Supplementary Information

Reduced Levels of Hspa9 Attenuates Stat5 Activation in Mouse B-cells

Kilannin Krysiak,[a,c] Justin F. Tibbitts,[a,c] Jin Shao,[a,c] Tuoen Liu,[a,c] Matthew Ndonwi,[a,c] and Matthew J. Walter[a,b,c,d]

Department of Medicine[a], Department of Genetics[b], Division of Oncology[c], Siteman Cancer Center[d], Washington University School of Medicine, St. Louis, MO.

SUPPLEMENTAL RESULTS

In this study we describe the effects of reduced *Hspa9* expression on murine hematopoiesis using a gene trap to disrupt *Hspa9*. We investigated the effect of the gene trap disruption of *Hspa9* on the expression of neighboring genes (*Ets1, Ctnna1,* and *Gm26109*). Microarray expression levels of *Ets1* and *Ctnna1* were not significantly different between *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ CFU-preB colony derived cells (N=3/genotype; *Ets1* 602±147 vs. 553±176; *Ctnna1* 205±19 vs. 214±41 arbitrary units) (*data not shown*). Two putative snoRNAs are predicted to reside within introns 10 (*Gm26109)* and 11 (*Gm22200*) of *Hspa9*. We were unable to detect expression of the putative snoRNA *Gm22200* in bulk bone marrow or B-cell fractions from wild-type mice by RT-PCR so it was not studied further. Gene trap insertion in *Hspa9*$^{+/-}$ mice does disrupt normal expression of the putative snoRNA, *Gm26109,* reducing its expression ~32% (N=3/genotype, *Hspa9*$^{+/+}$ 1±0.085 vs. *Hspa9*$^{+/-}$ 0.68±0.074 arbitrary units relative to a control *sno202*, p=0.008) (*data not shown*). shRNA-mediated knockdown of *Hspa9* did not affect *Gm26109* expression levels in YFP+ bone marrow cells (N=6-7/group, shLUC 1±0.3 vs. shHspa9 1.17±0.47 arbitrary units relative to a control *sno202*) (*data not shown*). *Gm26109* shares 83% identity with *SNORD63*, a human C/D box snoRNA of unknown functional significance located in intron 10 of human *HSPA9*. These results, in conjunction with the ability of *HSPA9* expression to rescue the reduction in B-cell colonies in *Hspa9*$^{+/-}$ mice (**Fig. S7B**), indicate that the reduction of *Hspa9*, not *Gm26109* levels, contributes to the B-cell phenotypes observed.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Hspa9 expression is reduced using an N-terminal Hspa9 antibody

Expression of Hspa9 in bone marrow and spleen of littermates was evaluated by Western blot using an N-terminal antibody and β-Actin loading control.

Figure S2: Organ cellularity, spleen size and CBCs are normal in *Hspa9*$^{+/-}$ mice

Hspa9$^{+/-}$ (*open circles*) and *Hspa9*$^{+/+}$ (*closed circles*) littermates were evaluated at the ages indicated in months for **A)** total cellularity of bone marrow (*left panel*, 4 leg bones) and spleens (*right panel)*, **B)** body (*left panel*) and spleen (*right panel*) weights, and **C)** complete blood counts from peripheral blood. No significant differences between genotypes were detected. PLT, platelets, *orange*; MCV, mean corpuscular volume, *green*; Hb, hemoglobin, *red*; WBC, white blood cells, *blue*.

Figure S3: Immunophenotyping of peripheral blood and spleen cells up to 12 months of age

A) Spleen cells of *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermates were analyzed by flow cytometry for immunophenotypic markers showing no difference between genotypes. Neutrophils (Gr1^{+}/CD115^{-}, *red bars*); B-cells (B220^{+}, *green bars*); monocytes (Gr1^{lo}/CD115^{+}, *orange bars*); T-cells (CD3e^{+}, *blue bars*) (N=3-6/genotype at each time point). **B)** The percent of red blood cell precursors (CD71$^{+/-}$/Ter119) in spleen and peripheral blood cell populations of *Hspa9*$^{+/-}$ (*open circles*) and *Hspa9*$^{+/+}$ (*closed circles*) littermates at 12 months of age are not different. ProEBs, proerythroblasts; BasoEBs, basophilic erythroblasts; PolyEBs, polychromatic erythroblasts; OrthoEBs, orthochromatic erythroblasts. Error bars represent mean ± SD.

Figure S4: Colony forming ability of erythroid and myeloid spleen and bone marrow progenitors from *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ mice are similar

Spleen cells from *Hspa9*$^{+/-}$ (*open circles*) and *Hspa9*$^{+/+}$ (*closed circles*) mice were plated in **A)** CFU-C media or **B)** mature BFU-E media (supplemented with only EPO) and colonies were enumerated on day 7 or 10, respectively. **C)** 10,000 bone marrow or 100,000 spleen cells from 12-month-old mice were plated in CFU-C media and BFU-E and CFU-E colonies were enumerated on day 7 and 3, respectively. BM, bone marrow; Spl, spleen

Figure S5: *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermate mice respond similarly to hematopoietic stress

A) Kaplan-Meier curve of overall survival for *Hspa9*$^{+/+}$ (*solid line*) and *Hspa9*$^{+/-}$ (*dotted line*) mice given weekly doses of 150mg/kg 5-fluorouracil (5-FU) (N=6/genotype, 6-8 months old). **B)** Two doses of 30mg/kg phenylhydrazine (PHZ) was used to induce hemolytic anemia in *Hspa9*$^{+/+}$ (*black line*) and *Hspa9*$^{+/-}$ (*red line*) mice >11 months old (N=5/genotype). Mice were bled at indicated intervals to monitor for red blood cell recovery (Hematocrit, HCT, *left*; mean corpuscular volume, MCV, *right*). **C)** A single, sublethal dose of radiation (500 rads) was given to 5-6 month old *Hspa9*$^{+/+}$ (*black line*) and *Hspa9*$^{+/-}$ (*red line*) mice and hematopoietic recovery was monitored by complete blood counts at indicated time intervals (N=5/genotype; white blood cell count, WBC, *left*; hemoglobin, Hb, *right*). fL, femtoliter. Statistical analysis by ANOVA and log-rank test. Error bars represent mean ± SD.

Figure S6: CFU-PreB colonies from *Hspa9*$^{+/-}$ bone marrow are significantly reduced when grown in media supplemented with mouse IL-7

Bone marrow from 13-19 week old mice was harvested and 100,000 cells were plated in CFU-PreB media containing recombinant mouse IL-7 (R&D systems, HSC009). Colonies were counted on day 7 ($p<0.001$).

Figure S7: Retroviral overexpression of HSPA9 rescues the reduction in CFU-PreB colonies in *Hspa9*$^{+/-}$ bone marrow

A) Western blot showing overexpression of HSPA9 cDNA in GFP+ sorted HEK293T cells transduced with MSCV-IRES-GFP control or HSPA9 overexpression vector (MSCV-HSPA9-IRES-GFP). Bone marrow from *Hspa9*$^{+/+}$ or *Hspa9*$^{+/-}$ mice was transduced with MSCV control (*white bars*) or HSPA9 overexpression (*grey bars*) vectors and harvested 8-10 weeks post-transplant. GFP+ **(B)** and GFP- **(C)** cells from each mouse were sorted and plated in CFU-PreB methylcellulose media. CFU-PreB colonies were counted on day 7. Statistical analysis by two tailed Student's t-test. NS, not significant. Error bars represent mean ± SEM.

Figure S8: Hardy fractions B-F are significantly reduced in spleens following knockdown of *Hspa9*

A) Percent of YFP+ cells in bone marrow and spleens of mice transduced with control shLUC vector (*filled squares*) or the Hspa9 knockdown vector, shHspa9 (*open squares*). **B)** Percent of Hardy fractions A-F within the YFP+ population of the spleen. *p<0.05, **p<0.01, ***p<0.001

Figure S9: *Hspa9*$^{+/-}$ B-cells isolated from CFU-PreB culture have an ~50% reduction in *Hspa9* mRNA expression

Bone marrow from *Hspa9*$^{+/-}$ (*open*) or *Hspa9*$^{+/+}$ mice (*filled*) was plated in CFU-PreB culture media. **A)** On day 7, colonies were counted and cells were isolated from methylcellulose. **B)** B220+ cells were sorted by FACS. The RNA isolated from these cells was hybridized to Mouse Gene 1.0 ST arrays (Affymetrix) and analyzed for mRNA expression. **C)** Levels of *Hspa9* mRNA were ~50% reduced in *Hspa9*$^{+/-}$ samples compared to *Hspa9*$^{+/+}$ samples (N=5/genotype). Statistical analysis by two tailed Student's t-test. Error bars represent mean ± SD.

Figure S10: B7 cell growth is IL-7 dose-dependent

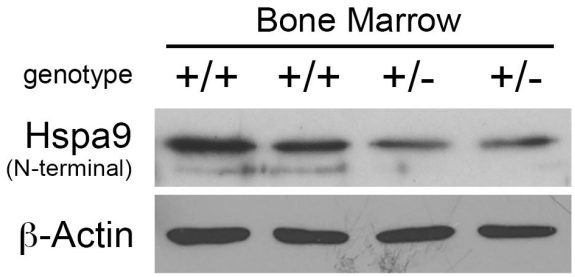
B7 cells were grown in media supplemented with varying concentrations of IL-7 (N=2/group).

Figure S11: Knockdown of *Hspa9* attenuates IL-7 mediated Stat5 activation independent of IL-7 concentration.

B7 cells were starved of cytokine overnight, spiked with various concentrations of IL-7 to stimulate phosphorylation of Stat5 and collected at times indicated. Stat5 phosphorylation was measured by intracellular flow cytometry. **A)** Representative flow cytometry histogram showing reduced pStat5 levels in *Hspa9* knockdown cells 5 minutes after B7 cells were stimulated with 1ng/mL IL-7 (*Blue*, *Hspa9*-targeting siRNA; *Red*, control siRNA; *Dashed lines*, IL-7 starved cells). **B)** The geometric mean of pStat5 fluorescence is depicted for cells treated with non-targeting control (*red lines*) or *Hspa9*-targeting siRNA (*blue lines*) following 1ng/mL IL-7 stimulation. Representative data is shown for 4 biological replicates. **C)** Independent experiment using two siRNAs with different levels of *Hspa9* knockdown showing cytokine starved B7 cells spiked with IL-7 at a concentration of 10 (*left*), 1 (*middle*) or 0.1ng/mL (*right*) to stimulate phosphorylation of Stat5 and collected at times indicated. Attenuation of Stat5 activation occurs with mild (*green*, siRNA 2) or more severe (*blue*, siRNA 1) knockdown of *Hspa9*, regardless of IL-7 concentration.

SUPPLEMENTAL FIGURES

Figure S1: Hspa9 expression is reduced using an N-terminal Hspa9 antibody



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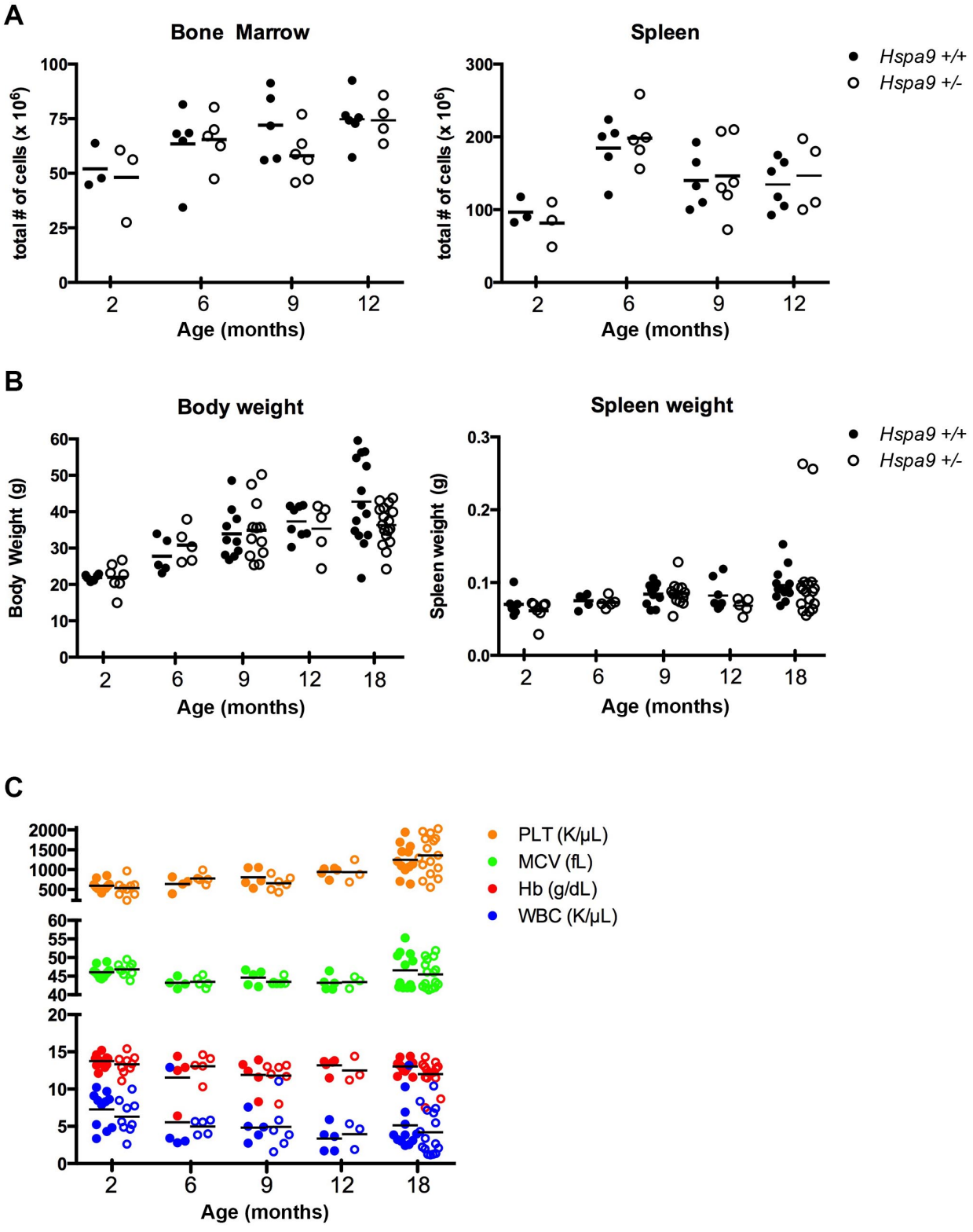

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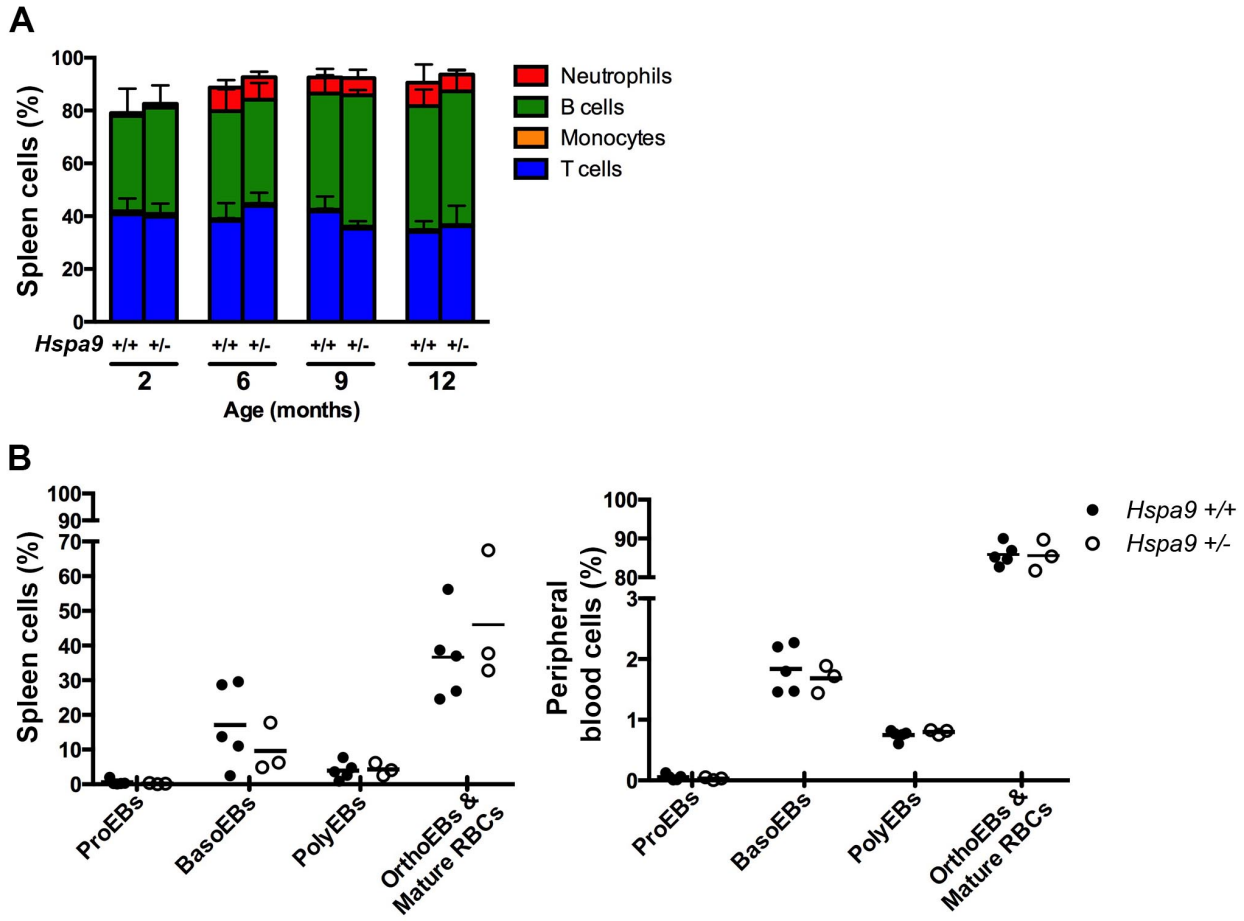

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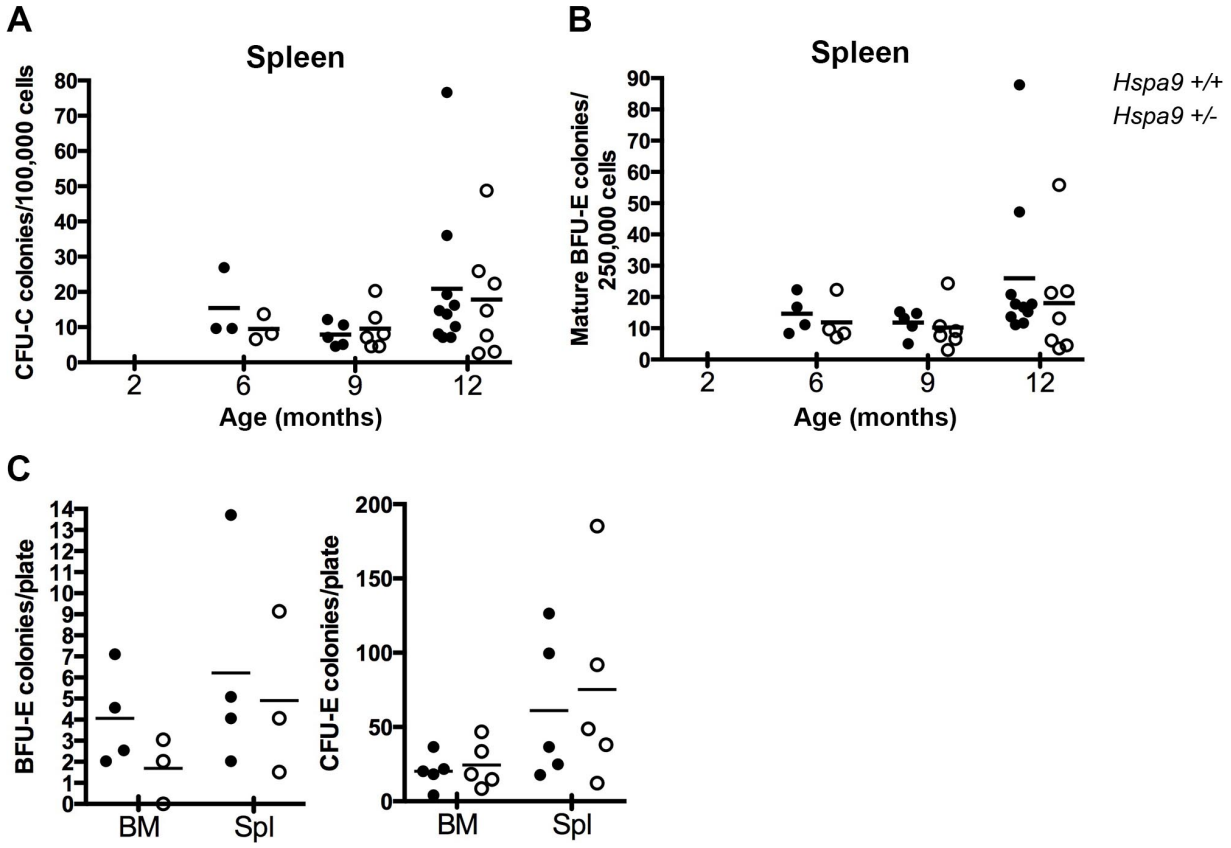

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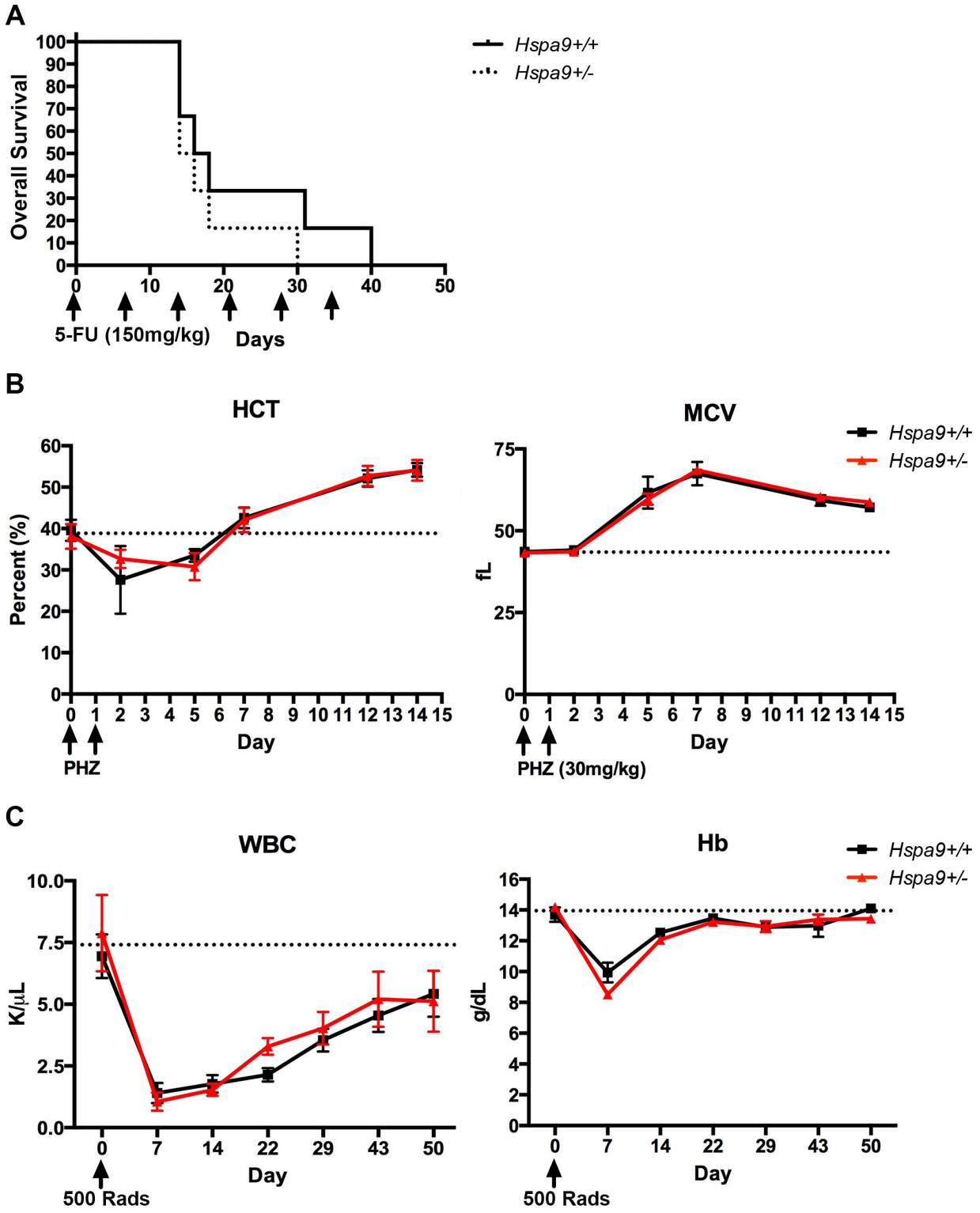

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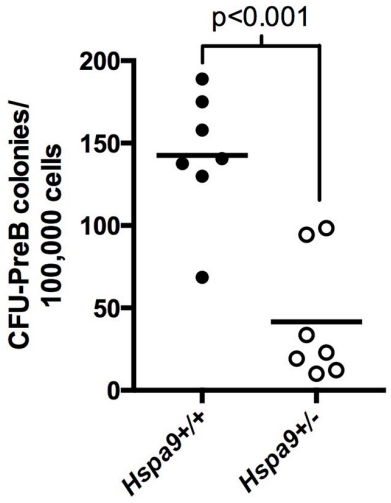

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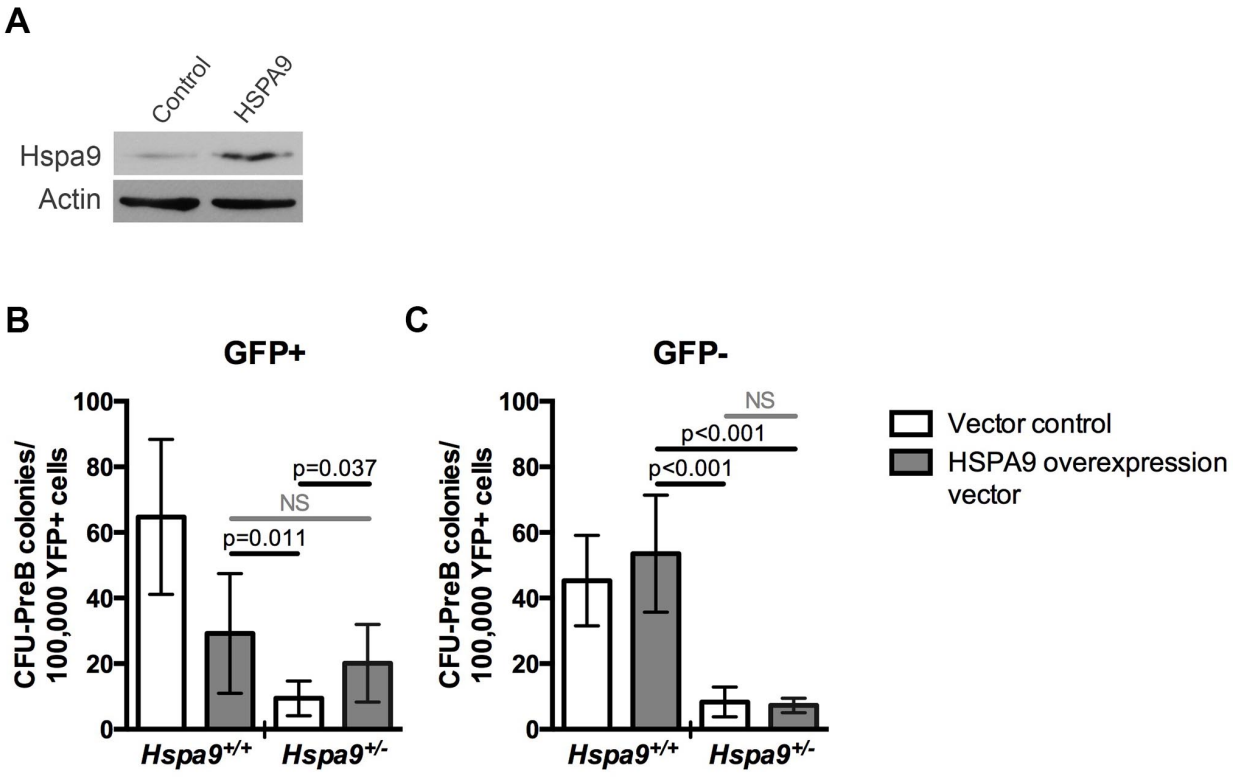

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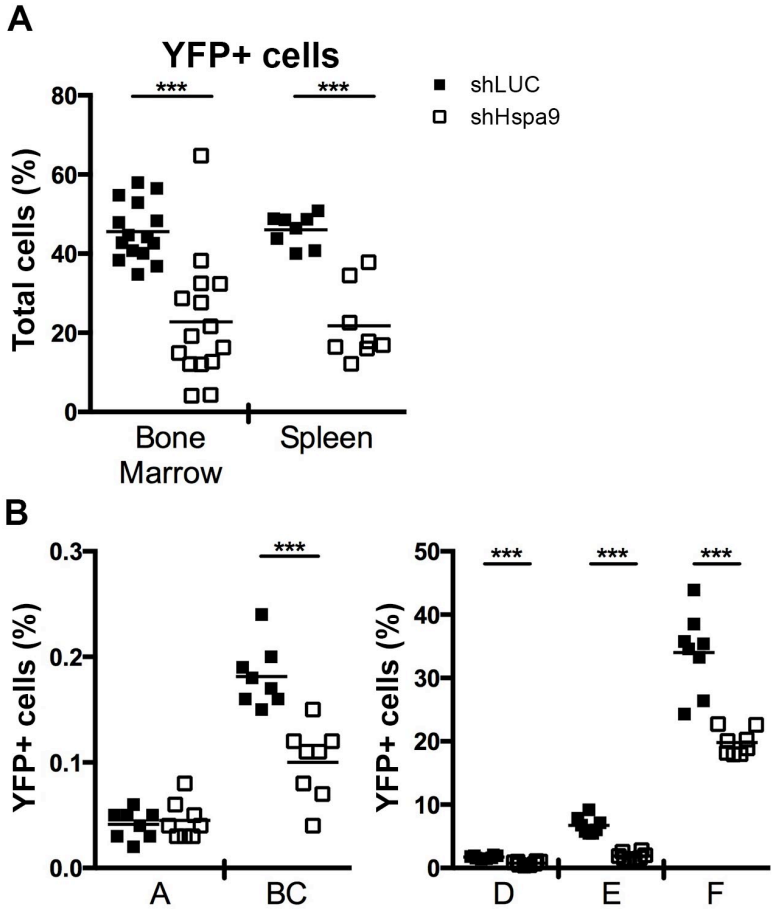

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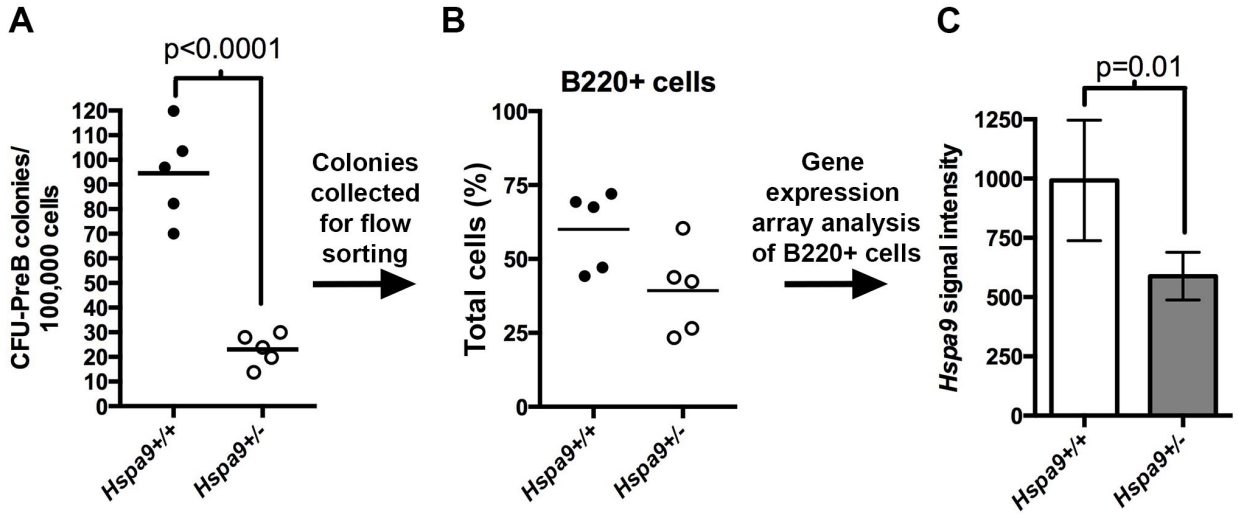

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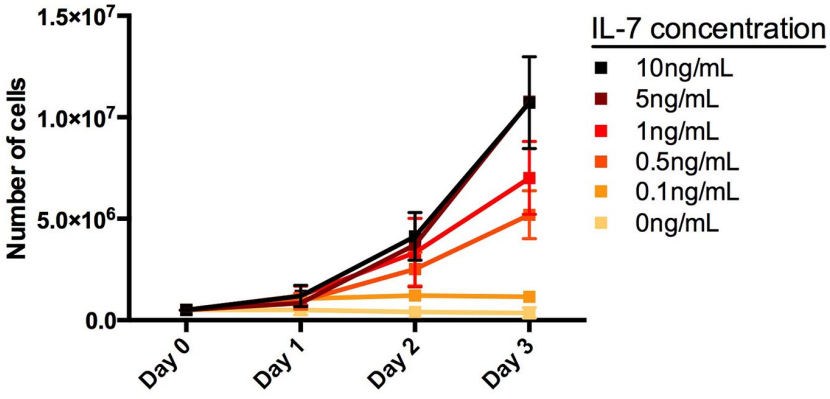

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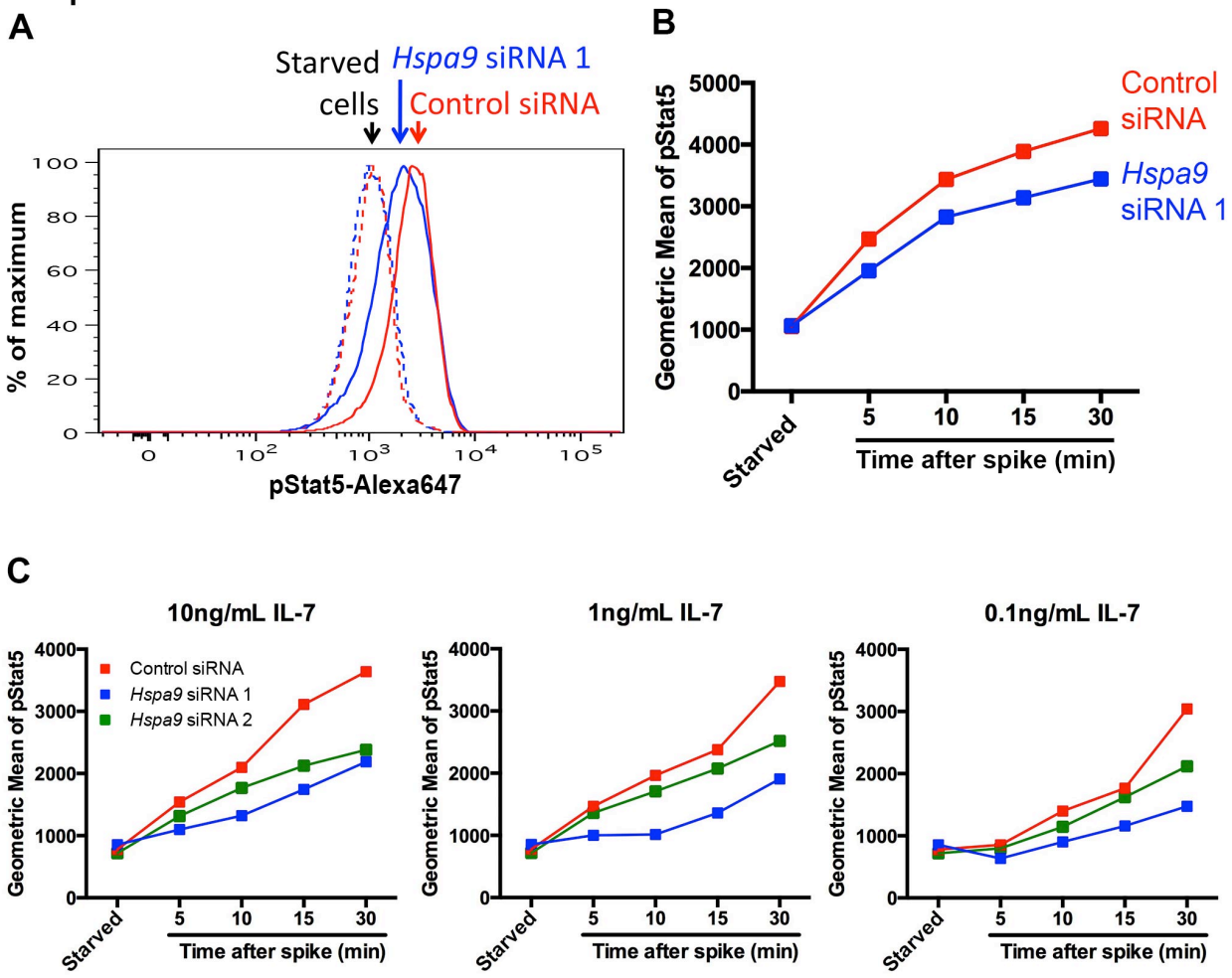

Table S1: Flow cytometry and Western blot antibodies

Antigen	Clone	Vendor
B220 (CD45R)	RA3-6B2	BD/eBioscience
CD115 (c-fms)	AF598	eBioscience
CD117 (c-Kit)	ACK2	eBioscience
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD135 (Flt3/Flk2)	A2F10	eBioscience
CD150 (SLAM)	TC15-12F12.2	Biolegend
CD16/32 (FcGamma)	93	eBioscience
CD19	1D3	BD
CD27	LG.7F9	eBioscience
CD34	RAM34	eBioscience
CD3e	145-2C11	BD/eBioscience
CD41	MWReg30	eBioscience
CD43	S7	BD
CD45.1 (Ly5.1)	A20	eBioscience
CD45.2 (Ly5.2)	104	BD
CD48	HM48-1	eBioscience
CD71	YTA74.4	ABD Serotec
Gr-1 (Ly-6G)	RB6-8C5	eBioscience
IgD	11-26c	eBioscience
IgM	II/41; eB121-15F9	BD; eBioscience
Igκ	187.1	BD
Igλ	R26-46	BD
IL7Rα	Not available	*
Ly6D	49-H4	BD
NK1.1	PK136	eBioscience
Sca-1 (Ly-6A/E)	D7	eBioscience
Stat5 (pY694)	47/Stat5pY694	BD
Ter119	TER119	eBioscience
Actin	AC-15	Sigma (A5441)
Hspa9 (C-terminal)	JG-1	Pierce (MA3-028)
Hspa9 (N-terminal)	polyclonal	Abcam (Ab23854)
Stat5	3H7	Cell Sig Tech (9358)
pStat5	D47E7	Cell Sig Tech (4322)

*Kindly provided by Deepta Bhattacharya

Table S2: Primers

Gene	Primer/probe	Vendor	Assay ID	Assay type	Forward primer (5' to 3')	Reverse primer (5' to 3')	Context sequence (5' to 3')	Citation
IL-6	FAM-MGB	Applied Biosystems	Mm00446190_m1	Gene Expression Assay				
Flt3-L	FAM-MGB	Applied Biosystems	Mm00442801_m1	Gene Expression Assay				
β-Actin	FAM-MGB	Applied Biosystems	Mm00607939_s1	Gene Expression Assay Control				
Hspa9	FAM-MGB	Applied Biosystems	Mm00477716_g1	Gene Expression Assay				
Gapdh	FAM-MGB	Applied Biosystems	Mm99999915_g1	Gene Expression Assay Control				
snoRNA-202	FAM-NFQ	Applied Biosystems	001232	Control miRNA Assay			GCTGTACTGACTTG ATGAAAGTACTTTT GAACCCTTTTCCAT CTGATG	
Gm26109	FAM-NFQ	Applied Biosystems	CS5IOYS	Custom ordered Small RNA Assay			GCATTTTATTCAACA CATCATTCTGAAAAT AGATGTGTAGAGAA ATGATAACTGAGCA CA	
Gm22200	FAM-NFQ	Applied Biosystems	CS6RM40	Custom ordered Small RNA Assay			GATGTATTTGTCACA TCATTCTGAAGGAA AGTTTGTGGTGACT TGTTATTACTGAGC ACA	
IL-7	SYBR Green	Applied Biosystems	---	Gene Expression Assay	TCTGCTGCCTGTC ACATCATC	GGACATTGAATTC TTCACTGATATTCA		PNAS 2005 Nov 15;102(46):16735-40
Gapdh	SYBR Green	Applied Biosystems	---	Gene Expression Assay Control	TGCACCACCAACT GCTTAG	GATGCAGG GATGATGTTC		PNAS 2005 Nov 15;102(46):16735-40
Hspa9		Sigma		3 primer genotyping for insert	AGACCACTGTTCA GATGACCATGG	TTAGAAGTCTGGA GCGGTCAATGC		
IST14901H6 (insertion)		Sigma		3 primer genotyping for insert	CCAATAAACCCTC TTGCAGTTGC			PNAS 2003 Nov 25;100(24):14109–14

Table S3: Genotypes of embryos derived from *Hspa9*$^{+/-}$ intercrossed mice

Embryonic Day	*Hspa9*$^{+/+}$	*Hspa9*$^{+/-}$	*Hspa9*$^{-/-}$	total
E9.5	2	5	0	7
E11.5	3	2	0	5
E12.5	4	7	0	11
E13	3	4	0	7
E14	3	2	0	5
Observed (Expected)	15 (8.75)	20 (17.5)	0 (8.75)*	35

*p=0.0011

Table S4: Immunophenotypic markers

Cell Type	Cell type by description in text	Flow cytometric markers
B-cell	B-cells	B220+
	Hardy Fraction A	B220+CD3e-CD11c-NK1.1-IgM-IgD-CD19-CD43+LY6D+
	Hardy Fraction BC	B220+CD3e-CD11c-NK1.1-IgM-IgD-CD19+CD43+
	Hardy Fraction D	B220+CD3e-CD11c-NK1.1-IgM-IgD-CD19+CD43-
	Hardy Fraction E	B220+CD3e-CD11c-NK1.1-IgM-IgD+
	Hardy Fraction F	B220+CD3e-CD11c-NK1.1-IgM+IgD+
Erythroid	proerythroblasts	$Ter119^{med}CD71^{hi}$
	polychromatic erythroblasts	$Ter119^{hi}CD71^{hi}FSC^{int}$
	basophilic erythroblasts	$Ter119^{hi}CD71^{hi}FSC^{hi}$
	reticulocytes	$Ter119^{hi}CD71^{lo}FSC^{lo}$
T-cell	T-cells	CD3e+
Myeloid	Neutrophils	Gr1+CD115-
	Monocytes	Gr1+CD115+
Progenitor populations	Common lymphoid progenitor (CLP)	B220-CD3e-Gr1-Ter119-CD27+Flk2+IL7Rα+Ly6D-
	Granulocyte-monocyte progenitor (GMP)	Lin-Sca-cKit+$FcγR^{hi}$CD34+
	Common myeloid progenitor (CMP)	Lin-Sca-cKit+$FcγR^{lo}$CD34+
	Megakaryocyte-erythrocyte progenitor (MEP)	Lin-Sca-cKit+$FcγR^{lo}$CD34-
Stem-cell enriched	KLS	Lin-Sca+cKit+
	SLAM	Lin-Sca+cKit+CD150+CD48-