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Supplemental Information

Mast Cells Condition Dendritic Cells to Mediate Allograft Tolerance

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Supplemental figures:

Figure S1 related to figure 1

Figure S2 related to figure 2: Migrating DCs 18 hours post-FITC painting are host derived.

Figure S3 related to figure 3: Suppression of T-cells by graft derived DCs from tolerized mice is dominant

Figure S4 related to figure 4: DC subsets do not show differential dependence on GM-CSF induced survival

Figure S5 related to figure 5: Distribution of MCs in *Csf2*$^{-/-}$ mice is normal

Figure S6 related to figure 6: Quantification of DC survival by trypan blue exclusion

Figure S7 related to figure 7: number of MCs in the graft after reconstitution

Supplemental Experimental Procedures:

Skin graft model: more detailed description of the protocol used for skin grafting which has been adopted from the reference given in the main text.

Chemicals and reagents: List of chemicals and reagents used in the experiments with their respective vendors.

Primers used for RT-PCR: List of primers used for RT-PCR

Immunohistochemistry: More extensive description of the various immunohistochemistry methods used for figures 2, 4 and 6.

MC analysis in *Csf2*$^{-/-}$ mice: Methods related to figure S5

Statistics: Comprehensive description of the various statistical methods used.

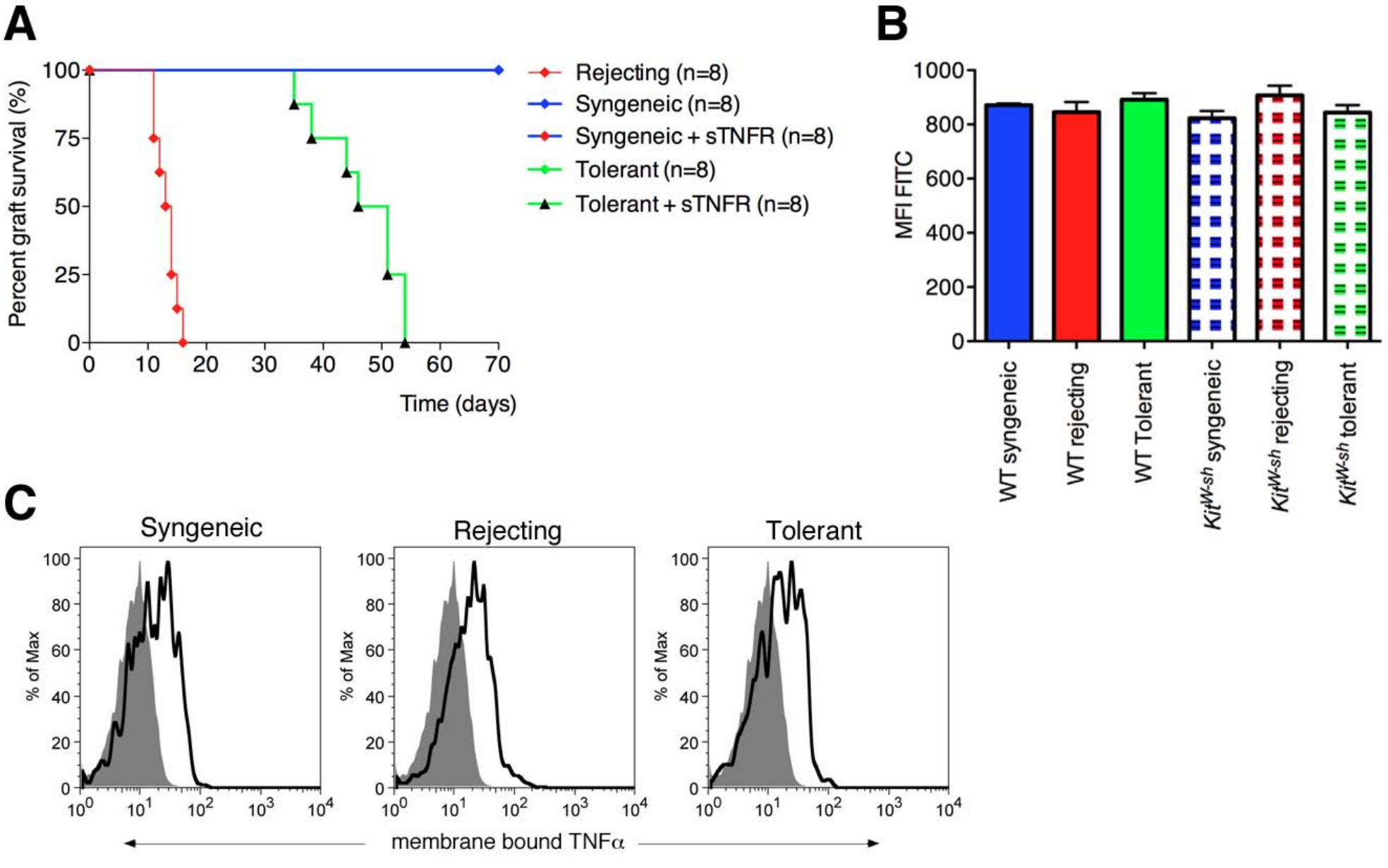
A
Percent graft survival (%)
100
75
50
25
0
0
10
20
30
40
50
60
70
Time (days)
Rejecting (n=8)
Syngeneic (n=8)
Syngeneic + sTNFR (n=8)
Tolerant (n=8)
Tolerant + sTNFR (n=8)
B
MFI FITC
1000
800
600
400
200
0
WT syngeneic
WT rejecting
WT Tolerant
Kit^W-sh syngeneic
Kit^W-sh rejecting
Kit^W-sh tolerant
C
Syngeneic
Rejecting
Tolerant
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
membrane bound TNFα

Figure S1 related to figure 1:

(A) Mice were grafted with either allogeneic skin (rejecting and tolerant) or syngeneic skin. At day 30 post-grafting all grafts in untreated mice with allogeneic grafts were rejected. Both syngeneic and accepted (tolerant) grafts were treated with 1µg of sTNFR-Ig at day 30 to block DC migration. Graft rejection was monitored for an additional 40 days. Combined data of 2 independent experiments. (B) Both WT and *Kit*$^{W\text{-}sh}$ were grafted and FITC painted 10 days later. After 18h MFIs of the graft derived (FITC^{+}) DC present in the dLN were determined. Pooled from multiple experiments from mice used in the main figures and presented as mean +/- SEM. (C) Histograms of syngeneic, rejecting and tolerant graft derived MCs stained for extracellular membrane bound TNFα. In short, skin grafts were collected at day 10 post-grafting and minced into small pieces. Single cell suspensions were obtained by digestion with DNAse, Liberase and Collagenase D for 3h at 37^{o}C with periodic mixing and subsequent homogenization by hand. Cells were initially gated on CD45^{+} lymphocytes and then subgated on cKit^{+}FceR1^{+} cells (see supplementary figure S7a for representative FACS plots). TNFα antibody was A488 conjugated (clone MP6-XT22, eBioscience) and a rat IgG1k-A488 (eBioscience) was used as isotype control. Shown are representative histograms from 1 out of 2 experiments.

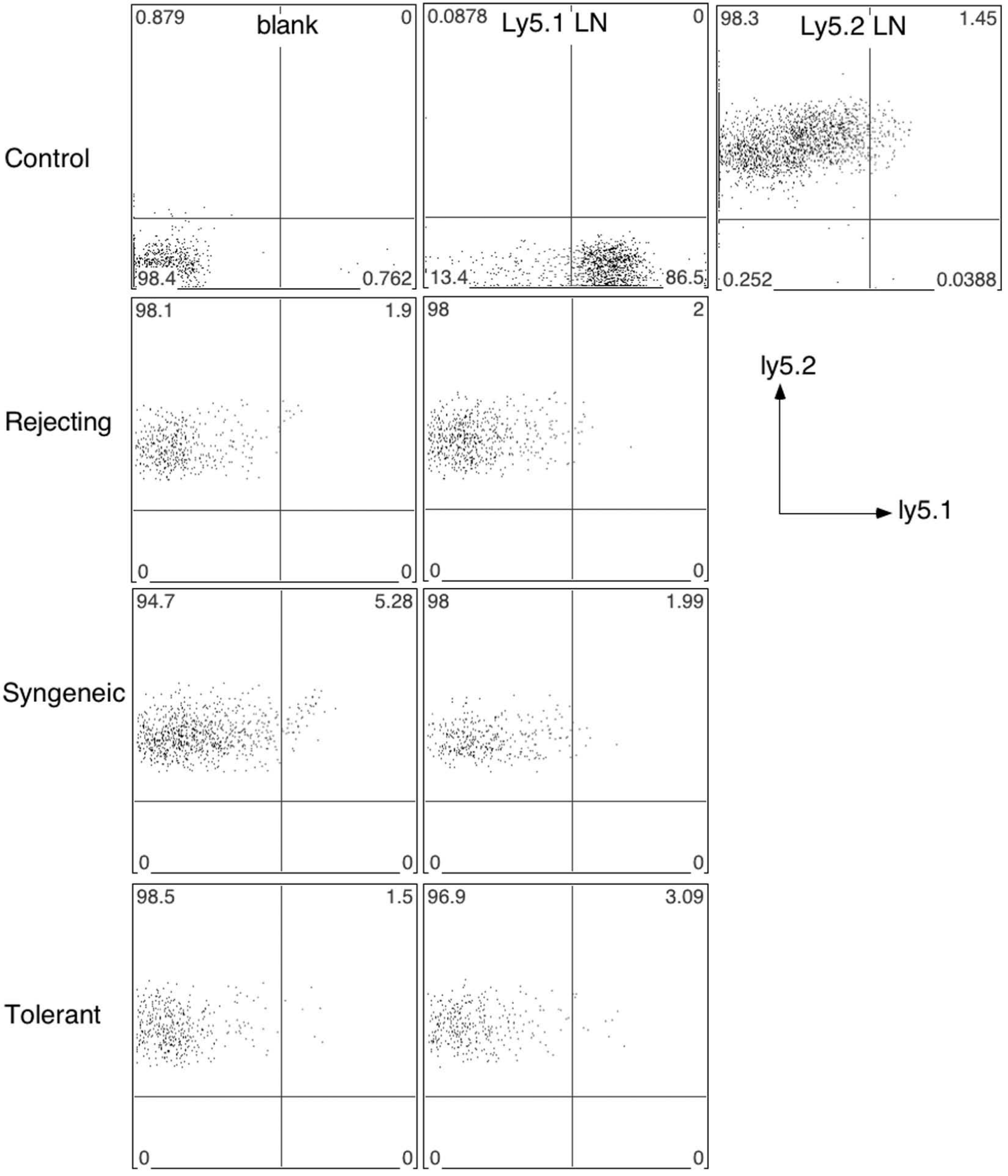
Control
Rejecting
Syngeneic
Tolerant
blank
Ly5.1 LN
Ly5.2 LN
0.879
0
98.4
0.762
0.0878
0
13.4
86.5
98.3
1.45
0.252
0.0388
98.1
1.9
0
0
98
2
0
0
94.7
5.28
0
0
98
1.99
0
0
98.5
1.5
0
0
96.9
3.09
0
0
ly5.2
ly5.1

Figure S2 related to figure 2: Migrating DCs 18 hours post-FITC painting are host derived.

Ly5.2 mice received Ly5.1 graft (B6 for syngeneic, F1 for rejecting and tolerant as described earlier). At day 10 post grafting FITC was applied to the graft and 18h later dLNs were collected. Shown are Ly5.1 and Ly5.2 positive cells within the graft derived $CD11c^{+}MCH\text{-}II^{high}FITC^{+}$ DCs. The top row comprises staining controls of B6 Ly5.1 (first two control figures) and Ly5.2 (ndLN of experimental mice). FACS plots show the minimum (left) and maximum (right) observed relative contribution of the Ly5.1 and Ly5.2 graft derived DCs from 2 independent experiments with a total of 3 mice in each group per experiment.

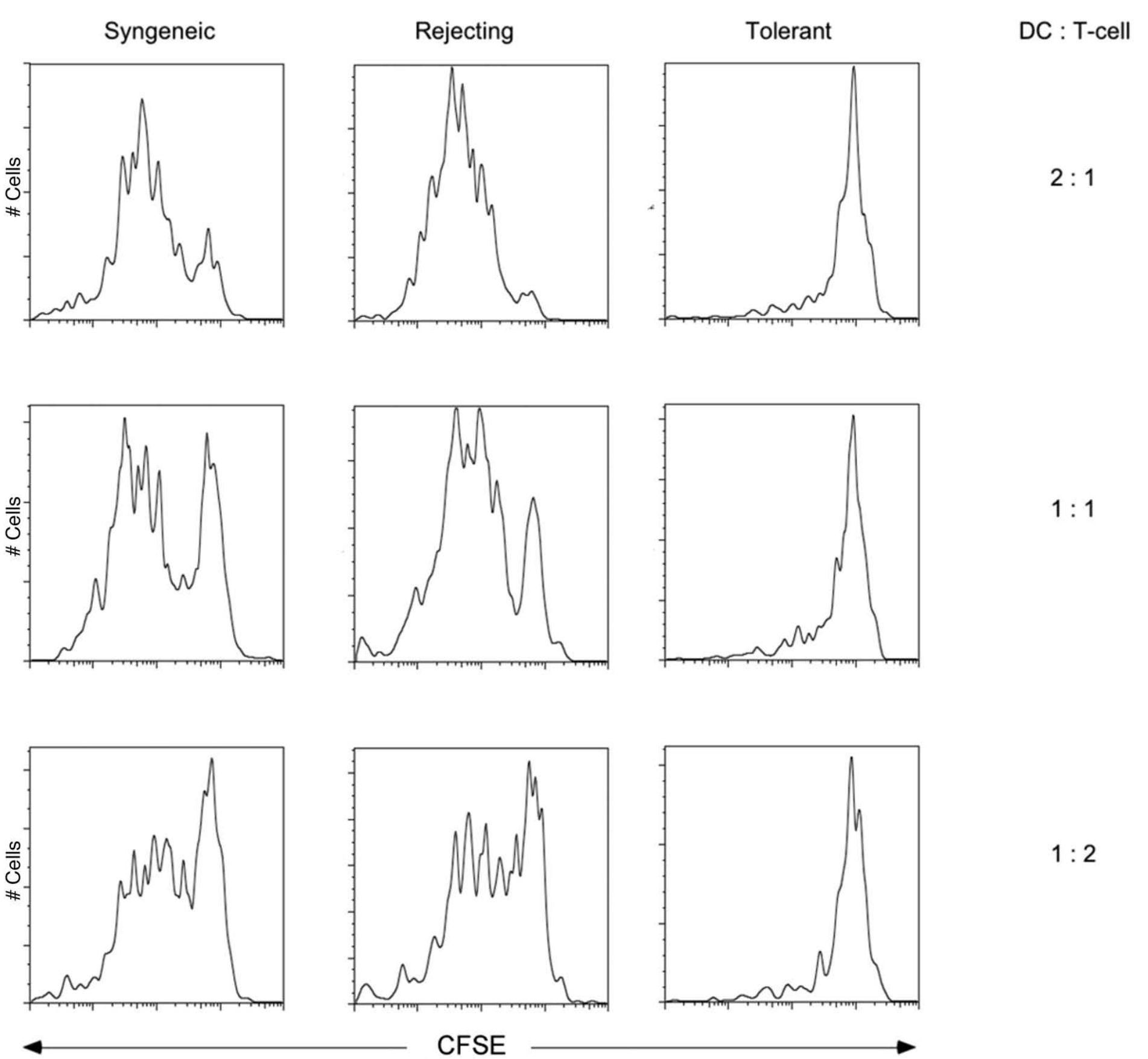
Syngeneic
Rejecting
Tolerant
DC : T-cell
Cells
2 : 1
Cells
1 : 1
Cells
1 : 2
CFSE

Figure S3 related to figure 3: Suppression of T-cells by graft derived DCs from tolerized mice is dominant

DCs from mice with syngeneic or allogeneic (rejecting and tolerant) graft were purified by positive selection with $CD11c^+$ beads (Miltenyi). After purification DCs were pulsed ex vivo with OVA peptide and co-cultured with CSFE labeled OVA-specific (OTII) T-cells. After 5 days CFSE dilution as measure of proliferation was determined by flow cytometry. Representative histograms of 2 independent experiments with 3 mice per group/experiment.

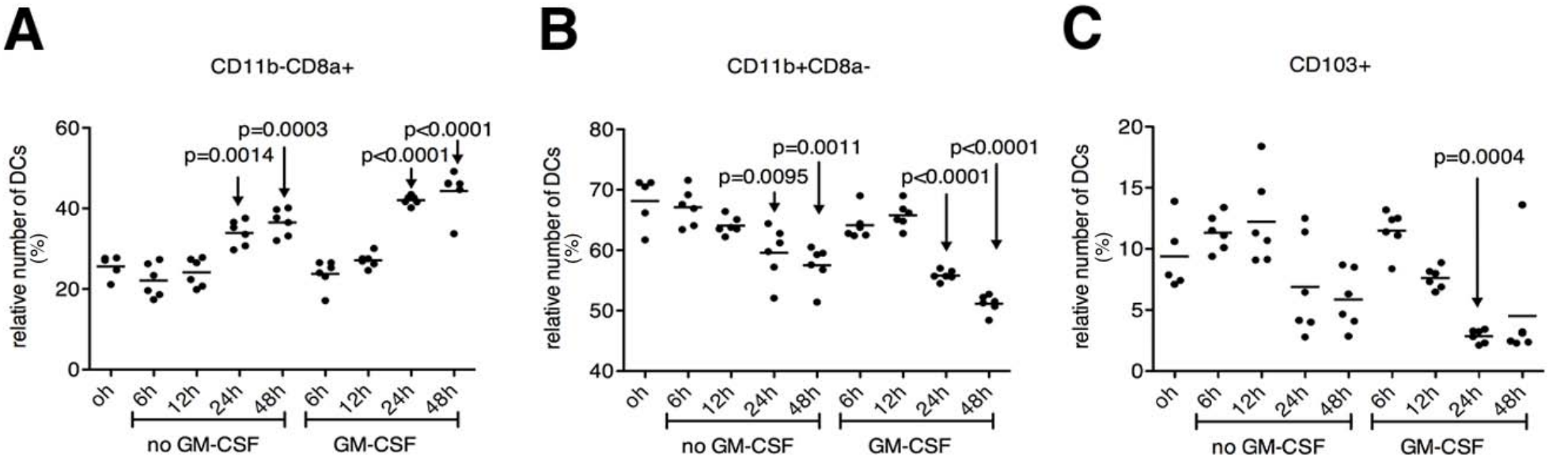
A
CD11b-CD8a+
relative number of DCs (%)
60
40
20
0
p=0.0014
p=0.0003
p<0.0001
p<0.0001
oh
6h
12h
24h
48h
6h
12h
24h
48h
no GM-CSF
GM-CSF
B
CD11b+CD8a-
relative number of DCs (%)
80
70
60
50
40
p=0.0095
p=0.0011
p<0.0001
p<0.0001
oh
6h
12h
24h
48h
6h
12h
24h
48h
no GM-CSF
GM-CSF
C
CD103+
relative number of DCs (%)
20
15
10
5
0
p=0.0004
oh
6h
12h
24h
48h
6h
12h
24h
48h
no GM-CSF
GM-CSF

Figure S4 related to figure 4: DC subsets do not show differential dependence on GM-CSF induced survival

Since the $CD8a^{+}CD11b^{-}$, $CD8a^{-}CD11b^{+}$ and $CD103^{+}$ DC subsets showed significant difference in the dLN of the allografts in tolerized mice compared to both syngeneic grafts and rejecting allografts (figure 2e), survival responses to GM-CSF were evaluated in vitro. Splenic DCs were isolated and the relative contribution of the subsets was evaluated in the presence or absence of 20ng/ml GM-CSF at different time points for 48h. Experiments have been performed twice with each symbol representing one mouse. Each symbol is the mean of a triplicate measurement. Statistics shown are in comparison to the initial composition at 0h time point.

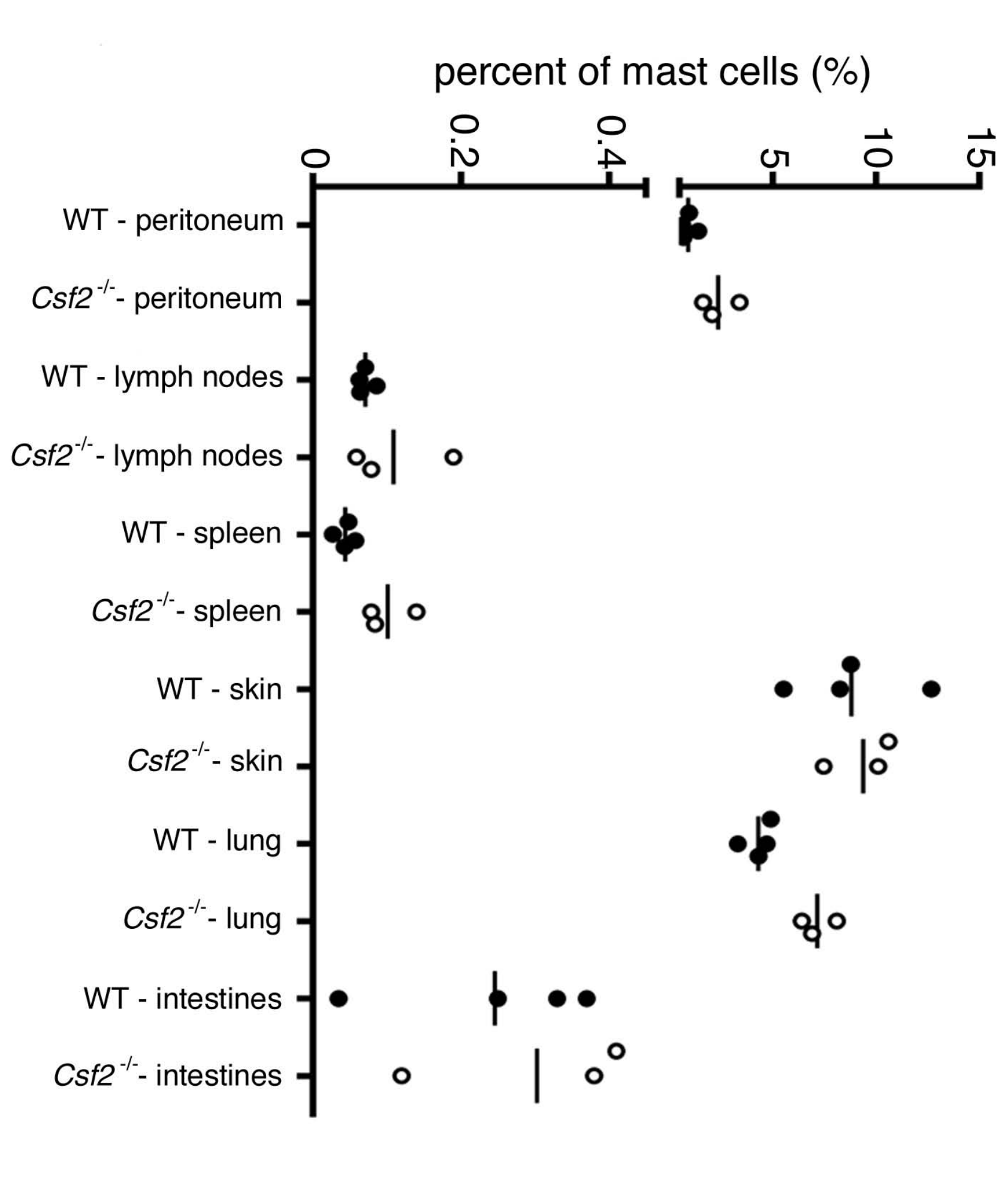
percent of mast cells (%)
0
0.2
0.4
5
10
15
WT - peritoneum
Csf2-/- - peritoneum
WT - lymph nodes
Csf2-/- - lymph nodes
WT - spleen
Csf2-/- - spleen
WT - skin
Csf2-/- - skin
WT - lung
Csf2-/- - lung
WT - intestines
Csf2-/- - intestines

Figure S5 related to figure 5: Distribution of MCs in *Csf2*$^{-/-}$ mice is normal

In order to determine whether the absence of GM-CSF would have an effect on the MCs under steady state conditions, several location known to harbor MCs were evaluated. The relative contribution within these locations showed no difference between WT and *Csf2*$^{-/-}$. Thus, both the development and the distribution of MCs in the *Csf2*$^{-/-}$ mice seem not to be affected. Pooled data from 2 independent experiments in which each symbol represents an individual mouse.

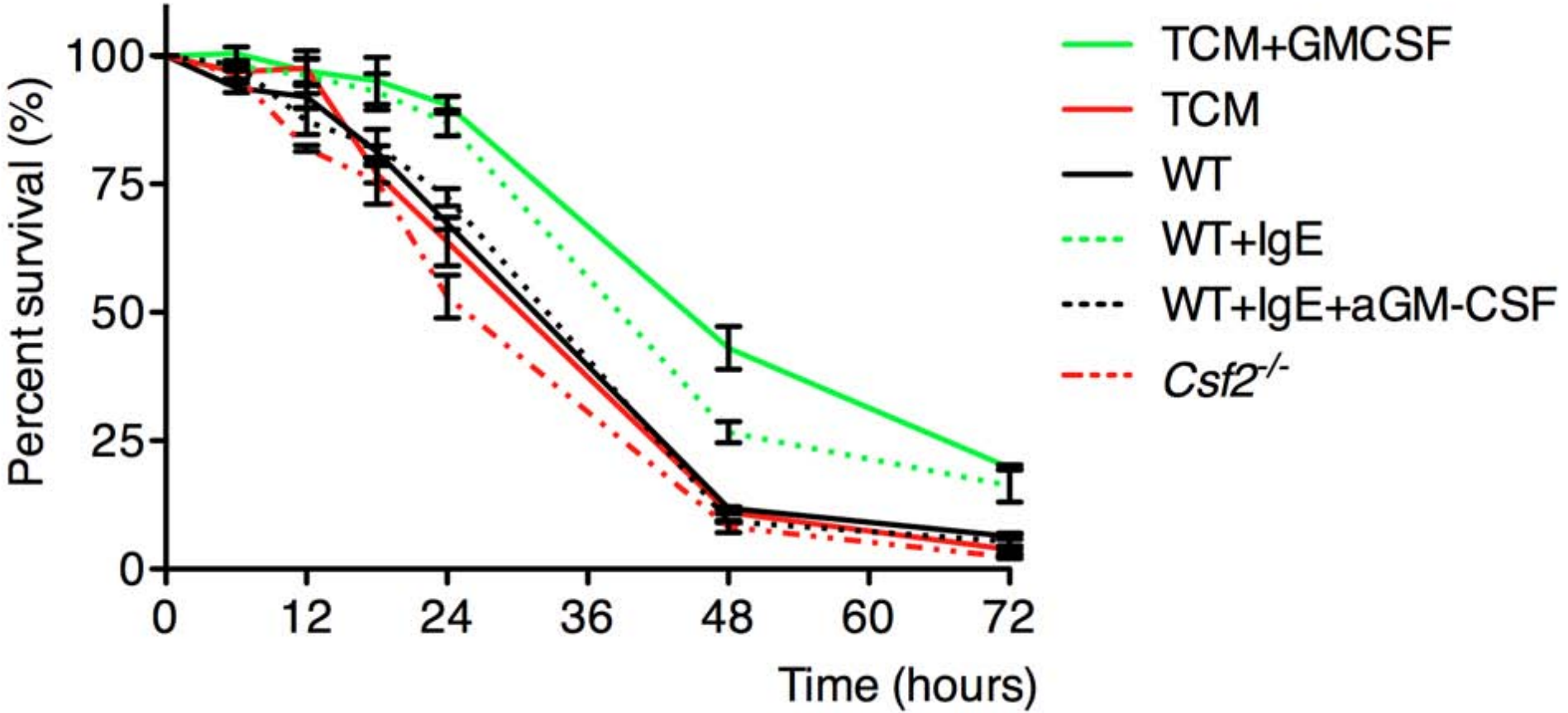
Percent survival (%)
100
75
50
25
0
0
12
24
36
48
60
72
Time (hours)
TCM+GMCSF
TCM
WT
WT+IgE
WT+IgE+aGM-CSF
Csf2-/-

Figure S6 related to figure 6: Quantification of DC survival by trypan blue exclusion

Supernatant of BMMCs was obtained from unstimulated MC (WT) or MC stimulated with recombinant DNP specific IgE (WT+IgE) and was used to maintain bead purified splenic DCs in vitro. For 72h DCs were monitored for survival by trypan blue exclusion. To confirm that GM-CSF produced by MCs could increase survival of the DCs, neutralizing GM-CSF antibody was added (WT+IgE+aGM-CSF). As positive control, complete RPMI was supplemented with 20ng/ml GM-CSF (TCM+GMCSF) as commonly used for bone marrow DC cultures. For negative controls, complete RPMI was used without pre-conditioning by MCs or addition of recombinant GM-CSF (TCM) or MCs from the *Csf2*$^{-/-}$ mouse. Data presented as mean +/- SEM are pooled from 4 independent experiments and had duplicates within each experiment.

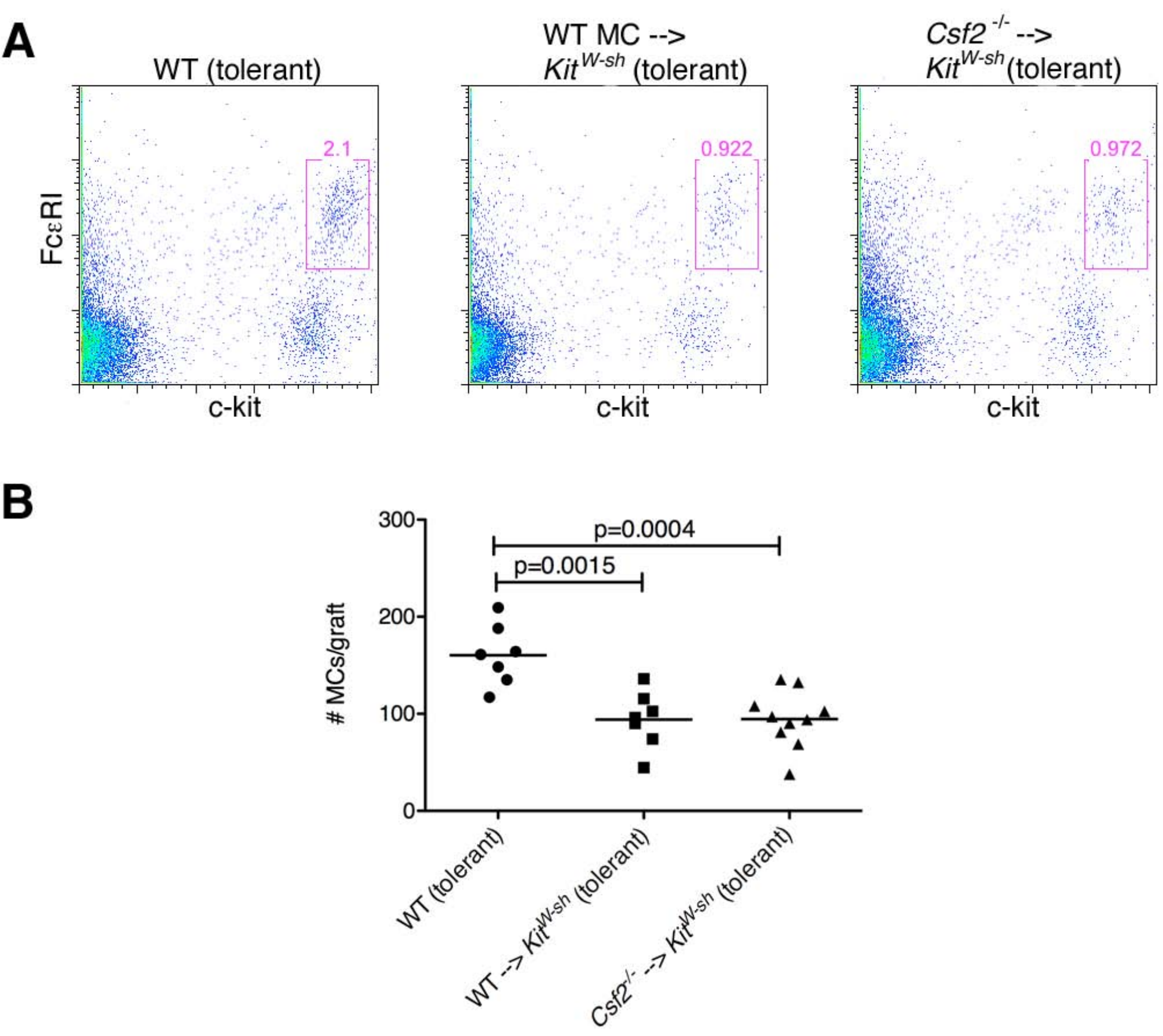
A
WT (tolerant)
WT MC --> Kit W-sh (tolerant)
Csf2 -/- --> Kit W-sh (tolerant)
FcεRI
c-kit
2.1
0.922
0.972
B
MCs/graft
300
200
100
0
p=0.0004
p=0.0015
WT (tolerant)
WT --> Kit W-sh (tolerant)
Csf2 -/- --> Kit W-sh (tolerant)

Figure S7 related to figure 7: number of MCs in the graft after reconstitution

Grafts from tolerized WT mice, tolerized mice reconstituted with WT BMMCs and tolerized mice with *Csf2*$^{-/-}$ BMMC were analyzed for successful reconstitution. A. Representative FACS plots showing the phenotype of the MC. Gates shown have been used to quantify the number of MCs in skin grafts. B. Quantification of number of MCs recovered from the grafts in which each symbol represents a single mouse. Mice that failed to reconstitute are omitted from the figure.

Supplemental Experimental Procedures

Skin graft model

Skin grafting was performed following the procedure described previously (Quezada, et al. 2005). In brief, at day -7, $4x10^7$ T-cell-depleted $CB6F_1$ splenocytes (DST) were intravenously transferred into recipients along with three intraperitoneal injections of 250µg αCD154 monoclonal antibody (clone MR-1) on days -7, -5 and -3. At day 0 full-thickness tail skins from $CB6F_1$ (F_1) donors was transplanted onto the dorsal side of age-matched C57BL/6 recipients. Syngeneic controls were grafted with C57Bl/6 tail skin without prior treatment. In order to address whether the $FITC^+$ DCs were host or donor derived $Ly5.2^+$ mice were grafted with $Ly5.1^+$ C57Bl/6 or $CB6F_1$ skin. Grafts were monitored for rejection every other day for up to 60 days and were considered rejected when 80% of the original graft disappeared or became necrotic. For MC reconstitution experiments $Kit^{W\text{-}sh}$ received 6-8 week cultured WT or $Csf2^{-/-}$ BMMCs locally by 4 subcutaneous injections of 1.10^6 MCs (3 times once a week) around the area where the graft would be placed (adapted from Lu et al., 2006). Treatment with DST and αCD154 was performed 8 weeks later after which the mice received the skin transplant. At the end of each experiment mice were analyzed for the presence of MCs either in the skin when the graft was rejected or in the graft when the mice accepted the graft for the duration of the experiment. On average 80% of the mice were successfully reconstituted and mice that failed to reconstitute were excluded from the studies.

Chemicals and reagents:

FITC painting: 5mg/ml FITC (Sigma) in 1:1 acetone (Fisher Scientific) dibutylphthalate (Sigma). Other in vivo labeling reagents: Dextran-A647 (Invitrogen) and DQ-OVA (Molecular Probes). Diphteria toxin, Aluminium hydroxide and Hoegst 33258 were purchased from Sigma. Soluble TNF-Receptor (Etanercept) was obtained from ProSpec, USA. Monensin used for intracellular accumulation of GM-CSF from BioLegend. OVA peptide 332-229 (ISQ) was obtained from AnaSpec, San Jose, CA, USA. CFSE was purchased from Molecular Probes. T-cells were purified with the CD4 negative selection kit from StemCell with the addition of CD25 to deplete activated T-cells and T^{regs}. Cell death was analyzed by either trypan blue (Sigma) or mitotracker deep red (MitoTracker Deep Red 633, Invitrogen).

The following antibodies were used for phenotyping of the dendritic cells of the graft draining LN: CD11c-biotin (clone N418), CD326-A647 (clone G8.8), CD11b-PerCP (clone M1/70), SA-APC-Cy7 (BioLegend), CD103-PE (2E7), CD205PE-Cy7 (clone 205yekta), CD8alpha-PE-Cy5 (53-6.7), B220-A700 (clone RA3-6B2), GR1-PE (clone RB6-8C5) (BD Bioscience, Pharmingen). Antibodies used for histology: CD11c-APC (N418, Biolegend), cKit-A647 (2B8, in house labeling with A647, Molecular Probes) CD11c-PE (N418, Biolegend), GM-CSF-FITC (MP1-22E9, R&D), unlabeled “cold” GM-CSF (MP1-22E9, BioLegend), GM-CSF neutralizing antibody (MP122E9, R&D systems). DNP-IgE (kind gift of Dr. T. Waldschmidt, University of Iowa Department of Pathology, Iowa City, IA, USA). Recombinant IL-33 was obtained from eBioscience whereas recombinant GM-CSF and recombinant stem cell factor (SCF) came from Peprotec.

Primers used for RT-PCR

For analyses of the several genes involved in survival and apoptosis of cells the following primers were used; Bcl-XL forward: 5'-GAGAACGGCGGCTGGGACAC-3'; Bcl-XL reverse: 5'-AGTTGTGGTGGGGGCAGGGT-3'; Bcl-2 forward: 5'-CTGACGCCCTTCACCGCGAG-3'; Bcl-2 reverse: 5'-CAAAGGCATCCCAGCCTCCGT-3'; Bim forward: 5'-CCAGCCCTGGCCCTTTTGCT-3'; Bim reverse: 5'-GAGACTGTCGTATGGAAGCCATTGCAC-3'; Bad forward: 5'-CGGGAGGAAGGCGGTGGAGA-3'; Bad reverse: 5'-TAGCCCCTGCGCCTCCATGA-3'; c-Flip forward: 5'-GCAAGGCAGGAGACGCCCAC-3'; c-Flip reverse: 5'-CTCTGCTCCAAGCCGCCACC-3'; cIAP1 forward: 5'-CATCTTCTTGAGCAGCTGTTGTCCA-3'; cIAP1 reverse: 5'-GTGGCCAGGATCTGCCGCTG-3'; cIAP2 forward: 5'-CCCCGGAGCCCTGGAGGAAA-3'; cIAP2 reverse: 5'-AGGCTGATACCGCAGCCCACT-3'; Spi-6 forward: 5'ACGCTG-GATTGAGAAGCCGCA-3'; Spi-6 reverse: 5'-GTGCCTGAGATATCTGGACTGCCG-3'.

Immunohistochemistry

Draining lymph nodes were collected after 18h after FITC-painting at day 10 post-grafting and compared to graft draining lymph nodes 11 days post grafting without FITC painting. The lymph nodes were snap-frozen and embedded in OCT compound (Tissue-Tek, Optimal Cutting Temperature 4583). Thin 8µm tissue sections were briefly fixed in ice-cold 1:1 methanol/acetone for 5 minutes. Slides were blocked with 10% normal rabbit serum and stained with CD11c-APC (clone N418, Biolegend). For skin sections a similar protocol was followed.

Cytospots of BMMCs were made by carefully putting the cells on SuperFrost/Plus microscope slides (Fisher Scientific) to prevent degranulation due to spinning down as normally done with cytospins. After drying, cells were fixed with 10% formalin and stained with cKit-A647, FcεRI-PE, GM-CSF-FITC and Hoechst 33258 as nuclear stain. Controls were rat IgG2b-FITC (isotype) and pre-incubation with unlabelled GM-CSF of the same clone as used for staining (MP1-22E9, BioLegend).

MC analysis in *Csf2*$^{-/-}$ mice

Spleen, pooled lymph node, peritoneal lavage, intestines, lung and skin were analyzed for the presence of MCs. Lung, skin and intestinal samples were weighed and equal amounts were used for the analysis (275mg, 175mg , and 850mg, respectively). For lung, tissue was collected and minced into small pieces before being digested with DNase and Liberase (Roche) for 2 hours at 37^{o}C with periodic mixing. At that time tissue was further homogenized by hand and then put through a 70mm filter. For intestine, tissue was collected and cleaned to remove debri before being further processed in a similar way as has been done for lung samples. Spleens were injected with DNAse and Liberase and incubate for 30 min at 37^{o}C before manually pressing them through a 70mm filter. For peritoneal lavage fluid two times 5 ml of 37^{o}C PBS containing 5 Units of heparin and 1% FCS was injected in the peritoneal cavity. After 5 minutes the fluid containing the cells was recovered. For skin samples, tail skin was removed and minced into small pieces and digested with DNAse, liberase and Collagenase D for three hours at 37^{o}C with periodic mixing before being further homogenized by hand.

Statistics

All statistics have been calculated using the prism 4.03 software package (GraphPad Software, San Diego California USA). Survival data were analyzed using the Kaplan-Meier method, with the Wilcoxon rank test and the log-rank test used to verify the significance of the difference in survival between groups. Luminex data were analyzed by the immune monitoring laboratory of the Norris Cotton Cancer Center/Dartmouth Hitchcock Medical Center and are shown as mean +/- SD whereas other data are expressed as mean +/- SEM. The additional data were analyzed by one-tailed ANOVA and post-tested by Tukey analysis for multiple group analysis or student T test for 2 group analysis. Statistical significance was calculated for a 95% confidence interval ($P<0.05$). The exact p-values are denoted in the figures.