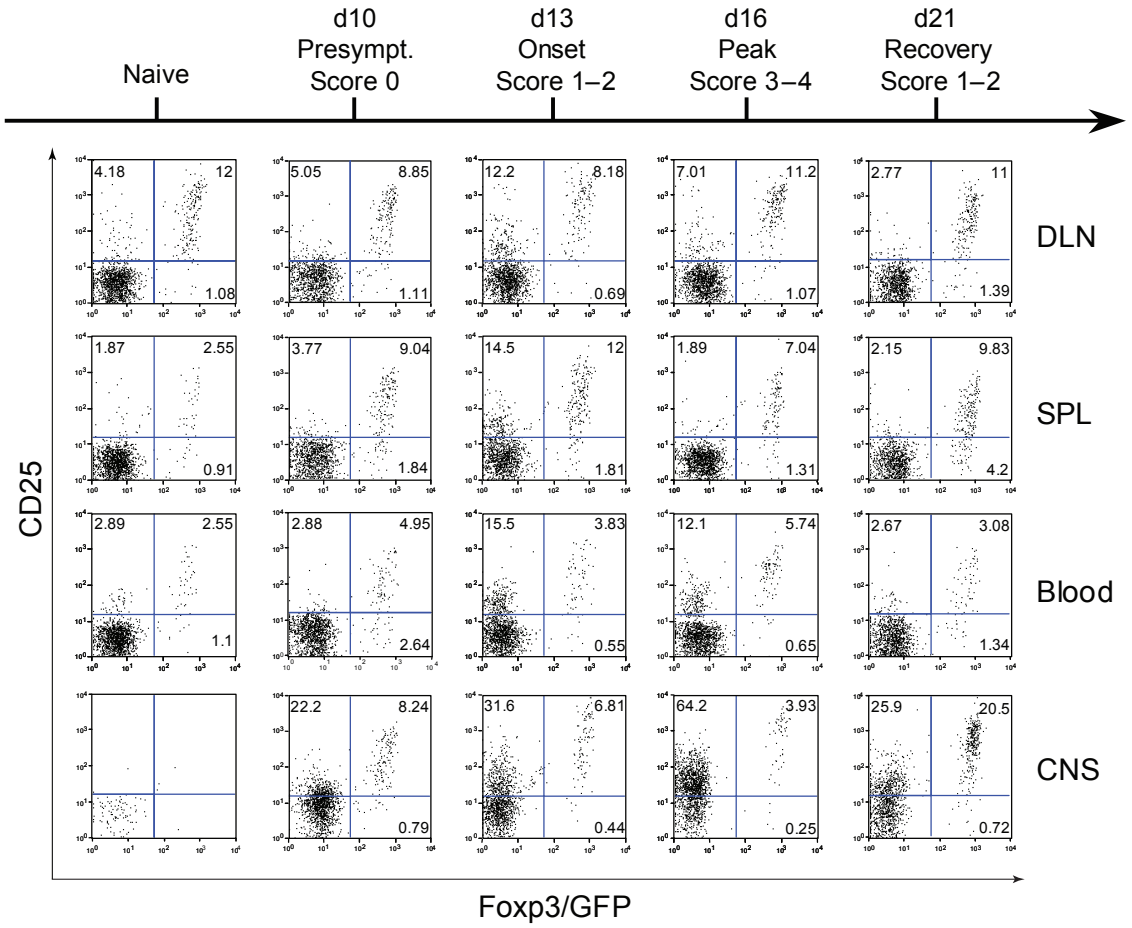
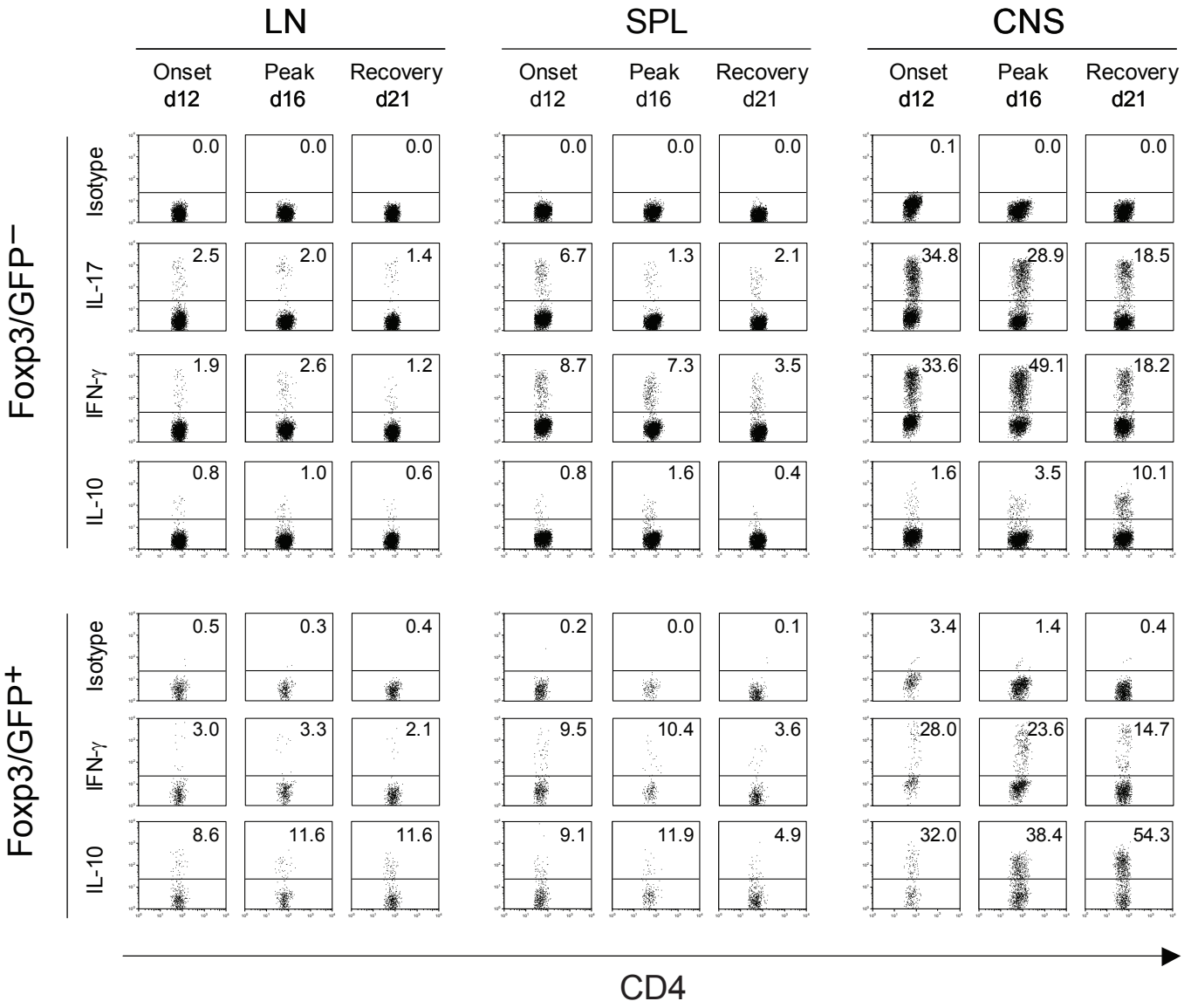
Supplementary Figure 1



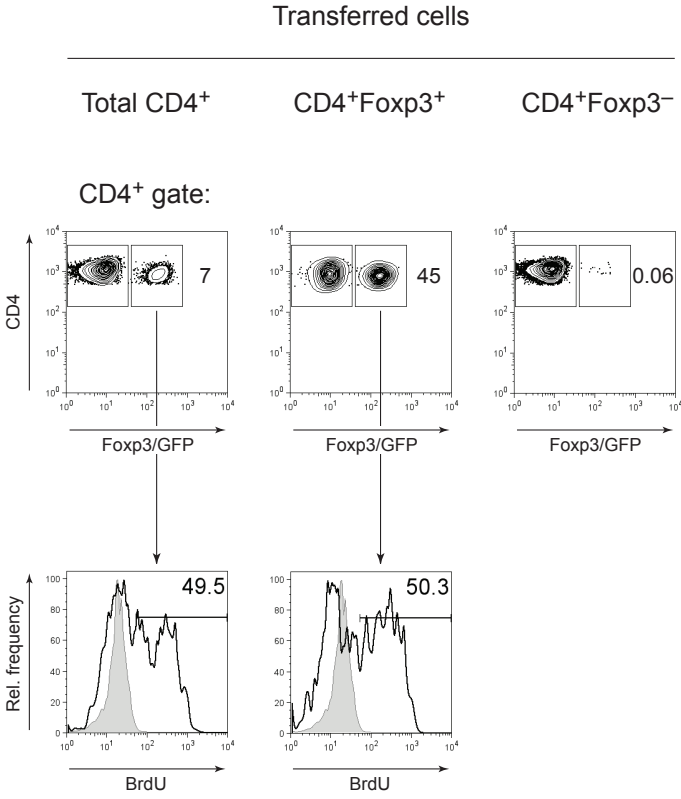
Supplementary Figure 1. T-reg population dynamics during EAE. Mononuclear cells were prepared from different compartments during EAE as indicated. Cells were stained for CD4 and CD25 and analyzed by flow cytometry. The panel illustrates the $CD4^+$ T-cell population relating the expression of Foxp3/GFP with the expression of CD25. Numbers indicate percentages.

Supplementary Figure 2



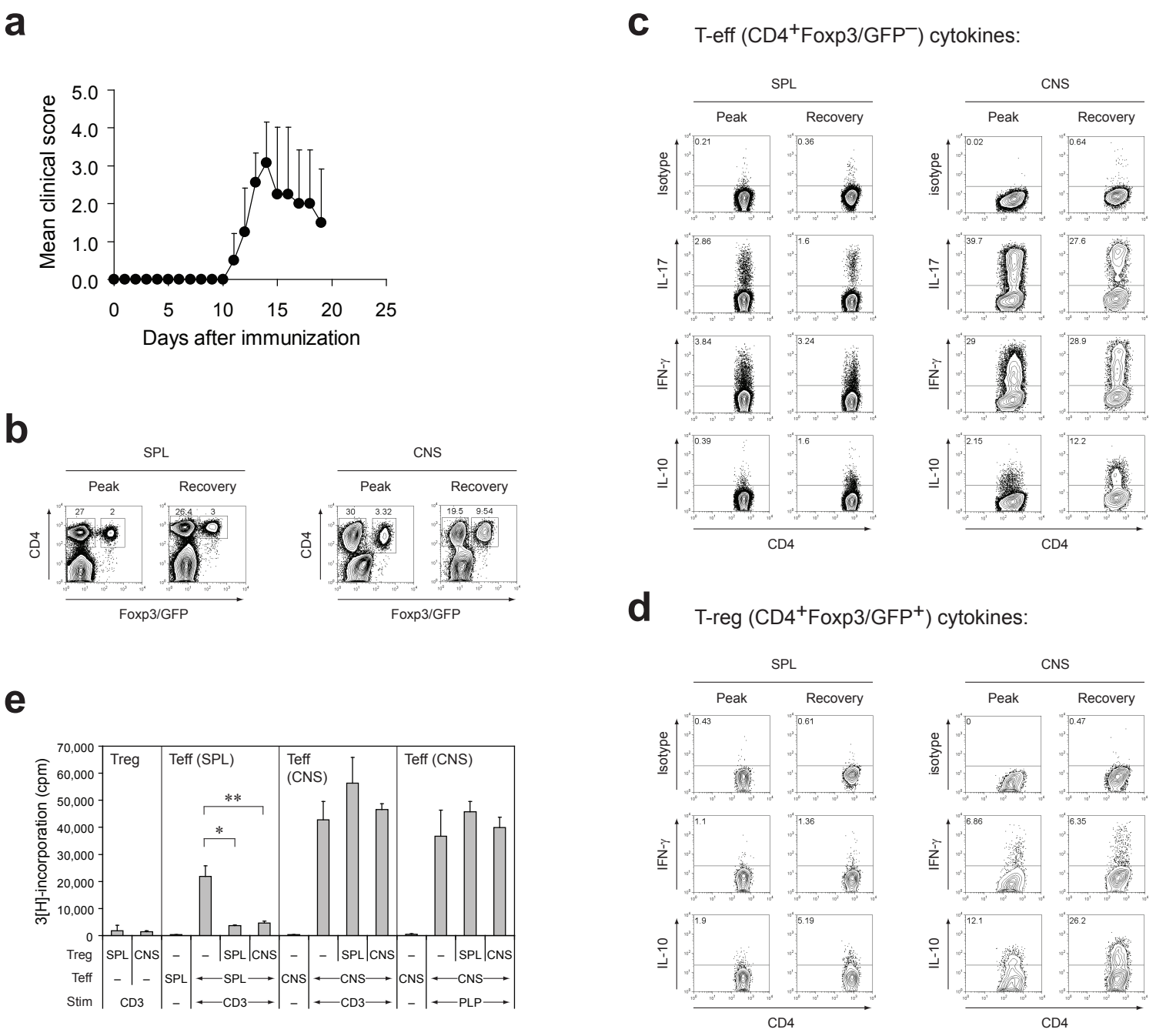
Supplementary Figure 2. Mononuclear cells were isolated from lymph nodes (LN), spleen (SPL), and CNS at different stages of EAE followed by *ex vivo*-stimulation with PMA/ionomycin and intracellular cytokine staining. Cytograms represent the profile of the indicated cytokines in T-eff ($CD4^+Foxp3/GFP^-$) and T-reg ($CD4^+Foxp3/GFP^+$) during EAE. Numbers indicate percentages.

Supplementary Figure 3



Supplementary Figure 3. Natural Foxp3$^+$ T-reg proliferate *in vivo*. *Rag2*$^{-/-}$ mice received 10x10^6 CD4$^+$ T-cells (containing a fraction of 8% Foxp3$^+$ cells), 1.5x10^6 FACS-sorted CD4$^+$Foxp3$^+$ T-cells, or 5x10^6 FACS-sorted CD4$^+$Foxp3$^-$ T-cells. After five days, the hosts were immunized with MOG_{35-55}/CFA followed by BrdU treatment. On d16 after immunization, the spleens were analyzed for Foxp3/GFP expression and BrdU incorporation. The percentage fractions in the corresponding gates are indicated. The histograms illustrate the BrdU incorporation within the CD4$^+$ Foxp3/GFP$^+$ gate. The shaded curves represent the CD4$^+$Foxp3/GFP$^+$ T-cell population recovered from a host that was not treated with BrdU (negative control).

Supplementary Figure 4



Supplementary Figure 4. T-eff and T-reg dynamics and function during the first disease episode in $PLP_{139-151}$ induced EAE. (**a**) SJL mice were crossed with *Foxp3gfp*.KI-mice and back-crossed onto the SJL genetic background for at least four generations (SJL *Foxp3gfp*.KI). In these mice, the expression of Foxp3 was linked to the expression of GFP as described for the original *Foxp3gfp*.KI-mice. EAE was induced by immunization with $PLP_{139-151}$/CFA and the disease course was followed for 20 days (n=9, +SD). (**b**) Splenocytes and CNS mononuclear cells were isolated from female SJL *Foxp3gfp*.KI-mice at the peak of disease (d14) and during recovery (d19) and stained for CD4. The percentages of $CD4^+Foxp3/GFP^-$ T-cells (T-eff) and $CD4^+Foxp3/GFP^+$ T-cells (T-reg) within the mononuclear cell populations of the spleen and the CNS are shown. (**c**, **d**) Mononuclear cells were prepared from SJL *Foxp3gfp*.KI-mice at the peak of disease and during recovery, stimulated *ex vivo* with PMA/ionomycin and stained for CD4 and intracellular cytokines. (**c**) Cytokine expression (percentages) in the T-eff gate ($CD4^+Foxp3/GFP^-$). (**d**) Cytokine expression (percentages) in the T-reg gate ($CD4^+Foxp3/GFP^+$). (**e**) T-eff ($CD4^+Foxp3/GFP^-$) and T-reg ($CD4^+Foxp3/GFP^+$) were isolated by FACS-sorting from the spleen and the CNS of SJL *Foxp3gfp*.KI-mice at the peak of disease (d14). Splenic T-eff and CNS-T-eff were compared for their susceptibility to suppression by spleen-derived or CNS-derived T-reg in anti-CD3-driven and $PLP_{139-151}$-specific proliferation assays. Mean 3[H]-thymidine-incorporation of triplicate cultures (+SD). *P<5x10^{-5}, **P<3x10^{-5}, t-test.

SUPPLEMENTARY METHODS

Generation of *Foxp3gfp*.KI mice. By gene targeting, a bicistronic cassette containing a viral internal ribosomal entry site (IRES)-linked EGFP gene followed by the polyadenylation sequence signal from SV40, was introduced just after the translation stop codon of the endogenous *Foxp3* gene and upstream of its polyadenylation sequence in the 3'-untranslated region. Therefore, this targeting strategy would in theory not alter the expression and regulation of the Foxp3 protein. In targeted Foxp3 expressing cells, EGFP expression is directly controlled by the endogenous regulatory elements of the *Foxp3* promoter. Mice bearing the *Foxp3* IRES-EGFP allele, named *Foxp3gfp*.KI mice, were confirmed by southern blotting and genotyped by PCR (data not shown). The *Foxp3gfp*.KI mice developed normally and remained as healthy as their *wildtype* littermates.

MHC class II (IA^b) tetramers. The cDNA constructs for the MOG_{35-55}/IA^b-monomers were made by overlapping PCR using plasmids encoding the IA^b α- and β-chains. The DNA sequences encoding the Fos and Jun dimerization domains were amplified by PCR from an IA^{g7} plasmid construct[1,2]. For construction of the α-chain cassette, the signal peptide and extracellular domain of the IA^b α-chain were amplified first. A linker containing a Sal I site, the Fos dimerization site, another linker, and a BirA biotinylation site with a 3'-end stop codon were added at the 3'-end. The carboxyl-terminal amino acids of the IA^b α-chain were 'ELTE', followed by 'VDGGGGG' (linker), 'LTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAA' (Fos dimerization site), 'SAGGG' (linker), and 'GLNDIFEAQKIEWHE' (BirA biotinylation site). For construction of the β-chain cassette, a long oligonucleotide encoding the MOG_{35-55} peptide

sequence, followed by a linker with a thrombin cleavage site[3] was inserted between the 3'-end of the β-chain leader peptide and the 5'-end of the IA^b β-chain by overlapping PCR. A Sal I containing linker followed by the Jun dimerization sequence and a stop codon were added to the 3'-end of the IA^b β-chain. The carboxyl-terminal amino acids of the IA^b β-chain leader peptide were 'GGDS', followed by 'MEVGWYRSPFSRVVHLYRNGK' (MOG_{35-55} peptide), 'GGGGS' (linker), 'LVPRGS' (thrombin cleavage site), and 'GGGGSGDS' (linker). The carboxyl-terminal amino acids of the IA^b β-chain were 'AWSK', followed by 'VDGGGGG' (Sal I-containing linker), and 'RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNH' (Jun dimerization site). Bgl II sites were engineered in at the 5'- and 3'-end of both the α- and β-chain DNA constructs. The IA^b α-cassette was cloned into the BamHI site of the pAcAB3 vector and the IA^b β-cassette was introduced into the Bgl II site of pAcAB3. MHC class II molecules were produced on a large scale as described[4].

The procedure for *ex vivo*-staining with MOG_{35-55}/IA^b-tetramers has been described in detail previously[4]. Briefly, single cell suspensions were incubated at a density of 10^7 cells/ml with neuraminidase (0.7 μU/ml, neuraminidase type X from *Clostridium perfringens*, Sigma) in serum free DMEM at 37 °C/10% CO_2 for 25 min before incubation with the IA^b multimers (30 μg/ml) in DMEM supplemented with 5 μM IL-2 and 2% FCS (pH 8.0) at RT for 3 h. After washing, cells were stained for 7-AAD (Molecular Probes) and the indicated surface molecules: CD4 (RM4-5), CD25 (PC61), CD62L (MEL-14), CD103 (M290), CCR5 (C34-3448), and isotype control mAbs (all BD Biosciences). The percentage of $tetramer^+$ cells was determined in the CD4 gate of live ($7\text{-}AAD^-$) cells. In order to control for unspecific binding, IA^s control-tetramers were used[4]. Stained cells were analyzed on a FACSCalibur machine (BD Biosciences) and data analysis was performed using FlowJo software (Tree Star, Stanford, CA, Version 6.3.3).

Cytokine production. T-cell suppression cultures were set up as described in the Methods section, supernatants were collected after 48 h, and cytokine concentrations were determined by ELISA (antibodies for IL-17 from BD Bioscience) or by cytometric bead array for the indicated cytokines (BD Bioscience) according to the manufacturers' instructions. For quantitative PCR, RNA was extracted from FACS-sorted cells using the RNAeasy columns (Qiagen, Valencia, CA). Complementary DNA was transcribed as recommended (Applied Biosystems, Foster City, CA) and used as template for quantitative PCR. Primer/probe mixtures for mouse IL-6 and mouse TNF-α were obtained from Applied Biosystems. The analysis was performed on the GeneAmp 5500 Sequence Detection System (Applied Biosystems). The gene expression was normalized to the expression of β-actin.

REFERENCES TO SUPPLEMENTARY METHODS

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2. Jang, M.H., Seth, N.P. & Wucherpfennig, K.W. Ex vivo analysis of thymic CD4 T cells in nonobese diabetic mice with tetramers generated from I-A(g7)/class II-associated invariant chain peptide precursors. *J Immunol* **171**, 4175-86 (2003).
3. Rees, W. et al. An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. *Proc Natl Acad Sci U S A* **96**, 9781-6 (1999).
4. Reddy, J. et al. Detection of autoreactive myelin proteolipid protein 139-151-specific T cells by using MHC II (IAs) tetramers. *J Immunol* **170**, 870-7 (2003).