Science Advances

Supplementary Materials for

Plasma iron controls neutrophil production and function

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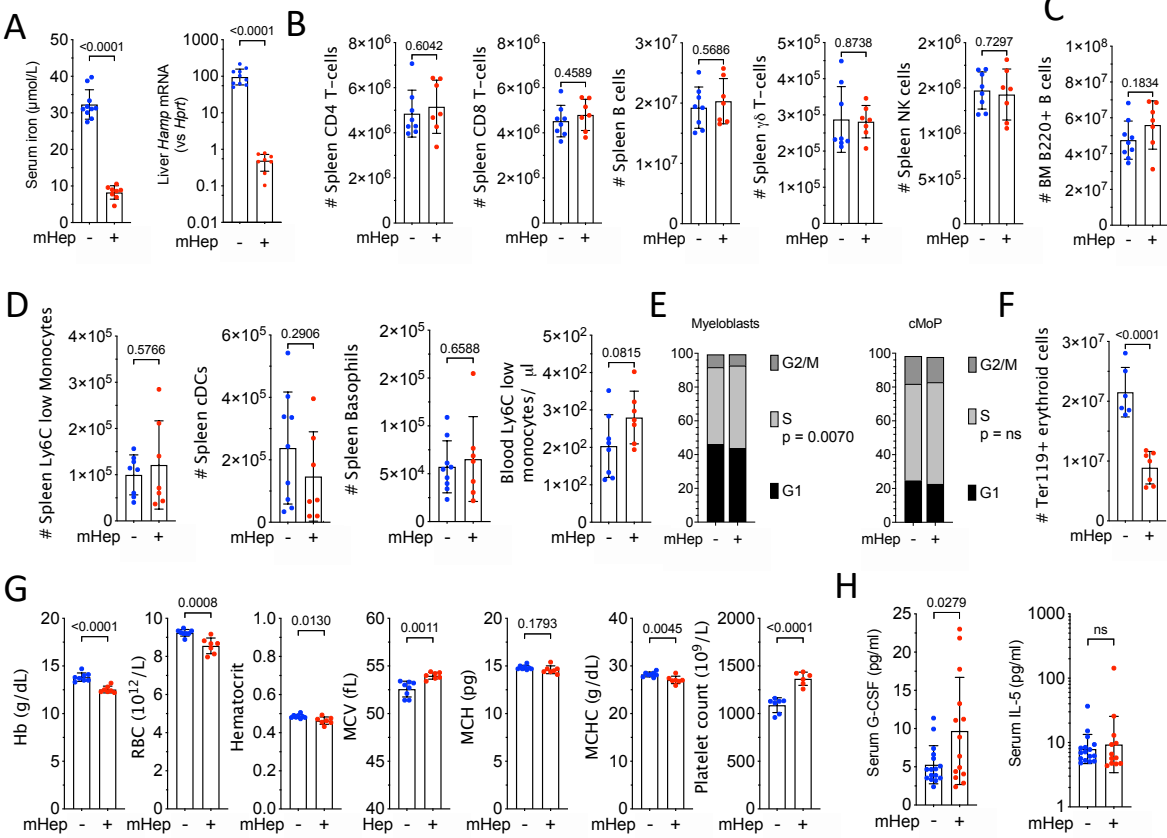**This PDF file includes:**

Figs. S1 to S4
Table S1

Figure S1

Figure S1

A) Serum iron and liver hepcidin mRNA in experiment described in Fig 1C, Representative of two independent experiments. Unpaired T-test. Serum iron, mean ± SD. Hepcidin mRNA, geometric mean ± geometric SD.

B) Number of splenic lymphocyte in experiment described in Fig 1C. Representative of two independent experiments. Unpaired T-test. Mean ± SD.

C) Number of B220+ B cells in the bone marrow in experiment described in Fig 1C. Unpaired T-test. Mean ± SD.

D) Number of specified splenic and blood myeloid cells in experiment described in Fig 1C. Representative of two independent experiments. Unpaired T-test. Mean ± SD.

E) Cell cycle distribution of myeloblast and cMoP in experiment described in Fig 1C. Representative of two independent experiments. Two way ANOVA, mean proportions of the whole.

F) Number of Ter119+ erythroid cells in the bone marrow in experiment described in Fig 1C. Unpaired T-test. Mean ± SD.

G) Red cell indices and platelet count were measured by sysmex in experiment described in Fig 1C. Unpaired T-test. Mean ± SD.

H) Serum G-CSF and IL-5 were measured by Luminex in experiment described in Fig 1C. 2-way ANOVA blocking on experimental repeat and reporting p-value treatment effect, combination of two independent experiments. G-CSF, mean ± SD. IL-5, geometric mean ± geometric SD.

Figure S2

A

α β T cells (% of live)

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

B cells (% of live)

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

NK cells (% of live)

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

Eosinophils (% of live)

0.0113
0.4822

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

CD115+ Monocytes (% of live)

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

Ly6C high monocytes (% of live)

0.0488
0.3302

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

Neutrophils (% of live)

<0.0001
<0.0001

| aGr1 | - | - | + | + |
| mHep | - | + | - | + |

B

C-kit BUV395 (y-axis)
Ly6G AF700 (x-axis)

I, II, III, IV, V

C

Granulocyte developmental stages (% of singlets)

0.0109   0.0055   <0.0001

% of singlets

Developmental stage: I, II, III, IV, V

□ Isotype SL220
□ Isotype mHep
□ aGr1 SL220
□ aGr1 mHep

D

Neutrophils / µL of blood

0.0188

| aGr1 | + | + |
| mHep | - | + |

E

Spleen eosinophils

0.0054
0.0099

aGr1	+	+	+
mHep	-	+	+
FAC	-	-	+

F

Serum iron (µmol/L)

<0.0001
<0.0001

| GCSF | - | + | + |
| mHep | - | - | + |

G

BM erythroblasts

<0.0001
0.0009

| GCSF | - | + | + |
| mHep | - | - | + |

H

Spleen Ly6G+ Neutrophils

<0.0001
0.0093

| GCSF | - | + | + |
| mHep | - | - | + |

Spleen CD101+ Neutrophils

<0.0001
0.0023

| GCSF | - | + | + |
| mHep | - | - | + |

I

Granulocytes / µL of blood

0.0134
0.0435

| GCSF | - | + | + |
| mHep | - | - | + |

J

CXCR4+ Granulocytes (% of blood granulocytes)

0.2301
0.0105

| GCSF | - | + | + |
| mHep | - | - | + |

K

CXCR4+ C-kit+ BM Granulocytes

0.2213
0.0046

| GCSF | - | + | + |
| mHep | - | - | + |

L

p = 0.0115
p = 0.0030

■ G2/M
□ S
■ G1

| GCSF | + | + |
| mHep | - | + |

M

Ly6C- Monocytes / µL of blood

0.0023
0.4562

| GCSF | - | + | + |
| mHep | - | - | + |

Spleen Ly6C+ Monocytes

0.0009
0.5388

| GCSF | - | + | + |
| mHep | - | - | + |

BM Ly6C+ Monocytes
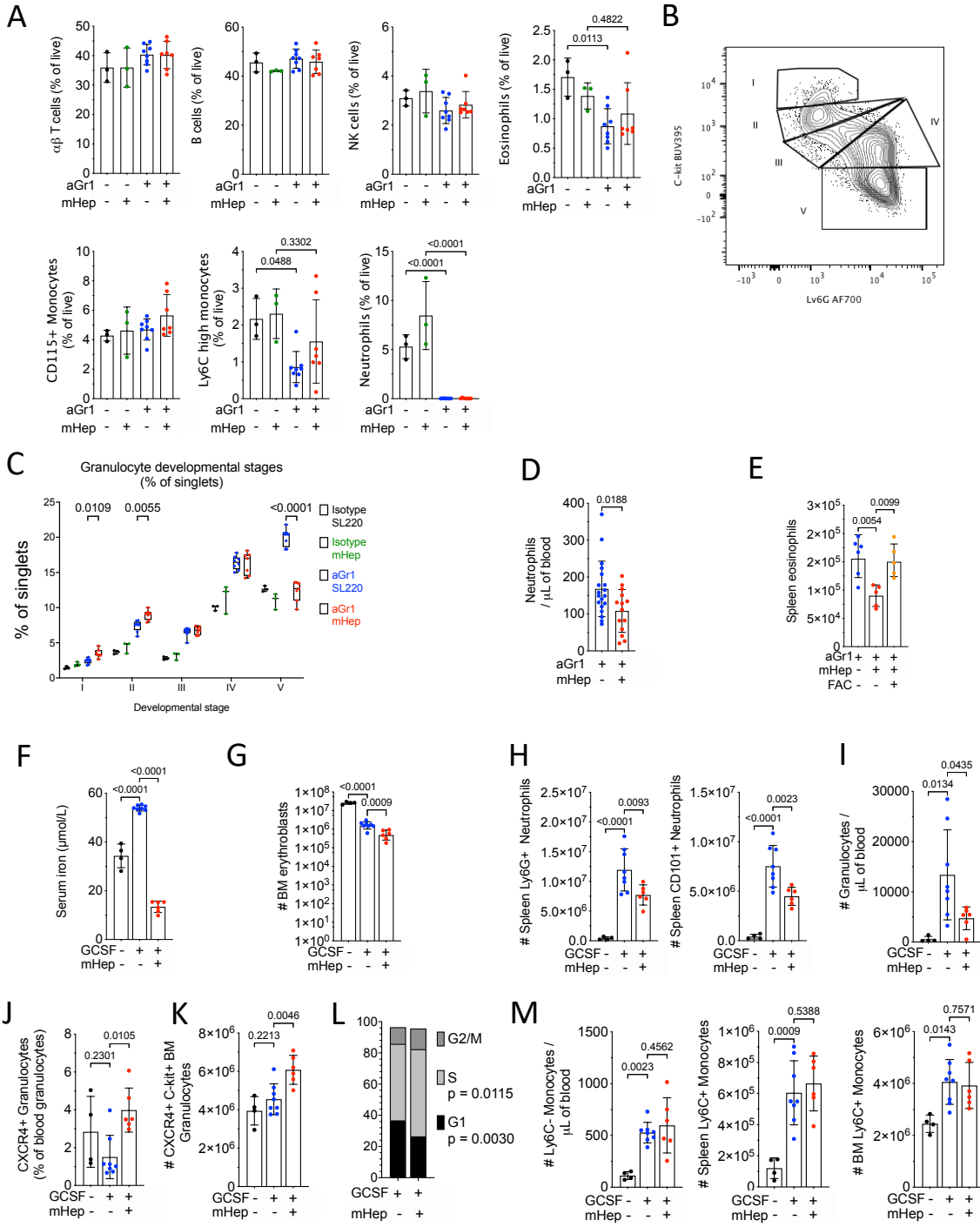
0.0143
0.7571

| GCSF | - | + | + |
| mHep | - | - | + |

Figure S2

A) Frequency of blood leukocyte subsets as a % of live events 24hrs after 10µg of aGr1 or isotype control as part of experiment outlined in Fig 2A. Two way ANOVA. Mean ± SD

B) Gating scheme to delineate stages of granulocyte differentiation (gated on Ly6C+, CD11b+, CD115- cells) in Fig 2B. From stage I (myeloblasts) to stage IV (mature neutrophils).

C) Bone marrow granulocyte differentiation trajectory in mice treated as in Fig S2A, representative to two independent experiments. Two way ANOVA. Mean, quartiles and range.

D) Blood neutrophil numbers in mice treated as in scheme 2A (combined from three independent experiments). Unpaired T-test. Mean ± SD

E) Spleen Eosinophil numbers in mice treated as in scheme 2C. One way ANOVA. Mean ± SD

F) Serum iron concentrations in experiment scheme 2E. One way ANOVA. Mean ± SD

G) Bone marrow CD71+ TER119+ erythroblast numbers in scheme 2E. One way ANOVA. Geo Mean ± GeoSD

H) Spleen Neutrophil numbers in experiment scheme 2E. One way ANOVA. Mean ± SD

I) Blood Ly6C int CD11b+ granulocyte numbers in experiment scheme 2E. One way ANOVA. Mean ± SD

J) % of granulocytes showing an immature CXCR4+ phenotype in experiment scheme 2E. One way ANOVA. Mean ± SD

K) Number of Bone marrow CXCR4+ c-kit+ neutrophil progenitors in experiment scheme 2E. One way ANOVA. Mean ± SD

L) Cell cycle distribution of CXCR4+ c-kit+ neutrophil progenitors in experiment scheme 2E. Two way ANOVA. Mean proportion of whole.

M) Monocyte numbers in blood, spleen and bone marrow in experiment scheme 2E. One way ANOVA. Mean ± SD

Figure S3 Functionality of myeloid cells in serum iron deficiency
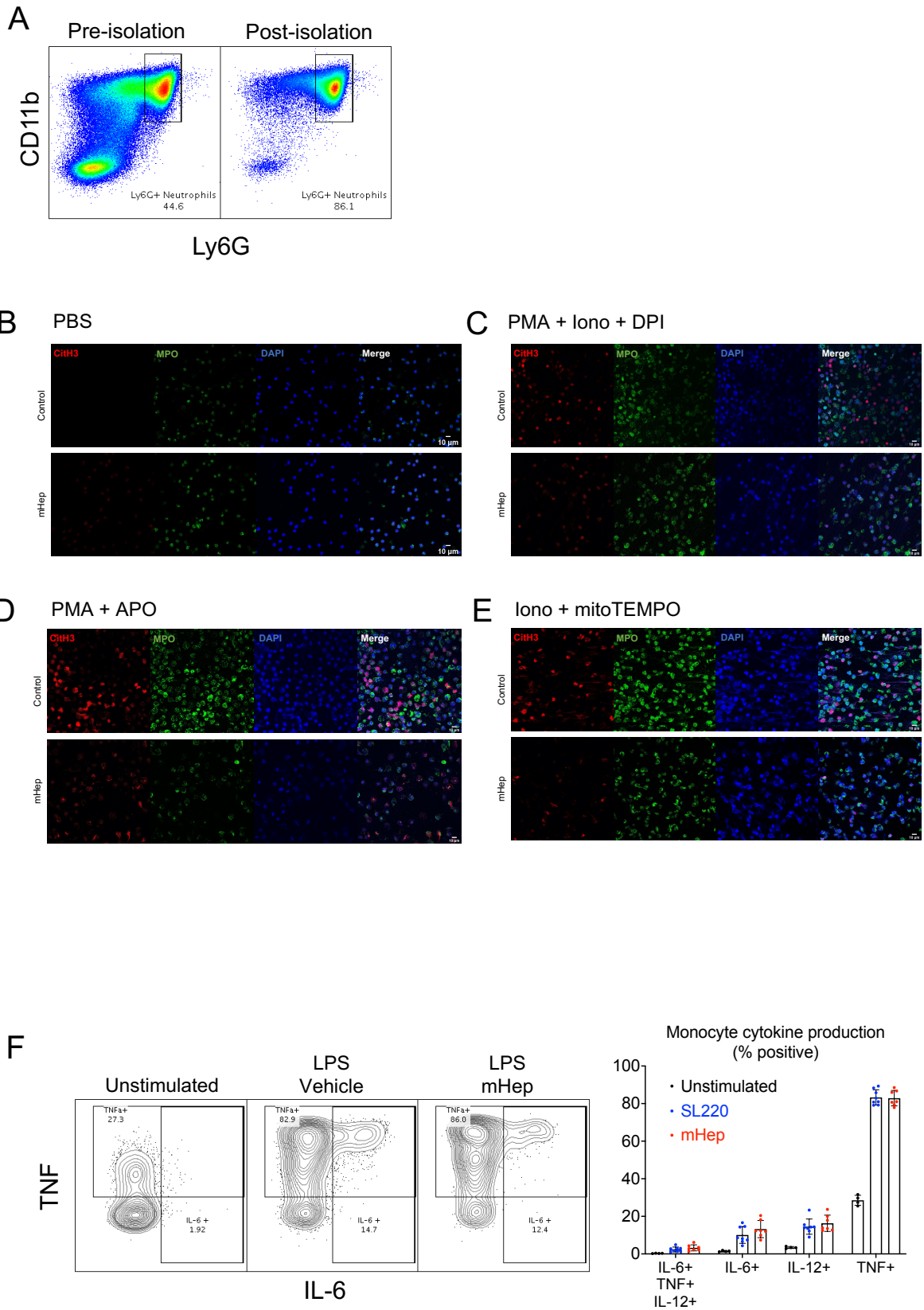
A

Pre-isolation

Post-isolation

CD11b

Ly6G+ Neutrophils
44.6

Ly6G+ Neutrophils
86.1

Ly6G

B   PBS

C   PMA + Iono + DPI

D   PMA + APO

E   Iono + mitoTEMPO

F

Unstimulated

TNFa+
27.3

IL-6 +
1.92

LPS
Vehicle

TNFa+
82.9

IL-6 +
14.7

LPS
mHep

TNFa+
86.0

IL-6 +
12.4

TNF

IL-6

Monocyte cytokine production
(% positive)

- Unstimulated
- SL220
- mHep

IL-6+
TNF+
IL-12+

IL-6+

IL-12+

TNF+

Figure S3
A) Enrichment purity of bone marrow Ly6G+ neutrophils (obtained from mice treated as in experiment scheme Fig 2A)

(B-E) Representative microscopy images of NETosis taken at 20X for the following conditions:
B) PBS treatment
C) PMA, Ionomycin and DPI
D) PMA and apocyanin (APO)
E) Ionomycin and mitoTEMPO

F) Intracellular cytokine staining within Ly6C+ CD11b+ monocytes after *ex vivo* LPS stimulation of whole splenocytes from mice treated as in experiment scheme 1C. One way ANOVA. Mean ± SD

Figure S4   Counteracting inflammatory hepcidin induction improves neutrophil production

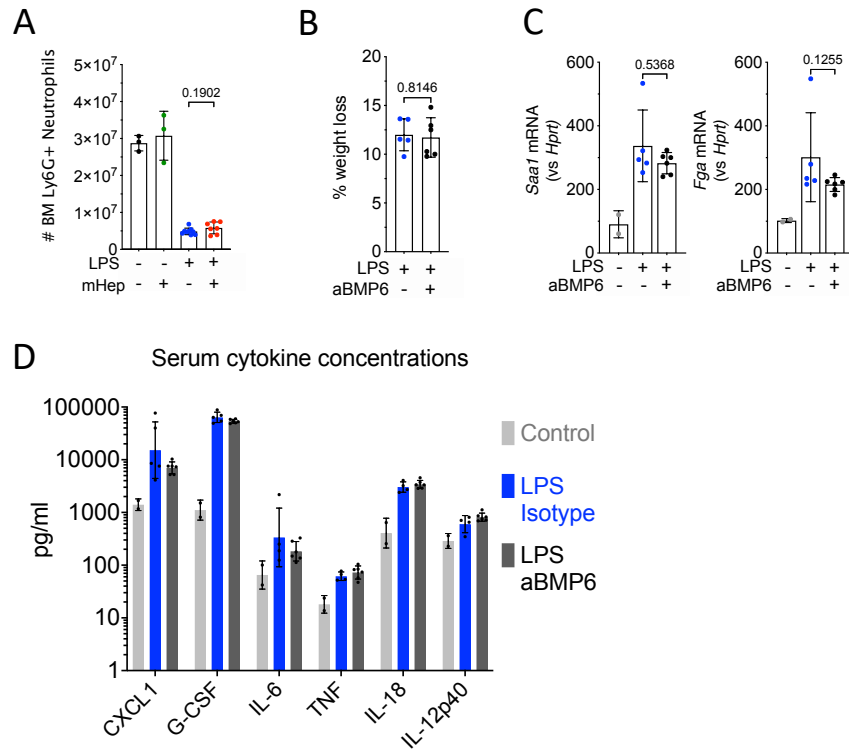## A

B

C

D

Serum cytokine concentrations

Figure S4
A) BM neutrophil numbers from mice treated as in 4A. t-test. Mean ± SD.
B) LPS induced weight loss from mice treated as in 4C. t-test. Mean ± SD. Representative of three independent experiments
C) Liver *Saa1* and *Fga* expression (Mann-Whitney test) from mice treated as in 4C. Mean ± SD. Non LPS treated control group was treated with aGr1 24hrs prior to culling to reflect the systemic iron environment prior to LPS injection. Representative of two independent experiments.
D) Serum cytokines measured by Legendplex from mice treated as in 4C. Mixed-effects model. Geometric Mean ± Geo SD.

Table S1. Flow cytometry antibodies used in the publication					
Conjugation	Marker	Clone	Supplier	Catalogue number	RRID
AF700	IgD	11-26c.2a	BioLegend	405729	AB_2563340
AF700	Ly6G	1A8	BioLegend	127621	AB_10640452
AF700	CD4	GK1.5	BioLegend	100429	AB_493698
APC	IL-12/IL-23 p40	C15.6	BioLegend	505205	AB_315369
APC	CD115	AFS98	BioLegend	135509	AB_2085222
APC	CX3CR1	SA011F11	BioLegend	149007	AB_2564491
APC	Ly6G	1A8	BioLegend	127613	AB_1877163
APC	Nk1.1	PK136	BioLegend	108709	AB_313396
APC	I-A/I-E (MHC class II)	M5/114.15.2	BioLegend	107613	AB_313328
APCcy7	CD90.2	30-H12	BioLegend	105327	AB_10613280
APCcy7	B220	RA3-6B2	BioLegend	103224	AB_313007
APCcy7	CD11b	M1/70	BioLegend	101226	AB_830642
APCcy7	CD19	6D5	BioLegend	115529	AB_830706
APCcy7	Ly6C	HK1.1	Biolegend	128025	AB_10643867
APCcy7	Ly6G	1A8	BioLegend	127623	AB_10645331
APCcy7	NK1.1	PK136	BioLegend	108723	AB_830870
APCcy7	Ter119	TER-119	BioLegend	116223	AB_2137788
Biotin	CD115	AFS98	BioLegend	135507	AB_2028401
Biotin	CD123	5B11	BioLegend	106003	AB_2124404
Biotin	CD71	RI7217	BioLegend	113803	AB_313564
Biotin	CXCR4	2B11	Invitrogen eBioscience	13-9991-80	AB_10609202
Biotin	CD115	AFS98	BioLegend	135507	AB_2028401
BUV395 Streptavidin			*BD Biosciences*	*564176*	AB_2869553
BUV496	B220	RA3-6B2	BD Biosciences	612950	AB_2870227
BUV805	CD8a	53-6.7	BD Biosciences	612898	AB_2870186
BV421	c-kit	2B8	BioLegend	105827	AB_10898120
BV421	CD11b	M1/70	BioLegend	101235	AB_10897942
BV510	CD11b	M1/70	BioLegend	101245	AB_2561390
BV605	B220	RA3-6B2	BioLegend	103243	AB_11203907
BV605	Ly6C	HK1.4	BioLegend	128035	AB_2562352
BV605	CD8a	53-6.7	BioLegend	100743	AB_2561352
BV785	CD11b	M1/70	BioLegend	101243	AB_2561373
FITC	CD11b	M1/70	BioLegend	101205	AB_312788
FITC	CD45	30-F11	BioLegend	103107	AB_312972
FITC	Ly6C	HK1.4	BioLegend	128005	AB_1186134
FITC	TNFa	MP6-XT22	BioLegend	506303	AB_315424

Conjugation	Marker	Clone	Supplier	Catalogue number	RRID
FITC	TCR β chain	H57-597	BioLegend	109205	AB_313428
PE	CD16/32	93	BioLegend	101307	AB_312806
PE	CXCR4	2B11	Invitrogen	12-9991-82	AB_891391
PE	FLT-3	A2F10	BioLegend	135305	AB_1877218
PE	IL-6	MP5-20F3	BioLegend	504503	AB_315337
PE	Ly6G	1A8	BioLegend	127607	AB_1186104
PE	Siglec F	S17007L	BioLegend	155505	AB_2750234
PE	Siglec H	551	BioLegend	129605	AB_1227763
PECy7	C-kit	2B8	BioLegend	105813	AB_313222
PECy7	CD101	Moushi101	invitrogen	25-1011-80	AB_2573378
PECy7	CD11c	N418	BioLegend	117317	AB_493569
PECy7	CD49b	HMa2	BioLegend	103517	AB_2566102
PECy7	Ly6C	HK1.4	BioLegend	128017	AB_1732093
PECy7	Sca-1	D7	Biolegend	108113	AB_493597
PECy7	TCR γ/δ	GL3	BioLegend	118123	AB_11203530
PE/dazzle	CXCR2	SA044G4	BioLegend	149317	AB_2750072
PE/dazzle	FcER1a	Mar-01	BioLegend	134331	AB_2687240
PE/dazzle	Ly6G	1A8	BioLegend	127647	AB_2566318
PE/dazzle	Siglec F	S17007L	BioLegend	155529	AB_2890716
PerCPCy5.5	Ly6G	1A8	BioLegend	127615	AB_1877272
PerCPCy5.5	B220	RA3-6B2	BioLegend	103235	AB_893356
PerCPCy5.5	CD16/32	93	BioLegend	101323	AB_1877268
PerCPCy5.5	MHC-II	M5/114.15.2	BioLegend	107625	AB_2191072
PerCPCy5.5	Ter119	TER-119	BioLegend	116227	AB_893638
PB	CD44	IM7	BioLegend	103019	AB_493682