Supplementary Figures and Table

Thymic self-reactivity selects natural interleukin 17-producing T cells that can regulate peripheral inflammation

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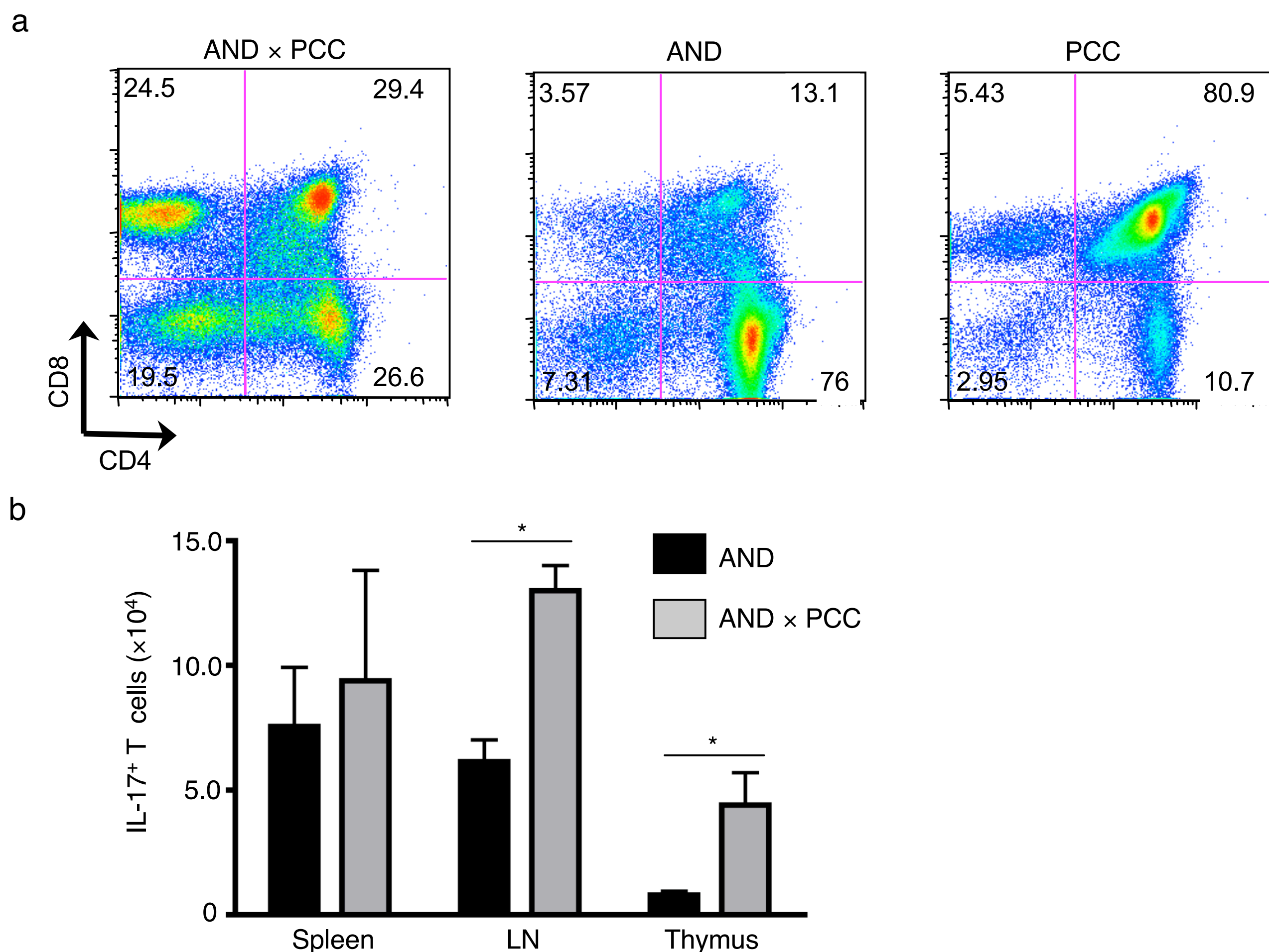
Supplementary Table 1: DTg and STg mice showed comparable survival at age 1 year.

Group	Alive at 1 year
B10.AND×PCC (M)	21/22
B10.AND×PCC (F)	12/12
B10.AND (M)	3/3
B10.AND (F)	3/3
B10.PCC (M)	10/11
B10.PCC (F)	6/6

M = male F = female. Right column indicates number of mice surviving to 1 year of age divided by the number of mice in the initial cohort.

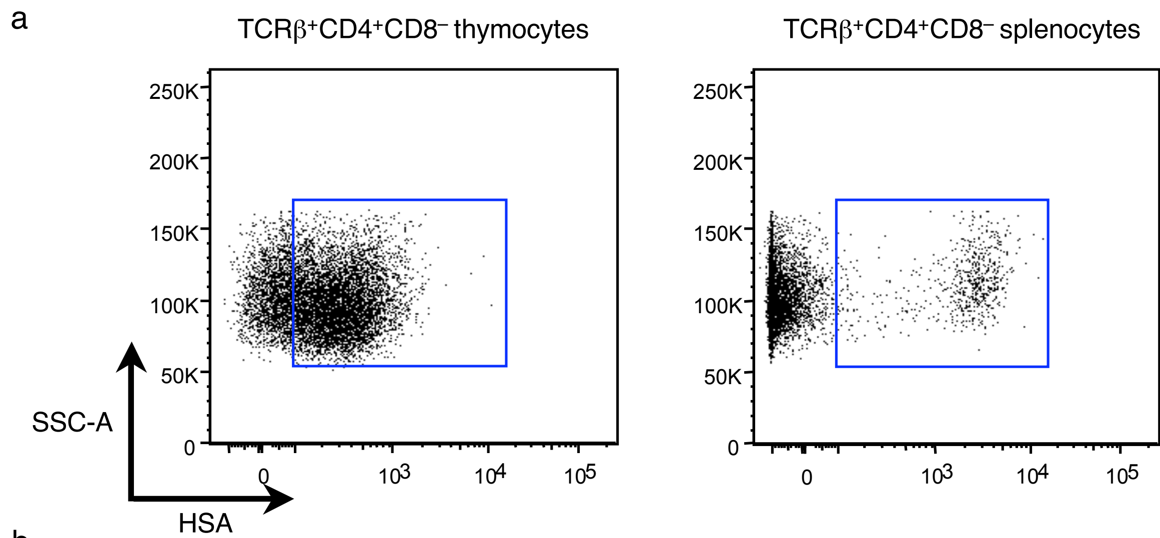
Supplementary Table 2: RT-PCR primer sequences

Name	Forward	Reverse
Actb	5'-AGAGGGAAATCGTGCGTGAC-3'	5'-CAATAGTGATGACCTGGCCGT-3'
Il17	5'-CTGGAGGATAACACTGTGAGAGT-3'	5'-TGCTGAATGGCGACGGAGTTC-3'
Ifng	5'-GATGCATTCATGAGTATTGCCAAGT-3'	5'-GTGGACCACTCGGATGAGCTC-3'
Il22	5'-CATGCAGGAGGTGGTACCTT-3'	5'-CAGACGCAAGCATTTCTCAG-3'
Il23r	5'-GCCAAGAAGACCATTCCCGA-3'	5'-TCAGTGCTACAATCTTCTTCAGAGGACA-3'
Il10	5'-CCCTTTGCTATGGTGTCCTT-3'	5'-TGGTTTCTCTTCCCAAGACC-3'
Rorc	5'-CCGCTGAGAGGGCTTCAC-3'	5'-TGCAGGAGTAGGCCACATTACA-3'
Rag1	5'-CATTCTAGCACTCTGGCCGG-3'	5'-TCATCGGGGCAGAACTGAA-3'
Hprt1	5'-CCAGCAAGCTTGCAACCTTAACCA-3'	5'-GTAATGATCAGTCAACGGGGGAC-3'



Supplementary Figure 1 Cellularity of the thymus is reduced in DTg mice while the total number of IL-17^{+} T cells is increased. (**a**) Percentages of CD4^{+}CD8^{+}, CD4^{+}CD8^{-} and CD8^{+}CD4^{-} cells of TCRβ^{+} thymocytes were measured by flow cytometry in DTg, STg AND and STg PCC mice, with data representative of 5 independent experiments. (**b**) Absolute numbers of TCRβ^{+}CD4^{+}CD8^{-}IL-17^{+} cells in the spleen, LN and thymi of DTg and STg mice were determined by counting numbers of cells in each organ and using percentages obtained by FACS staining ($P \leq 0.05$; 3 mice per group, 1 experiment).

a



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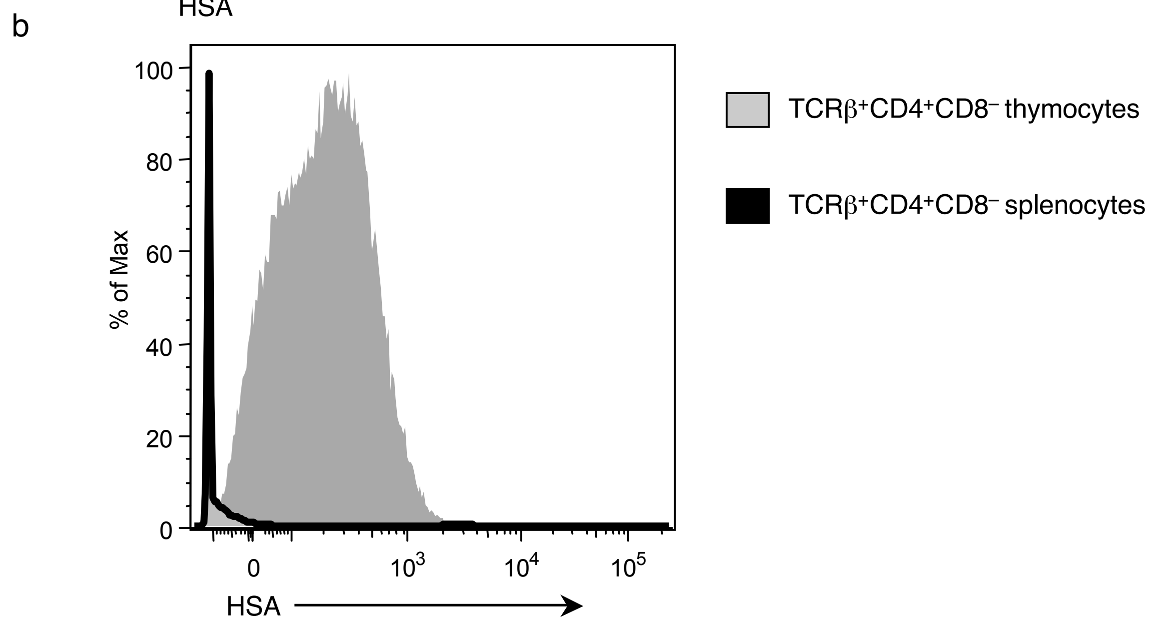

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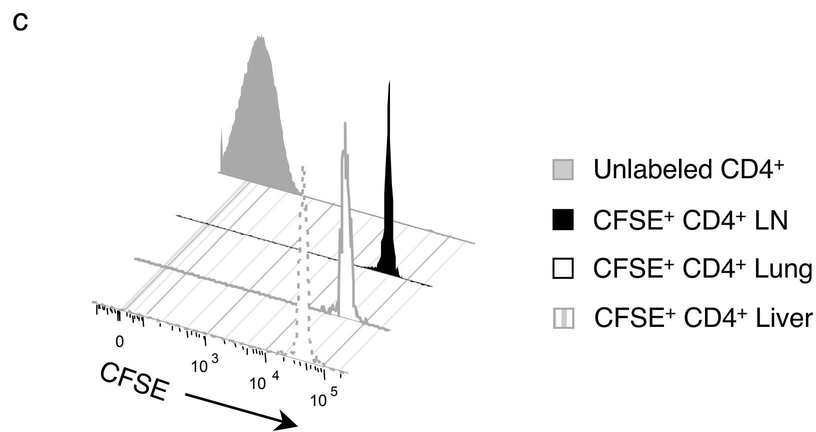
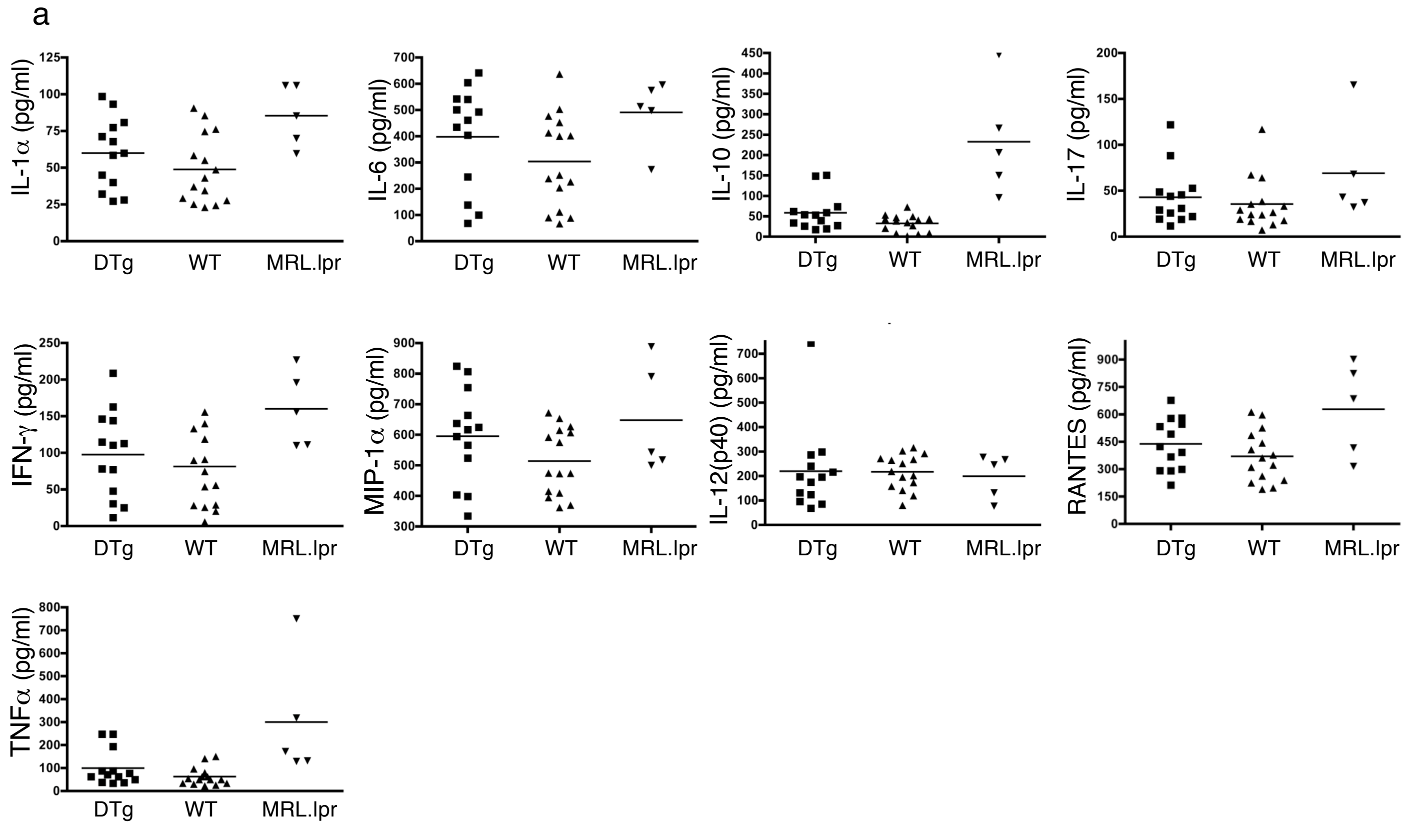


Marks *et al*. Supp. Figure 2

Supplementary Figure 2 HSA (CD24) positive thymocytes were sorted from DTg mice. (**a**) TCRβ+CD4+CD8–thymocytes from DTg mice were sorted based on HSA expression using splenocytes to determine gating. (**b**) Histogram of HSA expression on TCRβ+CD4+CD8– thymocytes (gray) compared to TCRβ+CD4+CD8– splenocytes (black). Data are representative of 3 (**a**,**b**) independent experiments. (**c**) Purified CD4+ DTg splenic and LN T cells labeled with CFSE were transferred to wild type recipients; five days following transfer, CFSE dilution of transferred cells in the LN, liver, lungs, and thymi of recipient mice was determined. Data are representative of 2 experiments, with 2-3 mice per experiment.

a



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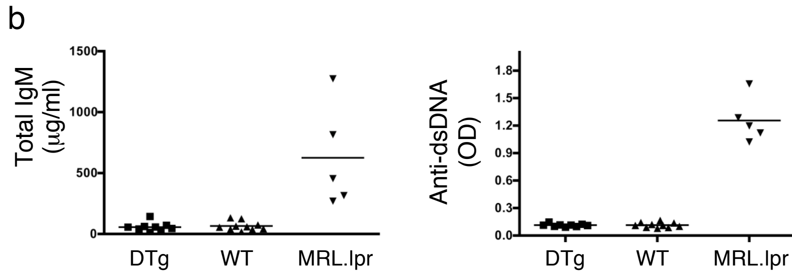

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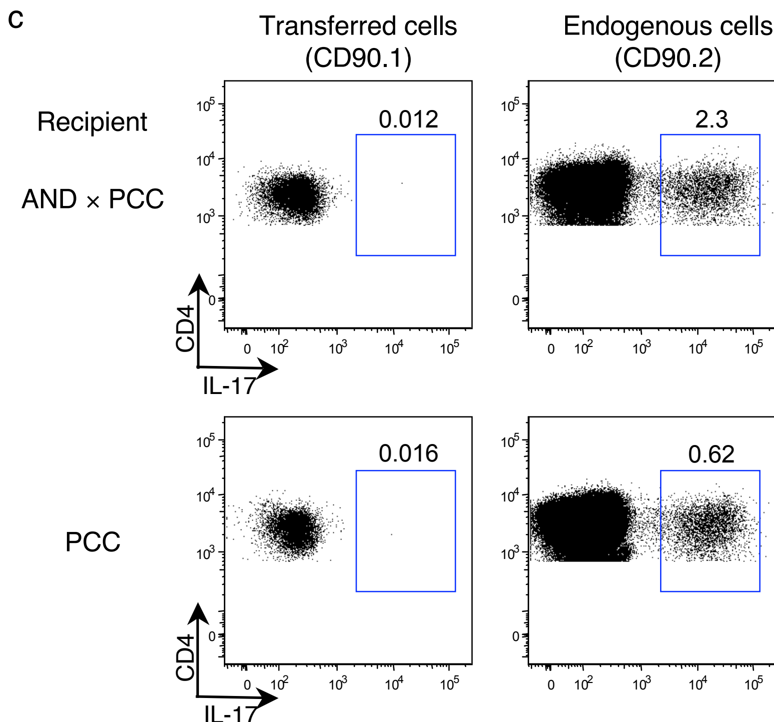
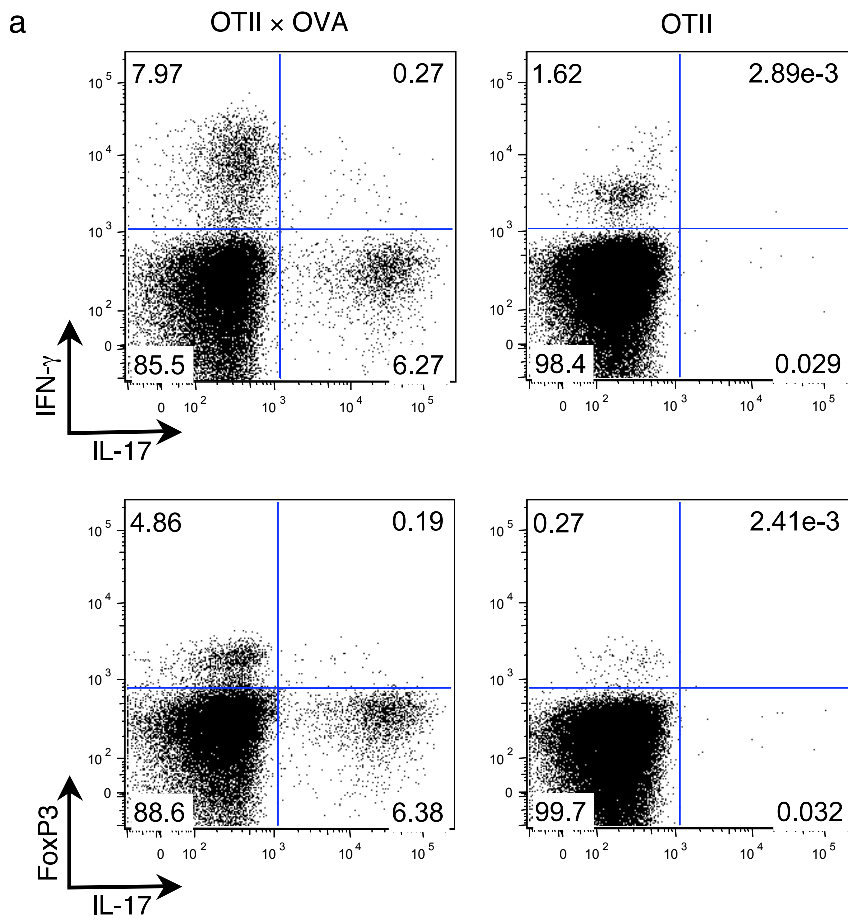
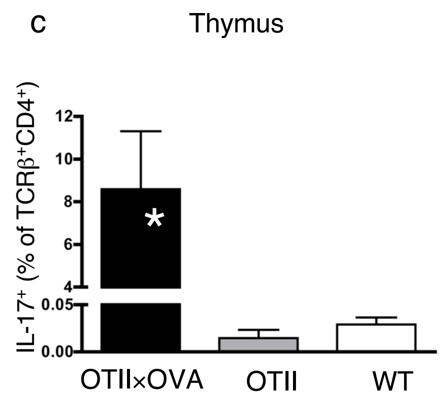
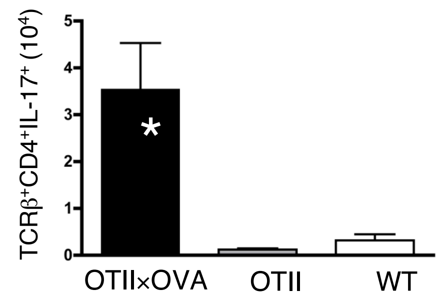


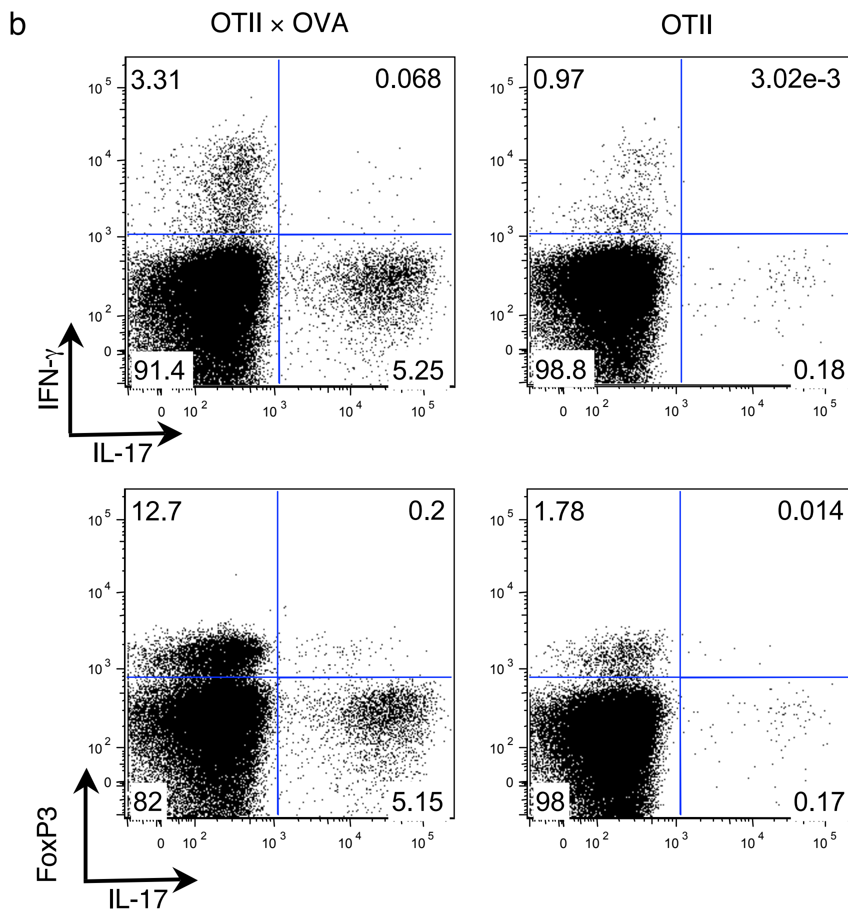
Marks *et al.* Supp. Figure 3

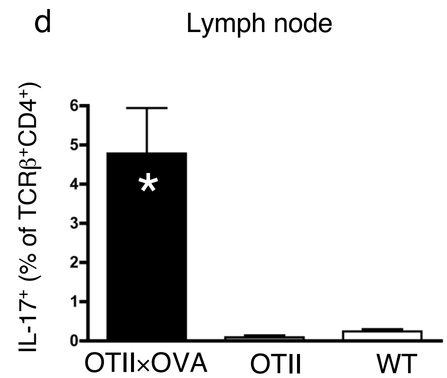
Supplementary Figure 3 DTg mice do not spontaneously develop autoimmunity and their peripheral environment does not promote T_H-17 cell development. (**a**,**b**) Serum concentrations of inflammatory cytokines and chemokines, including IL-1α, IL-6, IL-10, IL-17, IFN-γ, MIP-1α, IL-12(p40), RANTES and TNFα (**a**), and immunoglobulins, including IgM and anti-dsDNA (**b**) were calculated from DTg, wild-type and lupus-prone MRL/*Fas*^{lpr} mice (13 DTg, 15 wild-type, and 5 MRL mice, with 1 experiment). (**c**) Enriched CD4$^+$ T cells from AND.*Rag1*$^{-/-}$.CD90.1 mice were transferred to DTg and PCC STg CD90.2 mice and 5 days later the percentages of IL-17$^+$ cells among CD90.1 (transferred) and CD90.2 (recipient) TCRβ^+CD4$^+$ cells were determined by flow cytometry. Data are representative of 3 mice.





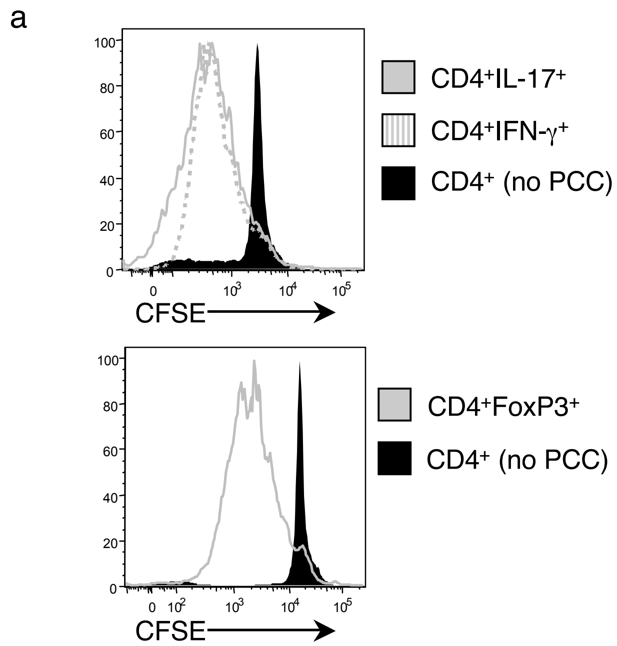


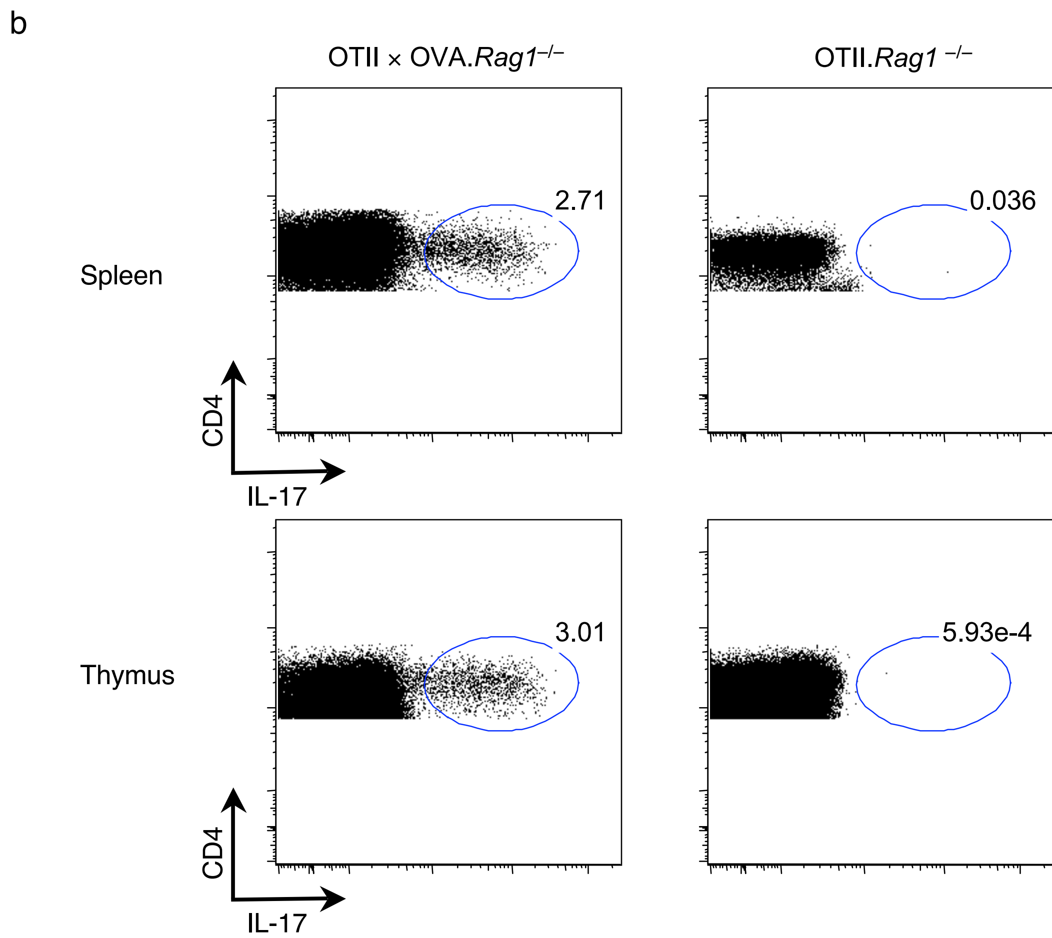




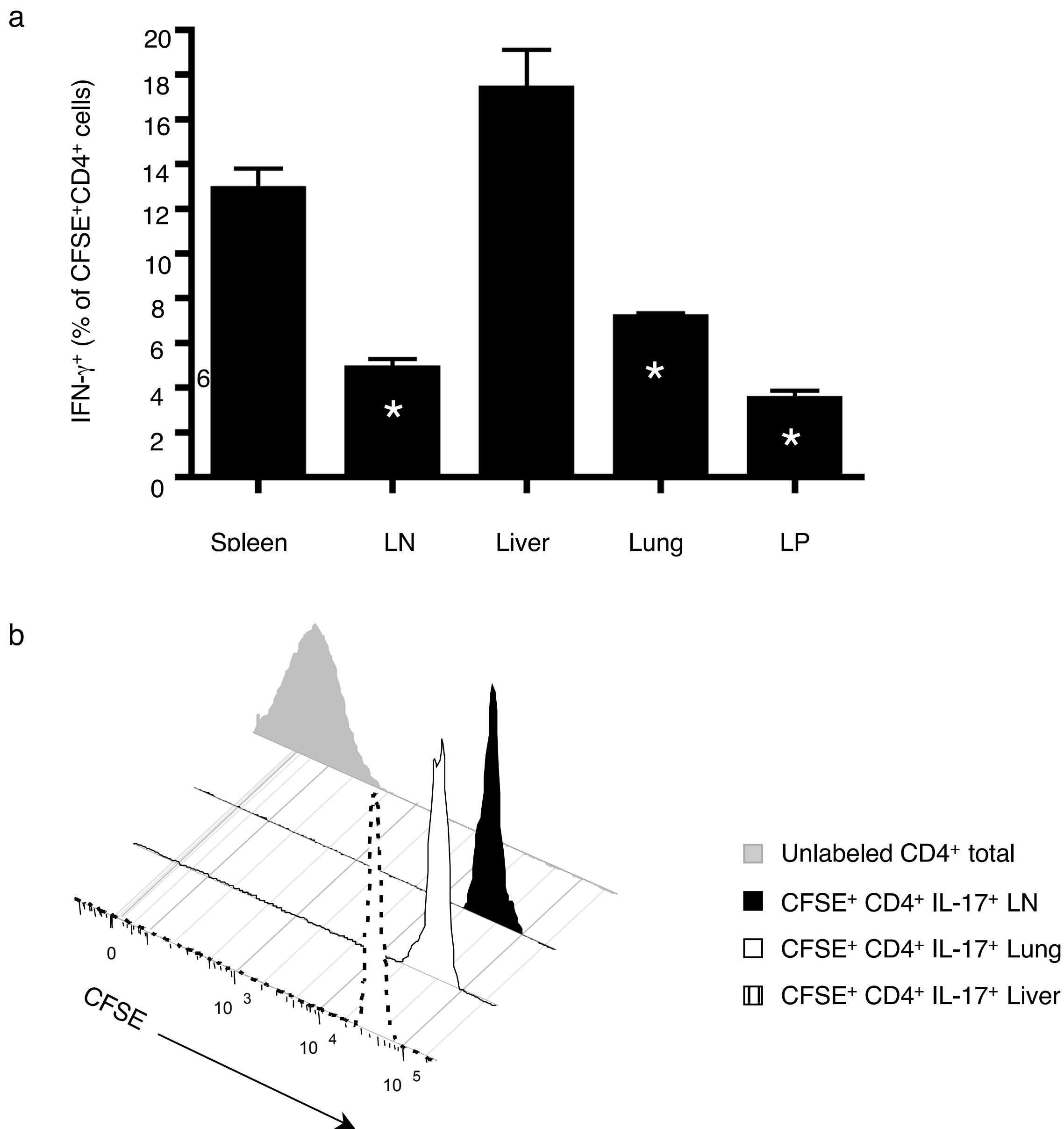
Marks *et al*. Supp. Figure 4

Supplementary Figure 4 Thymi and LN of OTII ×OVA DTg mice are enriched for IL -17+ cells. (**a,b**) The frequencies of IL-17+, IFN-γ+ and FoxP3+ cells among TCRβ+CD4+ T cells from the thymus (**a**) and the LN (**b**) were assessed by intracellular staining**.** (**c,d**) The percentage of IL-17+ among TCRβ+CD4+ cells and the absolute number of IL-17+ T cells from thymi (**c**) and the LN (**d**) of OTII ×OVA DTg, OTII STg, and wild -type mice are displayed ($P \leq 0.05$; 3 mice in each group). Data are representative of 3 (**a**,**b**) or 2 (**c**,**d**) independent experiments.

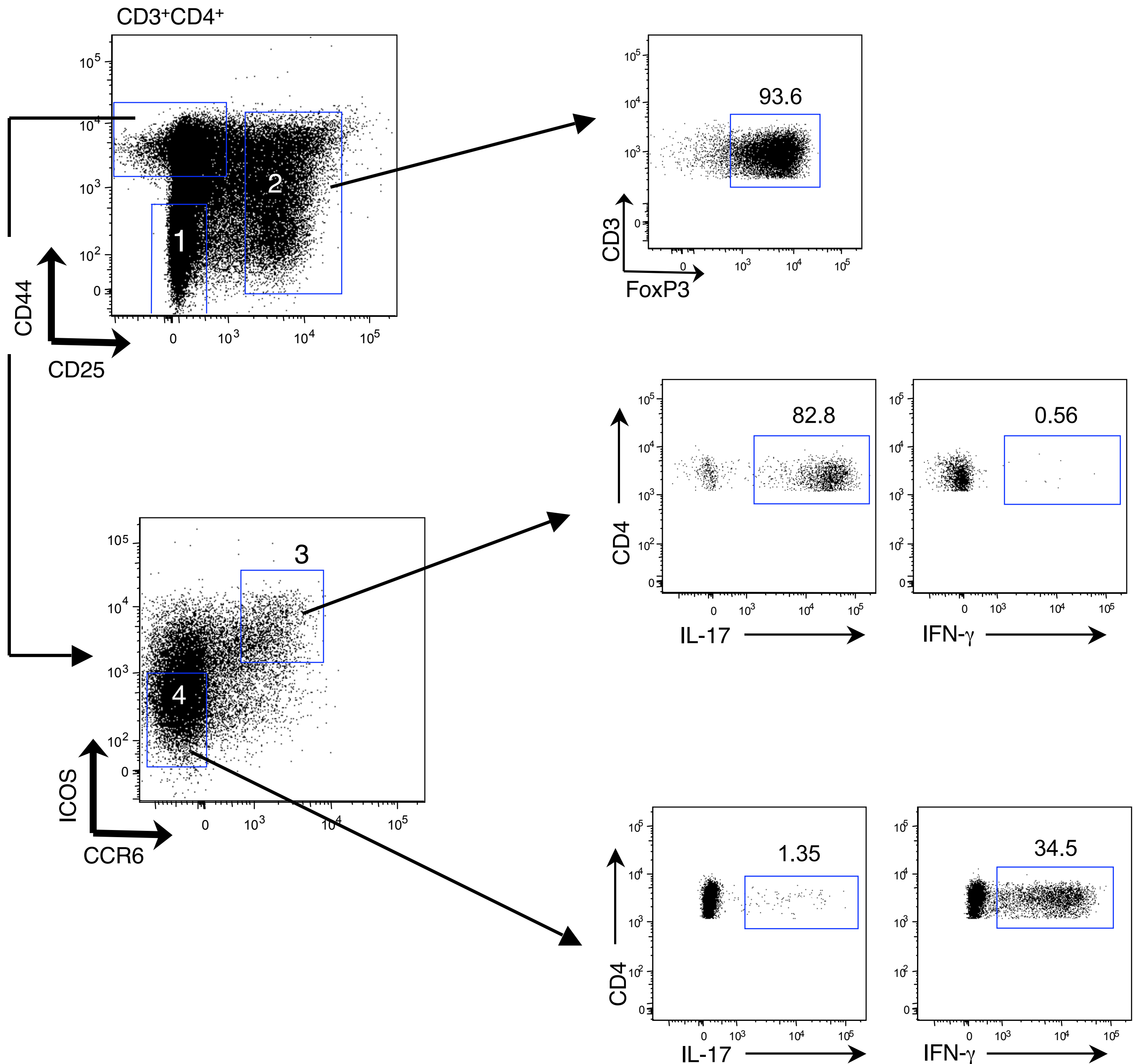

a
100
80
60
40
20
0
0
10³
10⁴
10⁵
CFSE
CD4+IL-17+
CD4+IFN-γ+
CD4+ (no PCC)
0
10²
CD4+FoxP3+


b
OTII × OVA.Rag1−/−
OTII.Rag1 −/−
Spleen
2.71
0.036
CD4
IL-17
Thymus
3.01
5.93e-4

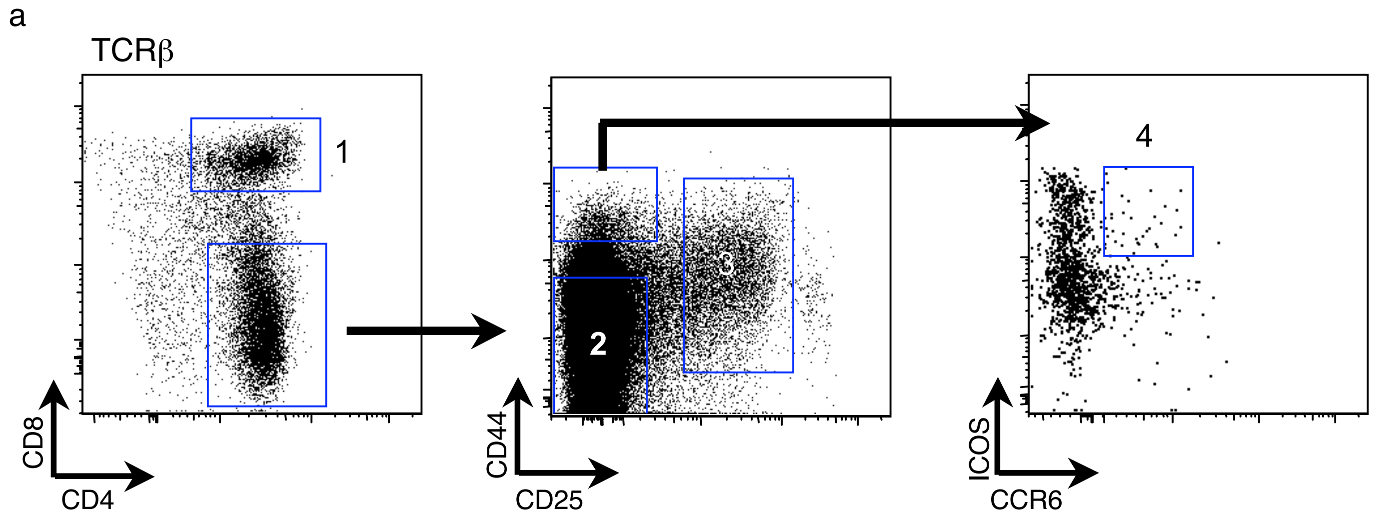
Supplementary Figure 5 T_H-17 cells proliferate in response to PCC and develop in *Rag1*$^{-/-}$ DTg mice. (**a**,**b**) Splenocytes from AND × PCC mice were enriched for CD4$^+$ T cells, labeled with CFSE and cultured for 3 days with 10μg/ml PCC_{88-14} peptide and T depleted splenocytes to evaluate proliferation of IL-17$^+$ (■ solid line), IFN-γ$^+$ (■ dotted line) or FoxP3$^+$ T cells (■ solid line) compared to unstimulated T cell cultures (■ solid line). (**b**) Percentages of IL-17$^+$ T cells of TCRβ$^+$CD4$^+$ in the spleen and thymus of *Rag1*$^{-/-}$ DTg and STg mice were measured by flow cytometry. Data are representative of 2 (**a**,**b**) independent experiments.

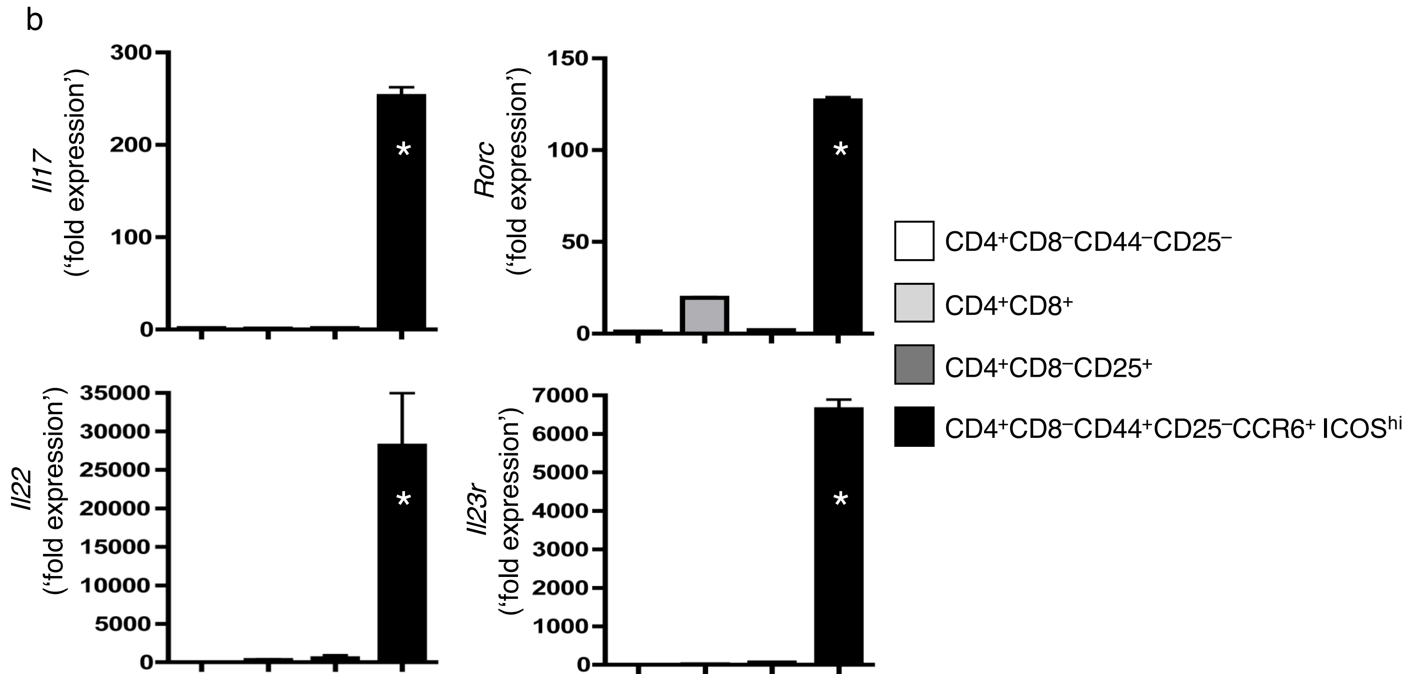


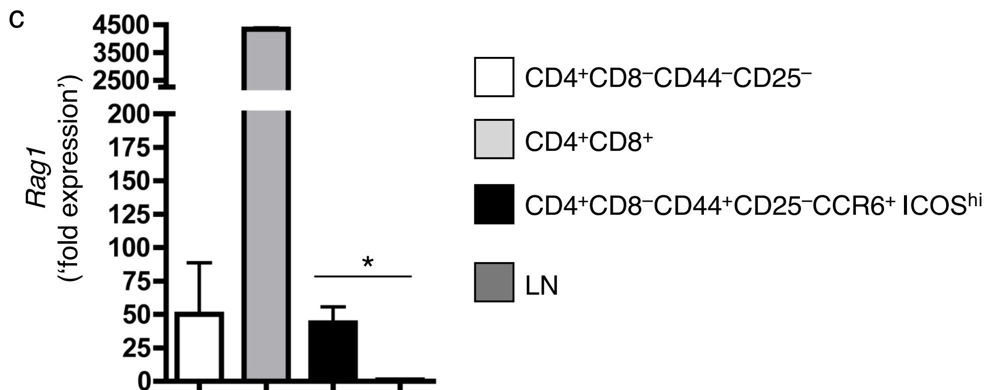
Supplementary Figure 6 Migration pattern of T_H1 cells from DTg mice. (**a**) Purified splenic and LN CD4$^+$ DTg T cells, labeled with CFSE, were transferred to wild-type recipients and percentages of IFN-γ^+ cells among CFSE$^+$CD4$^+$ cells were measured in the spleen, lymph nodes, liver, lung, and lamina propria at two days post transfer ($P \leq 0.05$, compared to spleen; 4 recipient mice examined, representative of 2 independent experiments). (**b**) Purified splenic and LN CD4$^+$ DTg T cells labeled with CFSE were transferred to wild type recipients and CFSE expression in CD4$^+$IL-17$^+$ cells in peripheral organs were evaluated two days later, with data representative of 3 independent experiments, each using 2-3 recipient mice.



Supplementary Figure 7 T_H-17 cells can be purified from LN and spleens of DTg mice. LN and spleen cells from DTg mice were sorted by gating on $CD3^+CD4^+$ cells, followed by separation into $CD44^-CD25^-$ (naïve), $CD25^+$ (T_R), and $CD44^+$ gates, with the latter further purified into $CCR6^+ICOS^{hi}$ (T_H-17) and $CCR6^-ICOS^{lo}$ (T_H1) populations. FoxP3, IL-17 and IFN-γ were measured by intracellular staining.

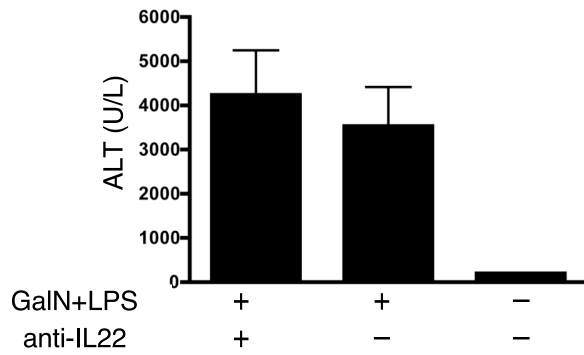




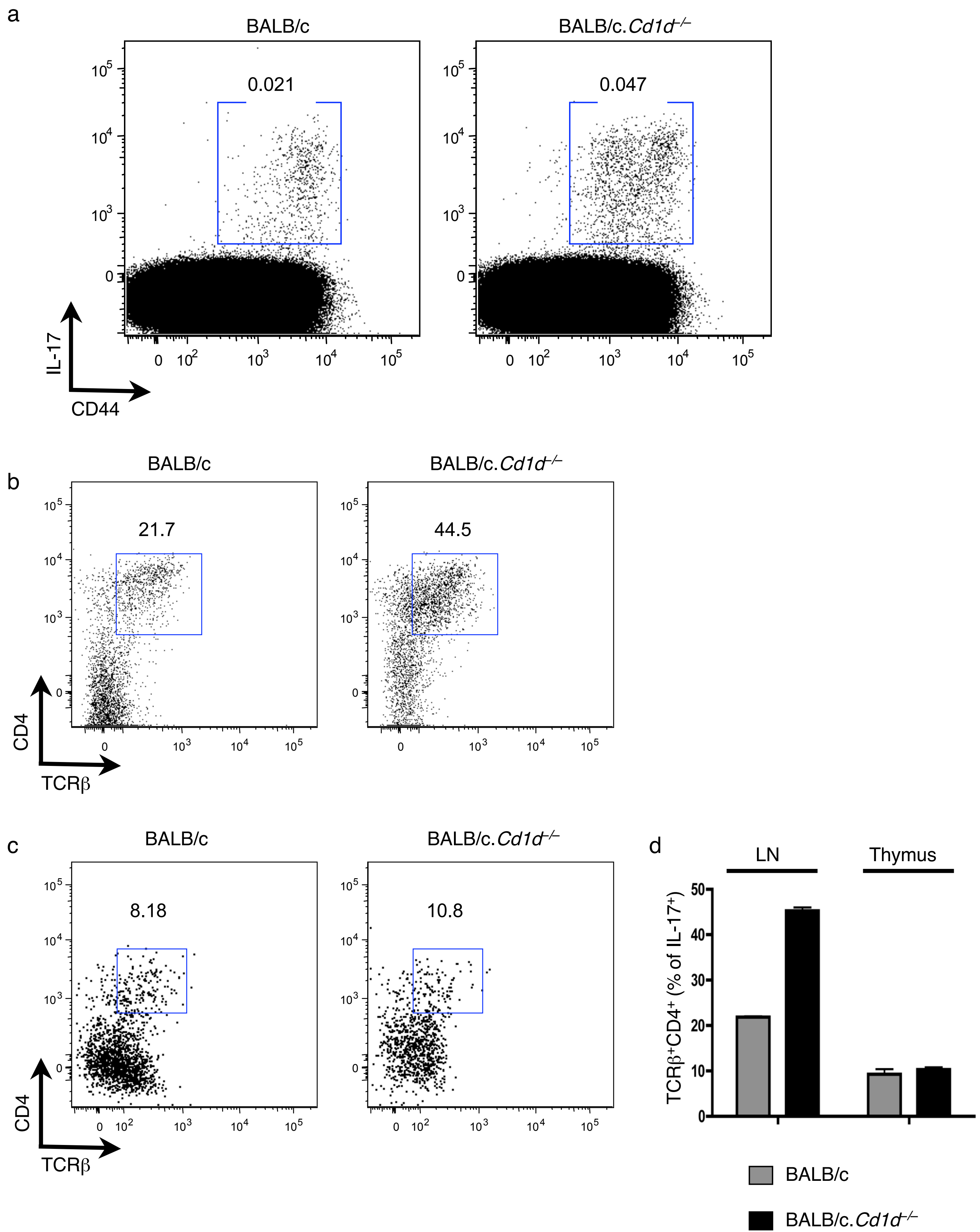


Marks *et al.* Supp. Figure 8

Supplementary Figure 8 T_H-17 cells from the thymus of DTg mice express RORγt, IL-23R, and IL-22, with RAG expression similar to CD4 SP T cells. (**a-c**) Thymocytes from DTg mice were sorted into the indicated subsets, followed by detection of mRNA expression using Q-PCR, with the ratio of gene to *Hprt* expression determined by the relative quantification method ($\Delta\Delta C_T$). mRNA expression in T_H-17 cells compared to DP, naïve and T_{reg} cells (**b**, $P \leq 0.05$; compared to 3 other groups,) or, for *Rag1* expression, to LN cells (**c**, $P \leq 0.05$, with data compiled from 3 replicates using 1 cell sort by flow cytometry) .



Supplementary Figure 9 IL-22 mediated protection from toxic hepatitis is dependent upon transfer of DTg T cells. Serum aspartate aminotransferase (ALT) was measured in sera taken from mice 5 h post GalN+LPS treatment, with or without 2h pre-treatment with an anti-IL-22 blocking antibody (4 mice per group, 1 experiment).



Marks et al. Supp. Figure 10

Supplementary Figure 10 T_H-17 cells develop in the absence of CD1d. (**a-d**) The frequency of IL-17 producing $TCR\beta^+CD4^+$ cells of the LN (**a**) or $TCR\beta^+CD4^+$ cells among the IL-17^+ cells in the LN (**b,d**) or thymus (**c,d**) was compared between wild type and CD1d-deficient mice (both on BALB/c background) using intracellular cytokine staining. Data are representative of 4 mice per group (**a**-**c**), with (**d**) a composite of those 4 animals.