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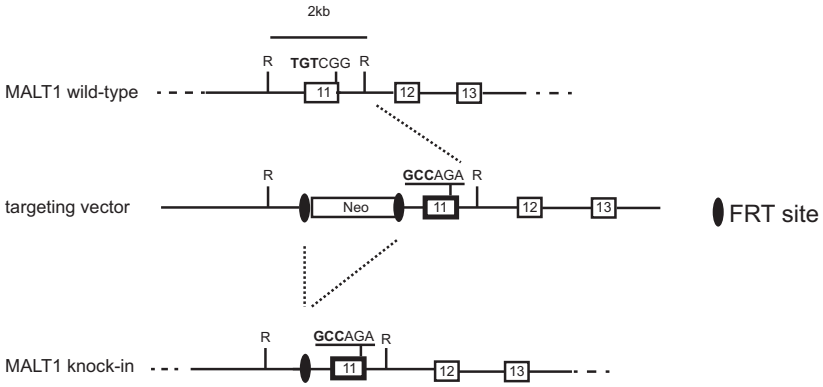

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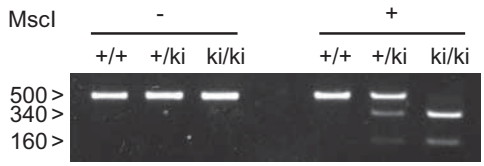

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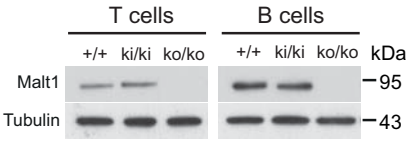
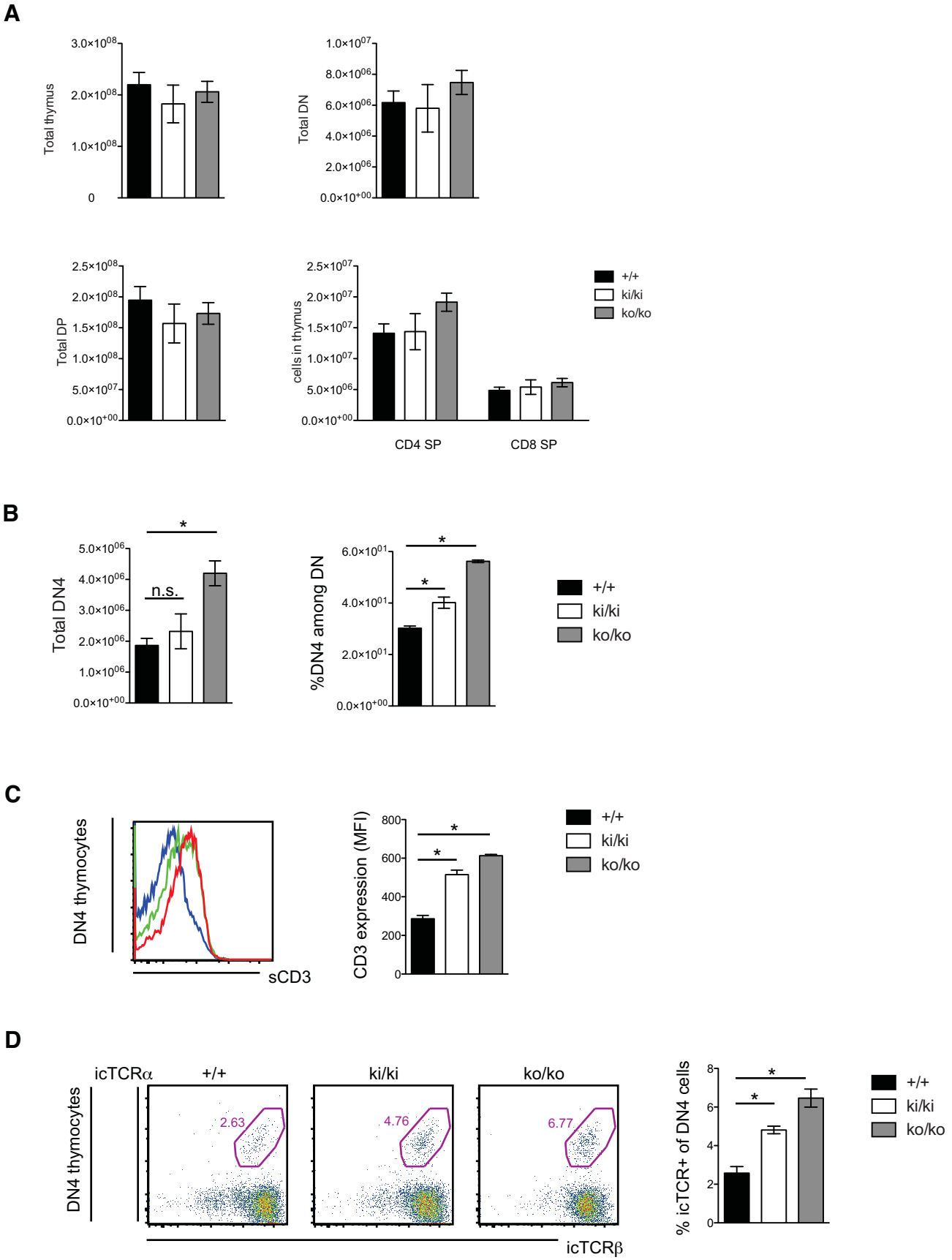
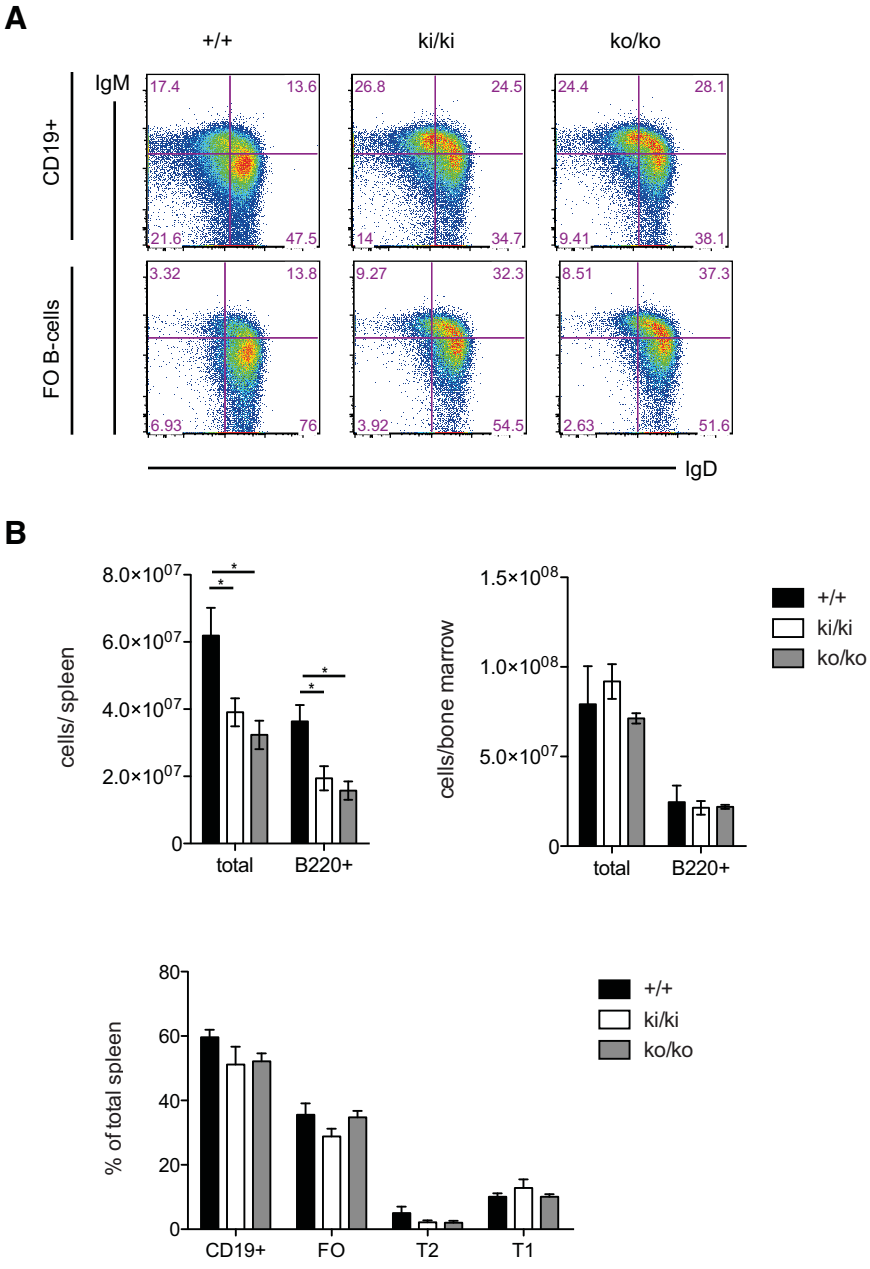


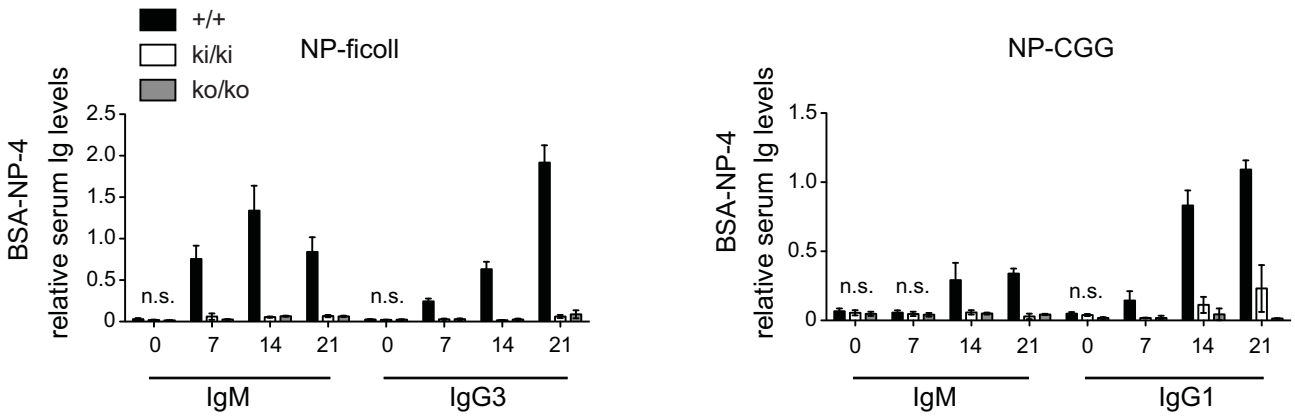
Jaworski et al., Figure S1



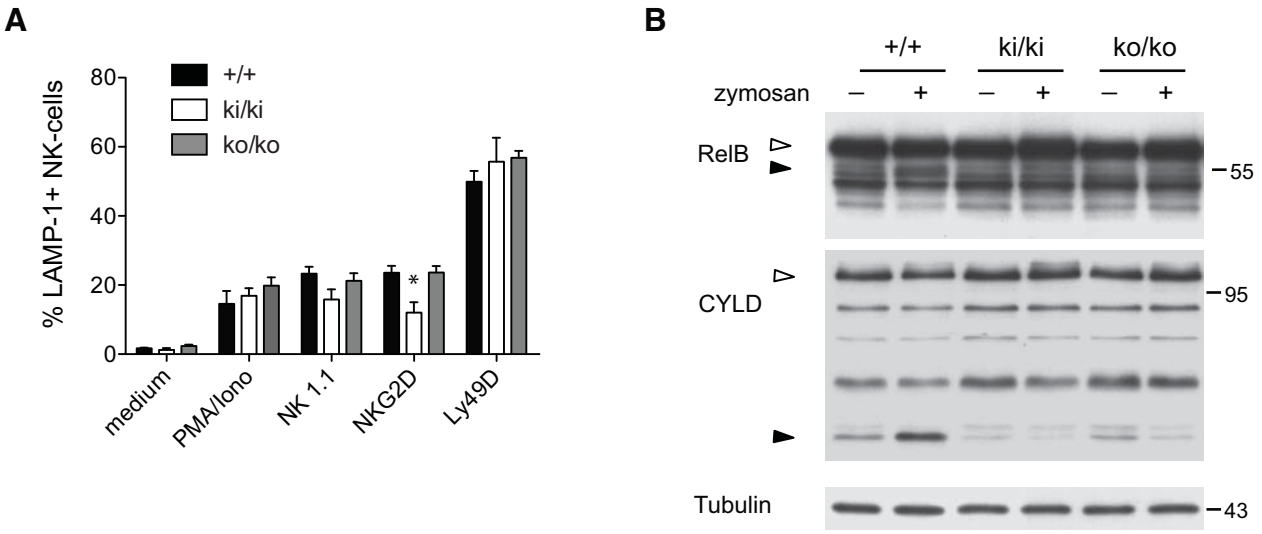
Jaworski et al., Figure S2



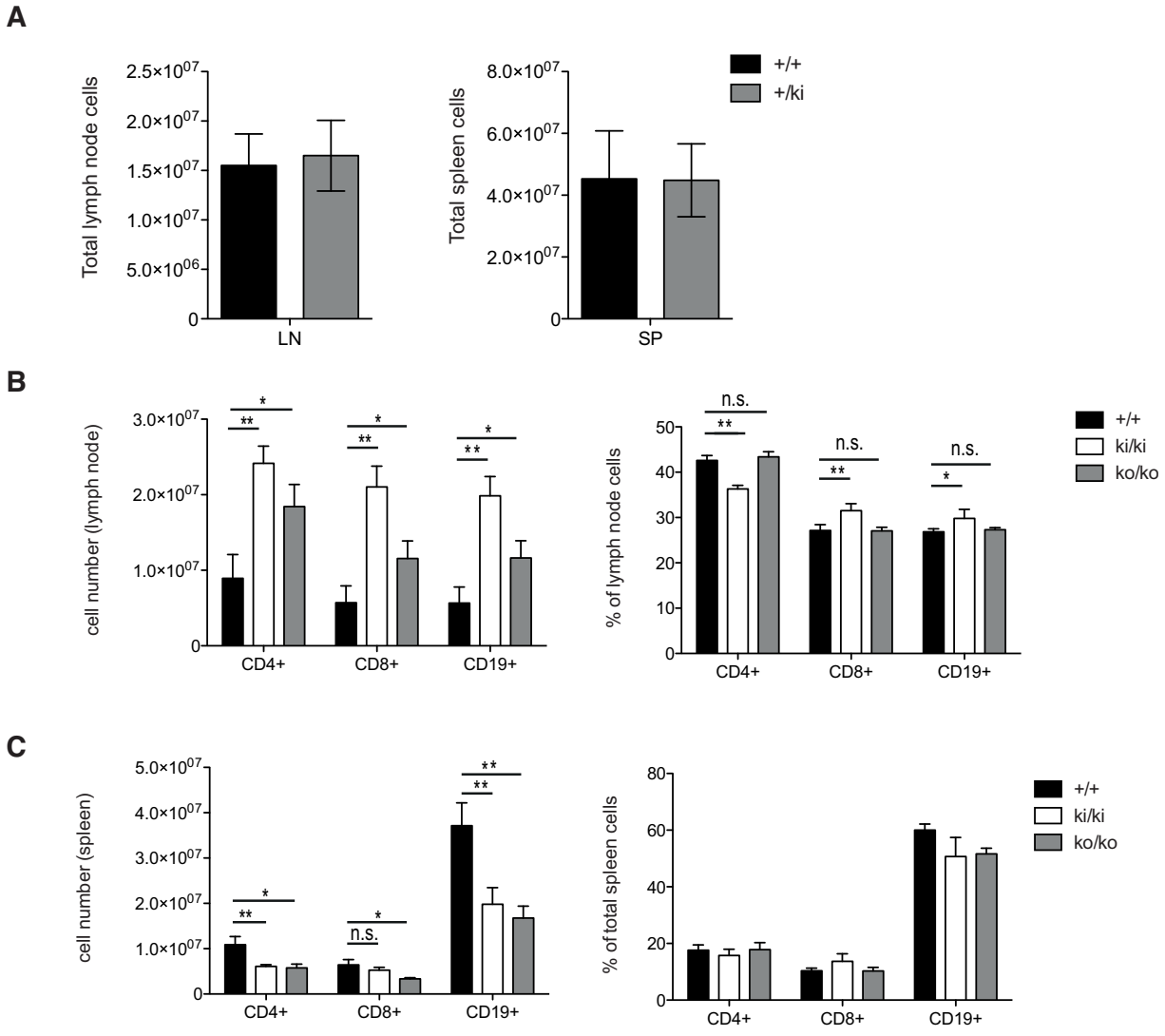
Jaworski et al., Figure S3



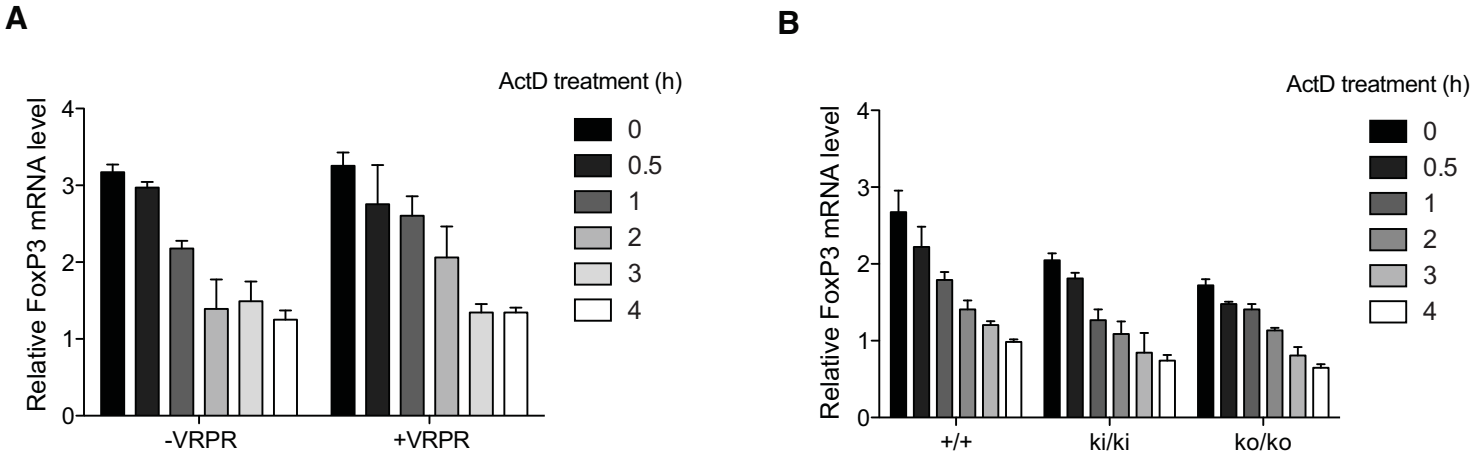
Jaworski et al., Figure S4



Jaworski et al., Figure S5

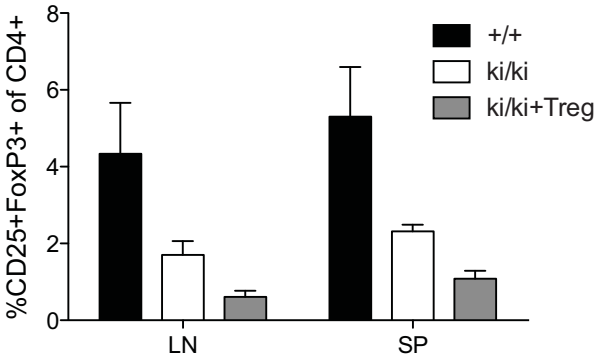


Jaworski et al., Figure S6



Jaworski et al., Figure S7

A



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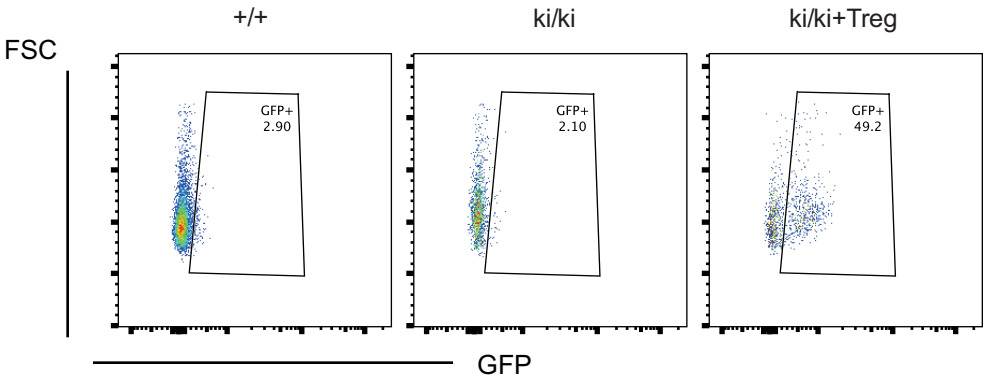

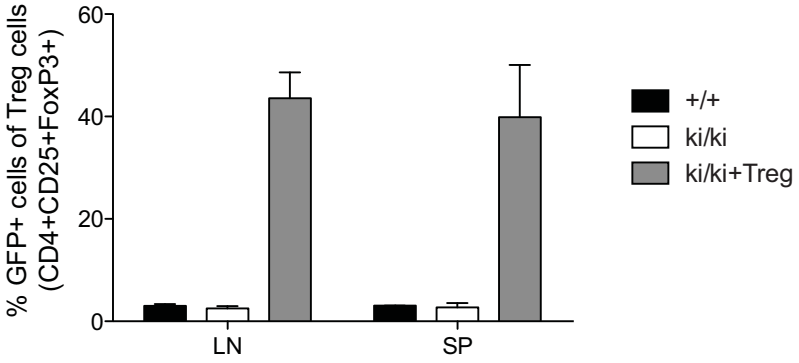

Jaworski et al., Figure S8

Supplementary figure legends

Figure S1. Generation of Malt1 C472A knock-in mice expressing a catalytically inactive form of Malt1.

A The targeting vector carries a TGTCGG -> GCCAGA mutation (nucleotides 168-173 of exon 11 of the MALT1 gene) leading to a C472A mutation in the Malt1 protein, and a neomycin resistance gene (Neo) that is flanked by flippase recombinase target (FRT) sites. In the Malt1 knock-in mice used for this study, the neomycin resistance cassette was removed by crossing to mice expressing the flippase recombinase (FLP). The position of naturally occurring EcoRI sites (R) is indicated.

B Wild-type mice (+/+), Malt1 C472A knock-in mice (ki/ki) and heterozygous littermates (+/ki) were genotyped by PCR amplification of a 500 bp fragment comprising the TGTCGG -> GCCAGA mutation of exon 11, which leads to a C472A mutation in the Malt1 protein and simultaneously generates a MscI restriction site that is absent in the wild-type gene. In the presence of the inactivating Malt1 mutation, digestion of the PCR fragment with MscI yields two fragments of 340 and 160 bp.

C Immunoblot analysis of peripheral T cells and B cells of wild-type (+/+), Malt1 C472A knock-in (ki/ki) and Malt1-deficient (ko/ko) mice for the levels of Malt1. Immunoblotting for tubulin served as loading control. Data are representative of two independent experiments.

Figure S2. **Analysis of thymocyte subpopulations in Malt1 knock-in mice**

A Analysis of the total number of thymocytes, double negative $CD4^-CD8^-$ (DN), double positive $CD4^+CD8^+$ (DP), and of $CD4^+$ or $CD8^+$ single positive (SP) thymocytes in 6 week-old wild-type (+/+), Malt1 C472A knock-in (ki/ki) and Malt1-deficient (ko/ko) mice. Thymocytes were stained with a lineage mix containing antibodies against B220, γδTCR, NK1.1, CD11b and Ter119, and further subdivided by staining against CD4 and CD8. One representative experiment out of four performed is shown with n≥3 for each genotype.

B-D Flow cytometric analysis of thymocytes of 6 week-old wild-type (+/+), Malt1 C472A knock-in (ki/ki) and Malt1-deficient (ko/ko) mice (n≥3) for the total number and percentage of $CD4^-CD8^-$ double negative (DN) cells with a $CD25^-$ $CD44^-$ phenotype (DN4) (B), the levels of surface CD3 expression on DN4 thymocytes (C), and the levels of intracellular TCRα and TCRβ chains (icTCRα and icTCRβ, respectively) (D). One representative experiment out of four performed is shown. $*P < 0.0002$, unpaired t-test.

Figure S3. Analysis of splenic B-cell subsets in Malt1 knock-in mice.

A Flow cytometric analysis of the expression of surface IgM and IgD on $CD19^+$ and $CD19^+CD24^{lo}CD23^{hi}CD21^{lo}$ follicular (FO) splenic B cells.

B Analysis of the numbers of total and $B220^+$ bone marrow and spleen cells, and of the percentage of $CD19^+$, follicular (FO), $CD19^+CD24^{hi}CD21^{hi}CD23^{hi}$ (T2) and $CD19^+CD24^{hi}CD21^{lo}$ (T1) B cells in wild-type (+/+), Malt1 C472A knock-in (ki/ki) and Malt1-deficient (ko/ko) mice (n≥3). One representative experiment out of six performed is shown. $*P < 0.005$, unpaired t-test.

Figure S4. Analysis of immunization-induced immunoglobulin (Ig) levels.

Analysis of total immunization-induced immunoglobulin (Ig) levels in the serum of wild-type (+/+), knock-in (ki/ki) or knock-out (ko/ko) mice (n = 8; 8 weeks old). Immunizations were performed using i.p. injection of nitrophenyl (NP)-Ficoll (d), and serum was analyzed for the presence of high affinity antibodies using BSA-NP-4. Bars represent means ± SD and differences were statistically significant with $P<0.01$ (unpaired t-test), unless indicated otherwise (n.s., not significant).

Figure S5. Stimulation-induced LAMP-1 upregulation by NK cells.

A Analysis of the percentage of NK cells producing LAMP-1 following stimulation with PMA and ionomycin (PMA+Iono) or agonistic antibodies directed against NKG2D, NK1.1 or Ly49D. Bars represent mean ± SD, *$P<0.05$ (unpaired t-test). Data are representative of two experiments (n=4).

B Immunoblot analysis of BMDCs stimulated with or without zymosan (100 μg/ml) for 90 min for the cleavage of the Malt1 substrates RelB and CYLD. Immunoblotting for tubulin served as a loading control. Data are representative of two experiments.

Figure S6. Analysis of lymphocyte subsets of lymph nodes and spleens of Malt1 C472A knock-in mice

A Analysis of the lymph nodes and spleens of Malt1-proficient (+/+) and heterozygous littermates carrying one Malt1 knock-in allele (+/ki) ($n\geq3$; 8 weeks old). Total numbers of lymph node and spleen cells are shown. One representative experiment out of three performed is shown.

B Flow cytometric analysis of the total numbers and percentages of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes in the lymph nodes (LN) (A) and spleens (B) of wild-type (+/+), C472A knock-in (ki/ki) and Malt1-deficient (ko/ko) mice (n≥3, 6 weeks old). One representative experiment out of 4 performed is shown.

Bars represent mean ± SD, *$P < 0.05$, ** $P < 0.005$ (unpaired t-test).

Figure S7. Assessment of FoxP3 mRNA stability.

A Naïve $CD4^+$ T cells of wild-type mice were stimulated with anti-CD3 and anti-CD28 (1 μg/ml each) under polarizing conditions (mTGF-β, 5 ng/ml; hIL-2, 100 U/ml) for 96 h, treated for additional 24 h with z-VRPR-fmk, and then treated with actinomycin D for the indicated times (n=3). FoxP3 mRNA levels were analyzed by qPCR and are shown relative to SDHA levels.

B Naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 (1 μg/ml each) under polarizing conditions (mTGF-β, 5 ng/ml; hIL-2, 100 U/ml) for 48 h, and upon addition of Actinomycin D, FoxP3 mRNA levels were analyzed by qPCR at different time points (n=3, for each genotype T cells from 3 mice were pooled before stimulation). FoxP3 mRNA levels are shown relative to SDHA levels.

Data in A and B are representative of two experiments.

Figure S8. Detection of adoptively transferred Treg cells in the hosts.

A Analysis of the percentage of total $CD25^+$ $FoxP3^+$ cells amongst $CD4^+$ T cells isolated from lymph nodes and spleens of mice of the indicated genotypes, with or without adoptive Treg cell transfer.

B Flow cytometric analysis (upper panel) and quantification (lower panel) of the presence of adoptively transferred GFP^+ Treg cells amongst total Treg cells in mice of the indicated genotypes.

Data in A and B are representative of two experiments (n=3, analyzed 4.5 weeks after transfer).