Supporting Information

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SI Materials and Methods

Animals. Murine embryonic stem cells (ES cells) bearing a gene trap insertion between the first and second exons of the stk4/Mst1 gene (cell line AJO315) were obtained from the Sanger Institute gene-trap resource center (http://www.sanger.ac.uk/PostGenomics/genetrap/), also accessed via (http://www.genetrap.org/). The insertion of the gene trap vector was mapped by PCR and confirmed by sequencing and Southern blot, as shown in Fig. 1*A*. The absence of additional insertions was established by the presence of a single band on a Southern blot developed with a probe generated entirely from within the gene trap vector (not shown). The heterozygous ES cells were injected into C57BL/6J blastocyts to produce chimeric mice by using standard methods. Resulting chimaeras were mated with C57Bl6/J mice to produce Mst1 mutant heterozygous mice. Heterozygous mice were backcrossed onto the C57Bl6/J strain for six generations. All comparisons were done using homozygous and wild-type littermate controls. All mice were housed in a temperature-controlled room at 22–24°C with a 12-h light/dark cycle and fed a standard laboratory chow diet and double-distilled water. Animal experiments were carried out in accordance with National Institutes of Health (NIH) guidelines for the Care and Use of Experimental Animals and were approved by the institutional Animal Care and Use committee of the Massachusetts General Hospital.

Mice Genotyping. Genomic DNA extracted from tail (postnatal 3–4 weeks) biopsies was screened for the presence of wild-type and mutant alleles by PCR. One forward primer based on the Mst1 genome sequence (primer a: 5′-GGG TCT TGC ACA GTG TCT CA -3′) is common for both wild-type and targeted alleles. Two reverse primers based on the Mst1 genome sequence (primer b: 5′-TGT CAA GCT CAT GAC TGC A –3′) and the targeting vector (primer c: GGGGATGTGCTGCAAGGCGA) were used for PCR to amplify 500- and 250-bp DNA fragments specifically for the wild-type and targeted alleles, respectively. The genotypes were confirmed by Southern blotting. Briefly, Southern blots of *Bam*H I-digested DNA probed with 5′ probe give rise to a wild-type 10-kb band or a disrupted 8-kb band. 5′ probe was amplified by the forward primer, GGG AAG GCT TGT GTT GAA T and reverse primer, TAG GGC GCA GTC AAG AAA C.

Northern Blot Analysis. Total RNA was extracted from 5×10^6 cells with TRIzol (Invitrogen). Twenty micrograms RNA was separated by electrophoresis in 2% denaturing formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N+, Amersham). RNA was cross-linked to the membranes by UV light. After prehybridization, the membrane was hybridized with the cDNA probes for Nore1B, labeled with ^{32}P by using a random primer DNA labeling kit (Qiagen). The probed membrane was then washed and exposed to radiographic film. Full-length Nore1B cDNA fragments were used as probes for Northern blot assay. The same membrane was reprobed with the Mst1 probe. The Mst1 probe was amplified by PCR using the primers as follows: Mst1 forward, CAC CCG TTT GTT AAG AGT G; reverse, TCA ATG GCA TCC AGG ATT GGC.

Cell Isolation and Stimulation. Single cell suspensions of thymus, spleen, and peripheral lymph nodes of Mst1-null and wild-type littermate control mice were made by disrupting the organs and then filtering them through 70-μm filters. Red blood cells were depleted by red cell lysis solution containing 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA. Total T cells were purified by CD90 microbeads, and naïve and memory T cell were isolated using the $CD4^+CD62L^+$ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's protocol. The overall purity of total T cells and the $CD4^+$/CD62Lhi subset was verified by cytofluorimetry always to be higher than 90 usually 95%. Before TCR stimulation, the cells were rested at 37°C for 2 to 3 h in RPMI 1640 complete medium containing 10% FBS, 25 mM Hepes, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (2-ME), 1% non-essential amino acid, and 2 mM sodium pyruvate. For the immunoblot studies shown in Fig. 5, two million purified T cells were incubated with 10 μg/ml anti-CD3 antibody and 10 μg/ml anti-CD28 antibody (clone 37.51, BD PharMingen) at 37°C for indicated time points. The cells were quickly spun down and washed with cold PBS once, then frozen on dry ice ready for immunoblot analysis. Following the same procedure, T cells were treated with either 50 ng/ml PMA (Sigma) and 1 μM Ionomycin (Sigma) or 0.5 mM H_2O_2 (Sigma).

Flow Cytometry. Single cell suspensions of spleen or lymph node were resuspended in PBS with 1% BSA and blocked with CD16/CD32 (Fcγ III/II Receptor) (BD PharMingen. The cells were then stained with the indicated antibodies, washed, and then fixed with paraformaldehyde. The lungs were flushed with cold PBS and excised. Single cell suspensions were obtained by disrupting the tissue with scissors followed by digestion in Blendzyme (Roche) and DNase for 45 min at 37°C and strained through a 70-mm filter. After lysis of red blood cells (RBC lysis buffer, Sigma), the cells were resuspended, counted, and stained for flow cytometry as above.

Antibodies were obtained from BD PharMingen. In some cases, the cells were permeablized and stained with an antibody to murine Foxp3 (eBiosciences) according to the manufacturer's protocol. Flow cytometry was performed on a BD FACScaliber and analyzed with Flowjo software (Treestar).

Ca^{2+} Flux Measurements. Five million lymphocytes isolated from mice 8 weeks of age were loaded with 5 μg/ml Fluo4 A.M. (reconstituted in anhydrous DMSO for a working concentration of 1 mg/ml; Molecular Probes/Invitrogen) for 30 min at 37°C with occasional mixing. The cells were washed briefly in complete media (IMDM containing 10% FCS+Fe^{+2}, 100 μM β-mercaptoethanol, and 50 μg/ml gentamicin) and were then stained with antibodies against CD4 and CD62L (BD Biosciences) for 10 min at room temperature. Cells were then washed and resuspended in complete medium, and then analyzed by flow cytometry (Becton Dickinson FACSCaliber machine and CellQuest Pro software, version 5.2). $CD4^+$ cell populations or CD4+CD62LHi cell populations were gated as indicated in Fig. S11*B*. Gated, unstimulated cells were collected for 30 s to establish a baseline reading, and were then stimulated with the addition of 2 μg of anti-CD3 (BD Biosciences), immediately followed by cross-linking with 5 μg of goat anti-Armenian hamster antibodies (Jackson ImmunoResearch). Ca + 2 fluxes were measured for eight minutes, followed by the addition of 0.5 μg/ml ionomycin (Sigma) for an additional 30 s. As indicated by the overlay in panel Y, lymphocytes from both wild-type (blue) and Mst1-deficient mice (red) had similar Ca^{2+} flux and response to the positive control (ionomycin). Data shown is representative of two independent experiments.

Immunoblotting. Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 10% glycerol, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM Na_3VO_4, complete Mini protease inhibitor mixture tablet (Roche), and 2 mM PMSF. After centrifugation, cell lysates from 1×10^6 were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blot was incubated with indicated antibodies and visualized by the ECL detection system (Amersham Pharmacia).

The following antibodies are used for immunoblot: anti-total or phosphor-ERK(Thr-202/Tyr-204,197G2), Jnk(Thr-183/Tyr-185), p38(Thr-180/Tyr-182), Akt (Ser-473,193H12), IKB α(Ser-32,14D4), PLCγ(Tyr-783), ZAP 70(Tyr-319), Lck(Tyr-505), YAP(Ser-127), and PAK1(Thr-423)/PAK2(Thr-402) are purchased from Cell Signaling Technology; Anti-CD3 (clone 145–2C11) and anti-CD28 (clone 37.51) are obtained from BD PharMingen; anti-phospho-FOXO1(Ser 212)/FOXO3(Ser 207) and anti-Actin are from Invitrogen and Sigma, respectively; anti-phospho-Tyrosine(pY, 4G10) was from Upstate; anti-Actin anti-phospho-Mob1(Thr-12), anti-phospho-Mob1(Thr 35), anti-Lats1, anti-phospho-Lats1/2 C-terminal (CT), anti-phospho-Lats1/2 activation loop (AL), anti-Mst1, anti-Mst2 and anti-phospho-Mst1/2(Thr-183/180) are described before (S1,S2).

Immunofluorescence Staining. Immunofluorescence staining of splenic sections was performed essentially according to Cariappa *et al.*, (S3) with minor modifications. Spleens were harvested and immediately frozen in OCT compound (Tissue-Tek). Six-μm thick sections were cut and stored at -80°C until use. All subsequent manipulations were done at room temperature. Sections were air dried for overnight, fixed in ice-cold acetone for 10 min, air dried briefly, blocked with 5% normal horse serum for 20 min, and incubate with biotin-anti-mouse IgM (R6–60.2, BD PharMingen) at 1:50 dilution for 1 h. After rinsed in PBS (three times, 5 min each), sections were then stained with a 1:500 dilution of streptavidin-Texas red (Jackson Immunoresearch Laboratories) and anti-MOMA-1-FITC (Serotec) for 1 h. Sections were rinsed in PBS (three times, 5 min each). and mounted with mounting medium (Vectashield). Digital images were acquired by using a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments) equipped with an Orca 100 CCD camera (Hamamatsu). Epifluorescence images were analyzed using IPLab version 3.2.4 image processing software (Scanalytics) and imported into Adobe Photoshop v7.0 (Adobe Systems) for production of the final figures.

T cell staining was performed as previously described (S4). Briefly, purified T cells were fixed with 4% PFA for 15 min at room temperature. Fixed cells were mounted on polyL-lysine-coated slides. Fixed cells were permeabilized for 5 min with 0.2% Triton X-100, and then blocked with 10% goat serum for 20 min. Cells were stained with antibody to LFA-1 a(M17/4, BD PharMingen) at 1:50 dilution for one and half an hour followed by Texas Red-conjugated goat antibody to Rat IgG (Jackson Immunoresearch Laboratories) at 1:500 dilution for 45 min. Unbound antibodies were removed by five washes with PBS plus 0.1% BSA. Stained cells were viewed by Nikon Eclipse 800 epifluorescence microscope.

Annexin V Assay. Annexin V staining was done after initial staining with anti-CD3 (PharMingen) and anti-CD62L with an annexin V staining kit (PharMingen). Cells were subsequently analyzed by flow cytometry on a FACScaliber.

Gene Expression Analysis and Real-time Quantitative PCR. $CD4^+$ cells were immunoisolated and the CD4/CD62Lhi and CD4/CD62Llo subsets were then purified by cell sorting. These cells and the excised mouse tissues were homogenized in TRIzol reagent (Invitrogen). After extraction with chloroform, the RNA was purified using RNeasy mini kit (Qiagen) and reverse transcribed using SuperScript III RT (Invitrogen). Real-time quantitative PCR was performed using Bio-Rad iQ SYBR Green Supermix in the BioRad iCycler iQ system (BioRad). β-actin RNA was used to standardize the total amount of cDNA. The sequence of primers was used as follows: Mst1 se:GGA AGC TTC CTC GCA ACT TTG; Mst1 as:GCT CAG GTG ACC TTC CCT GAA; CD25 se:AGA ACA CCA CCG ATT TCT GG; CD25 as:CTG TGG GTT GTG GGA AGT CT; CD4 se:CAC CTG GAA GTT CTC TGA CCA; CD4 as:AAA CGA TCA AAC TGC GAA GG; CD28 se:TGG CTT GCT AGT GAC AGT GG; CD28 as:GCT GGT AAG GCT TTC GAG TG; CD62L se:AAG CTG TGG GTC TGG ACA CT; CD62L as:AGC ATT TTC CCA GTT CAT GG; KLF2 se:AAC TGC GGC AAG ACC TAC AC; KLF2 as:TCC TTC CCA GTT GCA ATG AT; b7-integrin se:GGA CGA CTT GGA ACG TGT G; b7-integrin as:CGT TTT GTC CAC GAA GGA G; S1P1 se:GTG TAG ACC CAG AGT CCT GCG; S1P1 as:AGC TTT TCC TTG GCT GGA GAG; β-actin se:AAA TCG TGC GTG ACA TCA AA; β-actin as:TCT CCA GGG AGG AAG AGG AT. Other primers are as described in (S5). PCR specificity was examined by analyzing the melting-curves. Relative mRNA levels in Fig. 3*D* were determined by comparing the PCR cycle threshold between cDNA of CD25, Mst1, or CD4 and that of β-actin.

Assay of [^{3}H]-Thymidine Incorporation and Cytokine Production *in Vitro*. Two hundred μl of purified T cells at a concentration of 1×10^6 cells/ml in RPMI 1640 complete medium were added to 96-well plates precoated with 1 μg/ml of anti-CD3 with or without 1 μg/ml anti-CD28 antibody. Cells were cultured for 24 to 120 h at 37°C and pulsed with 1 μCi of [^{3}H]-thymidine/well for the final 10–12 h of culture. The plates were harvested using a Packard 96-well Filtermate Harvester and counted on a Packard Top Count Scintillation counter. Supernatants from day 1 and day 2 were taken for cytokine ELISA. IL-2, IL-4, and IFN-γ were done using ELISA Kits (BD Biosciences) according to the manufacturer's directions.

CFSE Labeling. $CD3^+$ T cells (2×10^6 cells/ml) were incubated with 5 mM CFSE in PBS containing 5% FBS for 10 min at 37°C. Unbound CFSE was quenched by using an excess of complete medium and incubation on ice for 5 min. Cells were washed three times in complete medium before cell culture. Analyses of CFSE profiles were performed on a FACS Calibur flow cytometer by using CellQuest software by gating on viable $CD3^+$ T cells.

Generation of Th1 and Th2 $CD4^+$ T Cells *in Vitro*. Naïve $CD4^+$ T cells were isolated from spleen and pooled cervical, axillary and inguinal lymph nodes from $Mst1^{-/-}$ and wild-type mice using a magnetic bead selection kit for $CD4^+/CD62L^+$ cells (Miltenyi). Purified $CD4^+$ T cells (1×10^6 cells/ml) were activated in the presence of α-CD3 (2 mg/ml; 2C11) and α-CD28 (1 mg/ml; both from BD PharMingen). Th1 cells were generated by activation of cells in the presence of 100 U/ml IL-12 (PeproTech) and anti-IL-4 (BD PharMingen) at inhibitory concentrations. Th2 cells were generated by activation of cells in the presence of 1,000 U/ml IL-4 (PeproTech) and α-IFN-gamma (BD PharMingen) at inhibitory concentrations. Media with 5–10 U/ml IL-2 (PeproTech) was added to the cells on day 2, and the cells were then split every day and used after 6 days in culture. Cytokine expression by intracytoplasmic staining was assayed by cytofluorimetry as in (S6 and S7).

Cell Adhesion Assay. Adhesion assays were done as described before (S8). Recombinant mouse ICAM-1 human IgG1 Fc was used to coat Nunc Maxi-Sorp 96-well plates. After blocking with 1% BSA, resuspended cells 5×10^6 in RPMI were labeled with 5 μM Calcein-acetoxymethyl Ester Diacetate (Calcein AM,

Calbiochem) and incubated at 37°C for 30 min. Labeled cells were washed twice with prewarmed RPMI and resuspended at 1×10^6 cells/ml. Fifty microliters of cell suspension per well was transferred to the coated plate in the presence of PMA (10 ng/ml), 2C11 (10 μg/ml), SLC (100 ng/ml), or no stimulus, then were incubated at 37°C for 30 min. Non-adherent cells were removed by four consecutive washes. Input and bound cells were measured in the 96-well plate using a fluorescence multiwell plate reader (Cytofluor4000; Applied Biosystems). The data are presented by the percentage of the number of bound to input cells.

Chemotaxis Assays. Chemotaxis assays using 24-well transwells (Costar; Corning) were performed as previously described (S9). Briefly, 0.5 ml of RPMI medium 1640 containing 200 ng/ml SLC/CCL21 was added to the lower chamber. One million splenic T cells in 150 μl of media were then added to the upper chamber of the transwell and incubated for 3 h at 37°C in 5% CO_2. The percentage of migration was calculated by dividing the number of cells in the lower chamber by the total cell input (one million), multiplying by 100. All chemotaxis data represent the average of triplicate well, done in duplicate experiments.

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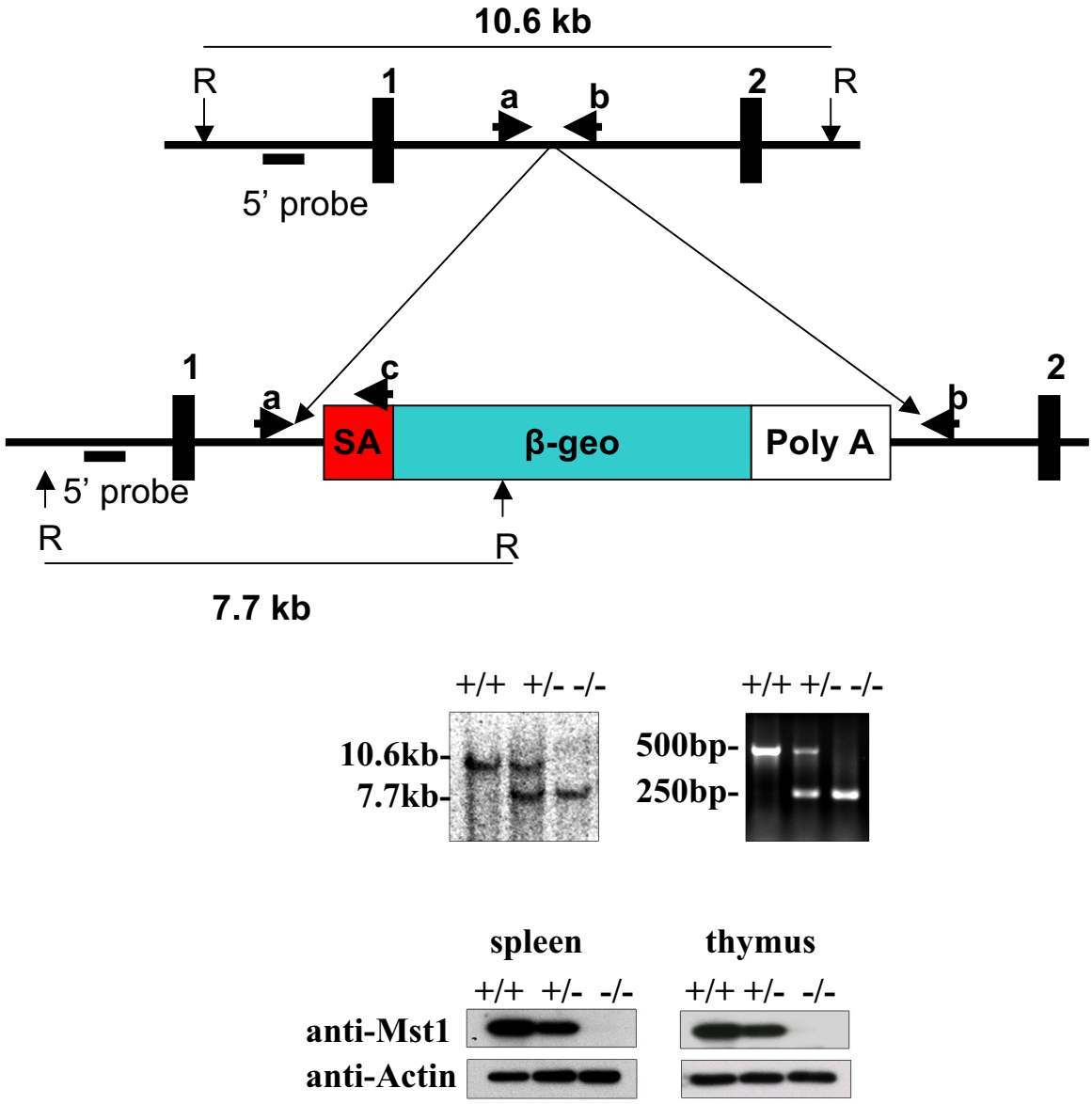

Fig. S1. Characterization of Mst1 gene trap mice. Top panel, Partial genomic sequence of the mouse Mst1 gene showing exons 1 and 2 (closed rectangles). Middle panel: Schematic representation of the gene trap vector targeted allele. The gene trap vector contains a splice acceptor (SA) sequence followed by the β-geo fusion containing the lacZ and neomycin transferase (neo) genes and a polyadenylation sequence (pA). The regions complementary to the PCR primers and to the Southern hybridization probe are indicated. Middle panels, Left-Southern blot of genomic DNA from mouse tails digested with *Bam*H1 and hydridized with the 5′ probe, The size of the fragments is indicated. Right, PCR was performed on DNA prepared from tail biopsies, using a mixture of primers a, b, and c as described in Methods. A 500-bp band is amplified for wild-type animals, a 250-bp band for homozygous mutants, and both bands for heterozygous animals. Bottom panels, Extracts of thymus (left) and spleen (right) from Mst1 wild-type (+/+), heterozygous deficient (+/−) and homozygous deficient (−/−) mice were immunoblotted with antibodies to Mst1 and beta-actin as indicated.

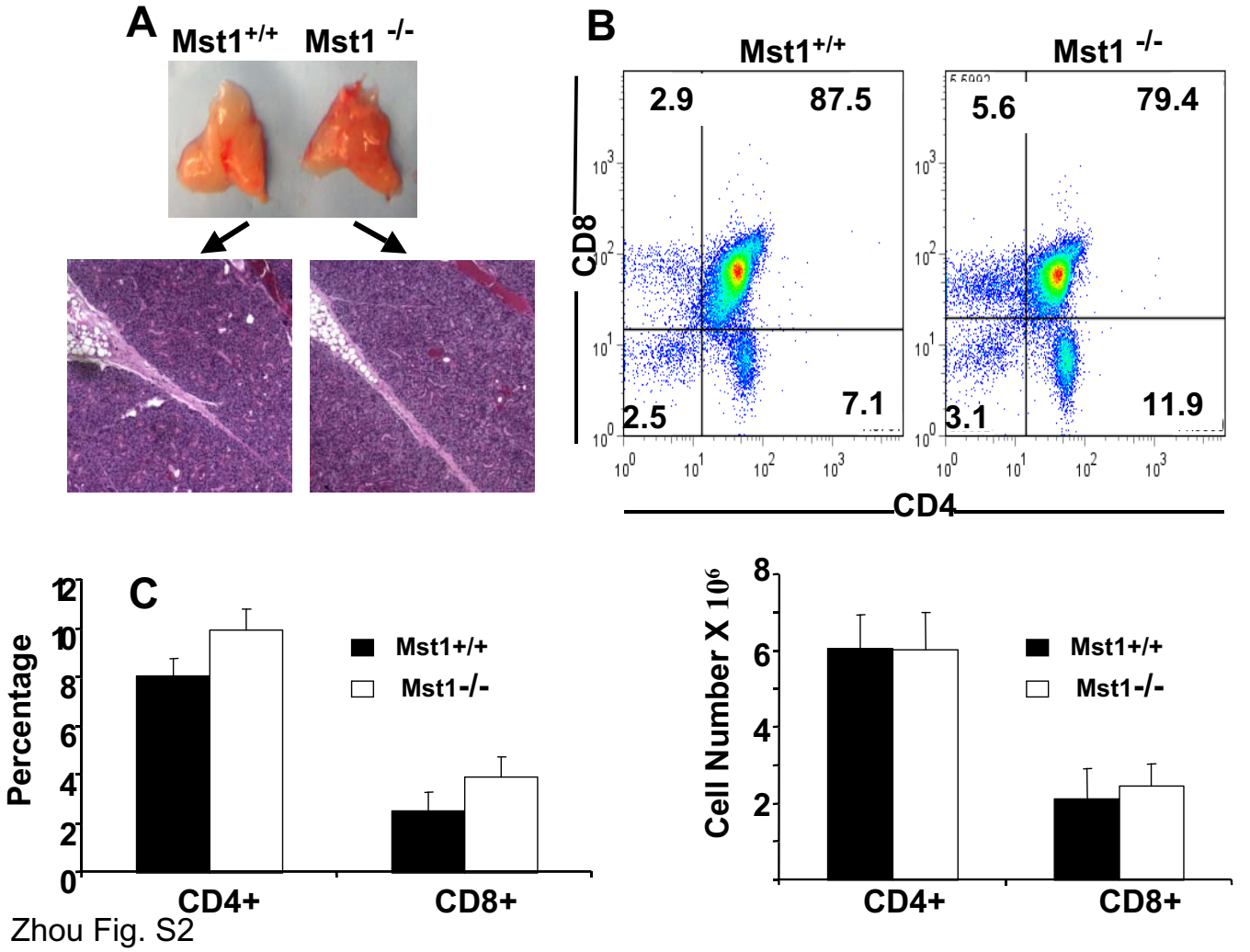

Fig. S2. (*A*) Thymic architecture in Mst1-null and wild-type mice. Upper, A comparison of the whole thymus from Mst1-null and wild-type mice. Lower, Representative sections of the thymus from wild-type (left) and Mst1-null (right) mice stained with H&E show normal architecture (original magnification 10×). (*B*) Representative dot plots of thymocytes isolated from Mst1-null and wild-type littermates stained with antibodies to CD4 and CD8. (*C*) Thymocyte composition in Mst1-null and wild-type mice. There is a non-significant increase in the mean percentage of $CD4^+$ and $CD8^+$ thymocytes in Mst1-null mice compared with wild-type mice ($P = 0.13$ and 0.25 respectively) but no difference in total numbers of these cells ($n = 6$ pairs of Mst1-null and wild-type littermates examined in two experiments).

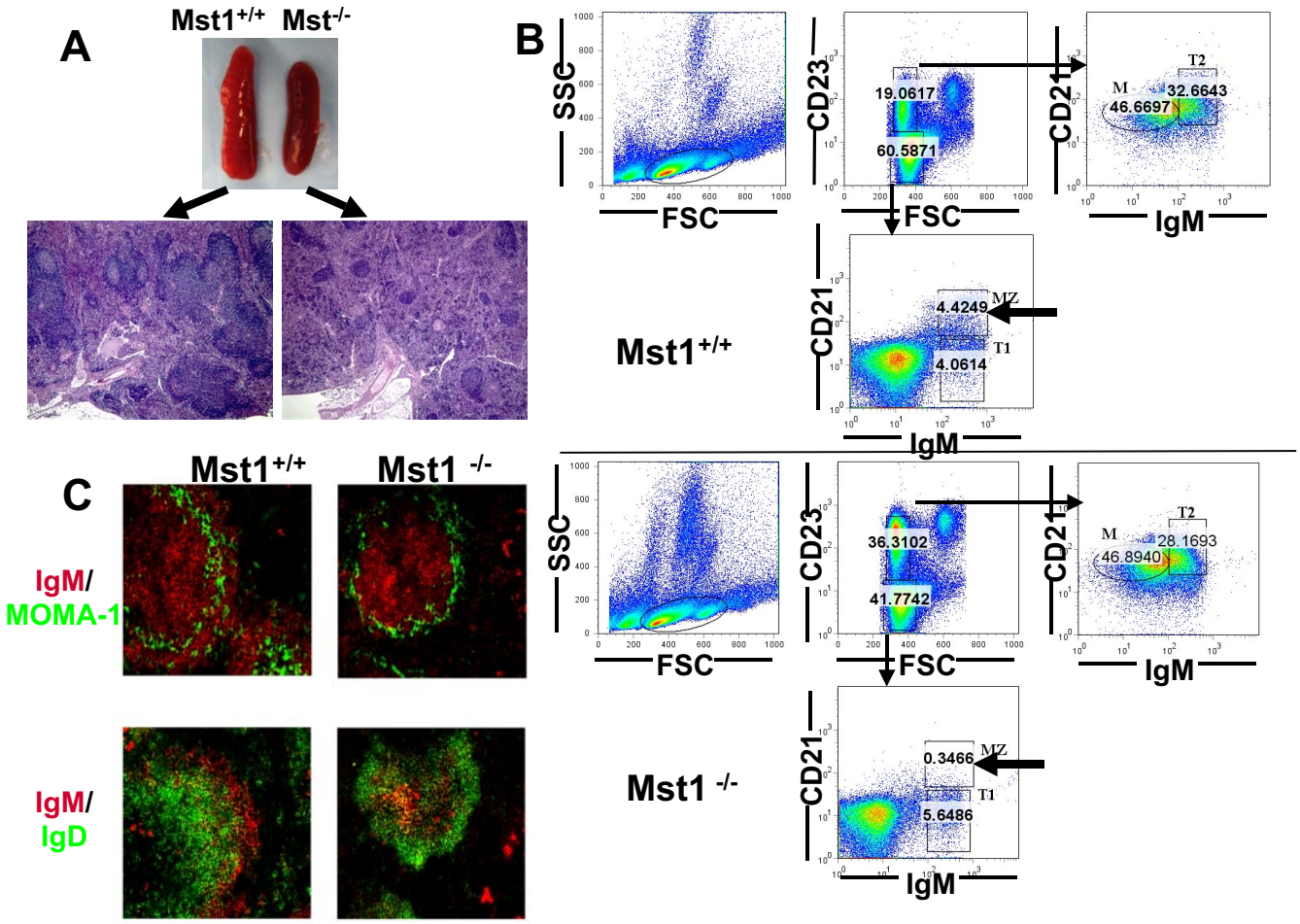

Fig. S3. Spleens from Mst1 null mice lack marginal zone B cells. (*A*) Comparison of the spleens of wild-type and Mst1 littermates. In the upper panel, the spleen of the Mst1-null mouse (right) is more deeply red than wild type. The representative H&E-stained sections (original magnification 10×) show that the Mst1 null spleen (right) has smaller and fewer lymphoid follicles (white pulp) and an expansion of the blood-filled spaces (red pulp) as compared with wild type. (*B*) Splenic B cell subsets in Mst1-null and wild-type mice. Representative dot plots of splenocytes isolated from Mst1-null and wild-type mice stained with antibodies to CD23, IgD, IgM, and CD21 and analyzed according to published protocol (10). Cells were first gated based on CD23 expression ($CD23^+$ or $CD23^-$) and then analyzed for CD21, IgD, and IgM expression. In Mst1-null mice, the MZ B cell population is significantly reduced when compared to wild-type littermates. Data shown is representative of three separate experiments. (*C*) Immunocytochemical staining of Mst1-null and wild-type spleen for IgM, IgD, and MOMA-1. In the upper panels, anti-MOMA-1FITC(green) marks the border of the follicle marginal zone; note the absence of biotin-IgM (visualized with Texas red-streptavidin) beyond the ring of MOMA-1 in the Mst1-null spleen (right). In the lower panels, the anti-IgM (red) extends beyond the IgD-rich (FITC;green) follicle in the wild type (left), but not in the Mst1 null (right).

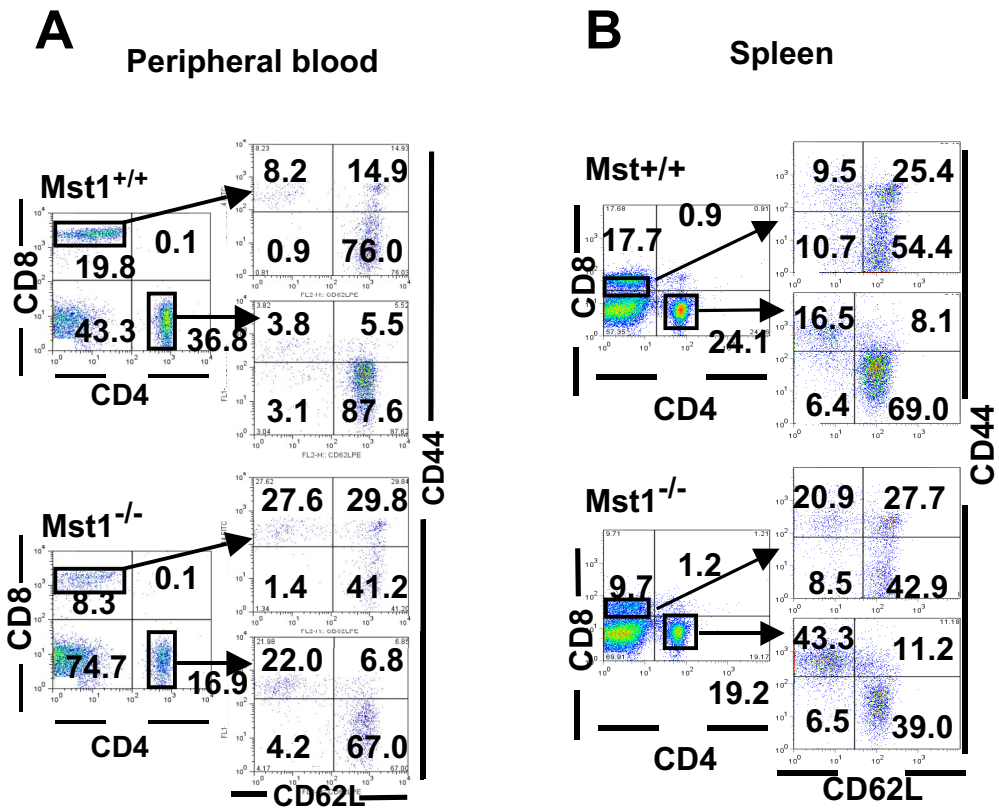

Fig. S4. T cell populations in the Mst1-null and wild-type circulation and spleen. Representative flow cytometric analysis of T cells isolated from the circulation (*A*) and spleen (*B*) of Mst1-null and wild-type littermates, using antibodies to CD4, CD8, CD62L, and CD44.

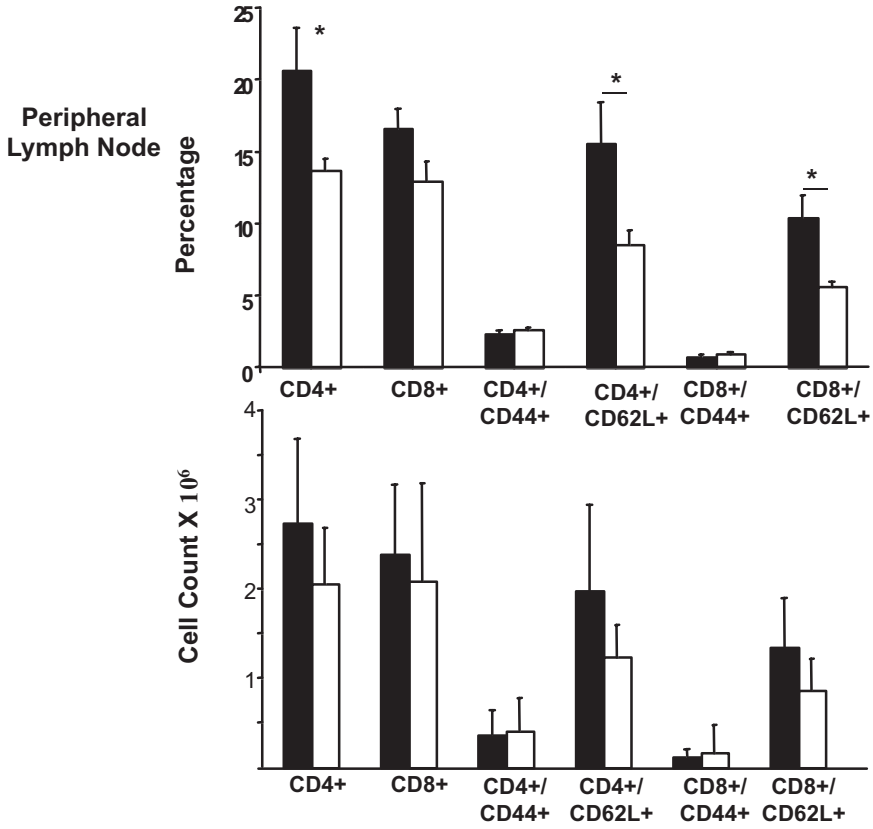

Fig. S5. T cell subsets in peripheral lymph nodes of Mst1-null and wild-type mice. Flow cytometric analysis of cells isolated from matched lymph nodes of Mst1-null and wild-type littermates, using antibodies to CD4, CD8, CD62L, and CD44. There is a consistent and significant reduction in the percentage of $CD4^+$, $CD8^+$, and especially of naïve $CD4^+$ and $CD8^+$ T cells in the peripheral lymph nodes of Mst1-null mice compared with wild-type mice ($n = 6$ pairs of Mst1-null and wild-type littermates examined in two experiments).

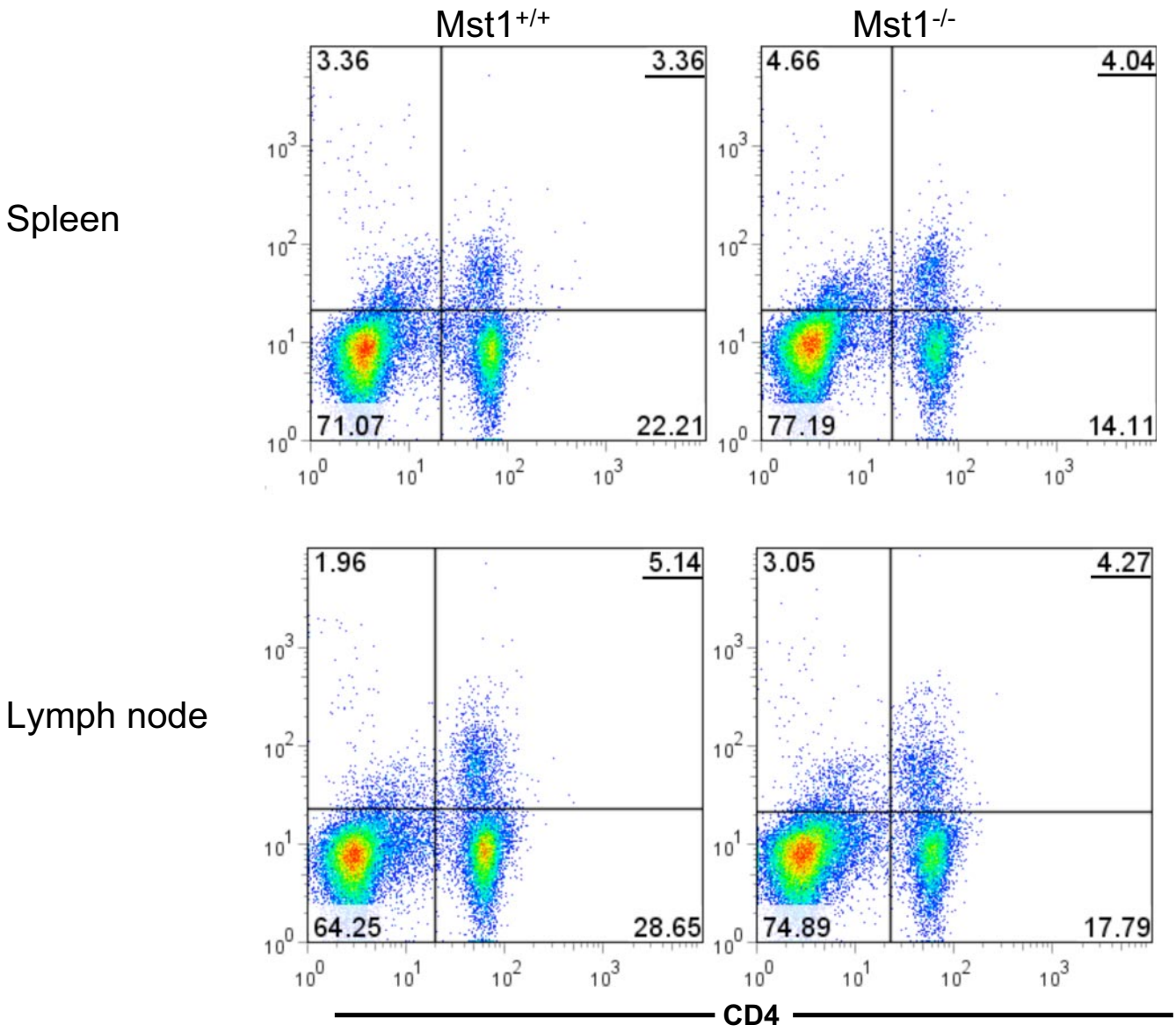

Fig. S6. The relative abundance of CD4$^+$/Foxp3$+$ cells in secondary lymphoid organs of Mst1-null and wild-type mice. Representative dot plots of cells isolated from spleen and lymph nodes of Mst1-null and wild-type mice, stained with antibodies to CD4, and Foxp3 and analyzed by flow cytometry.

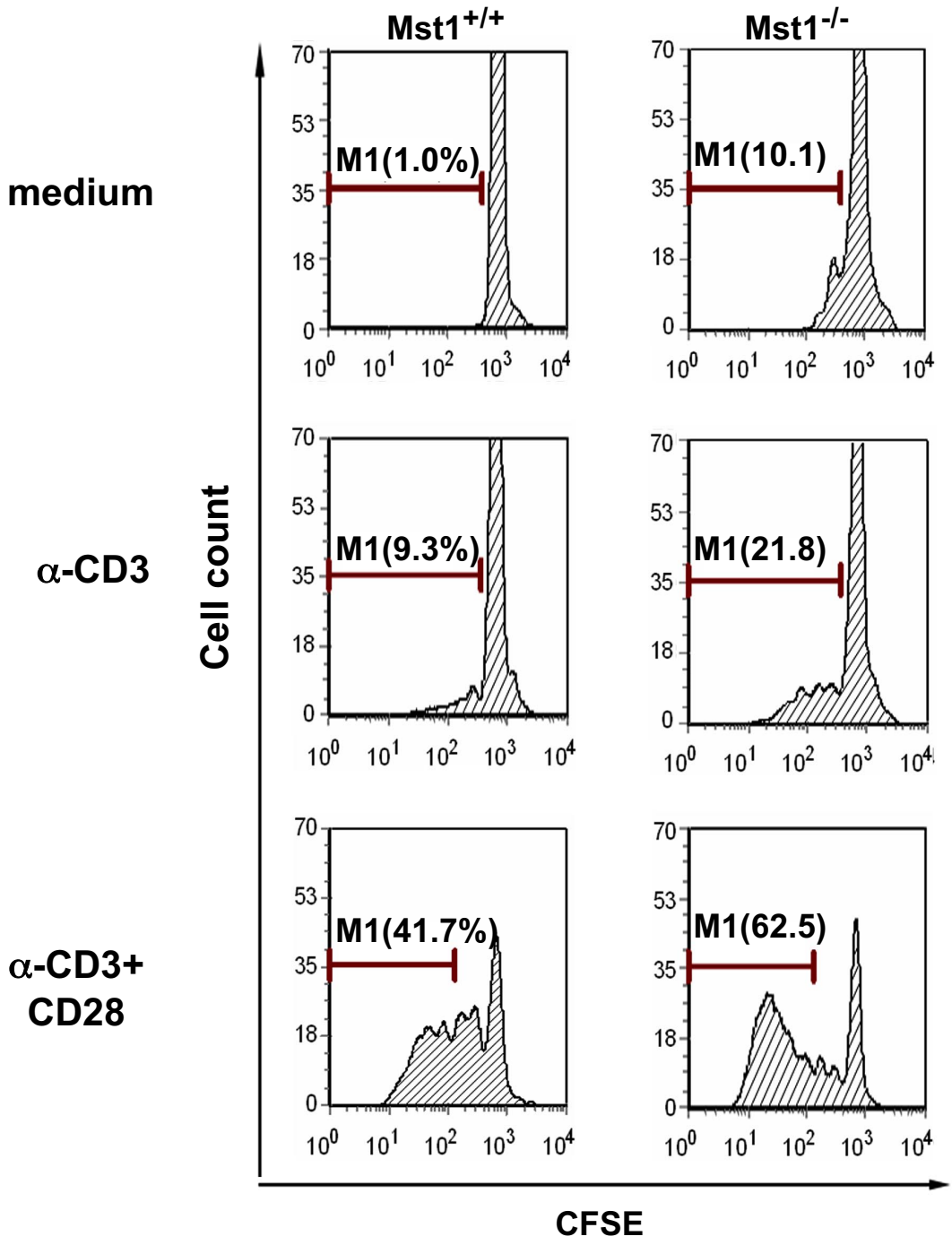

Fig. S7. Cell division in Mst1-null and wild-type T cells. Total splenic T cells were labeled with CFSE and were incubated without stimulus (upper panels) or stimulated with anti-CD3 (middle panels) or anti-CD3+ anti-CD28 (lower panels) for 48 h. Cells were collected and assayed for CFSE fluorescence. A second experiment gave similar results.

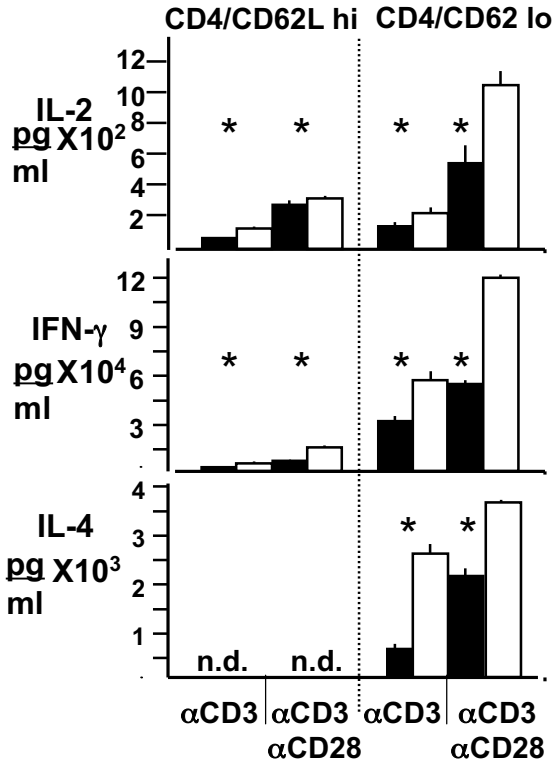

Fig. S8. Mst1-null CD4$^+$ cells exhibit enhanced cytokine production following activation. Culture supernatants from wild-type (black bars) or Mst1-null (white bars) cells were assayed for IL-2 and IL-4 on day 1 and for IFN-γ on day 2 after stimulation with α-CD3 or α-CD3+α-CD28; n.d., not detected. An asterisk indicates the difference is significant, $P < 0.05$ or less. Data are representative of four experiments.

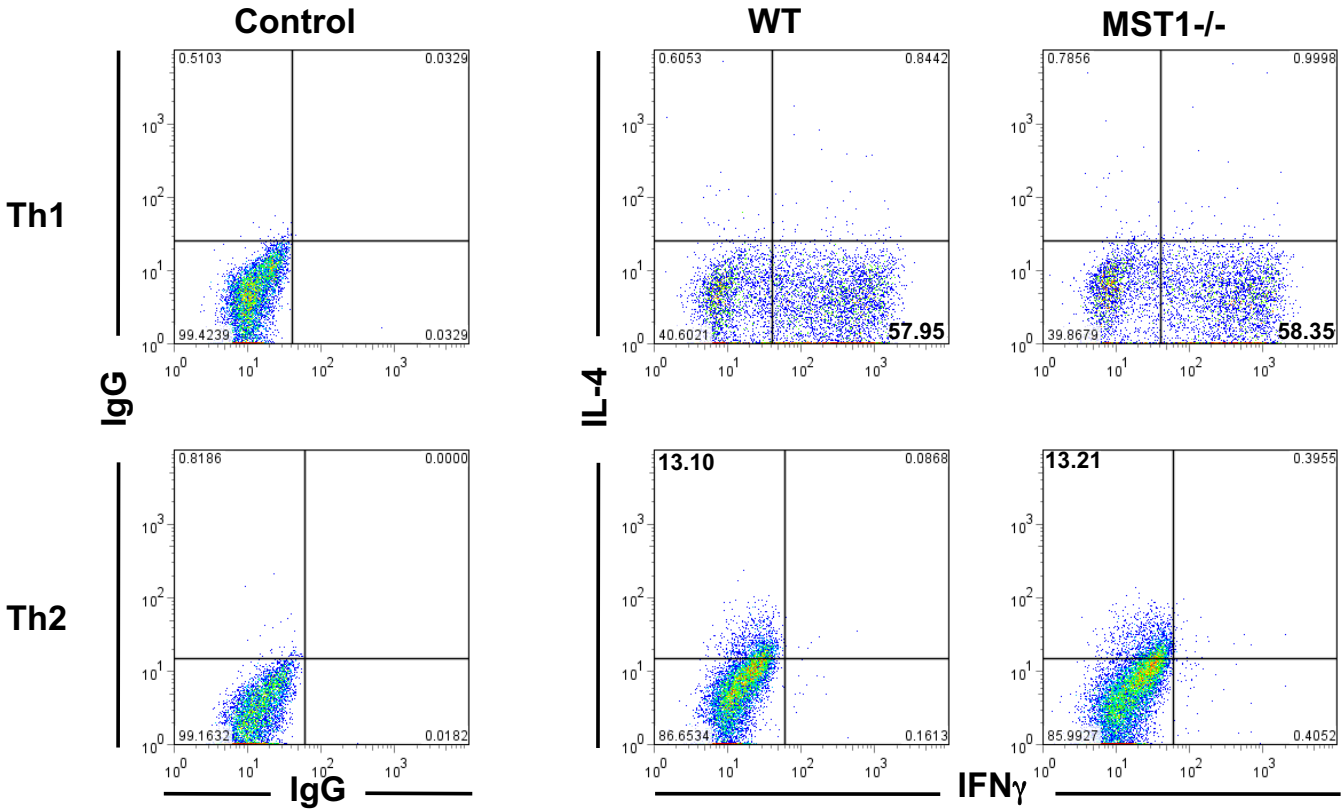

Fig. S9. Generation of Th1 and Th2 CD4$^+$ T cells in vitro. CD4/CD62Lhi cells from wild-type and Mst1-null mice were incubated *in vitro* for six days under conditions promoting polarization toward a Th1 or Th2 phenotype, as described in methods. Cytokine expression by intracytoplasmic staining was assayed by flow cytometry. Numbers indicate the percentage of cells in each quadrant, and are representative of three separate experiments.

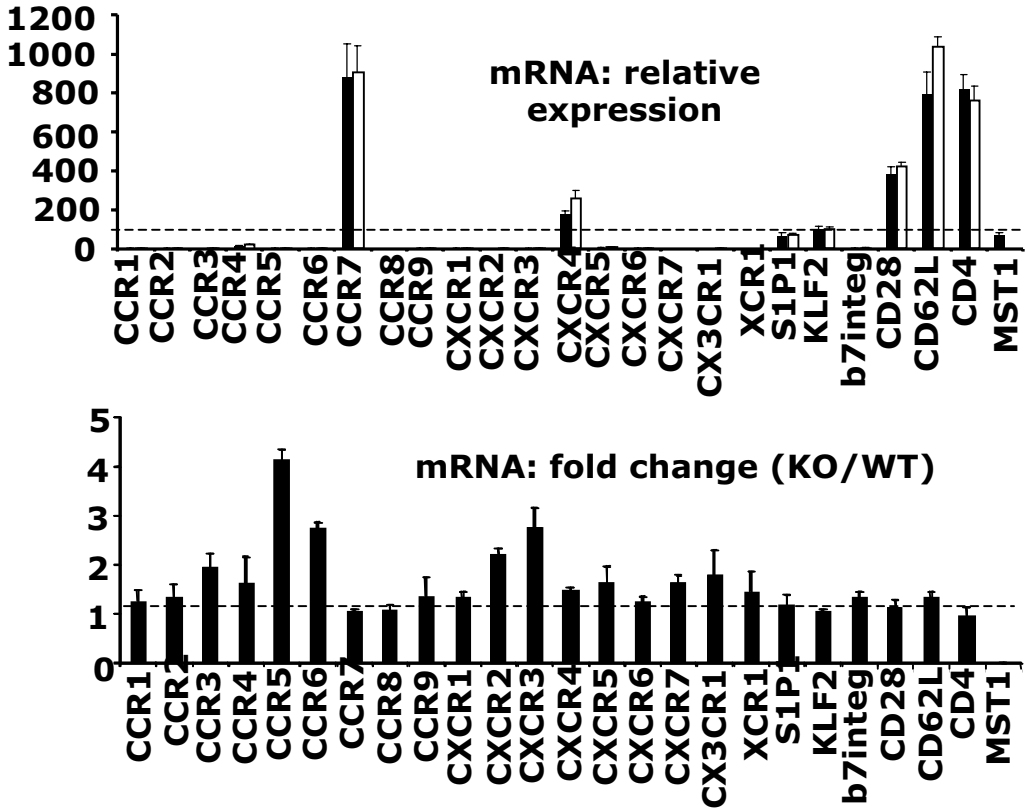

Fig. S10. Expression of mRNAs encoding KLF2, adhesion, and chemokine receptors in CD4/CD62Lhi T cells from wild-type and Mst1-null mice. Upper, Q-PCR measurements of the relative mRNA copy number, with the value for KLF2 in wild-type cells (black bars) set to 100. Lower, shows the ratio of mRNA expression with the value in the Mst1-null cells divided by that in the wild-type cells. Error bars indicate ±1 SD. Representative of two experiments.

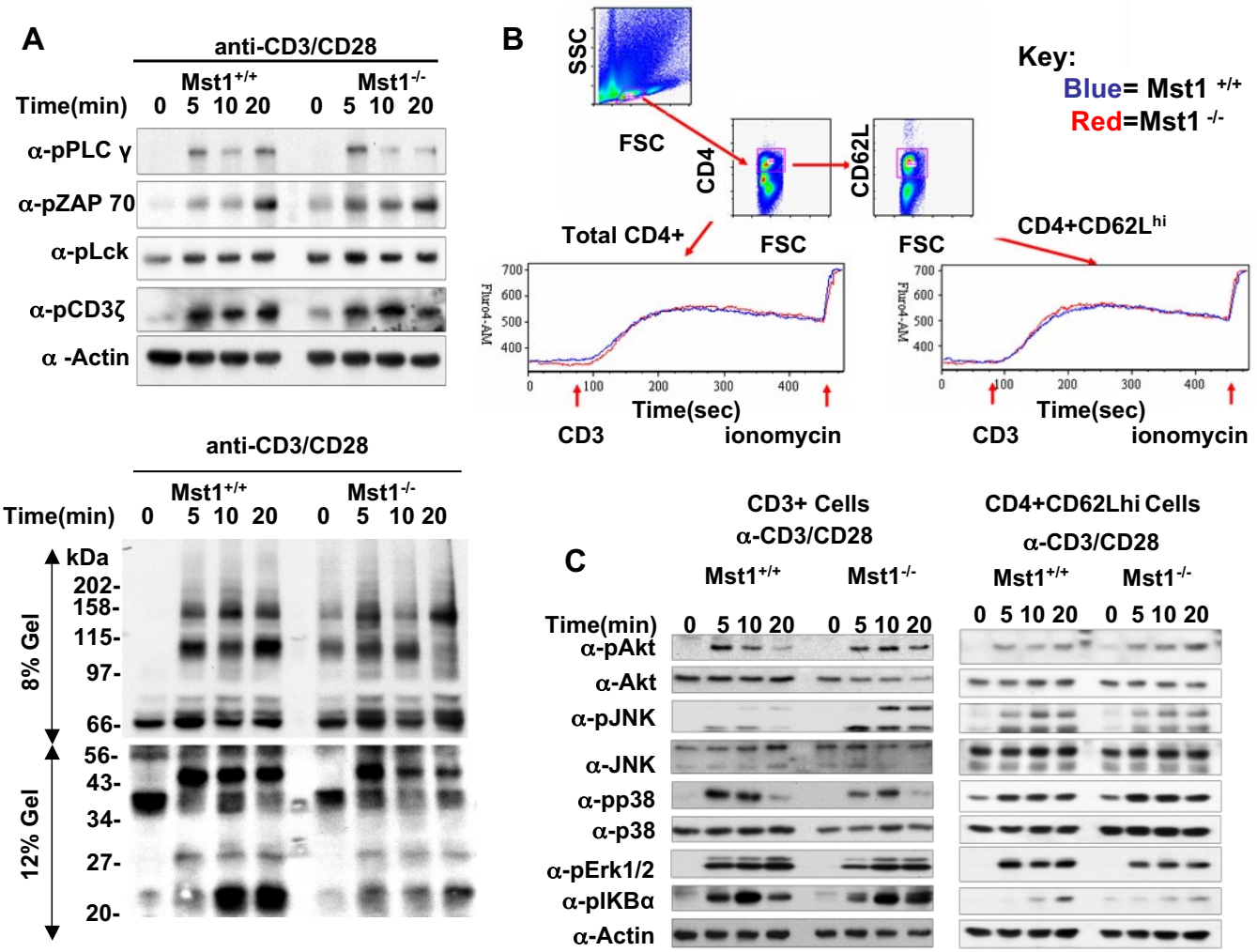

Fig. S11. Tyrosine phosphorylation, intracellular Ca^{++} and protein kinase activation in T cells from wild-type and Mst1 null mice. (*A*) Tyrosine phosphorylation in antiCD3 + anti-CD28 stimulated T cells from Mst1-null and wild-type mice. Lysates were prepared from antiCD3 + anti-CD28 stimulated splenic Mst1-null and wild-type T cells and immunoblotted with anti-phosphotyrosine specific antibodies specifically directed at PLC γ, ZAP-70, Lck, and CD3ζ (top panels) or at whole cell proteins separated on 8% and 12% SDS-polyacrylamide gels (bottom). (*B*) Calcium responses to CD3 cross-linking in $CD4^+$/CD62Lhi cells from Mst1-null and wild-type mice. Splenic T cells were loaded with Fura4 washed and labeled with anti-CD4 and anti-CD62L. After gating as indicated, a baseline signal was obtained for 0.5 min with the addition thereafter of anti-CD3 followed immediately by goat anti-Armenian hamster antibodies. After eight minutes, ionomycin was added and further signal was obtained. (*C*) Akt, MAP kinase, and IκB phosphorylation in Mst1-null and wild-type T cells. Splenic $CD3^+$ (left) and $CD4^+$/CD62Lhi (right) cells were isolated from Mst1-null and wild-type littermates. After resting *in vitro*, the cells were stimulated with anti-CD3 + anti-CD28; extracts prepared at the times indicated were subjected to immunoblot with the antibodies designated. Actin served as loading control. The middle lane contains M_r markers.