Stem Cell Reports

Supplemental Information

In Vivo Repopulating Activity Emerges at the Onset of Hematopoietic Specification during Embryonic Stem Cell Differentiation

Stella Pearson, Sara Cuvertino, Maud Fleury, Georges Lacaud, and Valerie Kouskoff

Supplemental Experimental Procedures

Gene expression analysis. For gene specific PCR, total RNA was extracted with an Rneasy plus kit (Qiagen) and reverse-transcribed with random hexamer using a SuperscriptIII kit (Life Technologies). The PCR reactions were performed using Biomix Taq (Bioline) and 0.2 mM of each primer. Real time PCR was performed on an ABI 7900 system (Applied Biosystem) using Exiqon universal probe library and primer designer (Roche): *β-actin* (F: tgacaggatgcagaaggag, R: cgctcaggaggagcaatg, probe #106) , *E47* (F: aagaggacaagaaggacctgaa, R: ttattggccatacgcctctc, probe #25) , *Pre-Tα* (F: ctgctttccggagcctct, R: gaggagcaggcgcagtag, probe #5) , *Rag1* (F: gaactgaagctcagggtagacg, R: gaactgaagctcagggtagacg, probe #16) , *Ebf1* (F: gaaacctctcggctccagta, R: caggattctttcgcaccatt, probe #17) , *Pax5* (F:acgctgacagggatggtg, R:ggggaacctccaagaatcat, probe #83). Expression data were calculated relative to *actin* controls as $2^{-\Delta ct}$.

Flow Cytometry. Cells were blocked with FcRγII/III antibody (24G2 supernatant) prior to staining with various combinations of antibodies as indicated in specific experiments (CD45.1-APC (17-0453), CD45.2-PE (12-0454), CD71-bio (13-0711), TER119-FITC (11-5921), GR1-bio (13-5931), CD11b-FITC (11-0112), B220-bio (13-0452), IgM-FITC (11-5890), CD4-FITC (11-0041), CD8-PE (12-0081), CD3-bio (13-0032), CD19-bio (13-0193), CD5-PE-CY7 (25-0051-81), TIE2-bio (13-5987), cKIT-APC (17-1171-83), CD41-PE (12-0411), ICAM2-FITC (53-1021), FLK1-bio (13-5821-85), CD144-APC (17-1441-82), CD105-bio (13-1051-81), CD40-bio (13-0401), CD45-FITC (11-0451), strep-PE-CY7 (25-4317) all from eBioscience). Analyses were performed on a FACSCalibur and cell sorts on a FACSAria (BD Biosciences). Data were analyzed with Flowjo (TreeStar), gating first on the forward scatter versus side scatter to exclude non-viable cells. Unstained and isotype controls were used for gating strategies.

Clonogenic assays. Cells were plated at a density of 2 x 10^4 cells/ml in 1% methylcellulose containing 10% plasma-derived serum (PDS; Antech), 5% protein-free hybridoma medium (PFHM-II; Gibco-BRL) and KL (1% conditioned medium), TPO (5 ng/ml), Erythropoietin (2 U/ml), IL-11 (25 ng/ml), IL-3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and IL-6 (5 ng/ml). LIF, IL3 and c-kit ligand were derived from media as described (Fehling et al., 2003). GM-CSF, M-CSF, G-CSF, TPO, IL-6 and IL-11 were purchased from R&D Systems.

Time lapse imaging. Sorted FLK1$^+$ cells were seeded on gelatinized Image-lock plates (Essen BioScience) in StemPro supplemented with the indicated cytokines, placed in the IncuCyte incubator system (Essen BioScience) and imaged every 15 minutes over a 4-day period in 5% CO2 at 37º C.

Lymphoid cells derivation. Sorted cKIT$^+$ cells were plated on irradiated OP9 or OP9-DL1 in IMDM supplemented with serum, IL7, KL and FLT3 as previously described (Schmitt et al., 2004). Cells were passaged weekly on fresh irradiated OP9.

Supplemental References

Fehling, H.J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G., and Kouskoff, V. (2003). Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development *130*, 4217-4227.

Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S., and Zuniga-Pflucker, J.C. (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol *5*, 410-417.

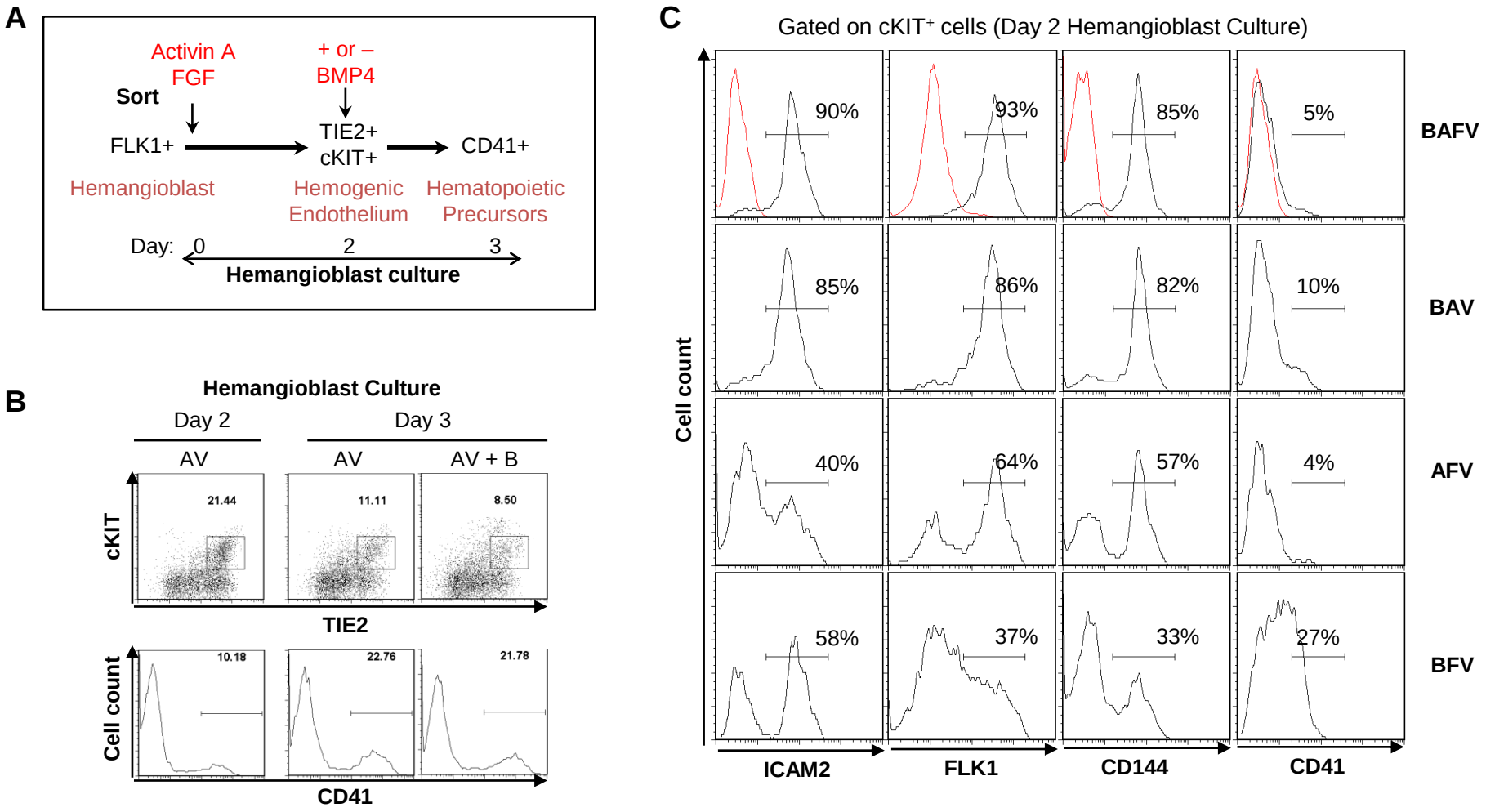

Figure S1, relating to figure 1: Endothelial characteristic of cKIT⁺ cells

(**A**) Schematic representation of the experimental strategy. FLK1⁺ cells sorted from day 3 EBs were seeded on gelatinized plate in serum-free media. Activin A, FGF and BMP4 were added or not as indicated. (**B**) Representative flow cytometry of cells analyzed after 2 or 3 days of culture in the indicated cytokines for the co-expression of TIE2 and cKIT which marks the hemogenic endothelium population and for CD41 expression marking the emergence of blood cells. (**C**) Representative flow cytometric analysis of the indicated cell surface markers on the cKIT⁺ population cultured for two days in the indicated cytokines. Red histograms indicate unstained controls. Data are representative of 3 independent experiments (B: BMP4, A: ActivinA, V: VEGF, F: FGF2).

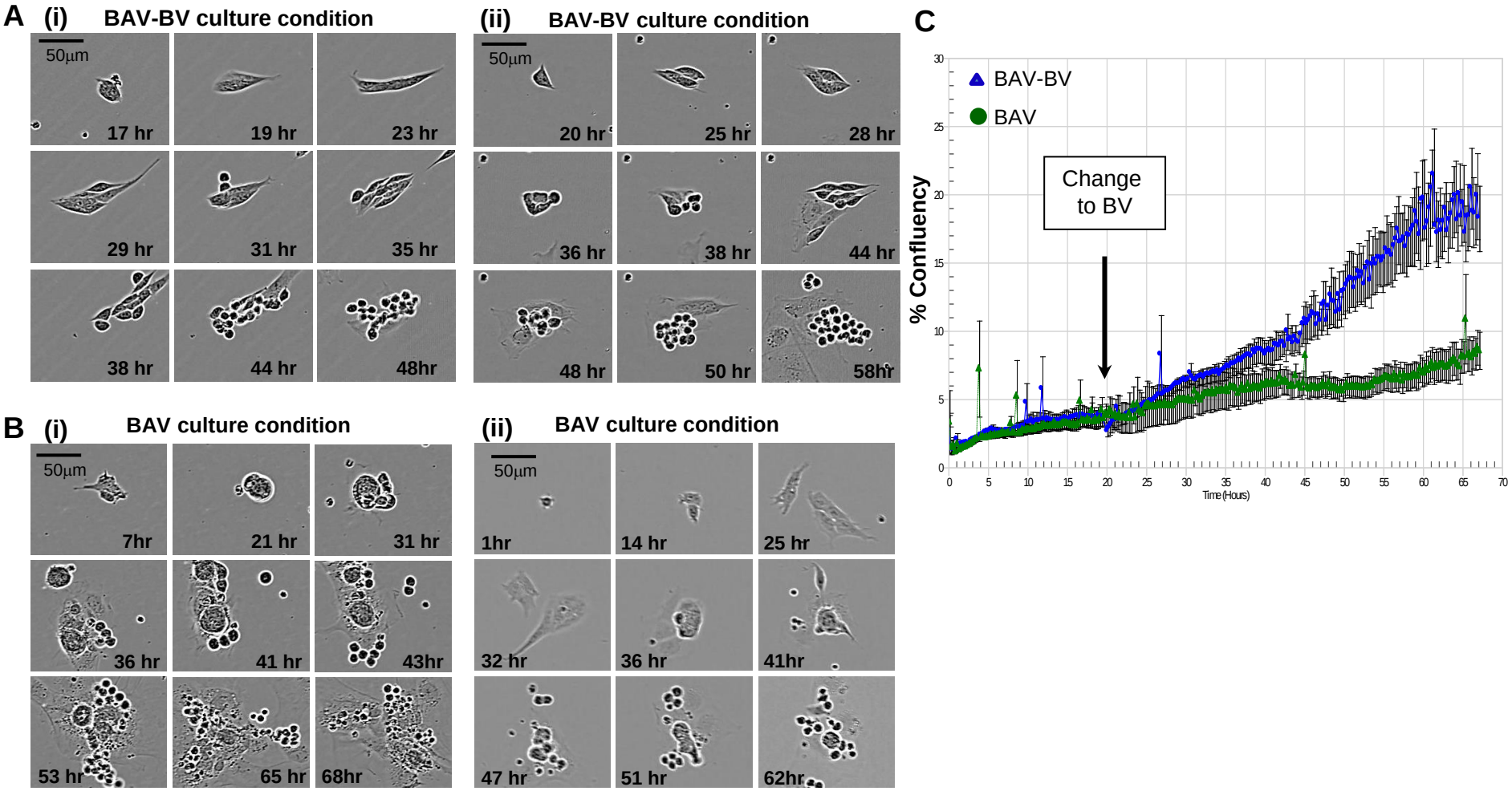

Figure S2, related to figure 3: Activin A impairs the expansion of emerging round floating hematopoietic cells

Further representative time lapse imaging of FLK1^{+} sorted cells cultured in serum-free media supplemented with (**Ai and ii**) BAV for the first day then switched to BV for the remaining time or with (**Bi and ii**) BAV for the entire culture. (**C**) Confluency over a 3-day period of FLK1^{+} cells cultured with BAV for the first day then BV for the remaining time (BAV-BV) or with BAV for the entire culture. Data were acquired with the IncuCyte software and are presented as a mean +/- SD of 30 individual measurements at each time point for each culture condition using a 15-minute time frame. Data are representative of 3 independent experiments (B: BMP4, A: ActivinA, V: VEGF, F: FGF2).

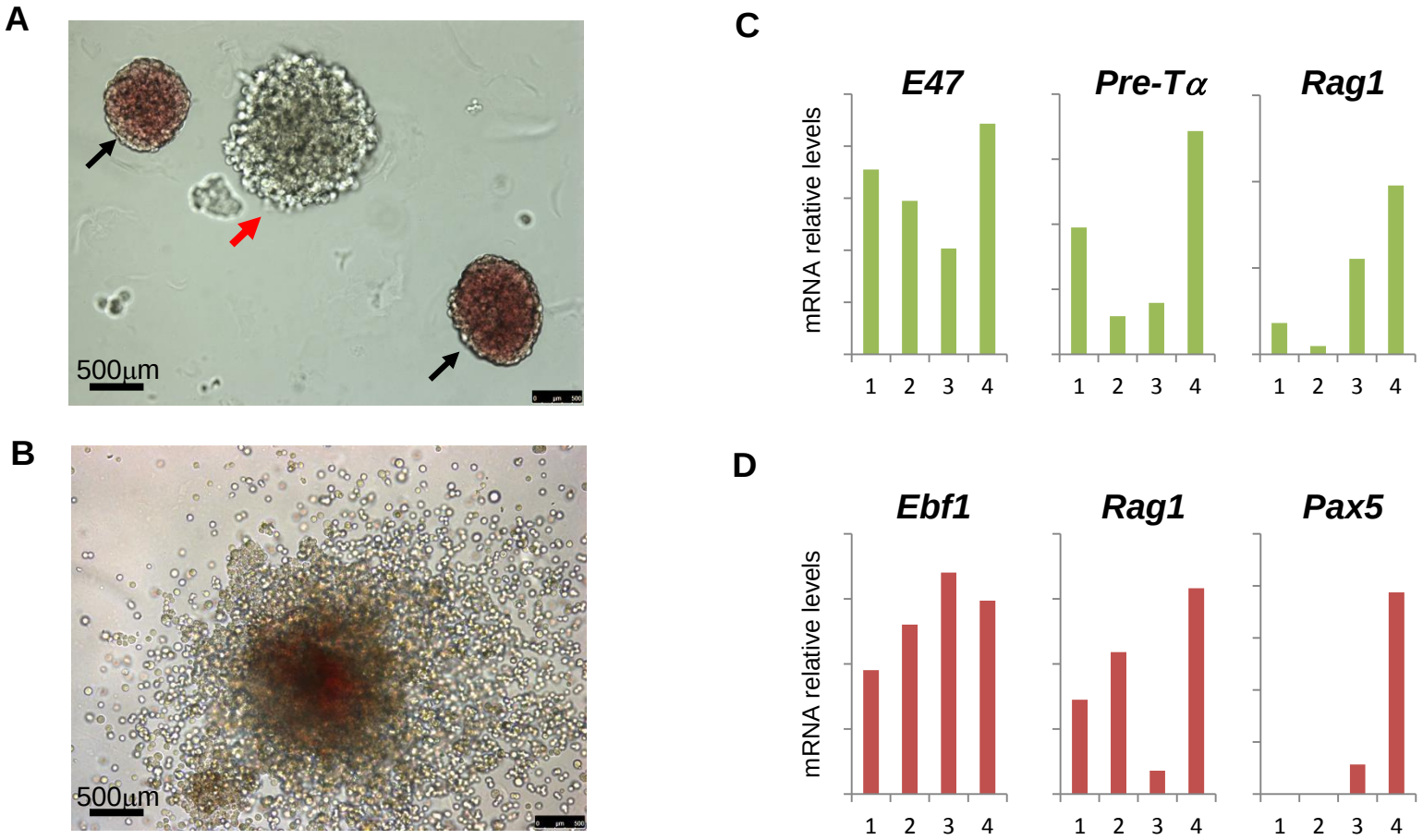

Figure S3, related to figure 4: Hematopoietic output of cKIT⁺ cells

(**A**) Representative picture of primitive colonies (black arrows) and definitive colonies (red arrow) taken 5 days after replating of cKIT⁺ cells. At this stage of the culture, cells within the definitive colonies are highly proliferative and not yet differentiating toward myeloid or erythroid lineages. (**B**) Representative picture of a multilineage definitive colony taken 10 days after replating of cKIT⁺ cells. (**C**) Real time PCR for the expression of the indicated genes in T cells derived from cKIT⁺ cultured on OP9-DL1. (**D**) Real time PCR for the expression of the indicated genes in B cells derived from cKIT⁺ cultured on OP9. Note that in two cases, the level of *Pax5* expression was below detection limit. Data are shown for 4 independent experiments (1 to 4) and mRNA expression data were calculated relative to *β-actin* controls as $2^{-\Delta ct}$.

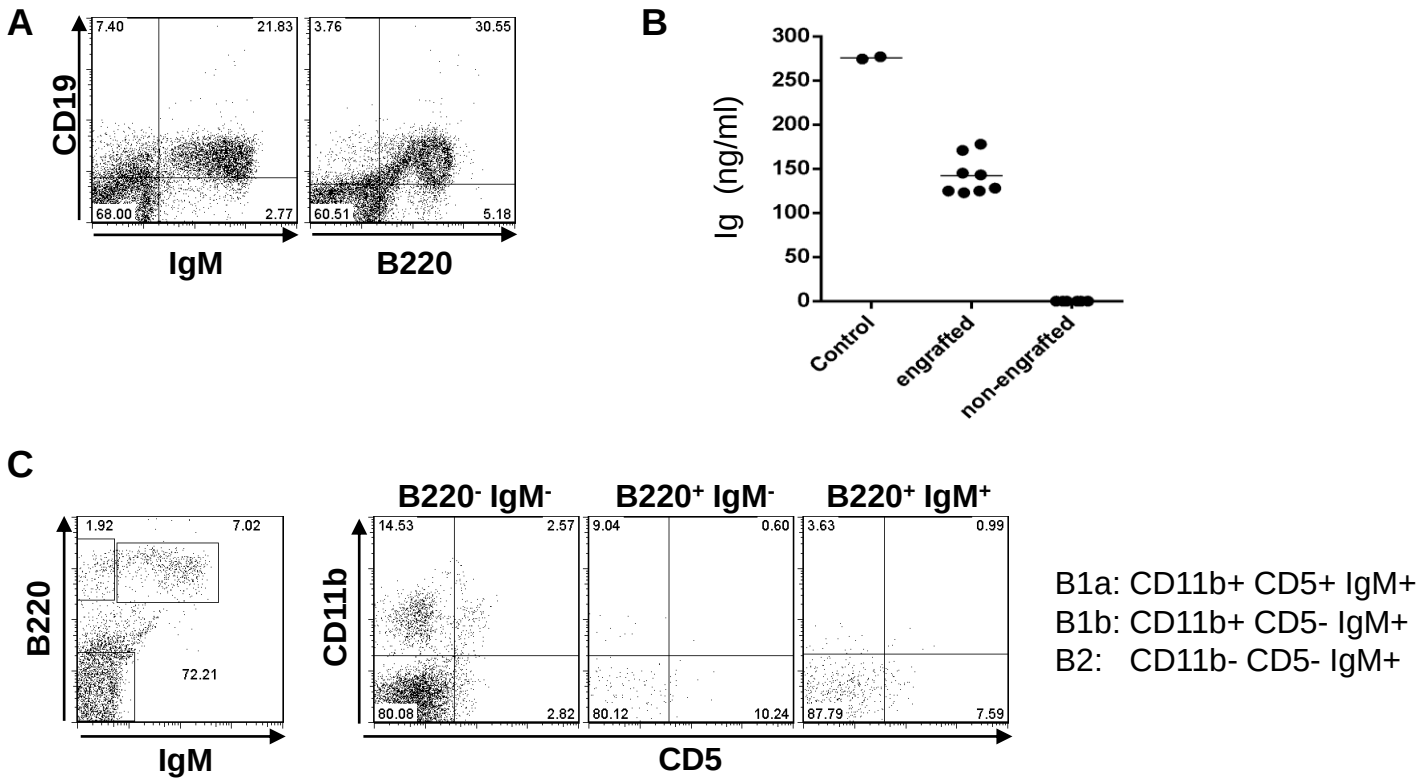

Figure S4, related to figure 5: B lymphocytes generated upon engraftment of ES-derived cKIT$^+$ cells are of the adaptive B2 lineage
(**A**) B cells in the spleen of engrafted mice co-express CD19, B220 and IgM. (**B**) Immunoglobulin concentration in the serum of control C57Bl/6 mice and NSG mice engrafted or not with ES-derived hematopoietic repopulating cells as measured by ELISA. Each dot represents an individual mouse. (**C**) Spleen cells of engrafted mice were co-stained for the expression of IgM, B220, CD11b and CD5. The expression of CD11b and CD5 which defines B1 B cell subsets was analyzed on B220$^-$IgM$^-$, B220$^+$IgM$^-$ and B220$^+$IgM$^+$. Data are representative of four independent mice .

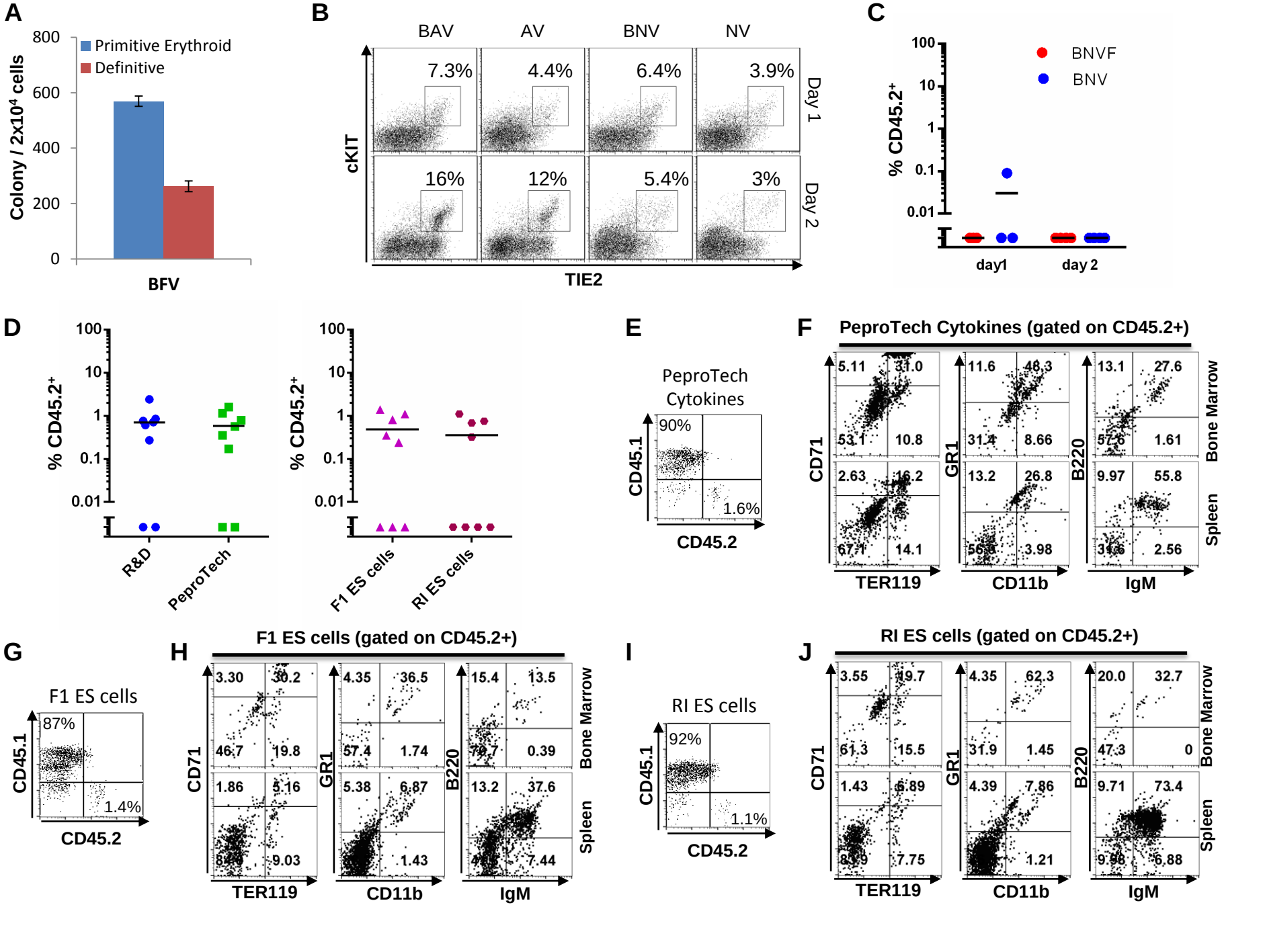
A
Colony / 2x10^4 cells
800
600
400
200
0
Primitive Erythroid
Definitive
BFV
B
BAV
AV
BNV
NV
7.3%
4.4%
6.4%
3.9%
16%
12%
5.4%
3%
Day 1
Day 2
cKIT
TIE2
C
% CD45.2+
100
10
1
0.1
0.01
BNVF
BNV
day1
day 2
D
% CD45.2+
R&D
PeproTech
F1 ES cells
RI ES cells
E
PeproTech Cytokines
CD45.1
CD45.2
90%
1.6%
F
PeproTech Cytokines (gated on CD45.2+)
CD71
GR1
B220
TER119
CD11b
IgM
Bone Marrow
Spleen
5.11
31.0
53.1
10.8
11.6
48.3
31.4
8.66
13.1
27.6
57.6
1.61
2.63
16.2
67.1
14.1
13.2
26.8
56.6
3.98
9.97
55.8
31.6
2.56
G
F1 ES cells
CD45.1
CD45.2
87%
1.4%
H
F1 ES cells (gated on CD45.2+)
3.30
30.2
46.7
19.8
4.35
36.5
57.4
1.74
15.4
13.5
79.7
0.39
1.86
5.16
5.38
6.87
13.2
37.6
9.03
1.43
7.44
I
RI ES cells
CD45.1
CD45.2
92%
1.1%
J
RI ES cells (gated on CD45.2+)
3.55
19.7
61.3
15.5
4.35
62.3
31.9
1.45
20.0
32.7
47.3
0
1.43
6.89
4.39
7.86
9.71
73.4
7.75
1.21
6.88

Figure S5, related to Table 1: Importance of cytokines in the formation of ES-derived repopulating cells

(**A**) At day 2 of culture in the indicated cytokines, cells were plated in clonogenic assay for hematopoietic precursors. Primitive colonies: primitive erythrocytes. Definitive colonies: all definitive colonies (macrophages colonies, macrophages/erythrocytes colonies, mix colonies and GM colonies). Data shown are representative of at least three experiments. Data are presented as the mean number of colonies from three dishes; bars represent SEM. (**B**) FLK1^{+} cells sorted from day 3 EBs were seeded on gelatinized plate in serum-free media supplemented with the indicated cytokine combinations (B: BMP4, A: ActivinA, V: VEGF, N: Nodal). Cells were analyzed by flow cytometry at day 1 and 2 for the co-expression of TIE2 and cKIT marking hemogenic endothelium. (**C**) Frequency of CD45.2^{+} cells in the blood of recipient mice 4 weeks after engraftment with cKIT^{+} cells derived from day 1 or day 2 culture containing BNV (blue circles) or BNVF (red circles) cytokines. Each point represents one mouse; number of mice and experiments are detailed in Table 1. (**D**) To assess the reproducibility of the protocol, *in vitro* differentiation followed by *in vivo* engraftment experiments were performed with cytokines obtained from a different supplier (R&D: blue circles; PeproTech: green squares) or with different ES cell lines F1 ESCs (purple triangles) and RI ESCs (brown circles). Frequency of CD45.2^{+} cells in the blood of recipient mice at 4 weeks is presented after engraftment with cKIT^{+} cells derived from day 1 in the indicated conditions. CD45.1 versus CD45.2 staining of peripheral blood samples at 4 weeks post engraftment using PeproTech cytokines for the *in vitro* differentiation (**E**), from *in vitro* differentiated F1 ESCs (**G**), and from *in vitro* differentiated RI ESCs (**I**). Each point represents one mouse; n=2, 4 mice per experiments. Bone marrow and spleen were harvested 12 weeks after engraftment and single cell suspensions were stained for CD45.1, CD45.2 and specific lineages markers. Flow cytometric analysis for lineage marker expression is shown on CD45.2^{+} ES-derived gated cells for bone marrow and spleen. CD71 and TER119 mark erythroid cells, CD11b and GR1 mark myeloid cells, IgM and B220 mark B lymphocytes. Data are presented for mice engrafted with cKIT cells derived using PeproTech cytokines for the *in vitro* differentiation (**F**), from *in vitro* differentiated F1 ESCs (**H**), from *in vitro* differentiated RI ESCs(**J**). For all reproducibility experiments n=2.

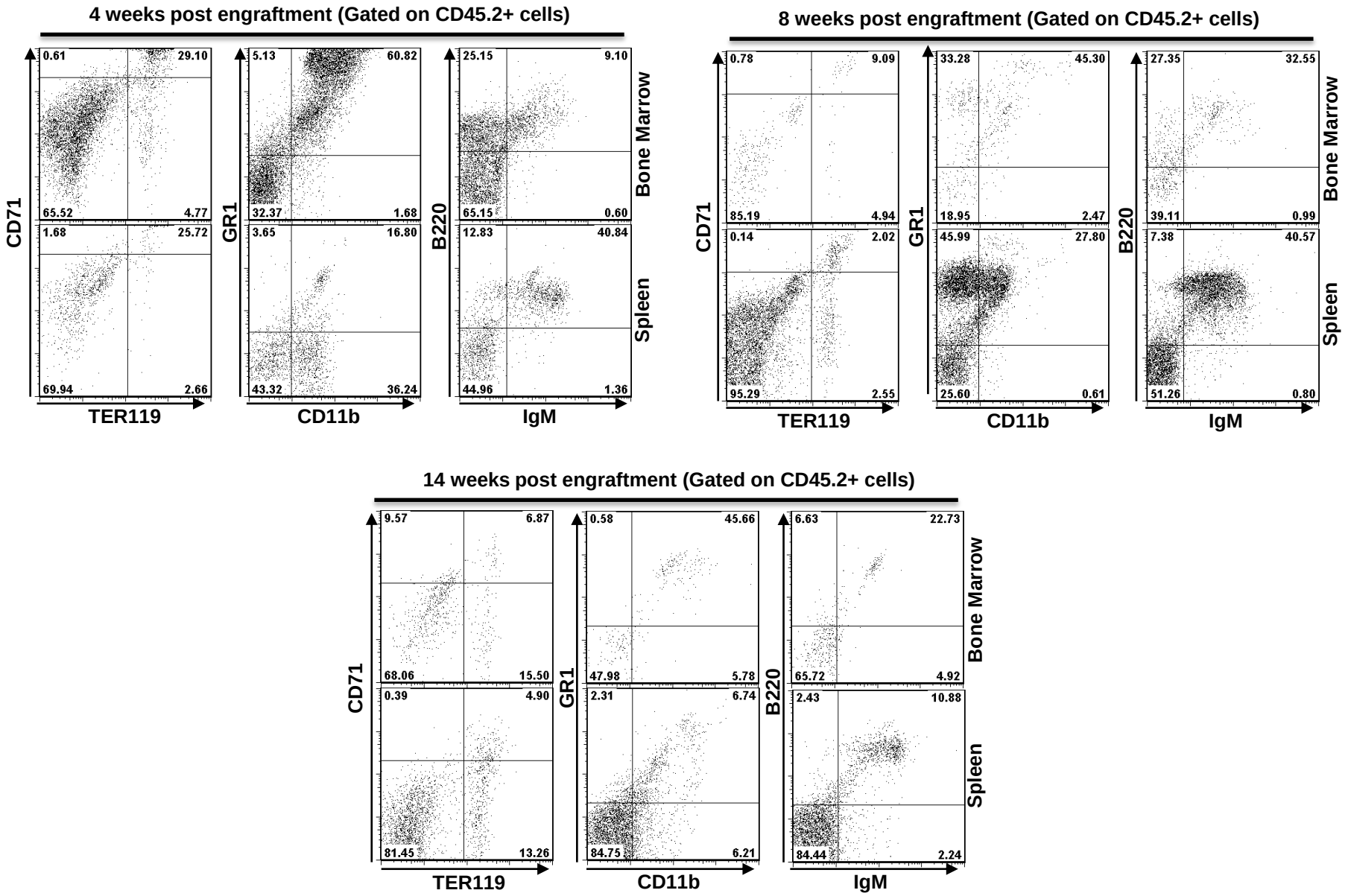

Figure S6, related to figure 6: Multilineage engraftment Bone marrow and spleen cells harvested 4, 8 or 14 weeks after engraftment were stained for CD45.1, CD45.2 and specific lineages markers. Flow cytometric analysis for lineage marker expression is shown on CD45.2$^+$ ES-derived cells for bone marrow and spleen. CD71 and TER119 mark erythroid cells, CD11b and GR1 mark myeloid cells, IgM and B220 mark B lymphocytes.