SUPPLEMENTARY APPENDIX

Ephrin/Eph receptor interaction facilitates macrophage recognition of differentiating human erythroblasts

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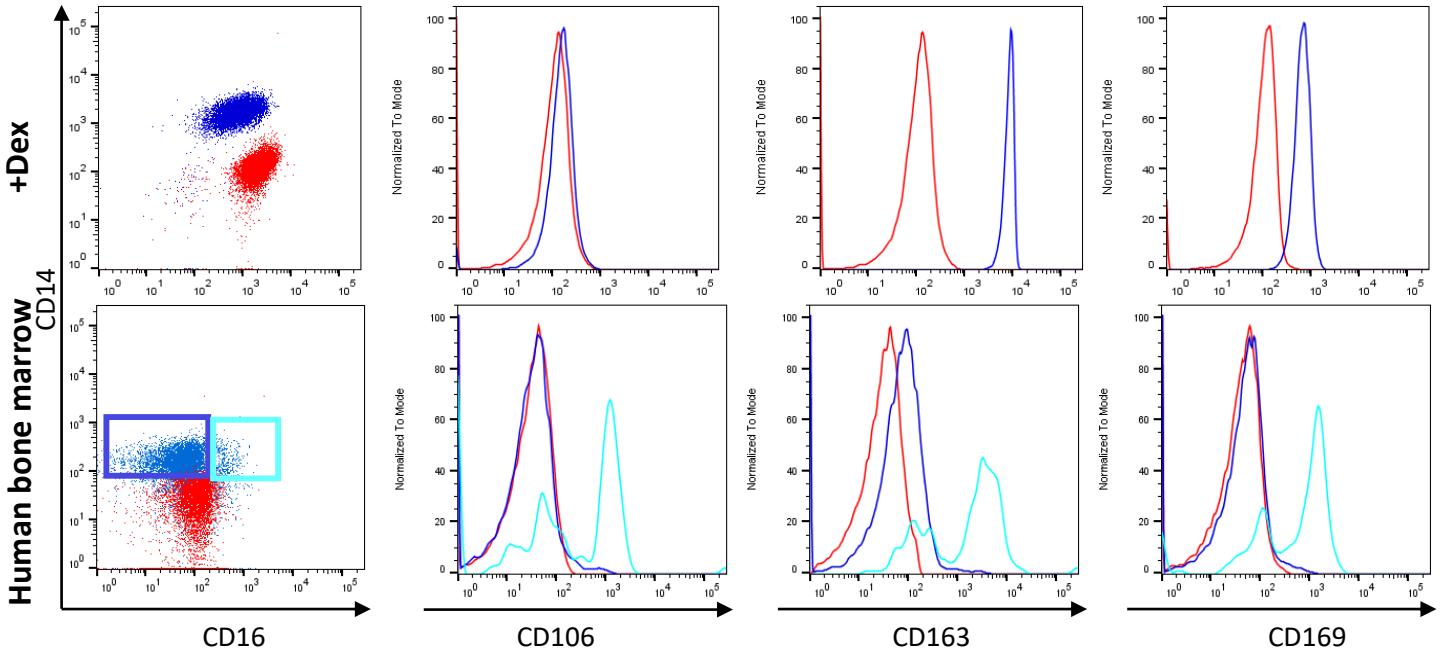
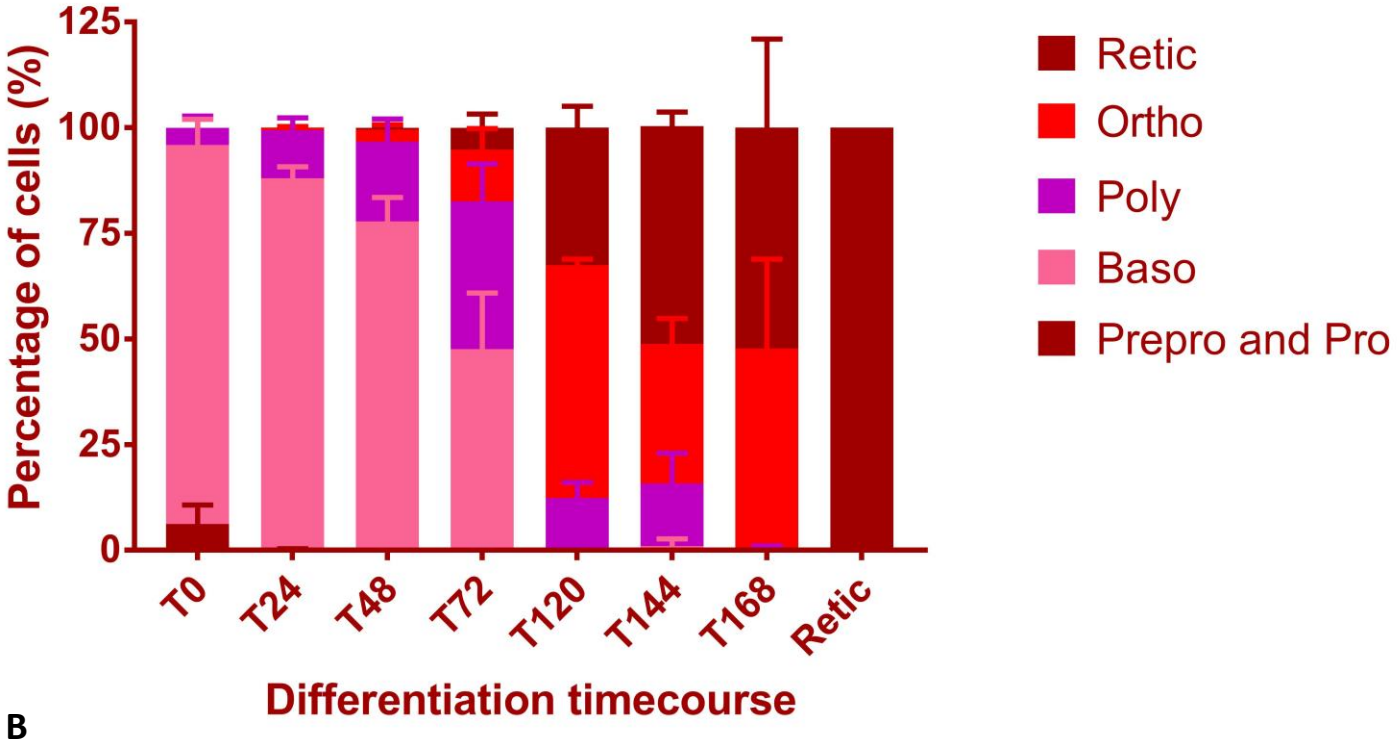
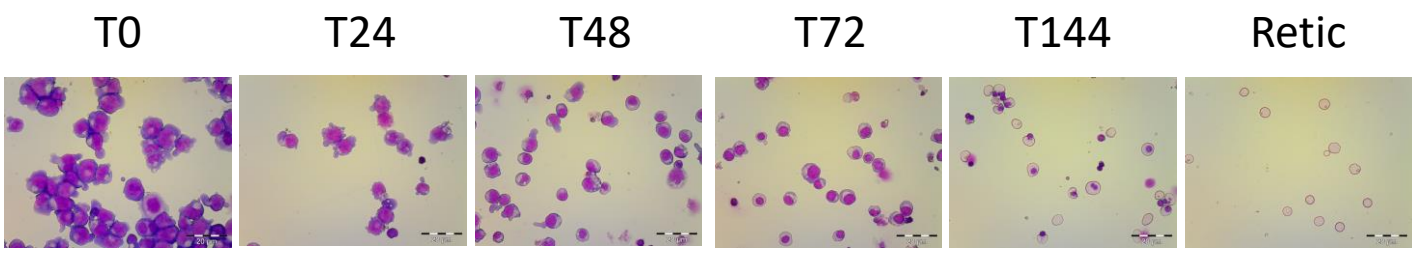
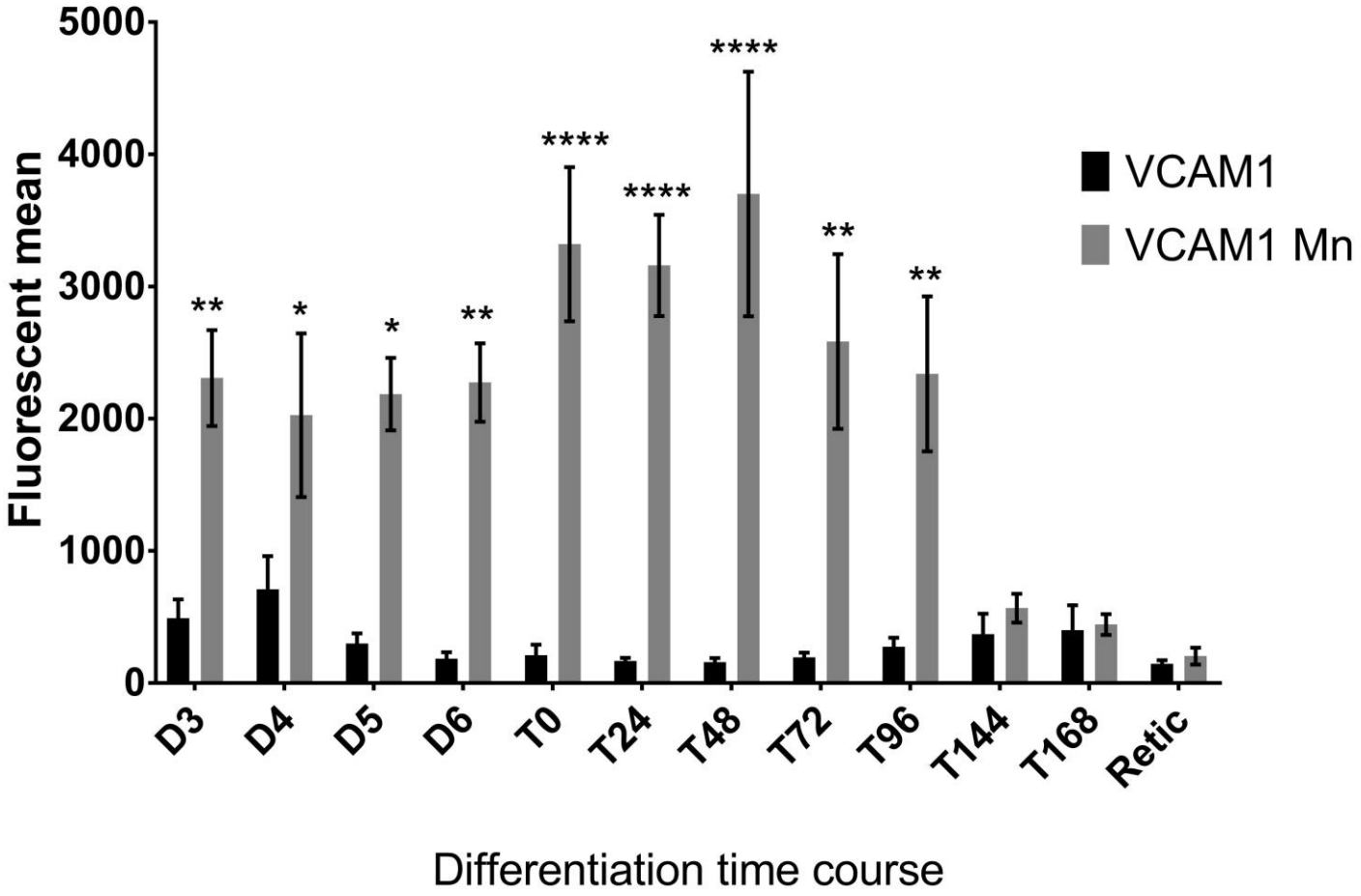

+Dex
Human bone marrow
CD14
CD16
Normalized To Mode
CD106
CD163
CD169

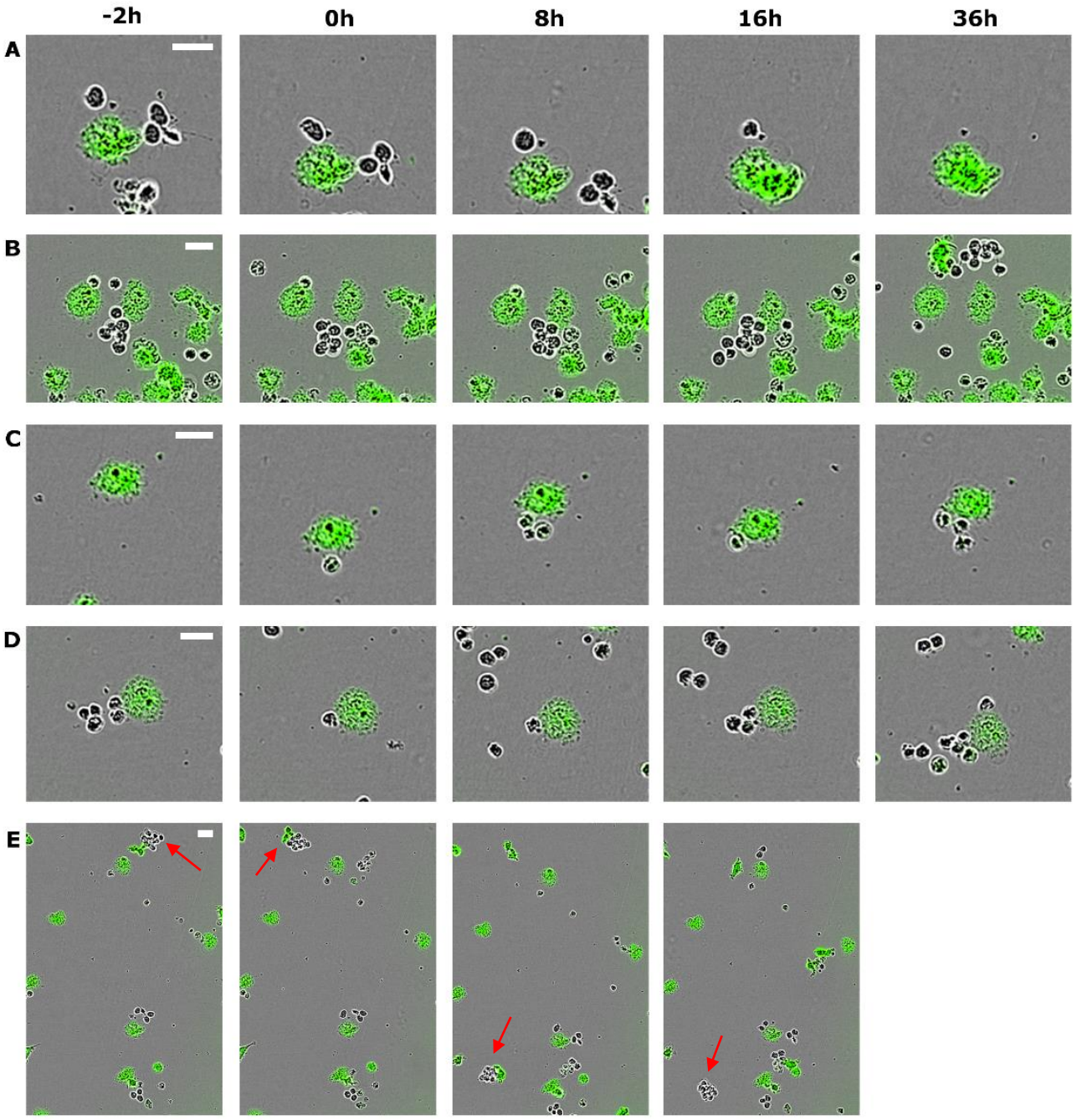
A


125
100
75
50
25
0
Percentage of cells (%)
T0
T24
T48
T72
T120
T144
T168
Retic
Differentiation timecourse
Retic
Ortho
Poly
Baso
Prepro and Pro

B


T0
T24
T48
T72
T144
Retic


5000
4000
3000
2000
1000
0
Fluorescent mean
VCAM1
VCAM1 Mn
**
*
*
**
****
****
****
**
**
D3
D4
D5
D6
T0
T24
T48
T72
T96
T144
T168
Retic
Differentiation time course


-2h
0h
8h
16h
36h
A
B
C
D
E

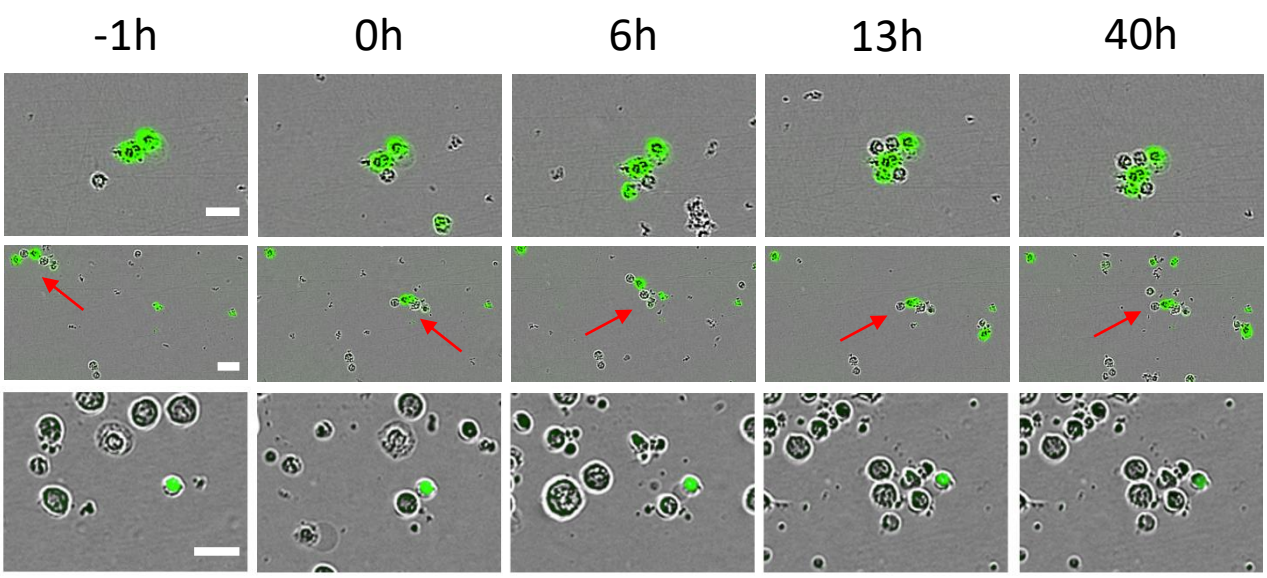

-1h
0h
6h
13h
40h

Figure S1 – +Dex macrophages have a similar phenotype to human bone marrow macrophages

Representative flow cytometry histograms of bone marrow macrophage cells and +Dex macrophages stained for CD14, CD16, CD106, CD163 and CD169. Dark blue box and lines are the $CD14^{+}CD16^{-}$ cells. The light blue box and lines are the $CD14^{+}$ $CD16^{+}$ cells.

Figure S2 – The different stages of erythropoiesis at different culture points present

A – Cytospins were captured for each day of the culture. 200 cells were categorised in each sample using morphological factors (n=6). B – Example pictures of cytospins for each day of culture.

Figure S3 – VCAM1-Fc binds to the integrins after Mn^{2+} activation

Graph showing the mean fluorescent intensity (MFI) obtained by flow cytometry of the constructs binding to erythroblasts throughout terminal differentiation (n=3). VCAM Mn is VCAM-Fc with manganese activation. The error bars represent the standard error of the mean. 2way ANOVA was performed as a comparison of binding compared to the IgG control (* ($p<0.05$); ** ($p<0.01$); **** ($p<0.0001$)).

Figure S4 - +Dex cells can form transient and long-lasting contacts with erythroblasts

A-E - +Dex macrophages (labelled with Cell Tracker green) were grown from PBMC. The erythroblasts were added at a ratio of 10:1. The excess erythroblasts were gently removed by washing 16 hours after. The cells were imaged every hour. These are examples of cells binding each other either in a transient (A, B and E) or non-transient manner (C and D). The arrow in red in E indicates a macrophage which moves with the erythroblastic cells until it detaches from them. The time indicated is in relation to the cells binding for the first time. Scale bar in all images represents 20µm.

Figure S5 – Bone marrow form these same transient and long-lasting contacts in this method

Bone marrow macrophages (labelled with Cell Tracker green) were sorted for the $CD14^{+}CD106^{+}$ cells. The erythroblasts were added at a ratio of 10:1. The excess erythroblasts were gently removed by washing 16 hours after. The cells were imaged every hour. These are examples of cells binding each other in a long-lasting manner. The arrow in red indicates a group of macrophage and erythroblast moving along together. Scale bar in all images represents 20µm.

Antibodies

Monoclonal antibodies used were Pacific Blue conjugated CD14 (BD Pharmingen, Franklin Lakes, New Jersey, US), FITC conjugated CD16 (Miltenyi, Bergisch Gladbach, Germany), PE conjugated CD163 (Miltenyi), APC conjugated CD169 (Miltenyi), PE conjugated CD106 (VCAM1; Biolegend, San Diego, California, US), VioBlue conjugated CD34 (Miltenyi) and PE conjugated CD36 (Miltenyi). Polyclonal mouse anti-EphB6 (Abnova, Taipei City, Taiwan) and rabbit anti-EphA4 (Abcam, Cambridge, UK), anti-GAPDH (Santa Cruz, Dallas, Texas), anti-EphB4 (Novus Biologicals, Abingdon, UK) and anti-ephrin-B2 (Novus Biologicals) were used for immunoblotting. HRP-conjugated rabbit anti-mouse, swine anti-rabbit and rabbit anti-goat (DAKO, Santa Clara, US) for immunoblotting. Monoclonal mouse anti-human CD29 (Clone HUTS-21; BD Pharmingen) was used to detect the active form of integrin β1 for flow cytometry. Anti-mouse PE secondary was purchased from Biolegend. Recombinant mouse ephrin-B2-Fc chimera protein and recombinant human VCAM1-Fc chimera protein were purchased from R&D systems (Minneapolis, Minnesota, US). Human IgG whole molecule (Jackson Immunoresearch, West Grove, Pennsylvania, US) were used as a control. Goat anti-human IgG+IgM 647 (Jackson Immunoresearch) was used to cluster the Fc constructs in the binding experiment.

Human cell culture

HEK293T cells (human embryonic kidney 293 cell line) and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX (Invitrogen Ltd, Carlsbad, California, US) containing 10% (v/v) fetal calf serum. Cells were incubated at 37°C in 5% CO2.

$CD34^+$ isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density purification (ρ = 1.077; Percoll; GE Healthcare, Chicago, Illinois) from healthy donors with informed consent given in accordance with the Declaration of Helsinki. The use of human donor peripheral blood progenitors was approved by the Bristol Research Ethics Committee (REC Number 12/SW/0199). Erythroblasts were

expanded and differentiated as described previously [35]. Reticulocytes were filtered using a 5µm Acrodisk Syringe filter (PALL, Port Washington, New York, US) to remove nuclei and nucleated cells.

Macrophage differentiation

The use of human donor peripheral blood progenitors for production of macrophages was approved by Bristol Research Ethics Committee (REC Number 12/SW/0199). PBMCs were isolated from peripheral blood in the same way as the $CD34^+$ cells. These cells are resuspended at a density of $3x10^6$/mL in Roswell Park Memorial Institute media (RPMI; Thermo Fischer, Waltham, Massachusetts, US) supplemented with 100 units/mL penicillin (Sigma-Aldrich, St Louis, Missouri, US), 10% foetal bovine calf serum and 25ng/mL M-CSF (Miltenyi), +/- 1µM dexamethasone (Sigma-Aldrich). The media was changed every third day and the selection for macrophages was conducted by adhesion selection, keeping only adherent cells. The macrophages were considered mature at day 7. To perform flow cytometry, the cells are scraped off the plate before being counted.

Erythroblast EphB4 and EphB6 lentiviral shRNA depletion

pLKO.1 EphB4 and EphB6 shRNA plasmids were designed by the Broad Institute and purchased from GE Healthcare (TRC number TRCN0000001774 for EphB4 knockdown and TRCN0000002018 the EphB6 knockdown). pLKO.1 scrambled was used as the scrambled control. Erythroblasts were transduced with lentivirus as described previously [36]. Briefly, cultured cells were counted and $0.5x10^6$ cells plated in 1mL of appropriate media including polybrene (Sigma-Aldrich) added at 8µg/mL. 50-100µL of concentrated virus was added drop wise to the cells and cells were incubated for 24 hours at 37°C in 5% CO2. After 24 hours, the virus treated cells were washed 3 times in PBS and re-plated in 2mL of fresh media and cultured as described above. The transfected erythroblasts were selected by puromycin treatment.

RT-PCR

RNA was extracted using the Quick-RNA Microprep (Zymo Research, Irvine, California, US) from $1x10^6$ cells at the indicated time point during differentiation. cDNA was synthesised from 100ng

DNase-treated RNA using Superscript III (Invitrogen). Real-time PCR was performed on 1μL of cDNA using fast cycling PCR kit (Qiagen, Hilden, Germany).

Western Blotting

Cell pellets of $1x10^6$ cells were lysed in 1% NP-40 lysis buffer. Proteins were resolved by SDS-PAGE and transferred to PVDF (Millipore, Burlington, Massachusetts, US) by western blotting. Membranes were blocked with 5% milk in TBS-T followed by incubation with primary antibodies as indicated in the figure legends followed by HRP-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (GE Healthcare) using an Amersham Imager 600 machine.

Flow cytometry and surface binding assay

For the surface binding assay, ephrin-B2-Fc construct or IgG control were pre-clustered at a ratio of 5:1 with Alexa 647 conjugated secondary for 45 minutes at room temperature. Then, $2×10^4$ cells were incubated with the ephrin-B2-Fc construct or IgG control for 30 minutes at 4°C. The cells were washed in PBSAG. For phenotyping the macrophages and erythroblasts, $1x10^5$ cells were incubated with directly conjugated antibodies in cell-type specific panels for 30 minutes at 4°C. Fluorescent signals were measured using a Miltenyi MacsQuant Analyzer 10 flow cytometer. Data was analyzed using FlowJo X.10.6 software (TreeStar Inc, Ashland, Oregon, US).

Cytospins

$5×10^5$ cells were cytospun onto glass slides, fixed in methanol, and stained with May-Grünwald-Giemsa stains according to the manufacturer's protocol. Images were taken using an Olympus CX31 microscope coupled to an Olympus LC30 camera using a 40× lens and cytospin counts were done by hand using Mousotron (Blacksun Software).

Statistical analysis

The statistical tests were chosen using SPSS (IBM, Armonk, New York, US). Further statistical testing was performed using GraphPad Prism (La Jolla, California, US). The test chosen is indicated in the appropriate figure legend.