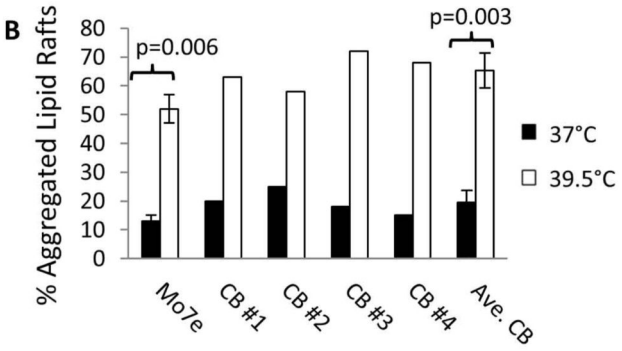
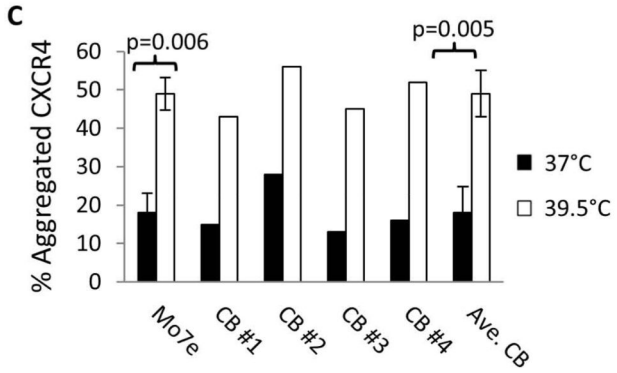


Figure 1. Heating CD34$^+$ CB and Mo7e cells significantly enhances their migration towards an SDF-1 gradient

(A) CB CD34$^+$ cells were incubated at 37°C or heated at 39.5°C for 1– 4 hours. After heating, cells were resuspended in media maintained at 37°C then allowed to migrate towards 50ng/mL of rhSDF-1α in the bottom well of a transwell plate for 4 hours at 37°C. Total cell migration was determined using flow cytometry. This was a representative of 2 experiments. (B) Mo7e and CD34$^+$ CB cells were either left at 37°C or heated at 39.5°C for 4 hours. After heating, cells were used in a transwell assay as described above in (A). * represents $p<0.01$ when comparing the migration of cells maintained at 37°C versus 39.5°C. (C) CD34$^+$ CB cells were either incubated at 37 or 39.5°C for 4 hours, resuspended in media maintained at 37°C and then allowed to migrate towards 0, 12.5, 25, 50, 100, or 200ng/mL of rhSDF-1α in the bottom well of a transwell plate for 4 hours at 37°C. This was representative of 2 experiments. * represents a $p<0.01$ when comparing the migration of cells maintained at 37°C versus 39.5°C. (D) CD34$^+$ CB cells were incubated at either 37 or 39.5°C for 4 hours. After heating, a transwell assay was performed as stated above in (A). This was a representative of 2 experiments. Data for transwell assays are the mean percent migration +/− the SEM of 3–5 wells each.







D

Fold Change in MFI of CXCR4 Compared to MFI at 37°C

Cell Type	1 Hr 39.5°C	2 Hr 39.5°C	3 Hr 39.5°C	4 Hr 39.5°C
Mo7e	-1.11	-1.08	1.01	1.42
CB #1	-1.55	-1.48	1.08	1.33
CB #2	-1.20	-1.15	1.18	1.36
CB #3	-1.23	-1.11	1.20	1.29
CB #4	-1.30	-1.40	1.07	1.48
Ave. CB	-1.39 ± 0.14	-1.29 ± 0.18	1.13 ± 0.07	1.37 ± 0.08

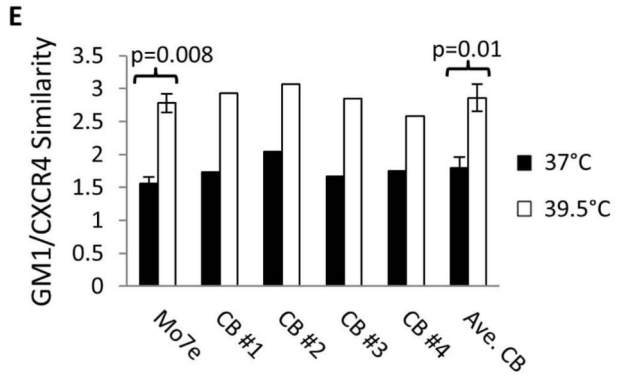


Figure 2. Heat significantly increases the percent of CD34$^+$ CB and Mo7e cells with aggregated lipid rafts and CXCR4 receptors on their cell surface

(A) Representative images of lipid raft (GM1) and CXCR4 staining on the surface of CD34$^+$ CB cells determined by CXCR4 and CTxB staining of GM1 obtained using ImageStream flow cytometry with images taken at 40× and analyzed using IDEAS software. The top panel represents diffuse GM1 and CXCR4 staining and the bottom panel represents an aggregated staining panel as determined by IDEAS software. (B) Percent aggregated lipid rafts (GM1) of Mo7e and CD34$^+$ CB cells incubated for 4 hours either at 37 or 39.5°C was determined by CTxB staining of GM1 within the plasma membrane region obtained by ImageStream flow cytometry and analyzed using IDEAS software. (C) Percent aggregated CXCR4 staining of Mo7e and CD34$^+$ CB cells that incubated for 4 hours either at 37 or 39.5°C was determined by CXCR4 staining obtained by ImageStream and analyzed using IDEAS software. (D) Fold change in CXCR4 mean fluorescent intensity (MFI) on the surface of Mo7e and CD34$^+$ cells from 4 different CB samples following 1, 2, 3, or 4 hours of heating to 39.5°C when compared to cells incubated at 37°C as determined by flow

cytometry. (E) The similarity scores of CXCR4 and GM1 overlay within the plasma membrane region of Mo7e and $CD34^+$ CB cells was determined from images taken following GM1 and CXCR4 staining and obtained by ImageStream flow cytometry then analyzed using IDEAs software.

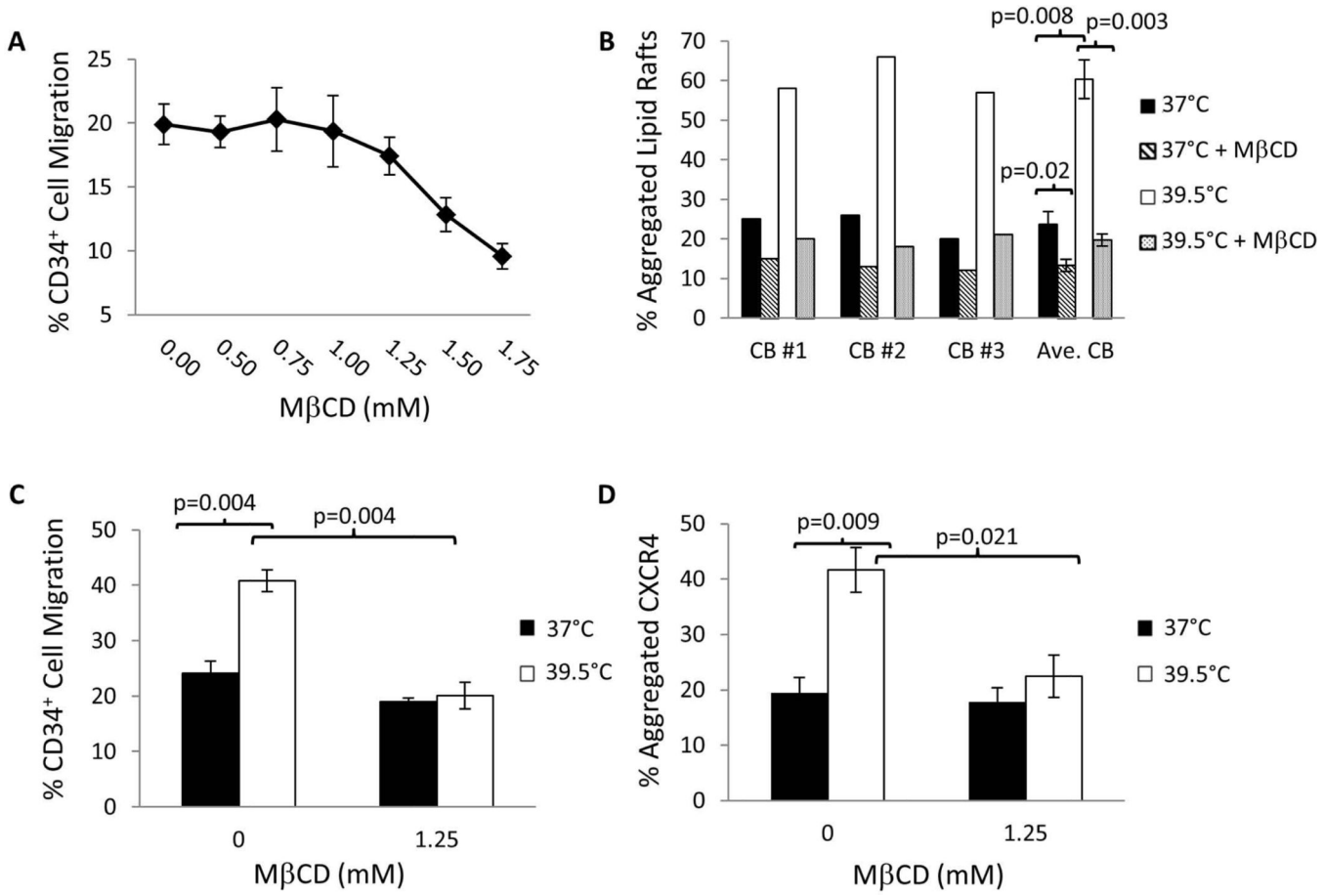


Figure 3. The enhancement in chemotaxis seen following heating of CD34$^+$ CB cells is dependent upon lipid raft aggregation

(A) CD34$^+$ CB cells were exposed to 0.50, 0.75, 1.00, 1.25, 1.50, or 1.75mM of MβCD for 30 minutes prior to being placed in a transwell assay with 50ng/mL rhSDF-1α in the bottom chamber and allowed to incubate for 4 hours at 37°C. Data are the mean percent migration +/− the SEM of 3 wells each. This was a representative of 2 experiments. (B) CD34$^+$ CB cells were incubated at 37 or 39.5°C for 4 hours. During the last 30 minutes, 1.25mM MβCD was added then cells were washed, stained with FITC CTxB, fixed, then ran on the ImageStream. Percent aggregated lipid rafts (GM1) was determined using IDEAS software. Data are the mean +/− the SEM of 3 CB samples. (C) CD34$^+$ CB cells were either left at 37°C or heated at 39.5°C for 4 hours. During the last 30 minutes 1.25mM MβCD was added then cells were washed and resuspended in media maintained at 37°C. Then transwell assays were performed as in (A). Data are the mean percent migration +/− the SEM for 3 wells each. This was a representative of 3 experiments. (D) Percent aggregated CXCR4 staining of CD34$^+$ CB cells incubated for 4 hours either at 37 or 39.5°C with 1.25mM MβCD added for the final 30 minutes was determined by CXCR4 staining obtained by ImageStream. Data are the mean +/− the SEM of 4 CB samples.

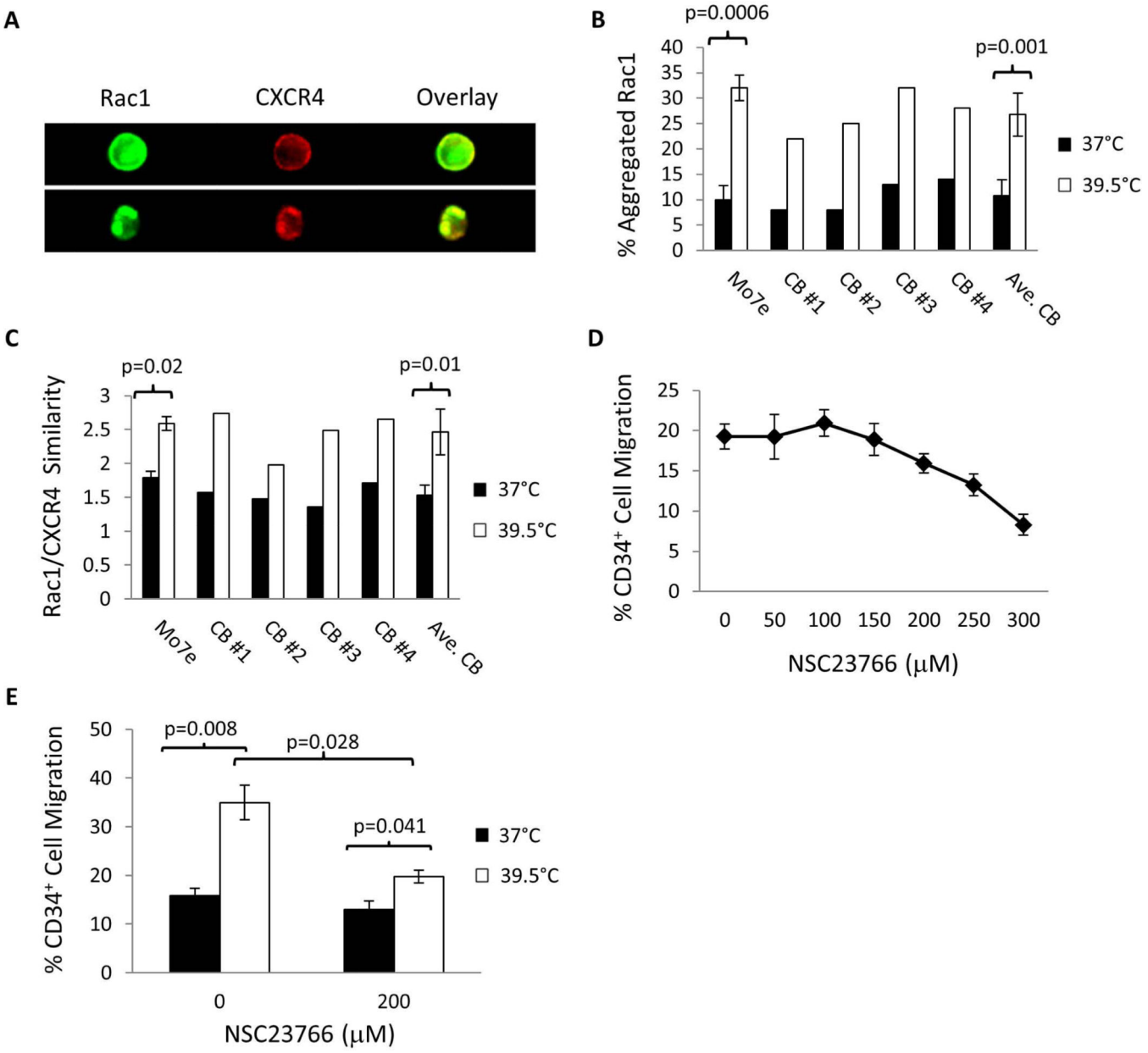


Figure 4. Activation of Rac1 is essential for the enhancement in migration seen following heat treatment of CD34$^+$ CB cells

(A) Representative images of Rac1 and CXCR4 staining on the surface of CD34$^+$ CB cells obtained using ImageStream with images taken at 40× and analyzed using IDEAS software. The top panel represents diffuse Rac1 and CXCR4 staining and the bottom panel represents an aggregated staining panel as determined by the software. (B) Percent aggregated Rac1 of Mo7e and CD34$^+$ CB cells incubated for 4 hours either at 37 or 39.5°C obtained using ImageStream. (C) The similarity scores of CXCR4 and Rac1 overlay within the plasma membrane region of Mo7e and CD34$^+$ CB cells was determined from images taken following GM1 and CXCR4 staining and obtained by ImageStream. (D) CD34$^+$ CB cells were exposed to 50, 100, 150, 200, 250 and 300μM of NSC23766 for 30 minutes prior to

being placed in a transwell assay with 50ng/mL rhSDF-1α in the bottom chamber and allowed to incubate for 4 hours. This was a representative of 2 experiments. (E) $CD34^+$ CB cells were either left at 37°C or heated at 39.5°C for 4 hours. During the last half an hour 200μM NSC23766 was added then cells were washed and resuspended in media maintained at 37°C then transwell assays were performed as in (D). This was a representative of 3 experiments. Data for transwell assays are the mean percent migration +/− the SEM percent migration for 3 wells each.

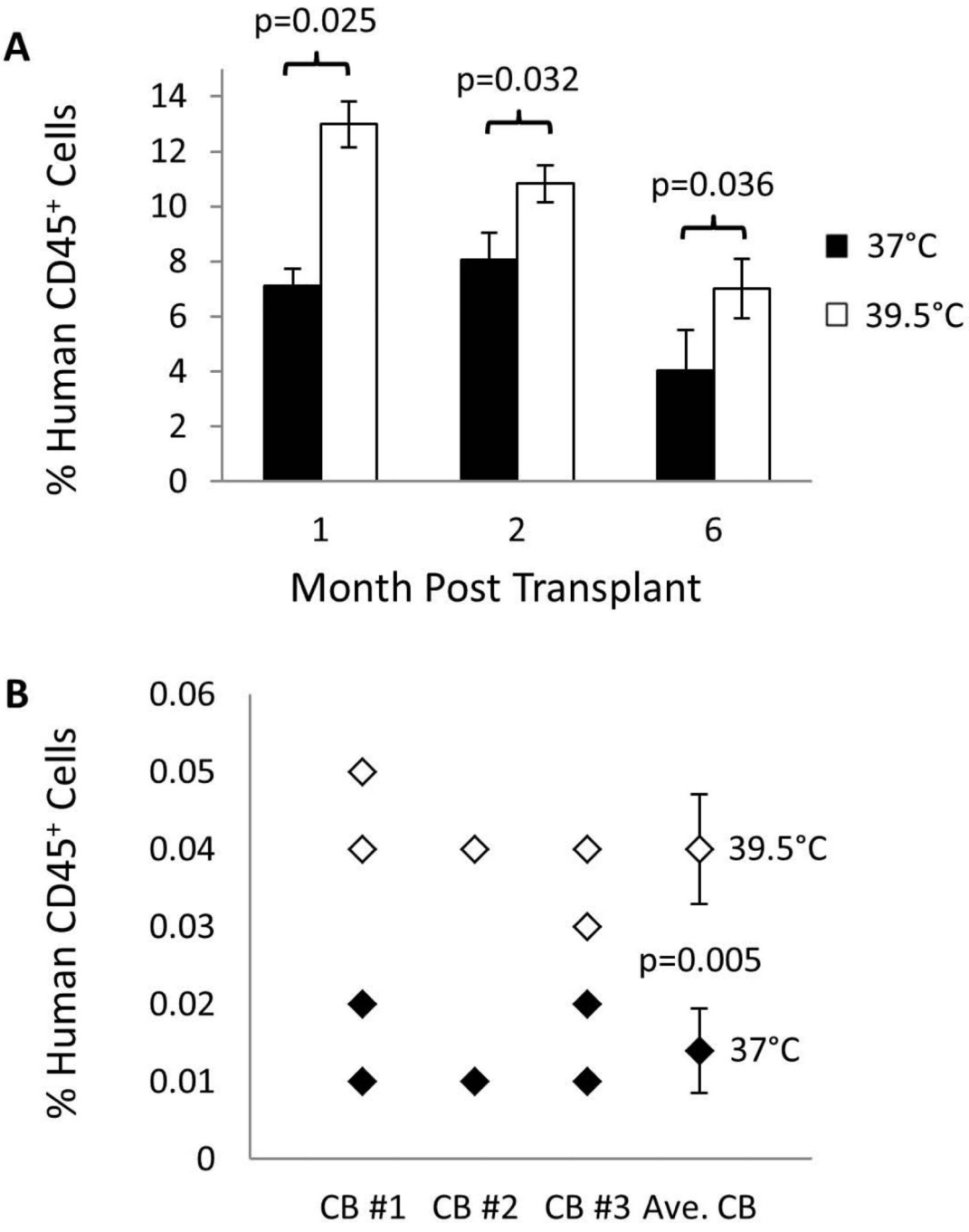


Figure 5. Heat treatment of human CD34$^+$ CB cells prior to transplantation into NSG significantly increases the percent human CD45$^+$ cell engraftment

(A) NSG mice received 3.5Gy total body irradiation followed one day later with 80,000 cord blood CD34$^+$ cells incubated for 4 hours at either 37 or 39.5°C immediately prior to i.v. injection. Percent human CD45$^+$ cells in the blood was determined by flow cytometry at 1, 2, and 6 months following primary transplantation. n= 5 NSG mice per group. This was a representative of 2 experiments. (B) NSG mice received 3.5Gy total body irradiation followed one day later with 500,000 cord blood CD34$^+$ cells incubated for 4 hours at either 37 (black points) or 39.5°C (white points) immediately prior to i.v. injection. Percent human

$CD45^+$ cell in the bone marrow was determined by flow cytometry 24 hours following injection. Each point represents an individual mouse.