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Retinoic Acid Regulates Hematopoietic Development from Human Pluripotent Stem Cells

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SUMMARY

The functions of retinoic acid (RA), a potent morphogen with crucial roles in embryogenesis including developmental hematopoiesis, have not been thoroughly investigated in the human setting. Using an in vitro model of human hematopoietic development, we evaluated the effects of RA signaling on the development of blood and on generated hematopoietic progenitors. Decreased RA signaling increases the generation of cells with a hematopoietic stem cell (HSC)-like phenotype, capable of differentiation into myeloid and lymphoid lineages, through two separate mechanisms: by increasing the commitment of pluripotent stem cells toward the hematopoietic lineage during the developmental process and by decreasing the differentiation of generated blood progenitors. Our results demonstrate that controlled low-level RA signaling is a requirement in human blood development, and we propose a new interpretation of RA as a regulatory factor, where appropriate control of RA signaling enables increased generation of hematopoietic progenitor cells from pluripotent stem cells in vitro.

INTRODUCTION

During embryonic development, retinoic acid (RA) acts as a morphogen, providing signals that instruct commitment of unspecified precursors toward separate cell fates, thereby helping to mediate tissue patterning and organogenesis (Duester, 2008; Kumar and Duester, 2011; Ross et al., 2000). As such, RA is also a potent teratogen capable of disturbing developmental processes, causing severe malformations of the fetus. RA is a signaling molecule derived from vitamin A (retinol), regulating cellular proliferation and differentiation, and is produced by cells that express the enzymes retinaldehyde dehydrogenase 1 (RALDH1), RALDH2, or RALDH3. A small molecule, diethylaminobenzaldehyde (DEAB), inhibits the activity of these enzymes and can be used to limit endogenous RA signaling (Moreb et al., 2012; Perz-Edwards et al., 2001; Russo and Hilton, 1988). When available, RA enters the nucleus to bind the retinoic acid receptor (RAR) family of nuclear receptors that, in turn, by forming heterodimeric complexes with the retinoid-X-receptor (RXR) family, localize to specific retinoic acid response elements (RAREs) in promoter regions of the genome to drive transcription of RA target genes. Modulation of RA in in vitro models of development provides a useful tool toward understanding commitment into tissues that depend either on high levels of RA, such as the developing ectodermal and endodermal derivatives (Murry and Keller, 2008) or on low levels of RA such as the posterior patterning of the lateral plate mesoderm (LPM) (Deimling and Drysdale, 2009). While several model organisms including Xenopus (Xenopus laevis), zebrafish (Danio rerio), and mouse (Mus musculus) have been used to study the effects of RA on embryonic development, similar studies have not been possible in the human setting for both technical and ethical reasons. In addition to its role in embryonic development, RA is also known to have strong effects on the hematopoietic lineage. However, the studies on the role of RA in regulating adult hematopoiesis have yielded opposing results. For example, RA signaling inhibition expands hematopoietic progenitors in both the human and murine setting (Chute et al., 2006; Muramoto et al., 2010), while other studies report increased maintenance and self-renewal upon adding RA to short- and long-term repopulating hematopoietic stem cells (HSCs) in the murine setting, mediated through activation of the RARγ receptor (Purton et al., 2000, 2006). These apparent contradictions likely reflect the increasingly complex nature of RA signaling that is emerging in the field, which is why a more contextual understanding might provide a better model to explain these previous findings. Indeed, work analyzing the effects of RA on different subpopulations of adult blood demonstrated that RA has a pleiotropic effect, enhancing the self-renewal of the HSC compartment while accelerating the differentiation of downstream progenitors (Purton et al., 1999). Several groups have created protocols that allow in vitro generation of hematopoietic cells from human pluripotent stem cells (Kardel and Eaves, 2012; Murry and Keller, 2008; Woods et al., 2011), with more
Figure 1. Retinoic Acid Signaling Inhibition Increases the Yield of Hematopoietic Progenitors with an HSC-like Phenotype from Human Pluripotent Stem Cells

(A) Schematic of pluripotent stem cell differentiation system used to model human hematopoietic development through mesoderm specification and blood commitment. RA inhibitors or RA was present continuously during the 16-day differentiation, except where otherwise stated.

(B) Representative FACS plots showing the hematopoietic population derived from pluripotent stem cells at day 16 of differentiation. FACS gates show blood (CD45/43+), hematopoietic progenitors (CD45/43−CD34+), and HSC-like immature progenitors (CD45/43−CD34hiCD38loCD90+CD45RA−/−). Gates are based on FMO controls with more stringent CD34hi and CD38lo gating based on cord blood hematopoietic stem and progenitor cell standards. Doublet exclusion and dead cell exclusion were done before applying the gates.

(legend continued on next page)
recent reports demonstrating the generation of definitive hematopoietic cells capable of differentiation into the lymphoid lineage (Kennedy et al., 2012; Timmermans et al., 2009). However, efficient generation of hematopoietic stem and progenitor cells, with functional properties of their in vivo counterparts, remains challenging, possibly due to the current limitations to how accurately the conditions of embryonic blood development can be reproduced in vitro. In this study, using a human pluripotent stem cell differentiation system, we model hematopoietic development and increase the generation efficiency of hematopoietic cells with an HSC-like phenotype by limiting RA signaling during directed pluripotent stem differentiation toward blood. RA signaling inhibition maintains immature progenitors in a less differentiated state and enables efficient commitment toward the hematopoietic lineage during multiple stages of the developmental process. Our results define the role of RA in hematopoietic differentiation and provide evidence that low levels of RA signaling promote hematopoietic development from pluripotent stem cells.

RESULTS

Decreased Retinoic Acid Signaling Increases the Generation of Hematopoietic Progenitors from Human Pluripotent Stem Cells

Using a 16-day embryonic stem cell (ESC)/induced pluripotent stem cell (iPSC) differentiation system based on our previously described protocol (Woods et al., 2011) to model human embryonic development toward the hematopoietic lineage, we assessed the effects of RA signaling on hematopoietic precursor lineage commitment, multilineage differentiation, and proliferation capacities (Figure 1A). We used established cell-surface markers of hematopoietic progenitors and HSCs and assessed their frequencies in our ESC/iPSC-derived hematopoietic cells (Figure 1B). The cord blood-based phenotypic markers were as follows: hematopoietic progenitors: CD45+CD34+, multipotent progenitors (MPPs): CD45+CD34+CD38−CD90−CD45RA−, and HSCs: CD45+CD34+CD38−CD90+CD45RA− (Majeti et al., 2007). The early pan hematopoietic marker, CD43 (Vodyianik et al., 2006), was included to ensure that all ESC/iPSC-derived blood cells were identified. Early observations such as accelerated differentiation of OP9 cells to adipocytes, and the presence of beating colonies (indicating the presence of cardiomyocytes) (data not shown) made us consider the possibility of elevated RA signaling in our cultures with a possible impact on final blood generation efficiency. Therefore, RA signaling was evaluated by means of both inhibition using the RA synthesis inhibitor DEAB and by adding RA. The relative proportions of viable hematopoietic cells, hematopoietic progenitors, and HSC-like cells were measured by fluorescence-activated cell sorting (FACS) analysis (Figure 1B). Viability was assessed by 7-aminoactinomycin D (7AAD) exclusion, and no change in total viability was observed after applying DEAB and RA. RA inhibition by DEAB significantly increased the frequency of HSC-like fraction by 2.3-fold while showing no significant change in the frequency of total hematopoietic cells or the more mature progenitor fraction (Figure 1C, left panels). This HSC-like cell increase was also seen when using the alternative pluripotent human stem cell lines H1 and HUES33 (Figures S1A and S1B). RA at the highest concentration of 1 μM, reported as a physiological concentration in the developing central nervous system and retina (Ross et al., 2000), resulted in complete abrogation of blood cell production (Figure 1C, upper right panel). Decreasing doses of RA concentration (0.1 μM and 0.01 μM) restored the presence of hematopoietic progenitors (Figure 1C). However, the frequency of HSC-like cells remained significantly decreased even at the lowest concentration (0.01 μM) (Figure 1C, lower right panel). To verify that DEAB and RA were indeed modulating RA signaling in our cultures, we measured the expression level of known RA target gene RAR-β and observed a significant decrease in expression with DEAB. At a low concentration of RA, RAR-β expression was enhanced (Figure 1D). In addition, to confirm the role of RA signaling inhibition by DEAB in increasing HSC-like cell frequency, we performed pluripotent stem cell differentiation experiments using the alternative RA signaling inhibitor LG1506, previously described to act as an inverse agonist (Safi et al., 2009), and observed an increase in phenotypic HSCs, similar to DEAB (Figure 1E). Taken together, these results demonstrate that limiting retinoic acid signaling during pluripotent stem cell differentiation increases the generation of more primitive hematopoietic progenitor cells.

Increased Generation of Hematopoietic Progenitors with Colony-Forming Capacity through Decreased Retinoic Acid Signaling

To evaluate the effects of RA signaling on the functionality of blood cells generated from human pluripotent stem
cells, we plated 20,000 bulk cells, harvested at day 16, into the colony-forming unit (CFU) assay to measure the output in cells capable of forming hematopoietic colonies when seeded in cytokine containing methylcellulose. Colony types were identified and scored according to their phenotypes (Figure 2A). Cytospin analysis confirmed the presence of several types of hematopoietic cells including macrophages, monoblasts, myeloblasts, neutrophils, and eosinophils (Figure 2B). RA inhibition by DEAB during the 16-day differentiation significantly increased the output of CFUs by 2.5-fold as compared to the DMSO control (Figure 2C, left panel). Compared to the DMSO control,
DEAB-treated cultures had a similar distribution of colony types including macrophages, granulocytes, mixed colonies, and erythroid colonies. DEAB-treated cells occasionally gave rise to colonies containing all lineages (GEMM) (Figure 2C, right panels). In case of differentiating cells treated with RA, we observed a complete lack of CFU potential on using 1 μM RA, confirming the block of hematopoietic cell production (Figure 2D, left panel). Lower concentrations of RA yielded hematopoietic colonies, with the lowest concentration at 0.01 μM showing a trend (p = 0.17) for an increase in the frequency of colonies. This is likely the result of higher numbers of more mature progenitors (CD34+CD38+) at the expense of the more immature progenitor fraction (CD34+CD38−) (Figure 2E), indicating that RA accelerates the maturation of hematopoietic progenitors. Of note, there were no erythroid colonies at any concentration of RA (Figure 2D, right panel). Together, these results demonstrate that RA signaling, in a concentration-dependent manner, decreases the generation efficiency of hematopoietic progenitors with colony-forming capacity from human pluripotent stem cells and that reduced RA levels enable a higher generation efficiency of more immature blood cells.

Retinoic Acid Inhibition Enhances the Lymphoid and Myeloid Differentiation Capacity of Hematopoietic Progenitors Generated from Human Pluripotent Stem Cells

We set out to assess if the observed increase in the immature progenitor fraction using DEAB, was directly related to the functional improvements seen with the colony assay. We analyzed the differentiation capacity of sorted blood subfractions using a range of different differentiation protocols. First, we sorted equal numbers of hematopoietic progenitor cells (CD45/CD43+/CD34+) that had been generated with or without DEAB and measured their CFU potential. We observed that cells generated with DEAB produced twice the amount of CFUs as compared to the DMSO control and produced a similar number of CFUs when compared to similarly sorted cord blood (Figure 3A). We then evaluated differentiation capacity of hematopoietic cells toward the lymphoid lineage. We analyzed the lymphoid commitment of hematopoietic progenitors by subculture on either the OP9 or the OP9DL1 stroma. By using OP9 stroma cells for the differentiation toward natural killer (NK) cells, we could evaluate the capacity of the HSC-like (CD34+CD38−CD90+) fraction as compared to the matured fraction (CD34+CD38−CD90−) (Figures 3B and S2). For reference, sorted cord blood cells were used as positive control. Of note, cord blood cells generally demonstrated >100-fold higher proliferative capacity compared to their in vitro-generated counterparts across these assays (data not shown). By examining events positive for the markers of NK cells, we observed that the CD90+ fraction was superior to the CD90− fraction (Figure 3B, lower left panel, and Figure S2) indicating that the HSC-like fraction of cells generated from human pluripotent stem cells represents the population with a robust capacity to differentiate toward the lymphoid lineage. For reference, the more mature CD38+ hematopoietic fraction (of cells generated in vitro) was evaluated but did not demonstrate any lymphoid or myeloid differentiation capacity (data not shown). NK cluster size from cells generated with or without DEAB showed no significant difference (Figure 3B, lower left panel); however, given the increase in HSC-like cells (Figure 1C), the total increase in NK generation from pluripotent stem cells was estimated to be 3-fold using DEAB. We then wanted to evaluate the lymphoid capacity of cells generated in presence of RA. Due to the low frequency of the HSC-like fraction as seen with RA, we evaluated the CD34+ population. By sorting out CD34+ cells, generated either with or without DEAB or RA, and subculturing them on OP9DL1 for 3 weeks, we observed the presence of non-myeloid (CD33/CD14−) CD7+ and CD45RA+ cells, similar to multipotent early lymphoid progenitors (Hao et al., 2001; Hoebeke et al., 2007; Larbi et al., 2014) (Figure 3C). We observed no significant difference in capacity to differentiate into these cells from hematopoietic progenitors generated with or without DEAB or RA, with a possible explanation due to the beneficial role of RA in T cell differentiation (Chenery et al., 2013). Together, these results demonstrate that inhibiting RA signaling with DEAB during the generation of hematopoietic cells from human pluripotent stem cells generates cells with the functional capacity to further differentiate toward the lymphoid and the myeloid lineage.

Retinoic Acid Inhibition Increases the Early Developmental Commitment toward the Hematopoietic Lineage

The observation that high-dose RA abrogated blood cell generation (Figure 1C) suggested that the developmental commitment toward the hematopoietic lineage might have been compromised at the level of a non-hematopoietic upstream precursor. Therefore, we reasoned that, during this process, RA inhibition by DEAB might have early positive effects, allowing more of the initial pluripotent cells to differentiate into hematopoietic mesoderm prior to blood production. Indeed DEAB, only during the initial 8 days of the protocol, established a significant positive effect on the final frequency of the HSC-like fraction that was less than the improvement seen when DEAB was applied throughout the protocol (days 0–16) (Figure 4A). Furthermore, measuring the recently characterized mesodermal precursor of the definitive hematopoietic lineage characterized by a cell-surface marker phenotype highly expressing
Figure 3. Retinoic Acid Inhibition Increases the Differentiation Capacity of Hematopoietic Progenitors toward Myeloid and Lymphoid Lineage

(A) CFU potential of 500 sorted progenitors (CD45/CD43+/CD34+) from DEAB-treated cultures (green) and DMSO control (gray) from four independent experiments (n = 4). CFU potential of progenitors similarly sorted from cord blood (CD45/CD43+/CD34+) is shown in red.

(B) Lymphoid/myeloid differentiation assay performed on iPSC-derived hematopoietic cells. FACS plots and bar graphs showing non-myeloid (CD33/CD14+), CD19-, CD56+, and CD56+/CD16+ NK fraction from wells seeded with 50 HSC-like cells (CD45/43+/CD34hi CD38lo CD90+/CD45RA-) (17 individual wells) cultured on OP9 stroma for 4 weeks. Left bar graph shows the cluster size of the NK fraction. Right bar graph shows the frequency of this population in the viable non-stroma fraction. See also Figure S2.

(C) FACS plots showing non-myeloid, CD7+, CD45RA+ multipotent early lymphoid progenitors from 3,500 sorted CD34+ cells cultured on OP9DL1 stroma for 3 weeks from one out of two independent experiments (n = 2). FACS plots are gated on the viable non-stroma fraction. Bar graph show the total output of differentiated cells from progenitors generated with DMSO, DEAB, or RA. FACS gates are based on FMO controls.

Data represent mean ± SEM. Asterisks indicate significant differences (*p < 0.05).
We evaluated if there was any beneficial effect of reduced subcultured hematopoietic progenitors in the adult setting. Modulation of RA signaling has earlier been described to affect the maintenance and expansion (Chute et al., 2011; Muramoto et al., 2010; Purton et al., 2000, 2006) of hematopoietic progenitors generated from pluripotent stem cells in vitro. We report that RA decreases blood generation efficiency of hematopoietic progenitors from human pluripotent stem cells in vitro.

DISCUSSION

In this study, we dissected the role of RA signaling in human hematopoietic development, using a human pluripotent stem cell differentiation system as an in vitro model, to generate hematopoietic progenitors. We have identified multiple stages in development that are influenced by RA signaling, where modulation of RA signaling directs the generation of hematopoietic cells from pluripotent stem cells in vitro. We report that RA decreases blood generation from human pluripotent stem cells. RA signaling inhibition before the onset of hematopoiesis establishes an increase in the final output of cells with an HSC-like phenotype that confirms the role of RA signaling in the early stages of blood generation. qRT-PCR analysis for genes marking specific developmental cell types identified the stages during development where decreased RA signaling increased commitment toward the hematopoietic lineage, including the initial commitment of pluripotent stem cells to mesoderm, and by increased specification toward hemangiogenic mesoderm, while reducing cardiac mesoderm differentiation. The suppressing effect of RA on...
Figure 4. Decreased Retinoic Acid Signaling Improves Early Developmental Commitment toward the Hematopoietic Lineage
(A) Frequency of the HSC-like fraction in cultures supplemented with DEAB (10 μM), during the first 8 days (n = 3) or during the full 16 days of differentiation (n = 7), as compared to DMSO control. Experiments are independent biological replicates.
(B) Frequency of the CD43^CD34^CD90^ mesoendothelial precursors of the hematopoietic lineage at day 8 for cultures treated with DEAB or RA (n = 4). See also Figure S3.
mesodermal commitment has been described in the murine embryo (Bain et al., 1996; Okada et al., 2004), and RA has been demonstrated to direct mesoderm toward cardiac tissue (anterior LPM) at the expense of hematopoietic development in *Xenopus* (Deimling and Drysdale, 2009), zebrafish (de Jong et al., 2010), and mouse (Szatmari et al., 2010), but this is the first time that these functions of RA have been verified in human development. Taken together, these results suggest that high but physiological levels of RA antagonize blood development and that substantially lower amounts of RA signaling relative to other tissues in the developing embryo are required to facilitate blood development. We show that RA accelerates the differentiation of generated hematopoietic progenitors to more mature blood cells, in agreement with findings in the adult human bone marrow where RA scavenging

Figure 5. Decreased Retinoic Acid Signaling Maintains the Primitiveness of Generated Hematopoietic Progenitors

(A) Representative FACS profiles of cells harvested at day 16 and after extended culture period at day 21. Cells were treated with or without DEAB at 10 μM during the whole culture period.

(B) Graphs showing fold changes in maturation of the hematopoietic population (CD45/43+) observed between day 16 and day 21 for cells treated with DEAB or DMSO from three independent experiments (n = 3). Data represent mean ± SEM. Asterisks indicate significant differences (*p < 0.05).

(C) Lineage map of the developmental process from pluripotent stem cell to hematopoietic cell. Adjacent wells of cultures treated with or without DEAB were analyzed at days 4, 8, 12, and 16 during differentiation by qRT-PCR for developmental genes including *BRACHYURY* (mesoderm), *MIXL1* (mesoderm), *SOX17* (endoderm), *SOX1* (ectoderm), and *NKK2.5* (cardiac mesoderm).

(D) Expression of developmental marker genes *BRACHYURY*, *NKK2.5*, and *CD38* for cultures treated with DEAB (n = 3) or RA (n = 2) as compared to DMSO control.

(E) Frequency (%) of beating colonies at day 15 during differentiation in the presence of DEAB compared to DMSO control (n = 2). Data represent mean ± SEM. Asterisks indicate significant differences (*p < 0.05, ****p < 0.0001).
stroma cells is crucial to prevent rapid differentiation of hematopoietic progenitors (Ghiaur et al., 2013). By restricting RA signaling using DEAB, we demonstrate a significant increase in the generation of HSC-like progenitors capable of lymphoid and myeloid differentiation (~3-fold). In this study, we performed transplantation experiments to assess the capacity of these cells to repopulate immunocompromised mice but found no significant difference in engraftment capacity in progenitors derived from human pluripotent stem cells (data not shown). Our results using DEAB may appear to contradict findings that RA can increase the progenitor output generated from human ESCs (Yu et al., 2010); however, Yu et al. only assessed the effect of RA on the general CD34⁺ progenitors and did not address the action of RA to differentiate and ultimately reduce the numbers of more immature hematopoietic progenitors; indeed, we observed that adding low amounts of RA increased total blood output but at the expense of the HSC-like progenitor fraction (the fraction with the highest lymphoid/myeloid differentiation potential). Our findings indicate that reduced RA signaling preserves the generated progenitor fraction from differentiation into more mature cell types, similar to the finding that DEAB delayed the differentiation of subcultured human cord blood and bone marrow HSCs (Chute et al., 2006). Regarding the role of RA signaling on blood development, a recent study on the cephalochordate Amphioxus (Branchiostoma lanceolatum), considered to be one of the most closely related invertebrates to the vertebrate family (Pascual-Anaya et al., 2013), reported that HSC-equivalent Pdvegfr⁺ Scl⁺ cells in cephalochordates to the vertebrate family (Pascual-Anaya et al., 2013), considered to be one of the most closely related invertebrates to the vertebrate family (Pascual-Anaya et al., 2013), reported that HSC-equivalent Pdvegfr⁺ Scl⁺ cells in
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The human ESC lines H1 and HUES 3 (obtained from WiCell and Harvard University, respectively, under material transfer agreements) and the iPSC cell line RB9-CB1 (derived from cord blood endothelial cells transduced with tetracycline-inducible lentiviral vectors expressing OCT4, SOX2, LIN28, KLF4, and C-MYC) (Woods et al., 2011) were used in this study. The lines were karyotypically normal and have previously been demonstrated to be pluripotent by in vivo teratoma assay and PCR. The pluripotent stem cell lines were routinely maintained and differentiated toward blood, as previously described (Woods et al., 2011), with some modifications. The modifications were as follows: prior to embryoid body (EB) formation, colonies of pluripotent stem cells were cultured an additional 24 hr to increase colony density. During the EB-formation stage, the 7-day culture was extended to 8 days with a 1.5-fold increase in media volume. For adherence plating of EBs, a coating of Matrigel was used instead of OP9 stroma. The pluripotent stem-cell-to-blood differentiation medium MesoTotal (Primorigen Biosciences), with and without DEAB, was added 24 hr after EB formation, and fresh media was added every 2 days throughout the differentiation until day 16 or day 21 (extended culture period). DEAB (Sigma), LG1506 (Tocris Bioscience), and RA (Sigma) were re-suspended in DMSO and added at specific concentrations to the differentiation media.

**EXPERIMENTAL PROCEDURES**

**Pluripotent Stem Cell Culture and Hematopoietic Differentiation**

Pluripotent Stem Cell Culture and Hematopoietic Differentiation
Flow Cytometry
Differentiated cells were washed in PBS before being singularized using TrypLE (Thermo Fischer Scientific), passed through a 21G needle, and filtered using 30 μm sterile Cup Filcons (BD Biosciences). Cells were treated with 7AAD to exclude dead cells. Cells were stained using the following anti-human antibodies: fluorescein isothiocyanate (FITC)-conjugated CD45 (eBioscience, 11-0459-42) and CD43 (BD Biosciences, 555475), phycoerythrin-cyanine (PE-Cy7)-conjugated CD34 (BioLegend, 343516), allophycocyanin-conjugated CD38 (BioLegend, 303510), CD33 (BioLegend, 303408), CD14 (BioLegend, 325608) Phycoerythrin (PE)-conjugated CD90 (BioLegend, 326110), CD19 (BioLegend, 302208), CD7 (BD Biosciences, 332774), V450-conjugated CD45RA (BD Biosciences, 560362), PE-Cy5-CD56 (BioLegend, 304608), BV605-CD45RA (BioLegend, 304133), and eFluor650-CD16 (eBioscience, 48-0168-42). Cells were acquired on a FACS Canto II (BD Biosciences) or sorted using a FACS Aria III (BD Biosciences). Analysis was done using FlowJo, version 9.4.10 (BD Biosciences). FACS gates are based on fluorescence minus one (FMO) controls unless stated otherwise.

CFU Assay
Single-cell suspensions of harvested differentiation cultures (20,000 cells per well) or, alternatively, sorted blood progenitor cells were plated in 2 ml of MethoCult H4435 (STEMCELL Technologies) in Falcon Tissue Culture six-well plates (Thermo Fisher Scientific). RA and DEAB were not added to the methylcellulose. Cells were incubated for 14 days in a humidified incubator at 37°C with 5% CO₂. Colonies were counted by bright-field microscopy.

Lymphoid Differentiation Assay
EBs obtained from pluripotent stem cells were differentiated on OP9 (Martin et al., 2008) and OP9-Delta1 (OP9D1) (Kennedy et al., 2012) stroma monolayer for NK cell and T cell differentiation, respectively, in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. To obtain NK cells, EBs were differentiated for 4 weeks in the presence of interleukin-3 (IL-3) (5 ng/ml), IL-7 (5 ng/ml), stem cell factor (SCF) (10 ng/ml), and Flt-3L (10 ng/ml) (all from PeproTech). Wells were scored positive if showing a cluster of more than ten NK cells. To obtain T cells, CD34⁺ hematopoietic progenitors obtained from the differentiating EBs were plated on OP9D1 cells and differentiated for 3 weeks in the presence of Flt-3L (5 ng/ml) and IL-7 (5 ng/ml). SCF (100 ng/ml) was applied only for the initial 5 days. The differentiating cells were collected (vigorous pipetting and filtering through a 40 μm nylon mesh) and re-plated every 7 days on fresh stroma. Fresh media and cytokines were replenished twice every 7 days. At the end of subculture, cells were collected and analyzed by flow cytometry. Cord blood cells, collected after informed consent at the Lund University hospital, were used as positive control and were prepared using Lymphoprep tubes (Axis-Shield), together with CD34⁺ enrichment using the Direct CD34 Progenitor Cell Isolation Kit together with MACS separation columns (both from Miltenyi Biotec).

Quantitative Real-Time PCR
RNA was extracted from differentiation cultures with the RNeasy Micro Kit (QIAGEN) according to the manufacturer’s specifications. The extracted RNA was transcribed into cDNA using random hexamers, dinucleotide triphosphates (dNTPs), 5 × first strand buffer, DTT, RNaseOUT, and SSIII (Thermo Scientific). The qRT-PCR analysis was carried out with SYBR green (Sigma) detection using Platinum Taq polymerase, SYBR green, and Rox reference dye, dNTPs (all from Thermo Scientific), and primers (Sigma) (for primer sequences, see Table S1) on an ABI 7900HT Fast Real Time PCR cycler (Thermo Scientific). Reaction efficiency was calculated for each primer pair with serial dilutions of cDNA template, and melting curve analysis was performed at the end of each reaction for detection of unspecific product amplification. Relative quantification of transcript levels were calculated using ΔC_T values normalized to β-ACTIN, and the fold change of gene expression was calculated relative to day 0 of differentiation.

Statistical Analysis
Statistical analysis was performed using an unpaired Student’s t test, and the results were considered to be statistically significant at p value < 0.05. All graphs depict mean ± SEM. The number of biological replicates is indicated by the n value. The graphs and statistical evaluation were done using GraphPad Prism (GraphPad Software).

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.01.009.

AUTHOR CONTRIBUTIONS
R.E.R., C.G., and N.W. designed experiments. R.E.R., C.G., R.M., S.S., and P.C. performed experiments. B.G. contributed unpublished data and expertise. R.E.R. and N.W. wrote the manuscript. C.G., R.M., S.S., P.C., and B.G. all provided critical evaluation of the manuscript. C.G. and R.M. share equal contribution to this manuscript. All experiments were performed in the laboratory of N.W.

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