The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development

Haiyan S. Li, Cliff Y. Yang, Kalyan Nallaparaju, Huiyuan Zhang, Yong-Jun Liu, Ananda W. Goldrath and Stephanie S. Watowich

Supplementary information

Figure S1. Sorting strategy for CDPs, pDCs, spleen and liver DC subsets.

CDPs (lin^- $CD117^{lo}$ $CD115^+$ $Flt3^+$) and pDCs ($CD11c^+$ $CD11b^-$ $B220^+$ PDCA-1^+) were sorted from BM cells following MACS-mediated depletion of lineage marker- (Gr1, CD11b, CD3, CD19, F4/80 and Ter-119) positive cells, using the indicated strategy. $CD8\alpha^+$ ($CD11c^+$ $B220^-$ $CD4^-$ $CD8\alpha^+$), $CD4^+$ ($CD11c^+$ $B220^-$ $CD4^+$ $CD8\alpha^-$), and $CD4^-$ $CD8\alpha^-$ ($CD11c^+$ $B220^-$ $CD4^-$ $CD8\alpha^-$) DCs were isolated from splenocytes following MACS-mediated depletion of lineage marker- (Gr1, CD3, CD19, F4/80 and Ter-119) positive cells. $CD103^+$ ($CD11c^+$ $B220^-$ $CD103^+$ $MHCII^+$) DCs were isolated from 37%/70% Percoll-enriched liver mononuclear cells following collagenase digestion.

Figure S2. Characterization of hematopoietic-*Stat5*$^{\Delta/\Delta}$ mice.

(A) Bone marrow cells were isolated from hematopoietic-*Stat5*$^{\Delta/\Delta}$ and *Stat5*$^{+/+}$ mice. Whole cell lysates were analyzed by immunoblotting, using antibodies specific for total STAT5 or tubulin. Results from 2 independent animals of each genotype are shown. (B) Hematologic parameters and WBC differential counts of hematopoietic-*Stat5*$^{\Delta/\Delta}$ and *Stat5*$^{+/+}$ mice, determined by automated counting.

Figure S3. Analysis of LN $CD103^+$ DCs.

Peripheral (LN) and mesenteric (mLN) lymph nodes were isolated from hematopoietic-*Stat5*$^{\Delta/\Delta}$ and *Stat5*$^{+/+}$ mice. Single cell suspensions were stained with antibodies for DC surface markers and analyzed by flow cytometry. MHC II and CD103 expression was

analyzed within the $CD11c^+$ $B220^-$ population, and is shown as a percentage of total LN cells. Data are representative of 2 mice per group.

Figure S4. Characterization of DC marker expression.

(A) Hematopoietic-*Stat5*$^{\Delta/\Delta}$ and *Stat5*$^{+/+}$ mice were treated by HGT with GM-CSF plasmid or empty pORF vector, as indicated. At d 7, liver mononuclear cells were enriched as described in Figure S1, and CD103 expression was analyzed within the $CD11c^-$ or $CD11c^+$ $B220^-$ populations, by flow cytometry, as shown. Data represent 2-6 mice/group. (B) Surface expression of CD24 and CD11b on $CD103^+$ DCs isolated from mice treated by HGT with GM-CSF plasmid or empty pORF vector, as indicated. $CD103^+$ DCs were defined as shown in Figure S1. (C) Liver $CD103^+$ DCs were purified by FACS as described in Figure S1 and treated with GM-CSF ex vivo. Cells were collected at d 0, d 1 and d 2, as indicated, and analyzed for CD11b expression. Data are representative of 3 independent experiments.

Figure S5. Analysis of the murine *Id2* and *Tcf4* promoter regions. The sequences encoding 2kb immediately 5' of the transcription start sites (arrow) of the murine *Id2* and *Tcf4* genes were identified from the Ensemble database. Putative transcription factor binding sites (underlined) were predicted by analysis with AliBaba and TF search software programs. The location of primers used for H3 ChIPs (Figures 3C, 3D) are shown in orange (sense) and blue (antisense).

Figure S6. Regulation of histone H3 modifications at *Tcf4* and *Id2* promoters.

(A) pDCs and $CD11c^+$ $CD11b^+$ DCs were isolated from mice that were unmanipulated (no HGT) or from animals following Flt3L or GM-CSF HGT, as indicated. Histone H3

modifications at the *Id2* and *Tcf4* proximal promoters in the vicinity of the STAT-consensus sites were determined by ChIPs using antibodies against H3K9ac, H3K4me3 and H3K27me3. Results were normalized to ChIPs for total H3 and presented as mean ± SEM. n=10-15. (B) pDCs were purified by FACS from BM cells cultured with Flt3L for 7 d, then stimulated with 5 μM CpGA for 0, 4, 8 and 16 h. *Id2* mRNA was analyzed by qPCR. (C, D) pDCs and $CD11c^+$ $CD11b^+$ DCs were isolated by FACS from HGT-treated hematopoietic-$Stat5^{\Delta/\Delta}$ mice, hematopoietic-$Stat3^{\Delta/\Delta}$ animals or appropriate controls, as indicated. Histone H3 modifications at the *Tcf4* and *Id2* proximal promoters in the vicinity of the STAT-consensus sites were determined by ChIPs using antibodies against H3K9ac, H3K4me3 and H3K27me3. Results were normalized to ChIPs for total H3 and presented as mean ± SEM. n=10-15. (E) Whole cell lysates were generated from total BM cells isolated from hematopoietic-$Stat5^{\Delta/\Delta}$ mice, hematopoietic-$Stat3^{\Delta/\Delta}$ animals or appropriate controls. Expression of H3K4me3 and total H3 was determined by immunoblotting. (F) Hematopoietic-$Stat5^{\Delta/\Delta}$ mice, hematopoietic-$Stat3^{\Delta/\Delta}$ animals or appropriate controls were treated with GM-CSF or Flt3L HGT for 7 d, as indicated. The abundance of H3K4me3 at the *Flt3*, *Gata-1*, *Irf8* and *Gapdh* promoters was analyzed in pDCs and cDCs as described in (C and D).

Figure S7. GM-CSF- and Flt3L-responsive expression of *Id2* and *Tcf4*.

(A, B) CDPs from hematopoietic-$Stat3^{\Delta/\Delta}$, hematopoietic-$Stat5^{\Delta/\Delta}$ mice and respective $Stat^{+/+}$ controls were purified and cultured in vitro in the presence of Flt3L or GM-CSF, as indicated. *Id2* and *Tcf4* mRNA was determined by qPCR. (C) Hematopoietic-$Stat5^{\Delta/\Delta}$ and $Stat5^{+/+}$ mice were treated by HGT with GM-CSF or pORF vector, as indicated. After 2 d, *Tcf4* mRNA was measured in CDPs by qPCR.

Figure S8. Characterization of D2SC/1 and D2SC/mFlt3 signaling responses to GM-CSF or Flt3L. (A) Expression of RFP and Flt3 in D2SC/1 or D2SC/mFlt3 cells was analyzed by flow cytometry. (B) *Id2* mRNA abundance was determined in D2SC/1 cells cultured with GM-CSF for 0, 2, 4 and 8 h; *Tcf4* expression was analyzed in D2SC/mFlt3 cells treated with Flt3L for 0, 2, 4 and 8 h. (C, D) STAT1, STAT3 and STAT5 activation was analyzed by immunoblotting to detect tyrosine-phosphorylated isoforms, using whole cell extracts from D2SC/1 and D2SC/mFlt3 cells stimulated with GM-CSF or Flt3L for 0.5, 2, 4 or 8 h, or left untreated, as indicated. IFN-γ, IL-6 or GM-CSF-stimulated whole cell extracts were used as positive controls for phosphorylated STAT1, STAT3 and STAT5, respectively. (E) ChIP assays were performed in D2SC/1 cells treated ± Flt3L for 1 h, using anti-STAT3 or IgG control antibody. Results are representative of 3 independent experiments.

Figure S9. Role for *Tcf4* in cytokine-driven DC development.

BM cells from *Tcf4*$^{+/-}$ or *Tcf4*$^{+/+}$ mice were cultured with Flt3L for 6 d. pDC and CD11c^{+} CD11b^{+} cDC generation was assessed within the CD11c^{+} population. n=8.

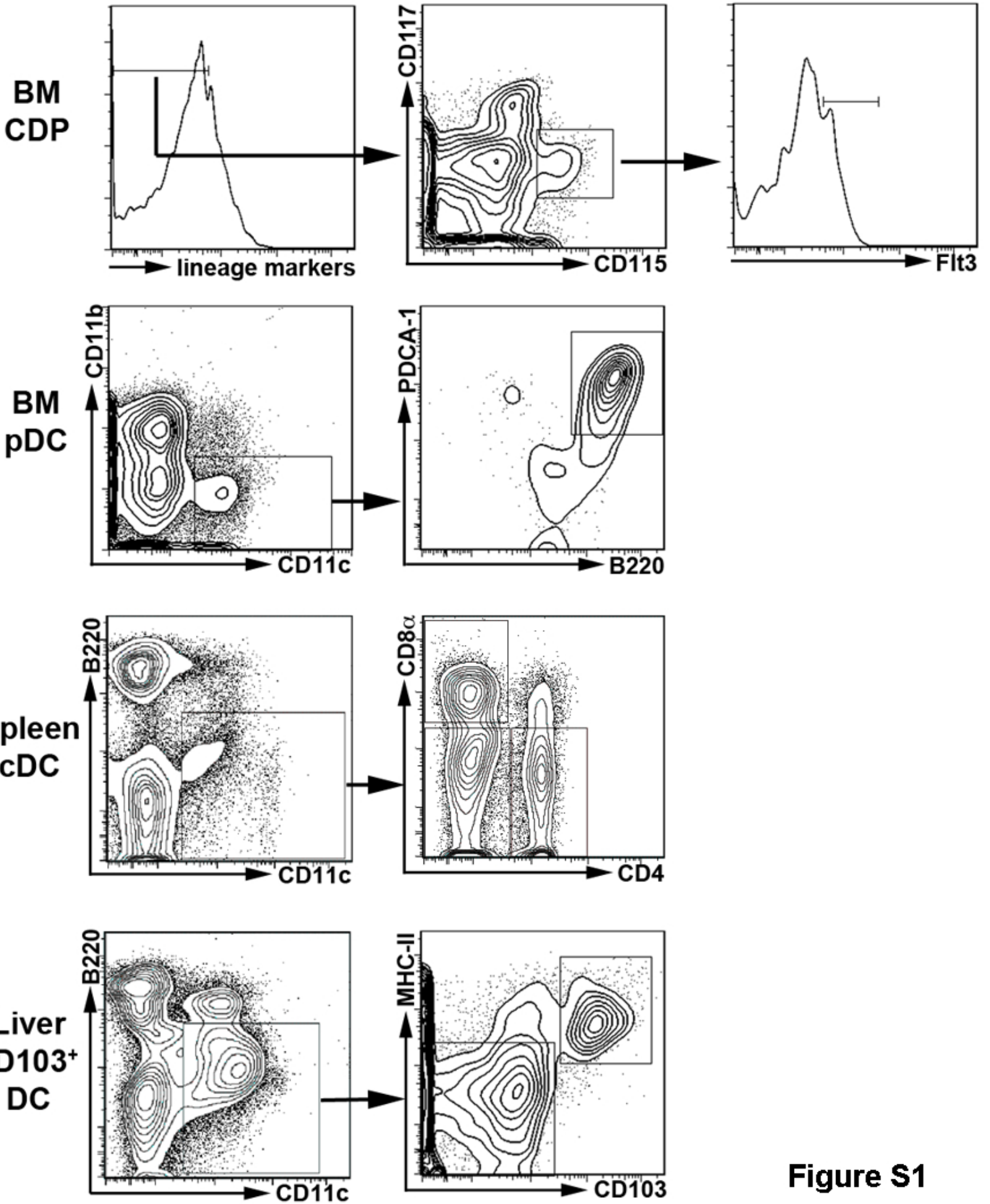
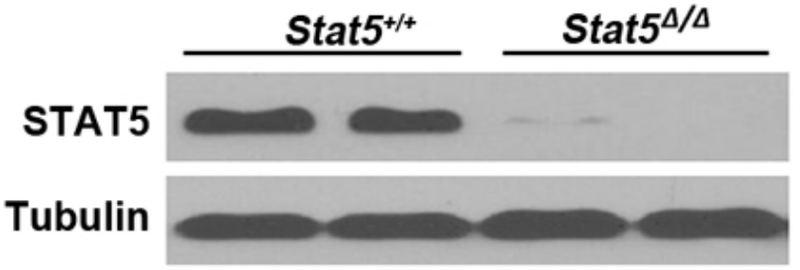

BM
CDP
lineage markers
CD117
CD115
Flt3
BM
pDC
CD11b
CD11c
PDCA-1
B220
Spleen
cDC
B220
CD11c
CD8α
CD4
Liver
CD103+
DC
B220
CD11c
MHC-II
CD103

Figure S1

A



B

Hematologic parameters and WBC differential counts of hematopoietic *Stat5*$^{\Delta/\Delta}$ mice

	Stat5$^{+/+}$	*Stat5*$^{\Delta/\Delta}$
Hematologic Parameters		
Hemoglobin (g/L)	152.7 ± 6.5	147.3 ± 1.7
Hematocrit (%)	53.8 ± 2.4	50.0 ± 2.4
WBC count (10^9/L)	3.91 ± 0.73	4.66 ± 0.69
RBC count (10^{12}/L)	10.9 ± 1.7	9.74 ± 0.94
Platelet count (10^9/L)	1413 ± 202.5	1233 ± 64.3
WBC Differential Count		
Neutrophil (10^9/L)	0.92 ± 0.15	1.19 ± 0.20
Monocyte (10^9/L)	0.07 ± 0.005	0.08 ± 0.03
Basophil (10^9/L)	0.02 ± 0.004	0.04 ± 0.02
Lymphocyte (10^9/L)	3.14 ± 0.97	3.32 ± 0.61
Eosinophil (10^9/L)	0.07 ± 0.02	0.09 ± 0.04

Results represent Mean ± SD of peripheral blood analysis of 4 hematopoietic Stat5$^{\Delta/\Delta}$ mice and their littermate control (*Stat5*$^{+/+}$).
WBC = white blood cell, RBC = red blood cell.

Figure S2

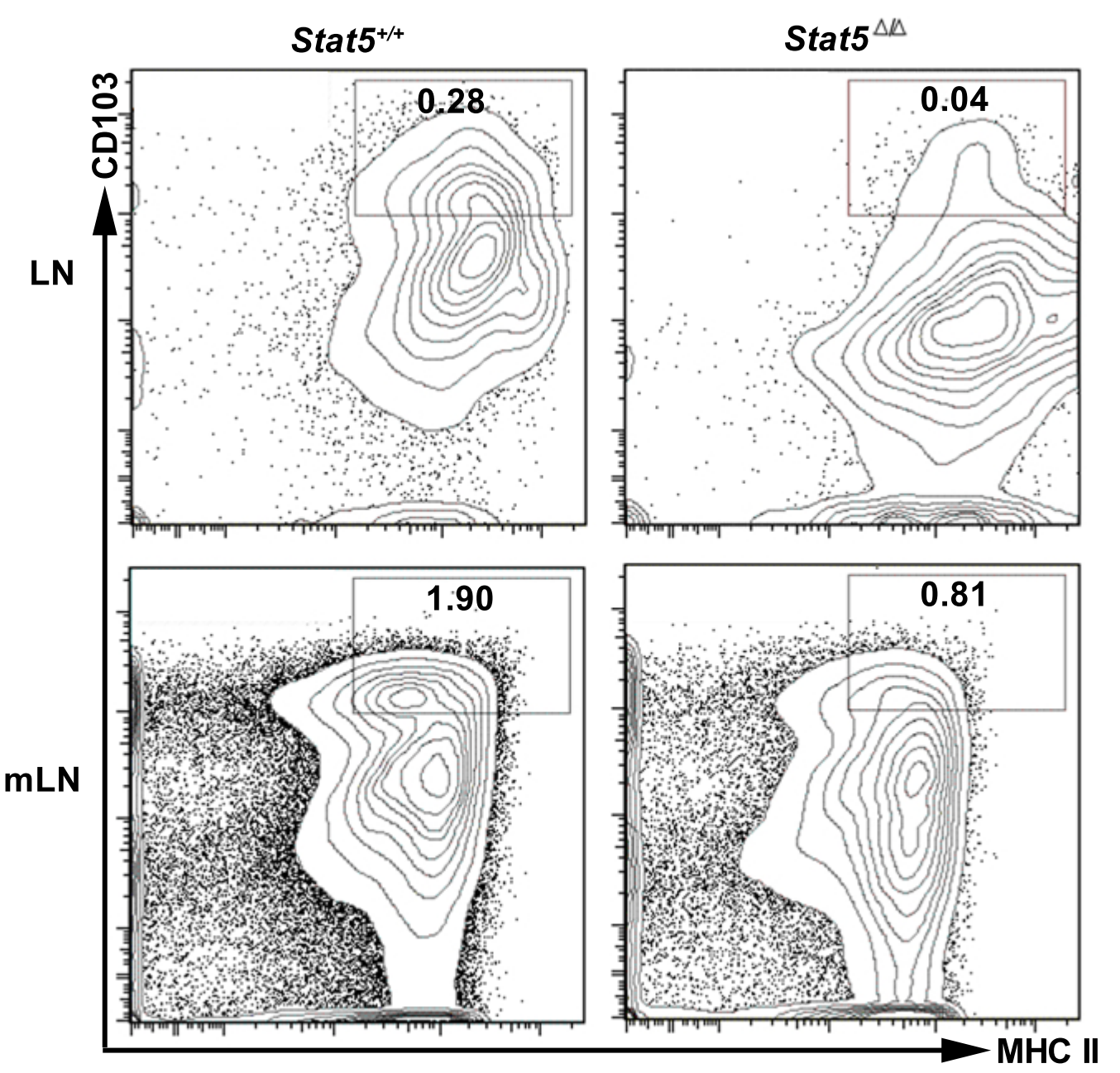


Figure S3

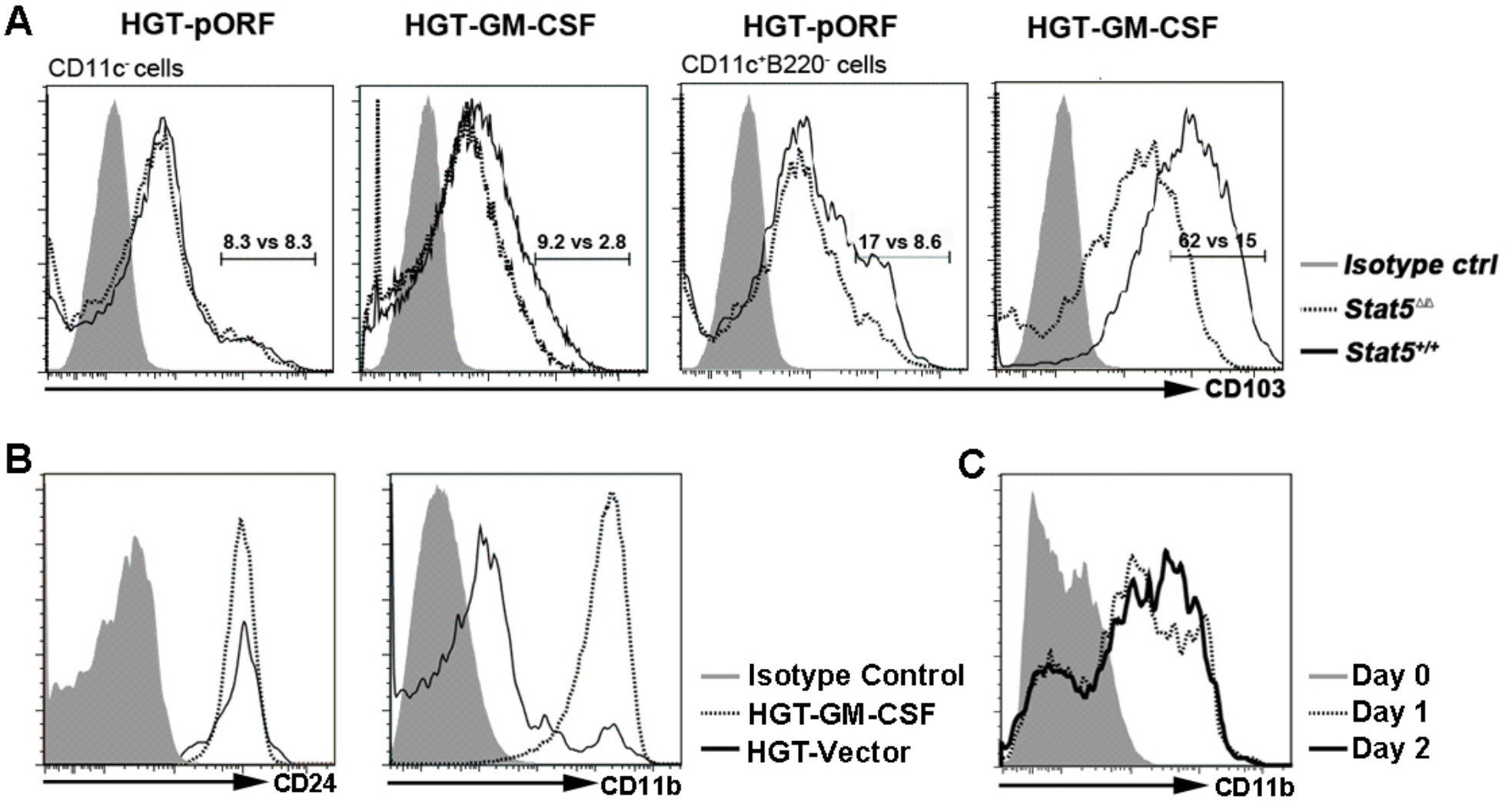

A
HGT-pORF
HGT-GM-CSF
HGT-pORF
HGT-GM-CSF
CD11c- cells
CD11c+B220- cells
8.3 vs 8.3
9.2 vs 2.8
17 vs 8.6
62 vs 15
Isotype ctrl
Stat5ΔΔ
Stat5+/+
CD103
B
CD24
CD11b
Isotype Control
HGT-GM-CSF
HGT-Vector
C
CD11b
Day 0
Day 1
Day 2

Figure S4

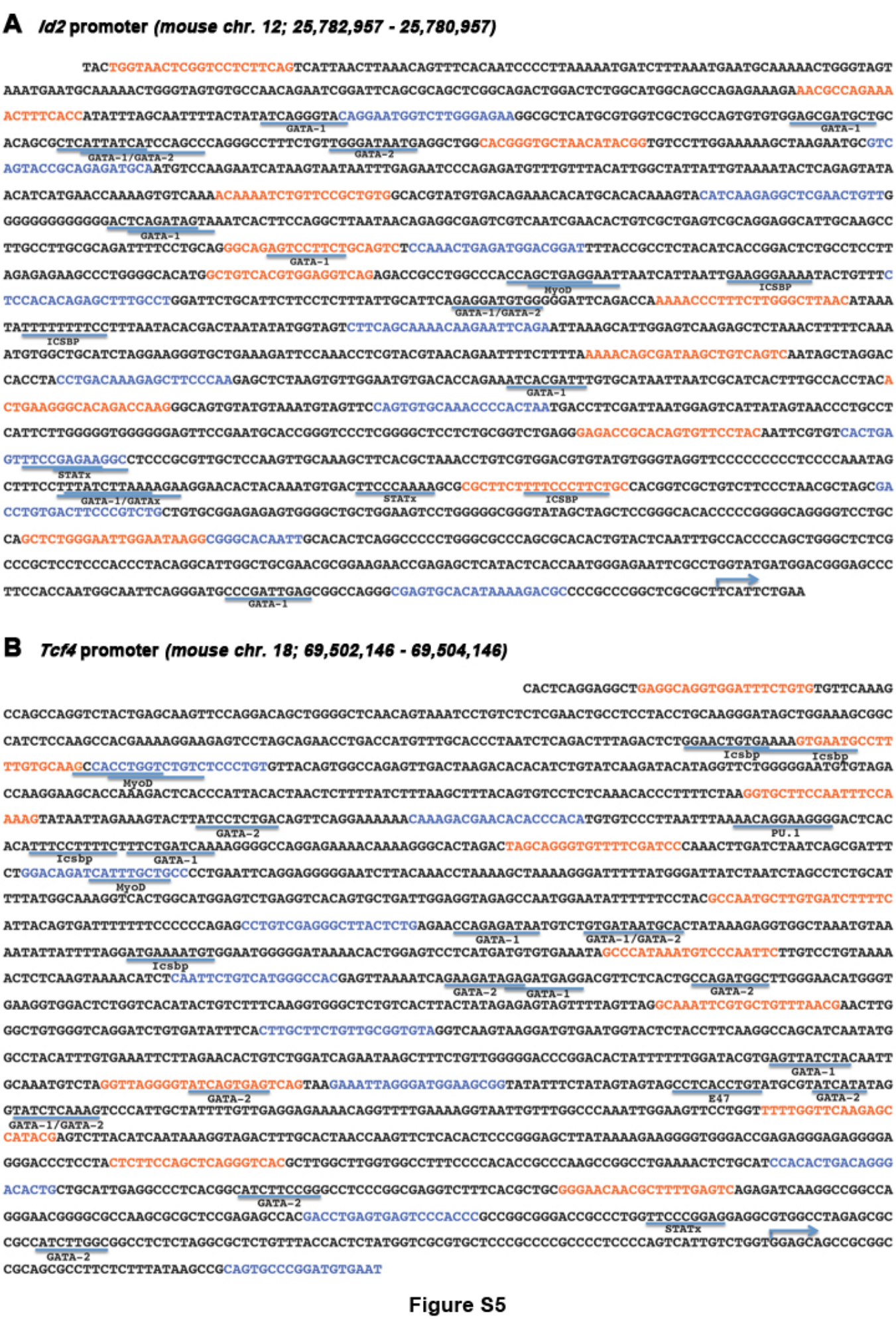

A Id2 promoter (mouse chr. 12; 25,782,957 - 25,780,957)
GATA-1
GATA-1
GATA-1/GATA-2
GATA-2
GATA-1
GATA-1
MyoD
ICSBP
GATA-1/GATA-2
ICSBP
GATA-1
STATx
GATA-1/GATAx
STATx
ICSBP
GATA-1
B Tcf4 promoter (mouse chr. 18; 69,502,146 - 69,504,146)
Icsbp
Icsbp
MyoD
GATA-2
PU.1
Icsbp
GATA-1
MyoD
GATA-1
GATA-1/GATA-2
Icsbp
GATA-2
GATA-1
GATA-2
GATA-1
GATA-2
E47
GATA-2
GATA-1/GATA-2
GATA-2
STATx
GATA-2

Figure S5

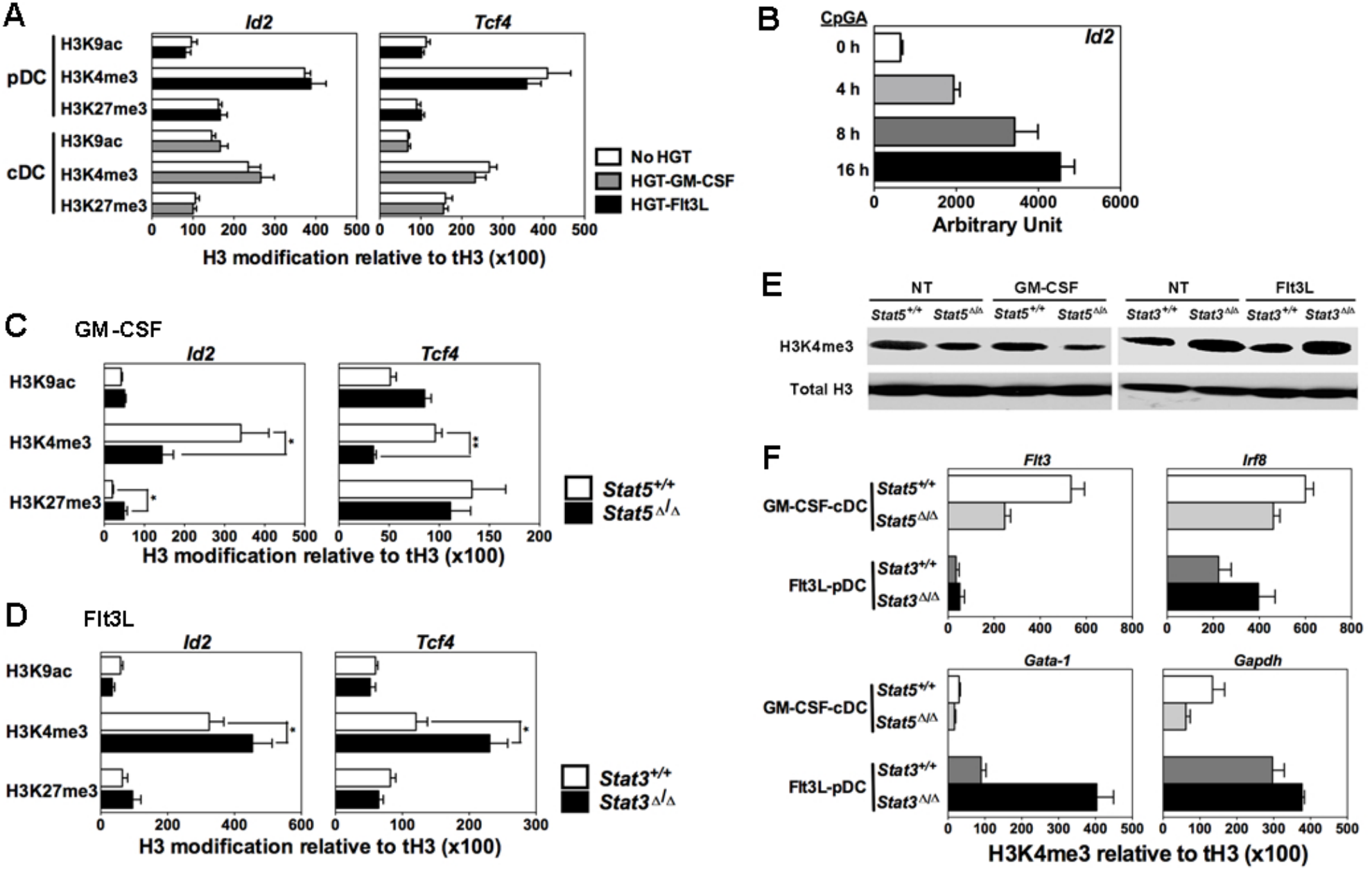

A
Id2
Tcf4
pDC
cDC
H3K9ac
H3K4me3
H3K27me3
No HGT
HGT-GM-CSF
HGT-Flt3L
H3 modification relative to tH3 (x100)
B
CpGA
Id2
0 h
4 h
8 h
16 h
Arbitrary Unit
C
GM-CSF
Stat5+/+
Stat5Δ/Δ
D
Flt3L
Stat3+/+
Stat3Δ/Δ
E
NT
GM-CSF
Flt3L
Stat5+/+
Stat5Δ/Δ
Stat3+/+
Stat3Δ/Δ
H3K4me3
Total H3
F
Flt3
Irf8
Gata-1
Gapdh
GM-CSF-cDC
Flt3L-pDC
H3K4me3 relative to tH3 (x100)

Figure S6

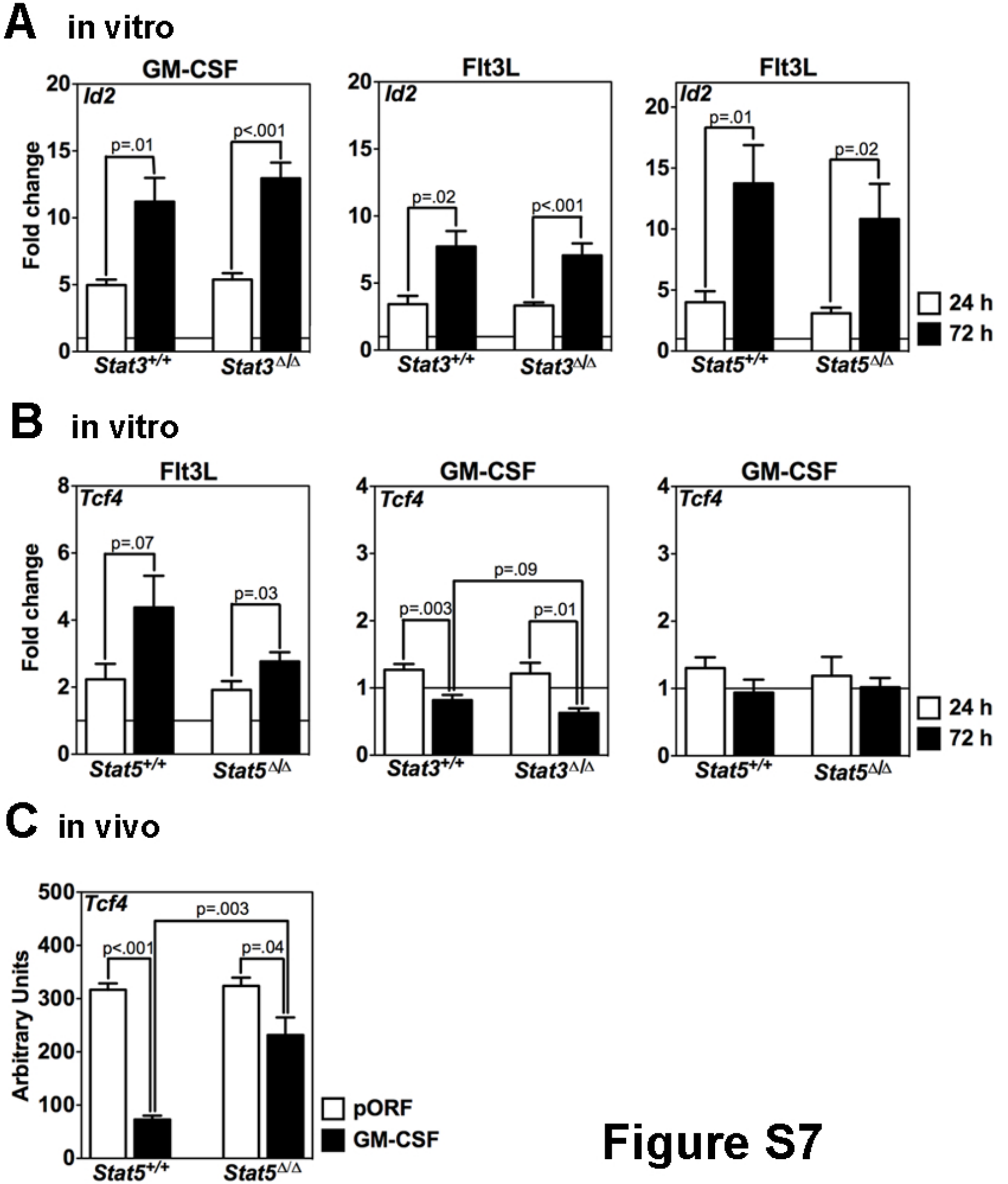

A in vitro
GM-CSF
Id2
Fold change
p=.01
p<.001
Stat3+/+
Stat3Δ/Δ
Flt3L
Id2
p=.02
p<.001
Stat3+/+
Stat3Δ/Δ
Flt3L
Id2
p=.01
p=.02
Stat5+/+
Stat5Δ/Δ
24 h
72 h
B in vitro
Flt3L
Tcf4
Fold change
p=.07
p=.03
Stat5+/+
Stat5Δ/Δ
GM-CSF
Tcf4
p=.09
p=.003
p=.01
Stat3+/+
Stat3Δ/Δ
GM-CSF
Tcf4
Stat5+/+
Stat5Δ/Δ
24 h
72 h
C in vivo
Tcf4
Arbitrary Units
p=.003
p<.001
p=.04
Stat5+/+
Stat5Δ/Δ
pORF
GM-CSF

Figure S7

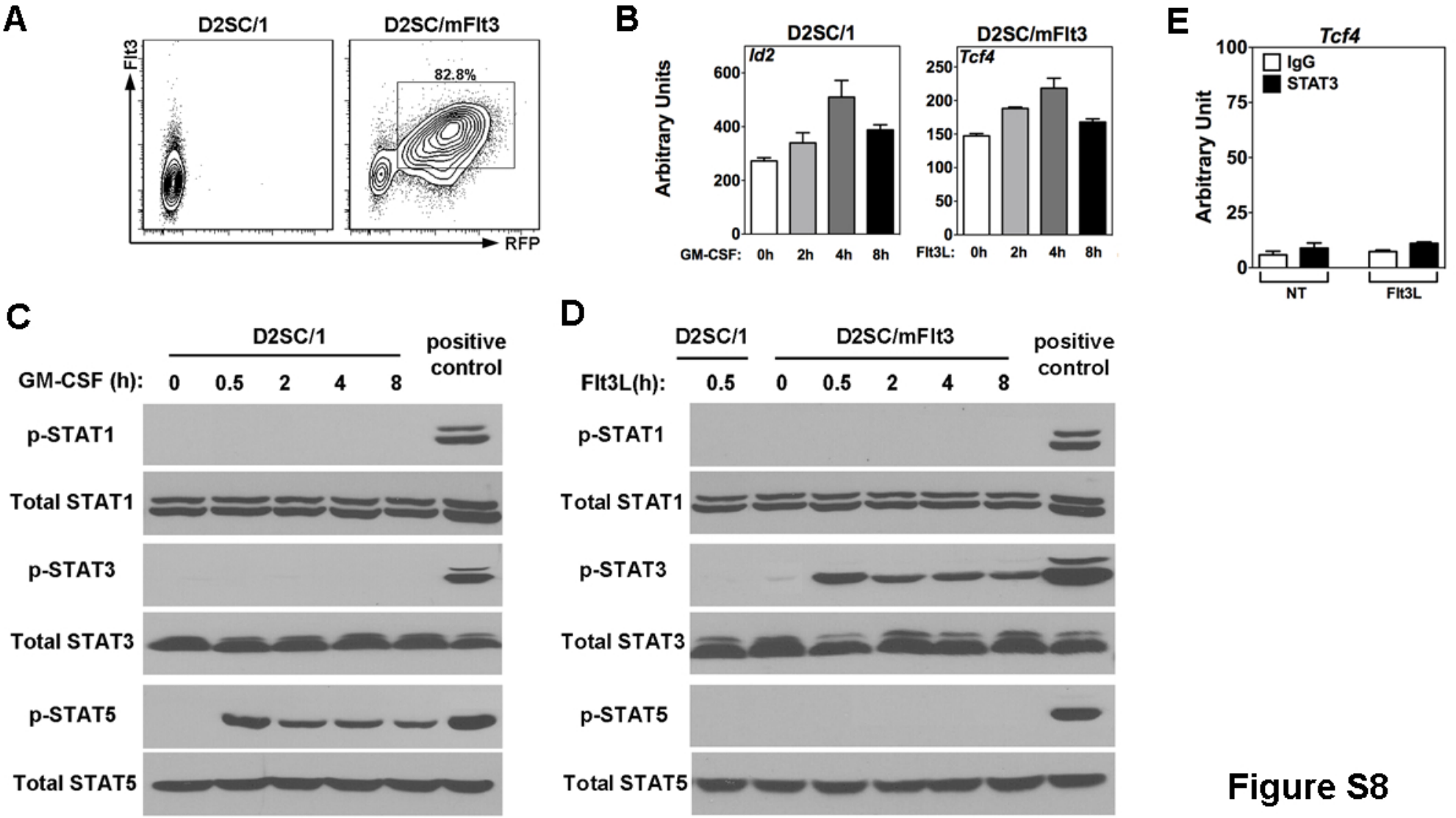

A
D2SC/1
D2SC/mFlt3
Flt3
RFP
82.8%
B
D2SC/1
D2SC/mFlt3
Id2
Tcf4
Arbitrary Units
GM-CSF: 0h 2h 4h 8h
Flt3L: 0h 2h 4h 8h
E
Tcf4
IgG
STAT3
Arbitrary Unit
NT
Flt3L
C
D2SC/1
positive control
GM-CSF (h): 0 0.5 2 4 8
p-STAT1
Total STAT1
p-STAT3
Total STAT3
p-STAT5
Total STAT5
D
D2SC/1
D2SC/mFlt3
positive control
Flt3L(h): 0.5 0 0.5 2 4 8
p-STAT1
Total STAT1
p-STAT3
Total STAT3
p-STAT5
Total STAT5

Figure S8

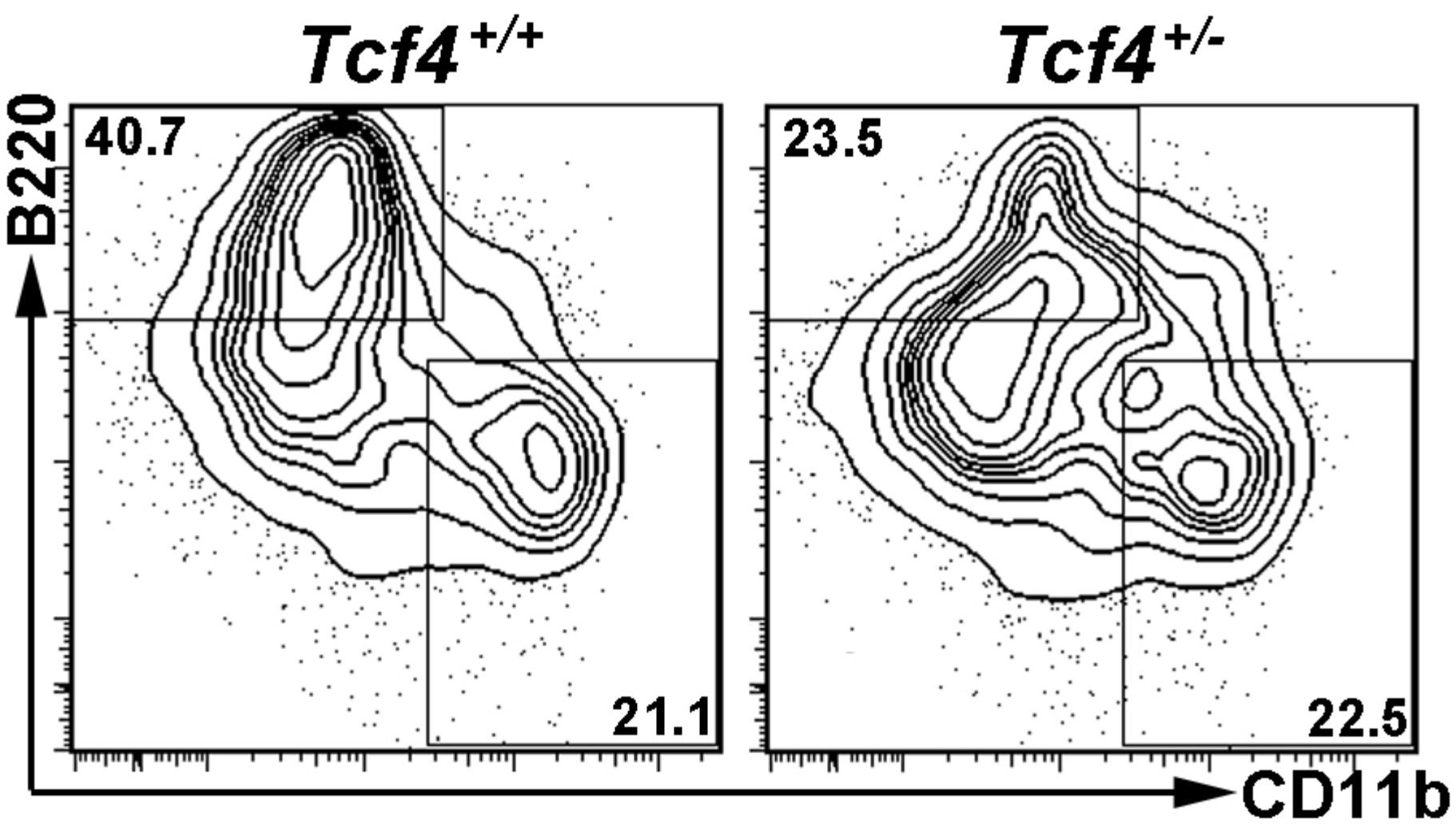


Figure S9

Table S1. Oligonucleotides used in this study.

Real-time PCR primers	
Tcf4 sense	AGACCAAGCTCCTGATTCTC
Tcf4 antisense	AGGCTCTGAGGACACCTTCT
Id2 sense	AAAACAGCCTGTCGGACCAC
Id2 antisense	CTGGGCACCAGTTCCTTGAG
***Tcf4* and *Id2* promoter cloning primers**	
Tcf4 sense (XhoI)	ATCTCGAGTGAAGGTGGACTCTG
Tcf4 antisense (HindIII)	ACAAGCTTGGCACTGCGGCTTAT
Id2 sense (XhoI)	ATCTCGAGTGGCTGCATCTAGGAAG
Id2 antisense (HindIII)	GCAAGCTTGCTTTCATGCTGCTCGT
EMSA oligonucleotides	
Tcf4 sense	CCCTGGTTCCCGGAGGAGGCG
Tcf4 antisense	CGCCTCCTCCGGGAACCAGGG
Id2 sense (distal)	CTGAGTTTCCGAGAAGGCCTC
Id2 antisense (distal)	GAGGCCTTCTCGGAAACTCAG
Id2 sense (proximal)	TGTGACTTCCCAAAAGCGCGC
Id2 antisense (proximal)	GCGCGCTTTTGGGAAGTCACA
ChIP primers [1]	
Tcf4 sense	CTCTTCCAGCTCAGGGTCAC
Tcf4 antisense	CTGCTCCACCAGACAATGAC
Id2 sense	TGTGCAAACCCCACTAATGA
Id2 antisense	CGCTTTTGGGAAGTCACATT

[1] Primer sequences for the ChIP assays performed in Figures 3C and 3D are indicated in Figure S5.