Supplementary Materials:

Materials and Methods:

Mice and Parasitic Infections: C57BL/6, B6.SJL-*Ptprc*^{a} (CD45.1) and OT-II TCR transgenic mice were purchased from Taconic. CBir1 Tg mice were produced by Dr. Charles Elson as described and back-crossed to CD45.1 expressing mice for three generations or Rag-/- mice for two generations (*15*). Foxp3^{EGFP} mice have been described previously (*32*). *T. gondii* Me49 C1 (RFP+) was the kind gift of M.E. Grigg (NIH/NIAID, Bethesda, MD) (*12*). Preparation of cysts and oral infection with this parasite was carried out as described previously (*12*). For TCR transgenic studies ~75 000 CBir1 Tg (CD45.1/45.1) and/or OT-II TCR (CD45.1/45.2) transgenic cells were transferred to congenic C57BL/6 (CD45.2/45.2) hosts i.v. and infected with 12-25 *T. gondii* cysts orally 24 hours later. For peptide stimulations mice containing CBir1 Tg T cells were injected i.p. with 50μg CBir$_{456\text{-}75}$ peptide (DMATEMVKYSNANILSQAGQ) or OTII peptide (ISQAVHAAHAEINEAGR) and 10μg LPS. For DSS experiments mice were treated for seven days with 2%DSS in sterile filtered water. All mice were maintained at and all experiments were performed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the National Institute for Allergy and Infectious Diseases (NIAID) and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NIAID Animal Care and Use Committee. Gender- and age-matched mice were used whenever possible.

Antibodies, tetramers and flow cytometry: All antibodies used for flow cytometry were purchased from eBioscience (San Diego CA) except for Ki67 (BD Biosciences, San Diego CA).

The following antibodies were used to discriminate cell surface phenotype: TCRβ (H57-597), CD4 (RM4-5), CD45.1 (A20), CD45.2 (104) CD44 (IM7), CD62L (MEL-14), Ly6c (HK1.4), CD27 (LG-7F9) and IL-7Rα (A7R34). Dead cells were discriminated in all experiments using LIVE/DEAD fixable dead stain (Life Technologies, Carlsbad CA) or DAPI and all stains were carried out in media containing anti-CD16/32 blocking antibody (clone 93, eBiosciences). For intracellular transcription factor staining, cells were fixed in Fixation/Permeabilization buffer (eBioscience) and stained in Perm/Wash buffer (eBioscience). The following antibodies were used for intracellular transcription factor staining: Foxp3 (FJK-16S), T-bet (eBio4B10) and Rorγt (B2D). Staining for intracellular cytokines has been described previously and was carried out using: IFN-γ (XMG1.2) and IL-17 (eBio17B7) antibodies (*12*). $CBir1_{464\text{-}72}$ Tetramers (YSNANILSQ) were created as described previously and conjugated to APC (*26*). T. gondii Me49 hypothetical protein tetramers (AVEIHRPVPGTAPPS) were made at the NIH Tetramer Core Facility and conjugated to APC (Atlanta, GA). All tetramer stains were carried out as described previously (*26*). *T. gondii* parasite burdens were calculated as percentages of cells positive for RFP from the spleen and siLP respectively. All flow cytometry was acquired on an LSRII FACS analyzer and cell sorting was carried out on a FACS Aria (BD Biosciences).

Cell isolation and stimulation: Cells from spleen, the lymph nodes and the small intestine lamina propria were isolated as previously described (*12*). For some CBir1 Tg transfer experiments, splenic CBir1 Tg cells were stained with 1μM CFSE (Life Technologies) for ten minutes at 37°C followed by an immediate wash in RPMI containing 10% Fetal bovine serum. For recovery of transferred CBir1 Tg cells from the spleen after *T. gondii* infection, CD4+ T cells were first isolated by either negative magnetic bead separation with a CD4 Isolation Kit II or positive selection by first staining with APC conjugated anti-CD45.1 antibody followed by anti-APC microbeads on an AutoMACS Pro instrument (Miltenyi, Auburn CA). For total cytokine

expression isolated cells were cultured for 3.5 hours with phorbol 12-myristate 13-acetate (50ng/mL) and ionomycin (2.5μg/mL) in the presence of Brefeldin A (1μg/mL) (GolgiPlug, BD Biosciences) and then fixed and assayed for cytokine expression by flow cytometry. For CBir1 peptide cytokine induction CD45.1+ T cells were isolated by MACS and cultured for 17 hours with MACS purified CD11c+ splenic dendritic cells that have been pulsed with CBir1 peptide. Brefeldin A (1μg/mL) was added for the last 5 hours of CBir1-specific stimulations, before cytokine expression was assessed by flow cytometry. For *T. gondii*-specific cytokine stimulations, splenic and siLP CD4 T cells were sorted from naïve and *T. gondii* infected mice by flow cytometry. Sorted CD4 T cells were cultured overnight with bone marrow derived dendritic cells (BMDCs) pre-loaded with soluble Toxplasma antigen (STAg) in the presence of Brefeldin A (1μg/mL) and assayed for cytokine expression by flow cytometry. For pre-loading of STAg, GM-CSF induced BMDCs were incubated with 5μg/mL STAg for five hours without brefeldin A, to allow for MHC loading, before subsequent co-culture with T cells. BMDCs were induced from total bone marrow by culture with GM-CSF (20ng/mL) for a minimum of 6 days and media was refreshed every 3 days. For peptide specific proliferation assays, splenocytes from TCR transgenic mice were isolated, labeled with CFSE and then cultured for 72 hours in the presence of MACS purified CD11c+ dendritic cells pulsed with peptide (CBir1 or OTII) (1μg/mL) or STAg (5μg/mL).

Immunofluorescence/Histology: For Fluorescence *In Situ* Hybridization (FISH), the small intestines were prepared by fixation in 60% Methanol, 30% Chloroform, 10 % Acetic acid, washed in 70% ethanol and embedded in paraffin. 5 μm longitudinal sections were hybridized to a bacterial 16S rRNA gene probe: [AminoC6+Alexa488]-GCTGCCTCCCGTAGGAGT-[AmC7~Q+Alexa488] as previously published (*33*). Sections were then visualized on a Leica

DM IRBE fluorescent microscope. Mouse ileal samples were fixed in 10% formalin and paraffin embedded. Slides were stained with hematoxylin and eosin and imaged by light microscopy.

Bacterial translocation: Mice were sacrificed and tissues isolated in a laminar flow hood under sterile conditions. Tissues were mechanically disassociated, diluted, plated directly onto tryptic soy agar plates and incubated at 37°C (Sigma-Aldrich). 24 and/or 48 hours later bacterial colonies were counted.

Analysis of 16S genes and CBir flagellin from mucosal samples: Small intestinal contents were washed from the lumen via the injection of PBS and the mucosal-associated bacteria was then isolated by vigorous washing in a petri dish. The mucosal associated bacteria was then centrifuged and immediately frozen. Bacterial genomic DNA was isolated using the QIAmp DNA stool Minikit (Qiagen, Germantown, MD). For CBir1 flagellin and eubacterial 16S ribosomal DNA measurements were analyzed by SYBR-Green RT-PCR (Bio-Rad, Hercules CA) using the following primers: CBir1 flagellin (Ann. Temp. 56°C)- Fwd-GCAACGGATG CAATTGATAC CATCA, Rev-AAGTACACCC TGGTTAGACT GGTTAGACTG. The amount of CBir1 flagellin is calculated by ΔCT method as compared to the total 16S ribosomal DNA in each mucosal-associated DNA sample. Eubacterial 16S ribosomal primers: Fwd-ACTCCTACGG GAGGCAGCAG T Rev- ATTACCGCGG CTGCTGGC.

Statistical Analyses: Where indicated, P values were determined by using the unpaired Student's t test. P values <0.05 were considered significant. All graphs show the mean +/- SEM.

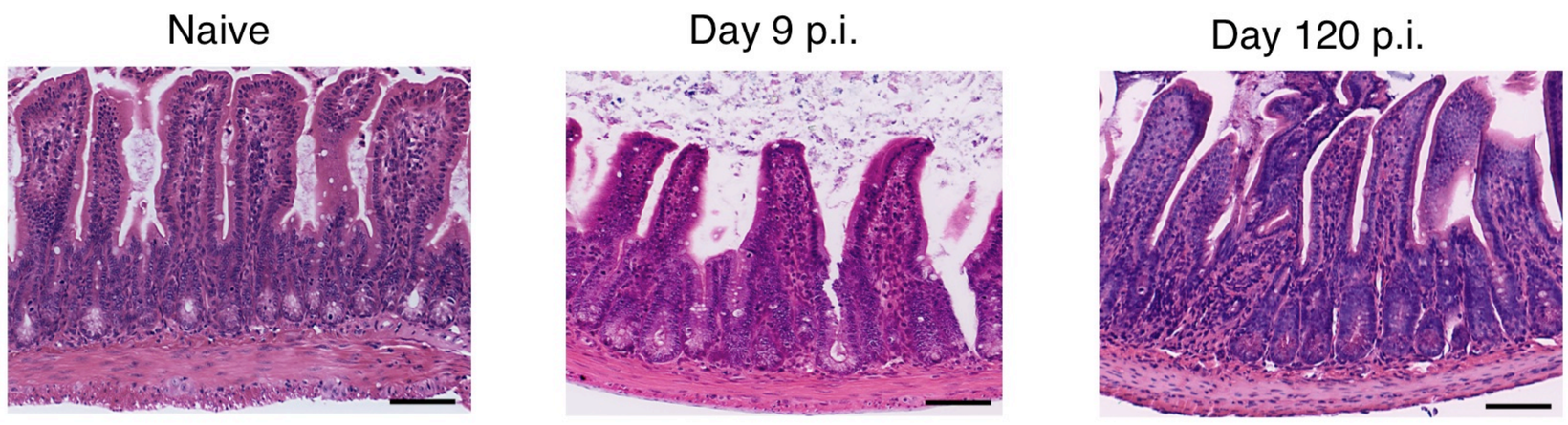


Figure S1

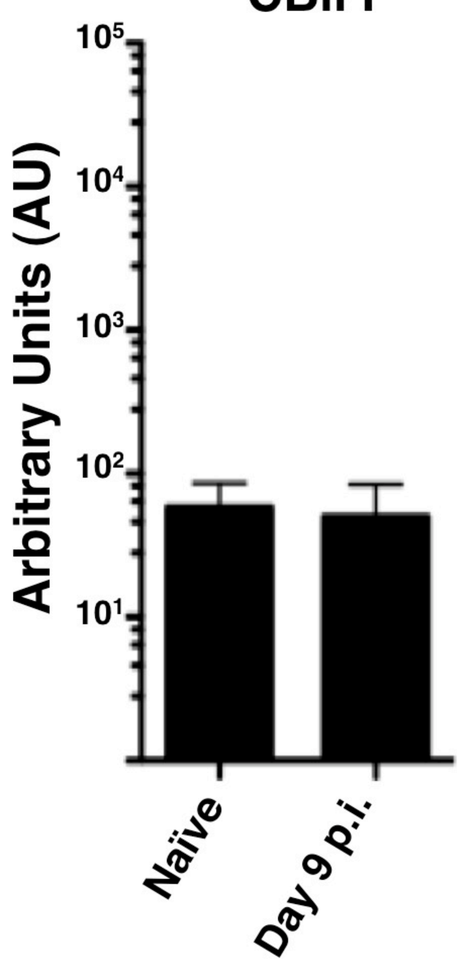

Mucosa-Associated Bacteria
(vs. Total 16s expression)
CBir1
Arbitrary Units (AU)
10^5
10^4
10^3
10^2
10^1
Naïve
Day 9 p.i.

Figure S2

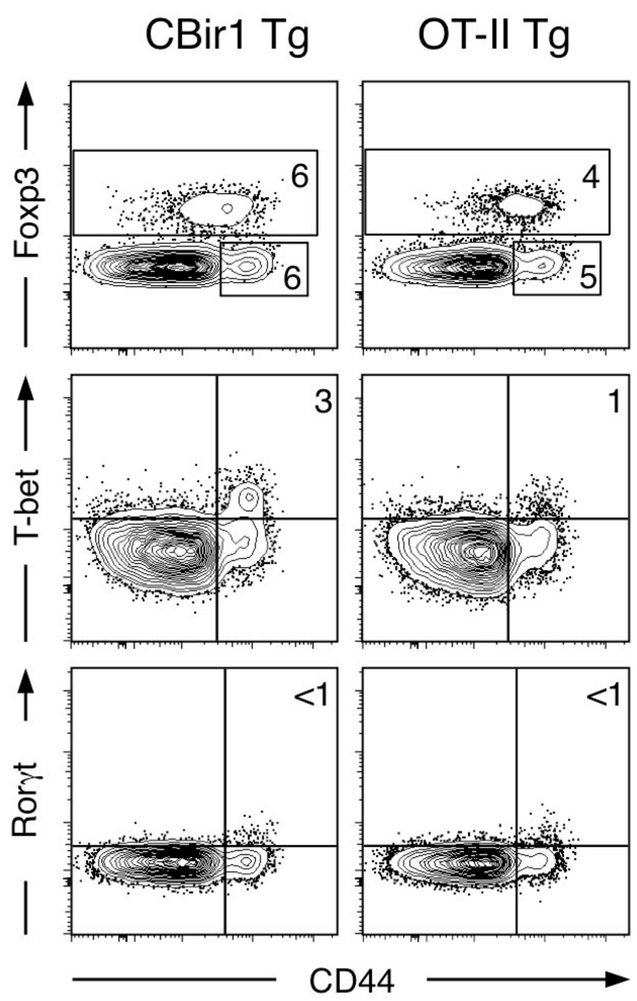
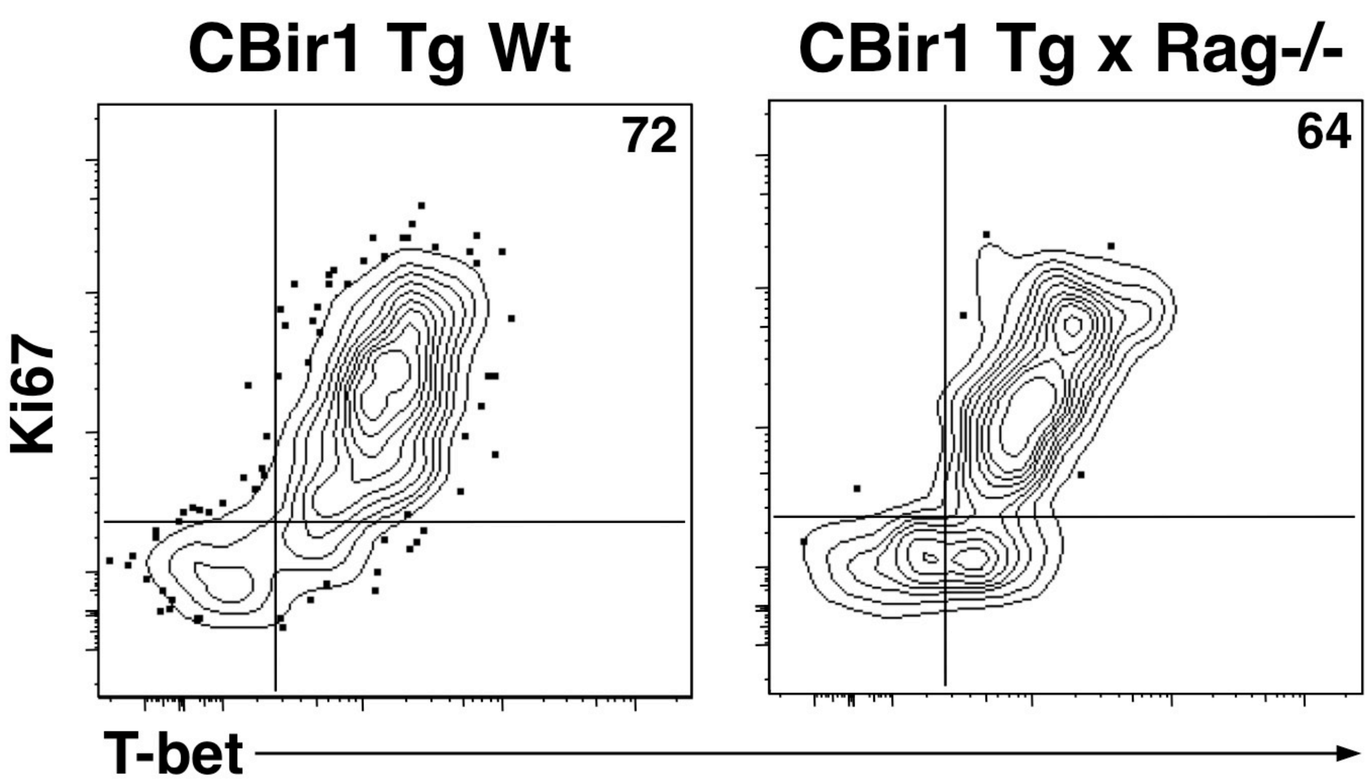


Figure S3



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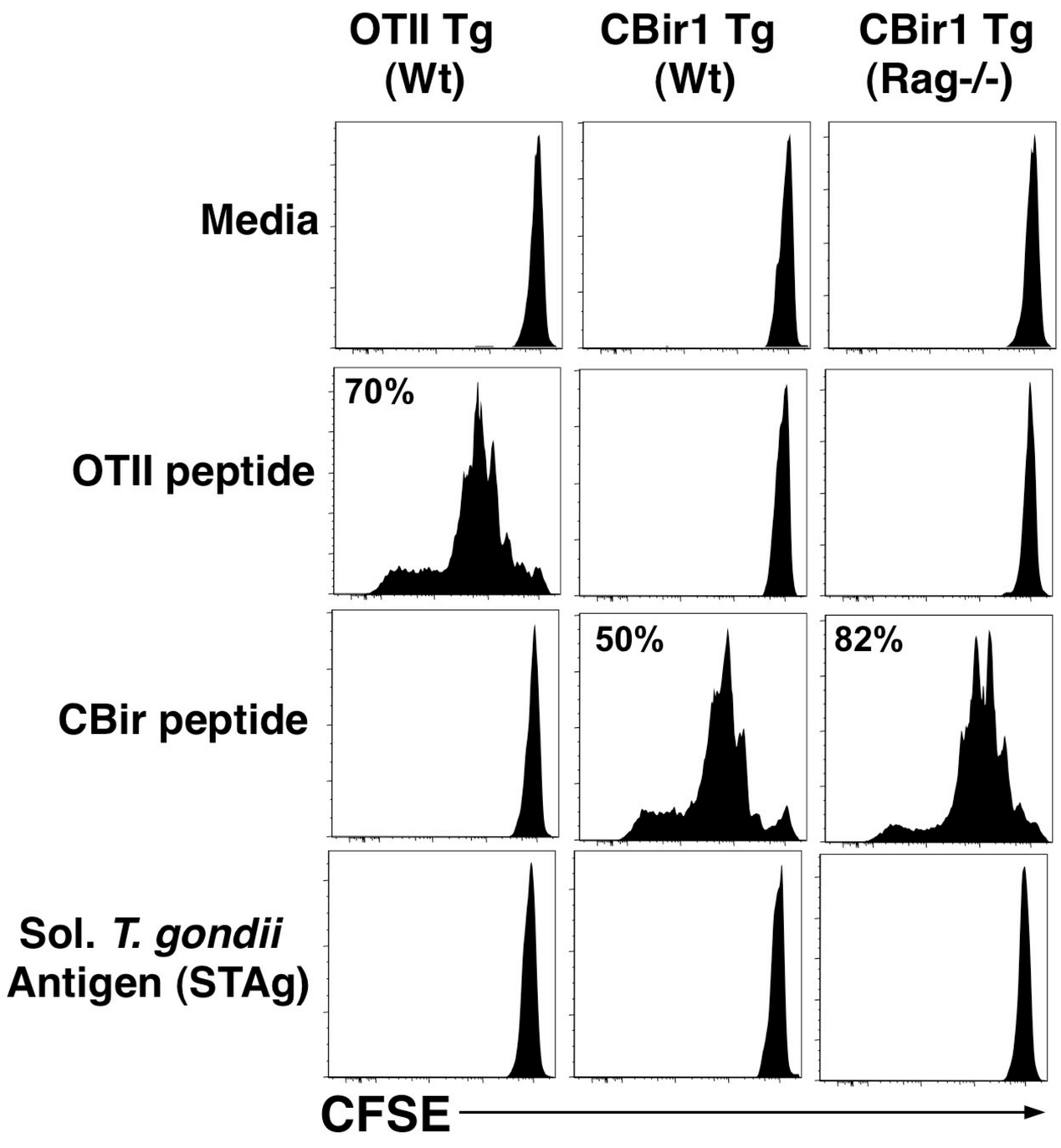

OTII Tg
(Wt)
CBir1 Tg
(Wt)
CBir1 Tg
(Rag-/-)
Media
OTII peptide
70%
CBir peptide
50%
82%
Sol. T. gondii
Antigen (STAg)
CFSE

Figure S5

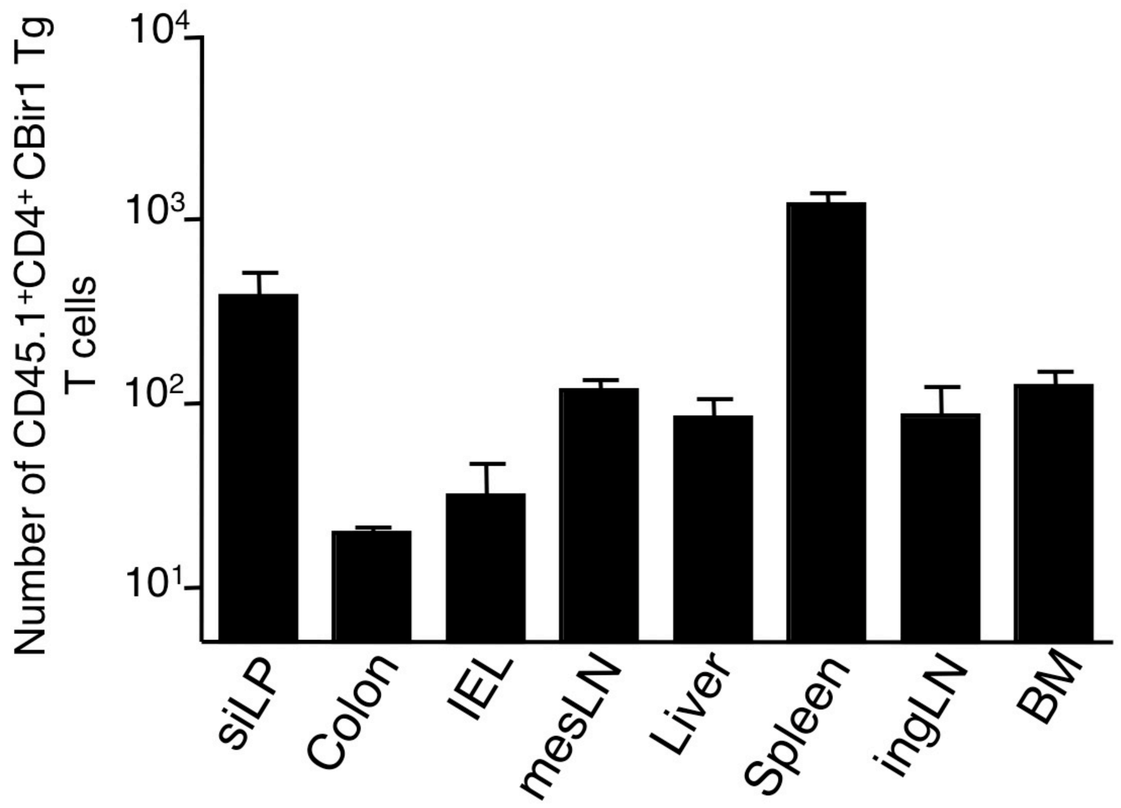
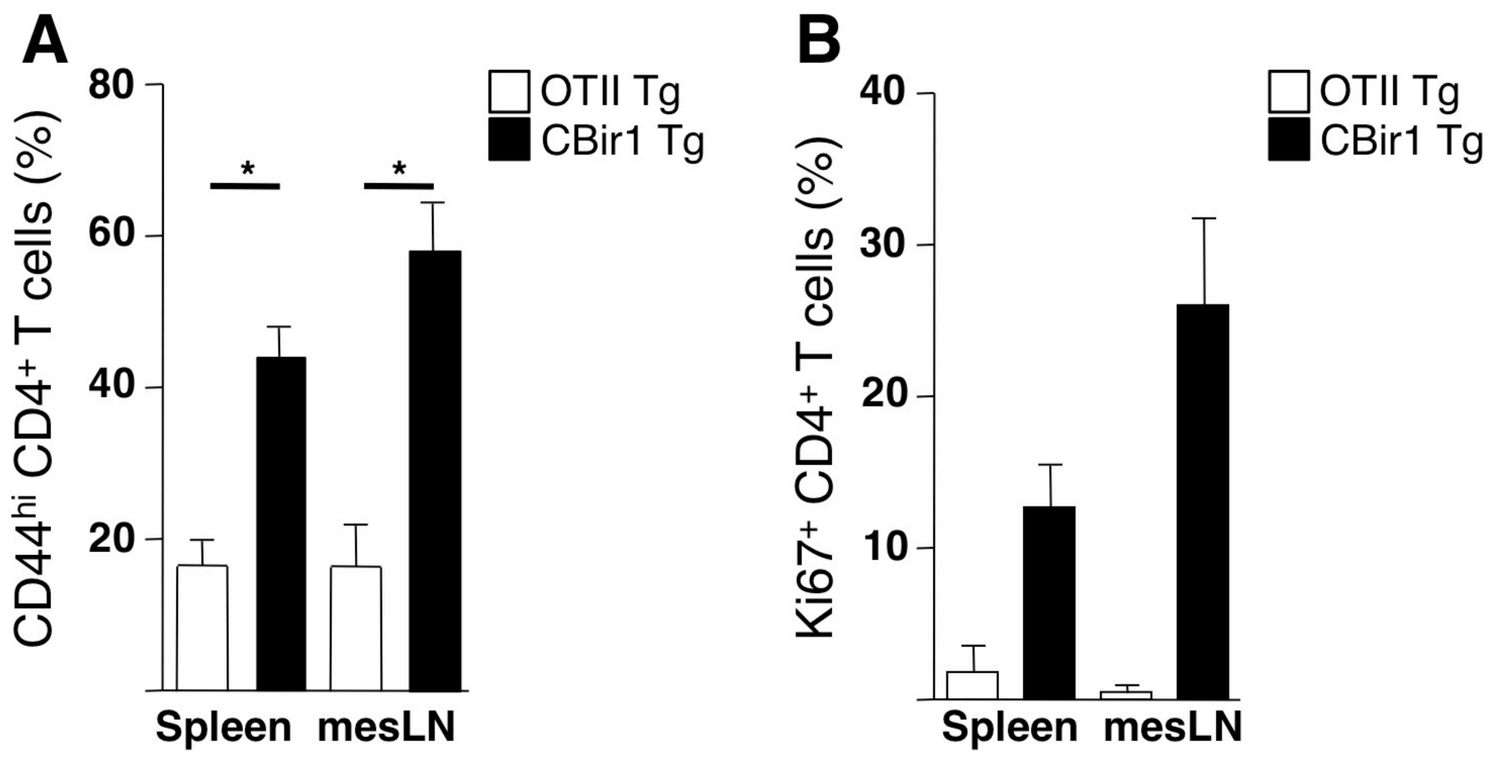

Number of CD45.1+CD4+ CBir1 Tg T cells
10^4
10^3
10^2
10^1
siLP
Colon
IEL
mesLN
Liver
Spleen
ingLN
BM

Figure S6


A
CD44hi CD4+ T cells (%)
80
60
40
20
*
*
OTII Tg
CBir1 Tg
Spleen
mesLN
B
Ki67+ CD4+ T cells (%)
40
30
20
10
OTII Tg
CBir1 Tg
Spleen
mesLN

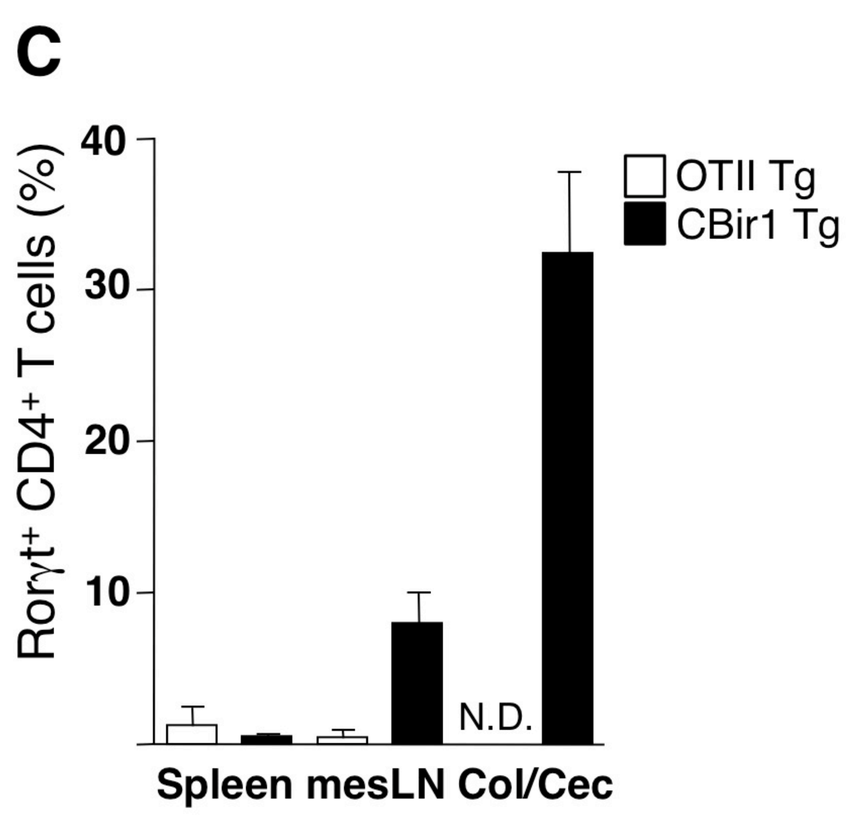

C
Rorγt+ CD4+ T cells (%)
40
30
20
10
OTII Tg
CBir1 Tg
N.D.
Spleen
mesLN
Col/Cec

Figure S7

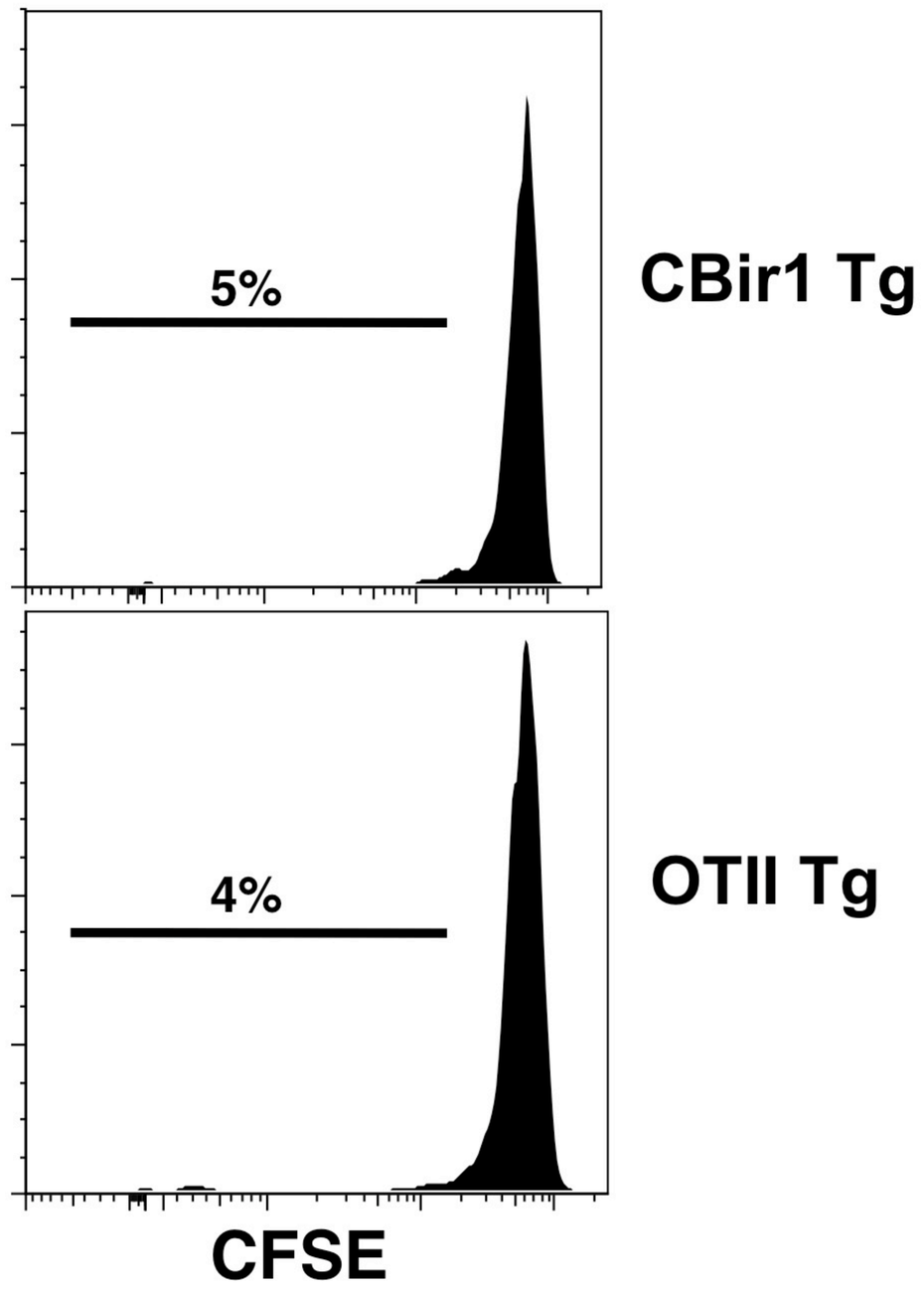


Figure S8

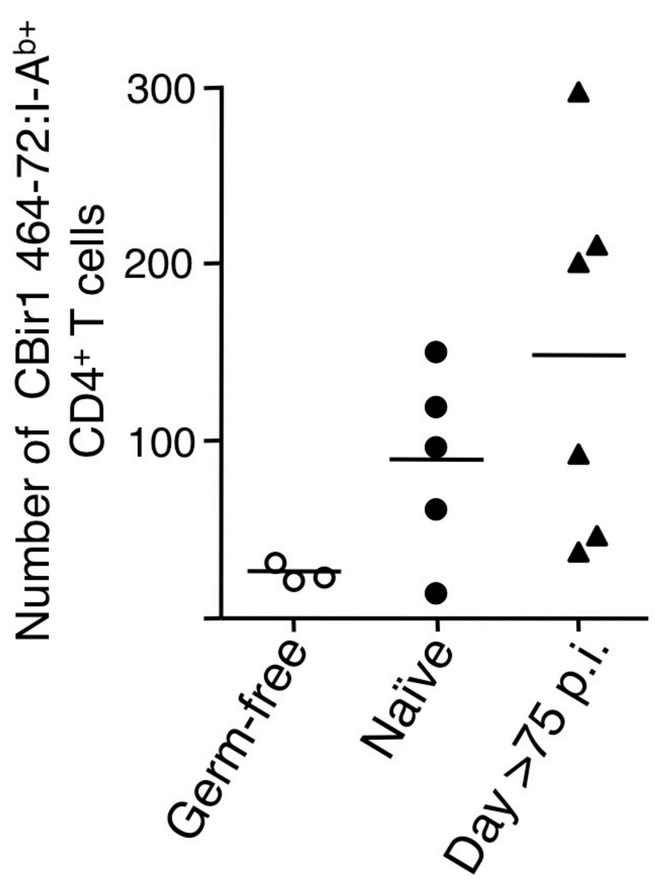

Number of CBir1 464-72:I-A^{b} CD4^{+} T cells
300
200
100
Germ-free
Naïve
Day >75 p.i.

Figure S9

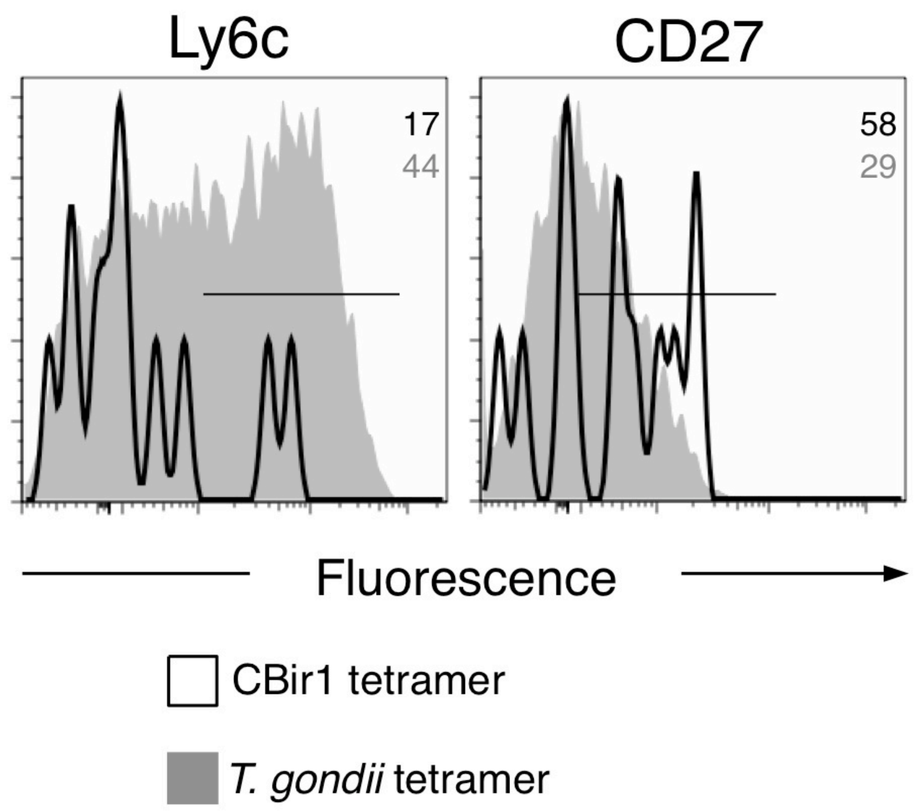

Ly6c
CD27
17
44
58
29
Fluorescence
CBir1 tetramer
T. gondii tetramer

Figure S10

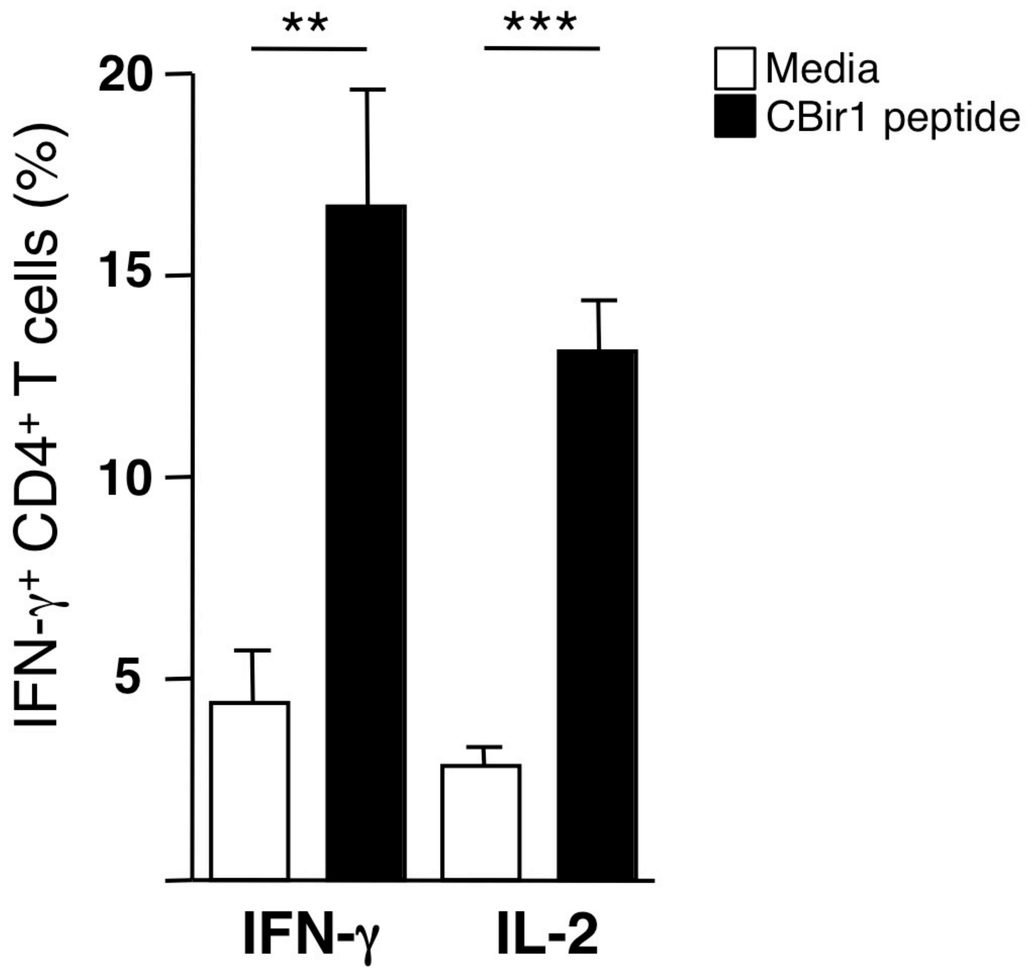


Figure S11

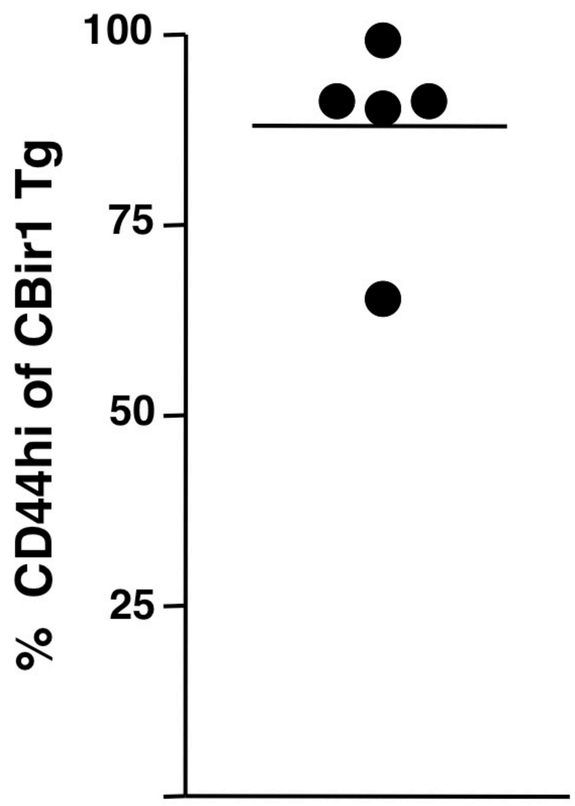


Figure S12

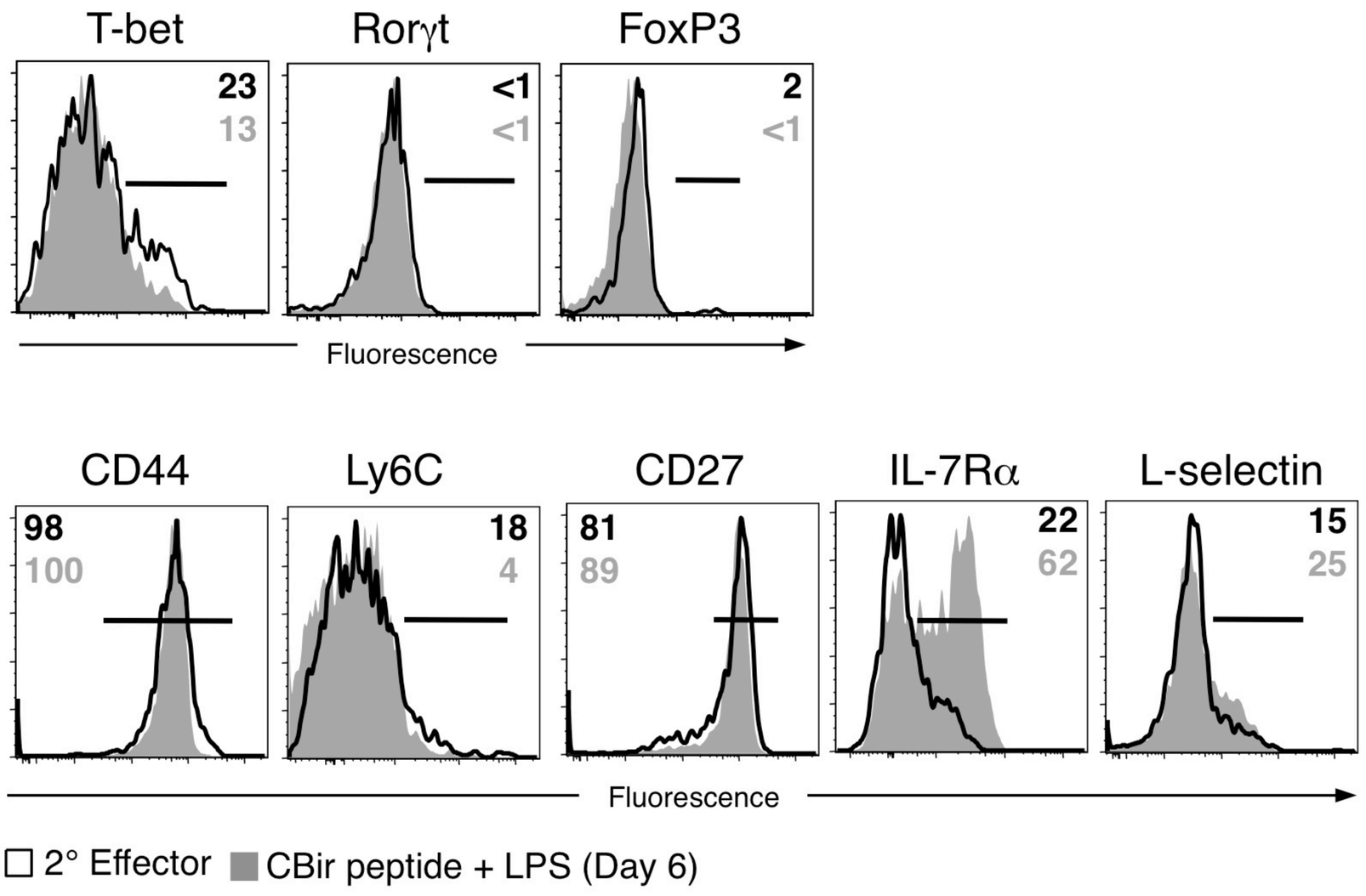

T-bet
Rorγt
FoxP3
23
13
<1
<1
2
<1
Fluorescence
CD44
Ly6C
CD27
IL-7Rα
L-selectin
98
100
18
4
81
89
22
62
15
25
Fluorescence
2° Effector
CBir peptide + LPS (Day 6)

Figure S13

Supplementary Figure 1 – Oral infection with *T. gondii* induces pathology in the ileum. C57Bl/6 mice were infected orally with 15 cysts of RFP-expressing *T. gondii*. Ileal tissue samples were isolated at the time indicated p.i. and stained with H&E. Original magnification x 20. Scale bar indicates 25μM.

Supplementary Figure 2 – CBir1 flagellin is present in naïve and *T. gondii* infected mice. Mucosa-associated bacteria were isolated from the LP, genomic DNA extracted and presence of CBir flagellin DNA sequences assessed by semi-quantitative PCR; n=3-6 mice per group. The bar graph indicates the relative presence of CBir flagellin with reference to total bacterial genomic DNA (Eubacteria-specific 16S).

Supplementary Figure 3 – Splenic CBir1 Tg T cells maintain a naïve (CD44lo/T-bet-/Rorγt-) phenotype. Splenocytes from CBir1 Tg and OTII mice were isolated and stained intracellularly for CD44, Foxp3, T-bet and Rorγt. All contour plots shown are gated on Live/Dead- CD4+ Vβ8+(CBir1 Tg) Vα2+(OTII). Data shown is representative of 2 separate experiments.

Supplementary Figure 4 – CBir1 Tg T cells on a Rag deficient background are activated during *T. gondii* infection. $5x10^4$ CBir1 Tg/Rag-/- (CD45.2) T cells were transferred into congenic (CD45.1) hosts that were subsequently infected orally with 15 cysts of *T. gondii*. Ten days p.i., single cell suspensions were prepared from the spleen and stained intracellularly for flow cytometry. Contour plots show expression of Ki67 and T-bet amongst CBir1 Tg Rag-/- (CD45.2+) CD4+ T cells compared to CBir1 Tg Wt controls. Plots shown are gated on Live/TCRβ+/CD45.2+. N=3 mice per group.

Supplementary Figure 5 – CBir1 Tg T cells do not respond to *T. gondii* derived antigens *in vitro.* Splenocytes (~50 000) from OTII Tg, CBir1 Tg and CBir1 Tg/Rag-/- mice were isolated, CFSE-labeled and co-cultured for 72 hours with DCs freshly isolated from naïve C57BL/6 mice in the presence of the antigen indicated above. (OTII/CBir1 peptide-1μg/mL, STAg-5μg/mL). Plots shown are gated on Live/TCRβ+/CD4+/CD8-.

Supplementary Figure 6 – Number of Activated ($CD44^{hi}$) CBir1 Tg T cells in various tissues post *T. gondii* infection. $7.5x10^4$ CBir1 Tg (CD45.1) T cells were transferred into congenic (CD45.2) hosts that were subsequently infected orally with 15 cysts of *T. gondii*. Eighteen days p.i. single cell suspensions were prepared from the spleen, ingLN, mLN, bone marrow, liver, peripheral blood, LP, IEL and colon and stained for flow cytometry; *n=3* mice per group.

Supplementary Figure 7 - CBir1 Tg T cells become activated and express Rorγt during DSS treatment. $7.5x10^4$ CBir1 Tg or OTII Tg (CD45.1) T cells were transferred into congenic (CD45.2) hosts that were subsequently treated orally with 2% DSS for seven days. 14 days after initiation of treatment single cell suspensions were isolated from the spleen, mesenteric lymph nodes (mesLN) and colon/cecum, stained and analyzed by intracellular flow cytometry. Bar graphs showing the frequency of CBir1 Tg T cells from DSS-treated mice expressing (A) CD44, (B) Ki67 and (C) Rorγt. CBir1

Tg/OTII Tg T cells are gated on Live/TCRβ+/CD4+/CD45.1+. All data shown is representative of three separate experiments.

Supplementary Figure 8 – CBir1 Tg T cells do not proliferate after transfer into chronic *T. gondii* infected mice. 10^5 CFSE-labeled CBir1 Tg (CD45.1/45.1) and 10^5 CFSE-labeled OTII Tg (CD45.1/45.2) T cells were transferred to congenic (CD45.2/45.2) mice at day 90 p.i. with *T. gondii*. Eight days later the mice were sacrificed and CFSE dilution on transferred CD4 T cells from the spleen was assessed by flow cytometry. Plots shown are gated on CD4+/CD8-/CD45.1+/ CD45.2+/-.

Supplementary Figure 9 – Number of $CBir_{464-72}$:I-A^b tetramer binding cells in naïve and *T. gondii* infected mice. Total lymphocytes were isolated from either naïve or day 75 *T. gondii* infected mice, enriched and stained for $CBir_{464-72}$:I-A^b tetramer binding cells.

Supplementary Figure 10 - Expression of Ly6c and CD27 on commensal and *T. gondii*- specific memory CD4 T cells. Histograms show expression of Ly6c and CD27 on CBir464-72:IA^b (black line) or *T. gondii* ME49 hp605-619:I-A^b (grey filled) from mice 75 days p.i..

Supplementary Figure 11 – Memory CBir1 Tg T cells make IFN-γ and IL-2 in response to peptide stimulation. $7.5x10^4$ CBir1 Tg (CD45.1) T cells were transferred into congenic (CD45.2) hosts that were subsequently infected orally with 15 cysts of *T. gondii*. 75 days p.i. CD45.1+ T cells were isolated by magnetic enrichment and co-cultured for 17 hours with dendritic cells pulsed with 1μg/mL CBir1 peptide. The final five hours of culture were carried out in media containing brefeldin A, after which cells were stained intracellularly for IFN-γ and IL-2. Bar graph shows expression of these cytokines in T cells gated on Live/TCRβ+/CD4+/CD45.1+. *n=4* samples per group.

Supplementary Figure 12 – Activated CBir1 Tg T cells can be identified in the peripheral blood of *T. gondii* infected mice. $7.5x10^4$ CBir1 Tg (CD45.1) T cells were transferred into congenic (CD45.2) hosts that were subsequently infected orally with 15 cysts of *T. gondii*. Eight days p.i. peripheral blood samples were taken, lymphocytes were isolated by gradient separation and stained for flow cytometry. Plots shown are gated on TCRβ+/CD4+. Data is representative of three separate experiments.

Supplementary Figure 13 - Phenotype of primary and secondary CBir1 Tg effector T cells. Either naïve hosts carrying ~10^4 naïve CBir1 Tg T cells (gray filled) or day 35 (1° memory) *T. gondii* infected mice (black line) were injected with CBir peptide (50μg) and LPS (10μg). Six days post-injection splenocytes were isolated and stained intracellularly for T-bet, Rorγt, CD44, Ly6c, CD27, IL-7Rα and L-selectin. Plots shown are gated on Live/TCRβ+/CD4+/CD45.1+. Shown are representative plots of three separate experiments.