Supporting Information

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SI Materials and Methods

Mice. DIETER double-transgenic mice allow tamoxifen-inducible presentation of three lymphocytic choriomeningitis virus (LCMV)-derived cytotoxic T lymphocyte (CTL) epitopes ($GP_{33–41}$/D^b, $GP_{34–41}$/K^b, and $NP_{396–404}$/D^b) and one β-galactosidase–derived CTL epitope (β-$gal_{497–504}$/K^b) by $CD11c^{high}$ dendritic cells (DCs) (1). P14 transgenic mice carry a transgenic T-cell receptor that recognizes the LCMV-gp–derived epitope GP33-41 in the context of H-$2D^b$ (2). SMARTA transgenic mice carry a transgenic T-cell receptor that recognizes the LCMV-gp–derived epitope GP61-80 in the context of I-A^b (3). FoxP3.LuciDTR-5 mice (4) allow the depletion of $FoxP3^+$ cells by diphtheria toxin. I-$A\alpha^{-/-}$ mice (5), PD-$1^{-/-}$ mice (6), $RAG1^{-/-}$ mice, and (7) CD11cDTR mice (8) have been described previously. All mice were obtained from the central animal facility of Johannes Gutenberg University of Mainz and were bred and maintained under specific pathogen-free conditions. All mice were on a C57BL/6J background. Experiments were performed with age- and sex-matched mice and were conducted with permission of the Landesuntersuchungsamt Rheinland-Pfalz (Protocol G08-1-002).

LCMV. LCMV strain WE (LCMV-WE) was propagated on L929 fibroblast cells at a low multiplicity of infection. LCMV titers were determined in spleen at 5 d after infection as described previously (9).

Antibodies and Flow Cytometric Analysis. Antibodies against mouse CD8 (YTS169.4), mouse CD4 (GK1.5), and mouse CTLA-4 (4F10) were purified from hybridoma culture supernatant using protein G Sepharose (Genscript). Tetrameric peptide–MHC complexes were generated and staining was performed as described previously (1, 10).

Fluorochrome-coupled antibodies for flow cytometry were purchased from eBioscience or BioLegend. Single-cell suspensions were incubated for 10 min with 0.5 μg/mL of anti-CD16/CD32 (clone 2.4G2) to block $F_c\gamma$ receptors, followed by staining with fluorochrome-coupled antibodies for 20 min at 4 °C. FoxP3 was detected using a FoxP3 Staining Kit (eBioscience) in accordance with the manufacturer's instructions.

Samples were analyzed with a, LSR-II Cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Peptides. LCMV-derived peptide GP33–41 (KAVYNFATM, H-$2D^b$) was purchased from NeoMPS in immunograde quality.

Generation of Bone Marrow Chimeras. Bone marrow (BM) was isolated by flushing tibias and femurs of donor mice with PBS. BM cells were depleted of T cells using anti-mouse pan T Dynabeads (Invitrogen) according to the manufacturer's instructions, mixed at the indicated ratios. Then 2–3 × 10^6 cells were injected i.v. into mice that had been lethally (9.5 Gy) irradiated using a ^{137}Cs source. Mice were given Borgal (1 mg/mL of sulfadoxin and 0.2 mg/mL of trimethoprim) in the drinking water for 3 wk and were rested for 6–8 wk after transplantation before being used in experiments. An equal take of mixed BM cell populations was verified by flow cytometric analysis of CD45.1 and CD45.2 expression on blood T lymphocytes.

DC Isolation and Phenotyping. Spleens and peripheral lymph nodes (pooled inguinal, brachial, axillary, and submandibular lymph nodes) from DIETER mice or MHC-$II^{-/-}$ + CD45.1 mixed BM chimeric mice were removed and digested with collagenase II (Worthington) and DNaseI (Sigma-Aldrich). Isolated cells were stained with antibodies against CD80, CD86, CD70, CD40, PDL-1, or the respective isotype controls, anti CD11c and anti-MHC class II, and analyzed by flow cytometry. DCs from DIETER mice were identified by gating on $CD11c^{high}$ and MHC class II^{high} cells. Isolated cells from MHC-$II^{-/-}$ + CD45.1 mixed BM chimeric mice were stained as described above, as well as for F4/80-APC, B220-APC, and CD90.2-APC to exclude non-DCs. DCs were identified as APC-negative, $CD11c^{high}$ cells. To distinguish between WT and MHC class II KO DCs, cells were stained for CD45.1. The median fluorescence intensities of expression markers CD80, CD86, CD70, CD40, and PDL-1 were calculated, and the median fluorescence intensities of the isotype controls were subtracted.

Regulatory T-Cell Isolation and in Vitro Suppression Assay. Splenic regulatory T cells from MHC-$II^{-/-}$ + CD45.1 mixed BM chimeric mice and WT mice were isolated using a biotinylated antibody against CD25 (clone 7D4) and streptavidin (SA) microbeads and LS columns (Miltenyi). $CD4^+$ T cells were isolated from WT spleens using biotinylated anti-CD4 (clone H129.19) and SA microbeads and LS columns (Miltenyi). $CD4^+$ T cells (Tconv) and Tregs from either WT mice or MHC $II^{-/-}$ + CD45.1 chimeric mice were incubated in Tconv:Treg cell ratios between 1 and 16 in round-bottomed 96-well plates. Mitomycin C-treated (60 μg/mL/10^7 cells for 30 min at 37 °C, washed three times) A20 B-cell lymphoma cells (2 × 10^3/well) as accessory cells, and anti-CD3 mAbs (145-2C11; 3 μg/mL) were used as stimulus in RPMI 1640 supplemented with 2 mM L-glutamine, 5 × 10^{-5} M β-mercaptoethanol, 10 IU penicillin, 100 μg/mL of streptomycin, and 10% FCS. After 48 h, [^{3}H]-thymidine was added to the cultures (0.5 μCi/well). [^{3}H]-Thymidine incorporation was assessed by scintillation counting after an additional 18 h of culture.

In Vivo Regulatory T-Cell Suppression Assay. $RAG1^{-/-}$ mice received 2 × 10^5 $CD4^+$ T cells i.v. that had been purified by MACS from the spleen of FoxP3.LuciDTR-5 mice that had been depleted of $FoxP3^+$ cells by injection of 1 μg of diphtheria toxin (Calbiochem). At the same time, some of the mice received 10^5 $CD4^+$ $CD25^+$ T cells purified as described above from C57BL/6 mice or MHC-$II^{-/-}$ + WT mixed BM chimeras. Mice were weighed weekly after cell transfer.

In Vivo Proliferation Assay. DIETER mice were adoptively transferred with 2 × 10^6 CFSE-labeled, MACS-purified $CD4^+$ T cells from SMARTA (CD90.1) TCR transgenic mice (CD90.1) or CFSE- labeled MACS-purified $CD8^+$ T cells from P14 TCR transgenic mice (CD45.1). Two days later, the mice were treated with tamoxifen and an agonistic antibody to CD40 (FGK45). As controls, mice were left untreated or infected with LCMV-WE. Seven days later, CFSE dilution of $CD90.1^+$ $CD4^+$ T cells or $CD45.1^+$ $CD8^+$ T cells was assessed in spleen and blood.

Induction of Antigen Presentation on DCs After Efficient Depletion of $FoxP3^+$ T Cells in Vivo. $Rag1^{-/-}$ mice were irradiated with 9.5 Gy and reconstituted i.v. with 70% BM from $RAG1^{-/-}$ DIETER mice and 30% BM from FoxP3.LuciDTR-5 mice, together with 2 × 10^5 $CD25^+$ T cells from FoxP3.LuciDTR-5 mice to prevent spontaneous autoimmunity (11). In the resulting BM chimeras, 70% of the DCs were of DIETER origin and could be induced to express the transgenic CTL epitopes, but all T cells were of FoxP3.LuciDTR-5 origin and could be efficiently

depleted of FoxP3$^+$ regulatory T cells by diphtheria toxin injection. At 8 wk after reconstitution, antigen presentation on mouse DCs was induced by injection of 2 mg of tamoxifen i.p., and FoxP3$^+$ Tregs were depleted by i.v. injection of 30 ng/g body weight of diphtheria toxin (Calbiochem) on days −1, 0, 1, and 3 or depleted of CD4$^+$ T cells by i.v. injection of 0.5 mg of GK1.5. On day 7 after tamoxifen injection, the frequency of D^b/GP33-41– and K^b/βGal497-505–specific T cells in the blood was determined by staining with tetrameric MHC–peptide complexes.

In Vivo Depletion of MHC Class II-Deficient DCs. To allow depletion of MHC class II-deficient DCs from MHC-II$^{-/-}$ + WT mixed BM chimeric mice, the CD11cDTR transgene (8) was crossed into MHC-II$^{-/-}$ mice. Mixed BM chimeras were generated by injecting 50% MHC-II$^{-/-}$ CD11cDTR and 50% WT BM into lethally irradiated C57BL/6 mice. Recipients were depleted of MHC-II$^{-/-}$ DCs by two weekly injections of 100 ng of diphtheria toxin (Calbiochem) starting at 3 wk after reconstitution. At 7 wk after reconstitution, mice were killed, and mononuclear infiltration in the pancreas was assessed on H&E-stained sections.

1. Probst HC, Lagnel J, Kollias G, van den Broek M (2003) Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8$^+$ T cell tolerance. *Immunity* 18: 713–720.
2. Pircher H, et al. (1990) Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature* 346:629–633.
3. Oxenius A, Bachmann MF, Zinkernagel RM, Hengartner H (1998) Virus-specific MHC class II-restricted TCR-transgenic mice: Effects on humoral and cellular immune responses after viral infection. *Eur J Immunol* 28:390–400.
4. Suffner J, et al. (2010) Dendritic cells support homeostatic expansion of Foxp3$^+$ regulatory T cells in Foxp3.LuciDTR mice. *J Immunol* 184:1810–1820.
5. Köntgen F, Süss G, Stewart C, Steinmetz M, Bluethmann H (1993) Targeted disruption of the MHC class II *Aa* gene in C57BL/6 mice. *Int Immunol* 5:957–964.
6. Nishimura H, Nose M, Hiai H, Minato N, Honjo T (1999) Development of lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141–151.
7. Mombaerts P, et al. (1992) RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869–877.
8. Jung S, et al. (2002) In vivo depletion of CD11c$^+$ dendritic cells abrogates priming of CD8$^+$ T cells by exogenous cell-associated antigens. *Immunity* 17:211–220.
9. Battegay M, et al. (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *J Virol Methods* 33:191–198.
10. Altman JD, et al. (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94–96.
11. Bénard A, Ceredig R, Rolink AG (2006) Regulatory T cells control autoimmunity following syngeneic bone marrow transplantation. *Eur J Immunol* 36:2324–2335.

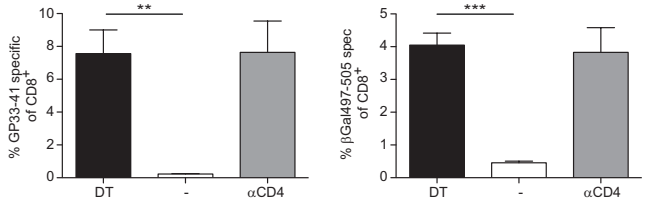

Fig. S1. Depletion of all CD4$^+$ cells or FoxP3$^+$ regulatory cells results in comparable CTL priming by steady-state DCs. Rag1$^{-/-}$ DIETER + FoxP3.LuciGDL-5 mixed BM chimeric mice were depleted of CD4$^+$ T cells (gray bars) or FoxP3$^+$ regulatory T cells (black bars) or left untreated (open bars), and antigen presentation on steady-state DCs was induced by tamoxifen injection. At day 7, the frequency of CD8$^+$ T cells specific for D^b/GP33-41 (*Left*) and K^b/βGal497-505 (*Right*) in the blood was determined by staining with tetrameric MHC–peptide complexes.

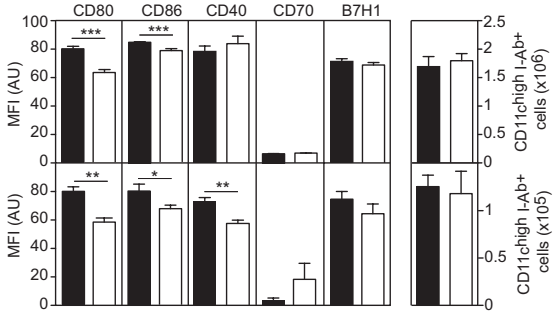

Fig. S2. DC activation, but no change in DC numbers, as a result of depletion of CD4$^+$ cells. C57BL/6 mice were injected i.v. with 0.5 mg of GK1.5 (closed bars) or isotype control (open bars), and expression of activation markers on splenic (*Upper*) and pooled lymph node (*Lower*) DCs was quantified by fluorescence-activated cell sorting analysis. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Median fluorescence intensities of five mice are shown. Results are representative of three independent experiments.

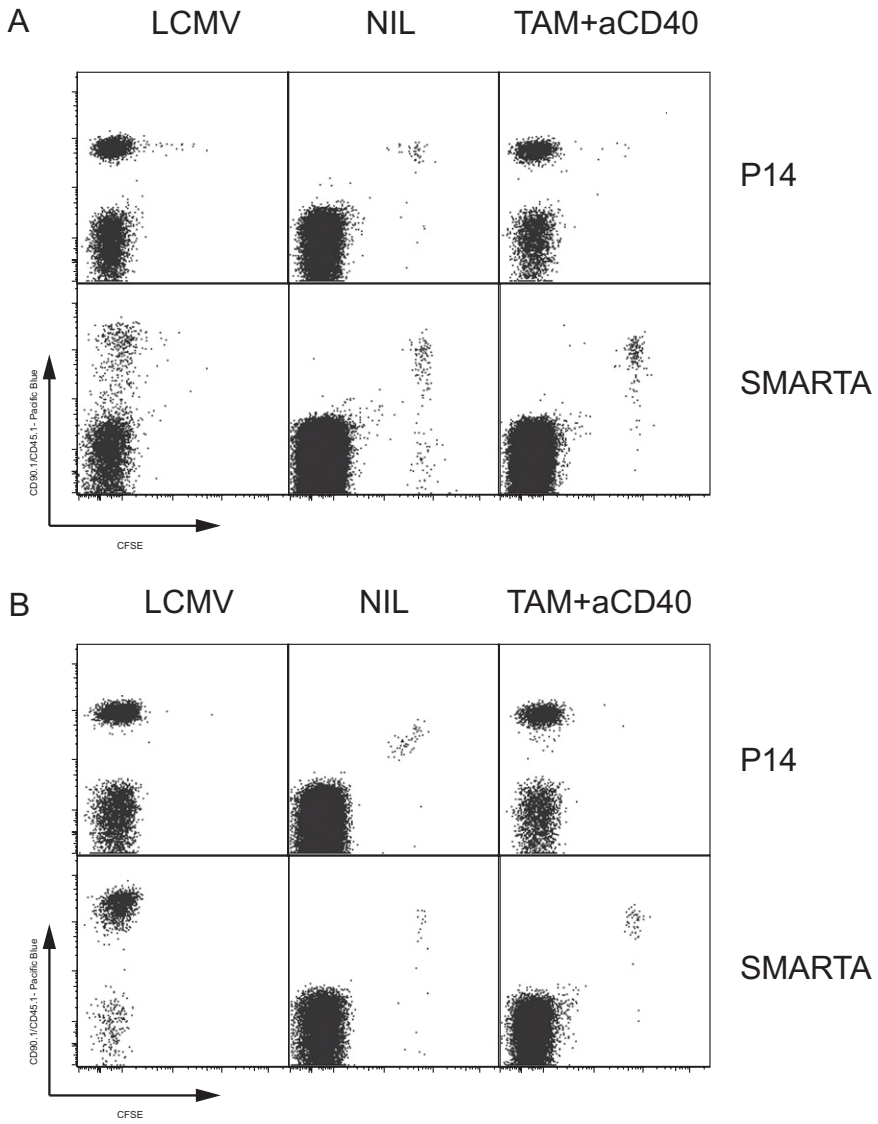

Fig. S3. MHC class I-restricted, but not MHC class II-restricted, transgenic epitopes are present in DIETER mice after tamoxifen induction. DIETER mice were adoptively transferred with CFSE-labeled SMARTA $CD4^+$ T cells (CD90.1) carrying a TCR specific for the I-A^b–restricted LCMV epitope GP61-80 or CFSE-labeled P14 $CD8^+$ T cells (CD45.1) specific for the H2-D^b–restricted LCMV epitope GP33-41. Both epitopes are part of the fusion protein expressed transgenically in DIETER mice after tamoxifen induction. Two days later, mice were treated with tamoxifen and an agonistic antibody to CD40 to induce expression of the antigen in activated DCs. As controls, some mice were left untreated or were infected with LCMV-WE. Seven days later, proliferation was assessed in spleen (*A*) and blood (*B*) by analyzing CFSE dilution. Plots are gated on $CD4^+CD90.1^+$ lymphocytes (SMARTA) or $CD8^+CD45.1^+$ lymphocytes (P14).

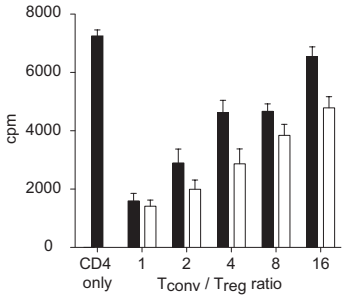

Fig. S4. $CD4^+$ $CD25^+$ regulatory T cells isolated from MHC-$II^{-/-}$ + WT BM chimeric mice show normal suppressive function in vitro. $CD25^+$ $CD4^+$ regulatory T cells from WT + WT (closed bars) and MHC $II^{-/-}$ + WT (open bars) mixed BM chimeric mice were isolated by magnetic cell sorting. Suppressive capacity of isolated Tregs was tested by incubation with $CD4^+$ T cells (Tconv) and anti-CD3 and quantitation of proliferation by ^{3}H-thymidine incorporation. One representative experiment out of two experiments is shown.

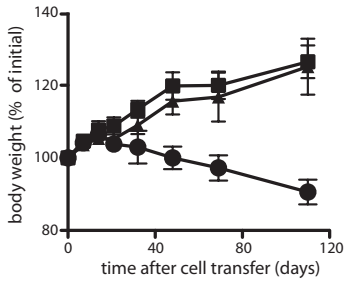

Fig. S5. CD4$^+$ CD25$^+$ regulatory T cells isolated from MHC-II$^{-/-}$ + WT chimeric animals suppress autoimmunity in vivo. RAG1$^{-/-}$ mice were injected with 2×10^5 CD4$^+$ T cells that had been depleted of FoxP3$^+$ cells, either alone (circles) or together with 1 10^5 CD4$^+$ CD25$^+$ cells isolated from MHC-II$^{-/-}$ + WT chimeras (squares) or WT mice (triangles). Body weight was monitored over the course of 15 wk.

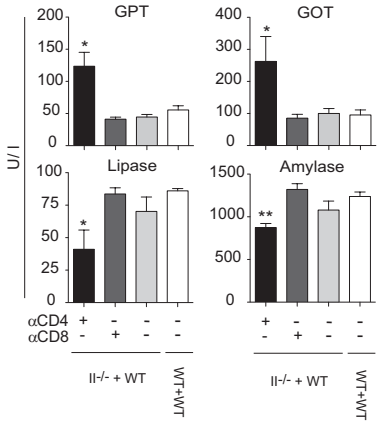

Fig. S6. Release of antigen-presenting cells (APCs) from CD4$^+$ T-cell control results in autoimmunity toward liver and pancreas. Serum levels of amylase, lipase, and transaminases in untreated or CD4-depleted MHC-II$^{-/-}$ + WT and WT + WT mixed BM chimeric mice at 7 wk after reconstitution.

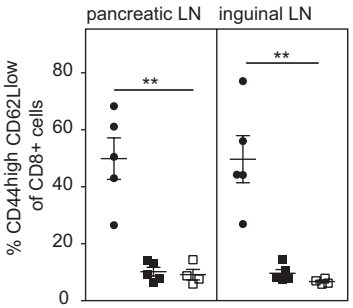

Fig. S7. Release of APCs from CD4$^+$ T-cell control results in CD8$^+$ T-cell activation. Percentage of activated (CD44^{high}CD62L^{low}) T cells among total CD8$^+$ T cells in pancreatic and inguinal lymph nodes at 7 wk after reconstitution. *$P < 0.05$; **$P < 0.01$. $n = 10$ per group. Results are representative of three independent experiments.

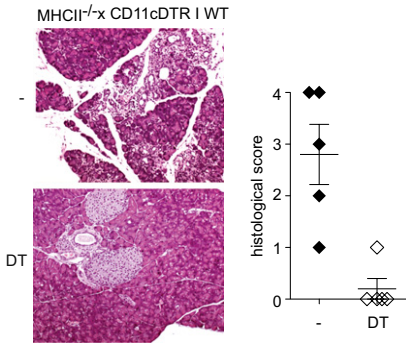

Fig. S8. Autoimmunity in MHC-II$^{-/-}$ + WT chimeras is driven by MHC-II$^{-/-}$ DCs. Histological analysis of the pancreas of CD11cDTRxMHC class-II$^{-/-}$ + WT mixed BM chimeric mice that had been depleted of MHC II-deficient DCs by diphtheria toxin treatment or control treatment.