values. Statistical significance was determined using a Mann-Whitney Test: *P<0.05, **P<0.01.

Figure 6. T_{FR} restrict the outgrowth of non-antigen specific clones in the germinal center. Flow cytometric contour plots **(a)** and graphs **(b)** of total GL-7^+CD95^+ germinal center B cells and **(c)** NP^+ germinal center B cells ten days after immunization of $Foxp3^{WT}$ and $Foxp3^{DTR}$ mice that have been treated with DT 6 days after NP-KLH immunization. Statistical analyses performed using Mann Whitney U-test. Experimental outline **(d)** of immunization and DT or saline treatment scheme of $Foxp3^{DTR}$ mice (n=8 per group) to examine the antigen specific immunoglobulin response over time, mice were bled prior to, and d10, d15, d20 and d28 after primary immunization. Mice were given a booster immunization 24 days after the primary immunization. **(e)** ELISA analysis of NP12 and NP2 antibodies in the experiment outlined in (d). Error bars represent the standard error of the mean from eight individual mice from one experiment, representative of two experiments. Statistical analyses in (e) were performed using a two-way ANOVA with Bonferroni post test to compare differences at each time point. Graphs and flow cytometric contour plots of NP^+ germinal center B cells **(f)**, total GL-7^+CD95^+ germinal center B cells **(g)** and NP^+ bone marrow plasma and memory cells **(h, i)** 21 days after NP-CGG immunization of chimeric mice generated by reconstituting *Rag2*$^{-/-}$ mice with a 1:1 mix of *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ fetal liver. Statistical analyses in (f, g, h and i) were performed using a one-way ANOVA with Bonferroni post test correction. Each symbol represents one mouse and horizontal bars represent median values. *P<0.05, **P<0.01, ***P<0.001.

Legends to Supplementary Figures

Supplementary Figure 1. Sorting strategy used to isolate different T cell subsets for gene expression analysis. The $CD4^+$ helper cell populations outlined below were sorted from Foxp3-GFP mice seven days after immunization with KLH in Ribi. The cells were sorted using the markers and nomenclature described in Figure 1.

Supplementary Figure 2. Expression of T_{FH} and T_{reg} associated molecules on T_{FR} cells. (a) C57BL/6 were immunized with SRBC and analyzed 7 days later by flow cytometry. Scatter plots show mean fluorescence intensity of intracellular CD40L, surface CD28, surface ICOS, surface GITR, intracellular CTLA4 and surface CD39 on T_{FH} (blue), T_{reg} (green) and T_{FR} (red) cells. Grey symbols represent a negative staining control (antibody isotype or cells from CD40L-deficient mice as indicated). **(b)** Percentage of T_{FH} (blue), T_{reg} (green) and T_{FR} (red) cells that express CD25 or KLRG1. **(c)** CD103 vs. Foxp3 expression on T_{reg} or T_{FR} cells. Each symbol represents one mouse and horizontal bars represent median values. Statistical significance was determined using a one-way ANOVA analysis with Bonferroni's multiple testing correction; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Figures are representative of at least 3 independent experiments.

Supplementary Figure 3. T_{reg} formation in the absence of CD28, B cells and SAP. Scatter plots of T_{reg} cells in mixed bone marrow chimeras seven days after SRBC immunization. Chimeras were generated by sub-lethally irradiating *Rag2*$^{-/-}$ mice and reconstituting their immune system with a 1:1 ratio of bone marrow cells from CD45.1 *Cd28*$^{+/+}$ and CD45.2 *Cd28*$^{-/-}$ mice or control CD45.1 *Cd28*$^{+/+}$ and CD45.2 *Cd28*$^{+/+}$**(a)**. Scatter plots showing the proportion of $CD4^+$ T cells that are $Foxp3^+$ in B cell-deficient μMT mice and wild type controls **(b)** or in *Sh2d1a*$^{+/+}$ and *Sh2d1a*$^{-/-}$ mice **(c)** seven days after SRBC immunization as determined by flow cytometry. Each symbol represents one mouse and horizontal bars represent median values. Statistical significance was determined using a Mann-Whitney Test: * $P<0.05$, ** $P<0.01$. Figures are representative of at least 3 independent experiments.

Supplementary Figure 4. Bcl-6 is required for T_{FR} formation but is dispensable for T_{reg} formation. Flow cytometric contour plots **(a)** and dot plots of T_{FH} **(b),** T_{FR} **(c)** and T_{reg} **(d)** cells in the mixed fetal liver chimeras of mice described below, seven days after SRBC immunization. Mixed fetal liver chimeras generated by sub-lethally irradiating *Rag2*$^{-/-}$ mice and reconstituting their immune system with a 1:1 ratio of fetal liver cells from CD45.1 *Bcl6*$^{+/+}$ and CD45.2 *Bcl6*$^{-/-}$ embryos or control CD45.1 *Bcl6*$^{+/+}$ and CD45.2 *Bcl6*$^{+/+}$. Each symbol represents one mouse and horizontal bars represent median values. Statistical significance was determined using a Mann-

Whitney Test: **P*<0.05, ***P*<0.01. Figures are representative of at least 3 independent experiments.

Supplementary Figure 5. T_{FR} derive from Foxp3$^+$ precursors. Representative flow cytometric contour plots and histograms of splenic CD4$^+$ T cells seven days after adoptive transfer of thymic T cells and SRBC immunization **(a)**. CD45.2 cells represent cells sorted from the thymus of Foxp3^{GFP} mice, in the top panel these are Foxp3$^-$CD4^{SP} thymocytes, the bottom panel represent Foxp3$^+$CD4^{SP} thymocytes. Flow cytometric contour plots showing expression of Helios and Foxp3 in the CD4$^+$ T cell population **(b)** and the CD4$^+$CXCR5^{high}PD-1^{high} T_{FH}/T_{FR} cell pool **(c)** seven days after immunization with SRBC. Proportion and total number of Foxp3$^+$ T_{reg} cells in Foxp3^{DTR} mice six days after immunization with SRBC and treatment on the same day with either 50μg/Kg DT or 0.9% saline, as determined by flow cytometry **(d)**. Each symbol represents one mouse and horizontal bars represent median values. Statistical significance was determined using a Mann-Whitney Test: **P*<0.05, ***P*<0.01.

Supplementary Figure 6. T_{FR} cells are suppressive *in vitro*. (a) CD4$^+$CXCR5^{high}PD-1^{high}Foxp3$^+$ T_{FR} cells and CD4$^+$CXCR5$^{low/int}$PD-1$^{low/int}$Foxp3$^+$ T_{reg} cells from Foxp3-GFP mice immunized with SRBC seven days previously were flow sorted and co-cultured with naïve CFSE-labeled CD4$^+$CD44^{low}Foxp3$^-$ cells activated with αCD3 and αCD28. T_{reg} or T_{FR} were co-cultured with T responder cells at the indicated T_{reg}:T responder or T_{FR}:T responder ratios. **(b)** CD4$^+$CXCR5^{high}PD-1^{high}Foxp3$^-$ T_{FH} cells and CD4$^+$CXCR5$^{low/int}$PD-1$^{low/int}$Foxp3$^-$ T_N/T_{EM} cells were also co-cultured with T responder cells to test whether these cells can also suppress proliferation. **(c)** Bar chart showing the percentage of cells that had undergone 2 or more divisions after 3 days of culture. Height of the bars represents the mean, and errors bars represent the range of expression in 3 biological replicates. Data are representative of 2 independent experiments.

Supplementary Figure 7. DT treatment post-immunization depletes T_{FR} cells and T_{reg}. Scatter plots showing the proportion and total number of T_{reg} and T_{FR} cells in spleens from Foxp3^{DTR} mice immunized with SRBC eight days previously (d0). Five

days after immunization the mice were treated with either DT or 0.9% saline. Statistical significance was determined using a Mann-Whitney Test: *P<0.05, ns= not significant. Each symbol represents one mouse and horizontal bars represent median values.

Supplementary Figure 8. Construction of chimeric mice in which T_{FR} cells can be selectively and inducibly ablated. Mixed bone marrow chimeras were generated by sub-lethally irradiation of *Rag2*$^{-/-}$ mice and reconstitution with a 1:1 mix of *Sh2d1a*$^{-/-}$ CD45.2 : Foxp3^{DTR} CD45.1 bone marrow and control *Sh2d1a*$^{+/+}$ CD45.2 : Foxp3^{DTR} CD45.1. Eight weeks after reconstitution chimeric mice were immunized with SRBC and treated with 50μg/Kg of DT on d-1, d2 and d5 relative to immunization. Spleen cells were analyzed on d8. **(a)** Flow cytometric dot plots showing CD45.1 and Foxp3 expression in CD4^{+}CXCR5^{high}PD-1^{high} cells from control and experimental chimeras. **(b-c)** Scatter plots showing the percentage of CD4^{+}Foxp3^{+} cells that derive from the CD45.2 compartment (b) and the percentage of CD4^{+}CXCR5^{high}PD-1^{high} cells that derive from the CD45.1 compartment (c) in control and experimental chimeras. Statistical significance was determined using a Mann-Whitney Test: *P<0.05. Each symbol represents one animal and horizontal bars represent median values.

Supplementary Figure 9. Selective reduction of T_{FR} cells results in the increase in T_{FH} cells, but not T_{reg} cells. Flow cytometric contour plots **(a)** and graphs demonstrating a specific reduction in T_{FR} cells **(b)**, and increased T_{FH} cells **(c)** in *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ chimeras compared with control *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ mice 21 days after NP-CGG immunization. Total number of Foxp3^{+}CD4^{+} T_{reg} **(d)** are comparable in all groups of chimeras. Statistical analyses were performed using a one-way ANOVA with Bonferroni post test correction. Each symbol represents one mouse and horizontal bars represent median values. *P<0.05.

Supplementary Figure 10. Th1 and Th2 cell numbers are not altered in mice with reduced T_{FR} cells. Flow cytometric contour plots and graphs of Tbet^{+}CD44^{high} CD4^{+} **(a)** and Gata3^{+}CD44^{high} CD4^{+} **(b)** T cells in the blood of *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ mice 14 days after

NP-CGG immunization. And splenic IFNγ^{+}CD44^{high} CD4^{+} **(c)** and IL-4^{+}CD44^{high} CD4^{+} **(d)** T cells in the same chimeras 21 days after immunization. Each symbol represents one mouse and horizontal bars represent median values.

Supplementary Figure 11. Splenic plasma cell number and NP titers are not altered in the absence of T_{FR} cells. Flow cytometric contour plots **(a)** and graphs of NP^{+}B220^{low} splenic plasma cells **(b)** 21 days after *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ mice are immunized with NP-CGG. ELISA analysis of anti-NP14 and anti-NP2 antibodies in the serum of the same mice 21 days after immunization. Each symbol represents one mouse and horizontal bars represent median values.

Supplementary Table 2.

Chimera	Genotype	Allotype	T_{FH}*	T_{reg}*	T_{FR}*
Control (intact T_{FR})	*Sh2d1a*$^{+/+}$	CD45.2	50	100	100
	Foxp3^{DTR}	CD45.1	50	0	0
Experimental (lacking T_{FR})	*Sh2d1a*$^{-/-}$	CD45.2	0	100	0
	Foxp3^{DTR}	CD45.1	100	0	0

* These numbers represent the percentage of cells expected to derive from the bone marrow cells in each of the chimeras after immunization and DT treatment.

Materials and methods

Mice

C57BL/6 (B6), B10.Br, *Bcl6*$^{-/-}$, Foxp3^{GFP}, *Sh2d1a*$^{-/-}$, Foxp3^{GFP} and TCR^{3A9} mice (ANU Bioscience Facility); B6, B6-CD45.1, μMT and OT-II (Central Biomedical Services, University of Cambridge); Foxp3^{GFP}, Foxp3^{CRE} x Rosa26-stop-flox and Foxp3^{DTR} mice on the C57BL/6 background (University of Leuven animal facility) and *Prdm1*^{gfp} and *Bcl6*$^{-/-}$ mice (Walter and Eliza Hall Institute, Melbourne). All experiments were performed according to the regulations of the UK Home Office Scientific Procedures Act (1986) and approved by the Australian National University Animal Experimentation Ethics Committee and the University of Leuven Animal Ethics Committee.

Immunizations and T_{reg} depletion

To generate thymus-dependent germinal center responses, 8-12 week old mice were immunized intraperitoneally with 2x10^9 sheep red blood cells (SRBC) (IMVS Veterinary Services, Australia or TCS bioscience, UK), or KLH (Sigma) emulsified in Ribi (Sigma). Foxp3$^+$ cells were deleted during a response to immunization by administering 50μg/Kg of diphtheria toxin (DT) in 0.9% NaCl intraperitoneally into Foxp3^{DTR} mice.

Antibodies for flow cytometry

Antibodies and streptavidin conjugates for flow cytometry were from BD Pharmingen except where otherwise indicated: anti-mouse B220-PerCP, B220-Pacific Blue, CD4-PerCP, CD4-Pacific Blue, ICOS-FITC (eBioscience), GL-7-FITC, GL-7 biotin, CD95-PE-Cy7, CD95-PE, CXCR5-biotin, PD-1-PE (eBioscience), PD-1-FITC

(eBioscience), streptavidin-APC, streptavidin-PE-Cy7, CD45.2-FITC, B220-APC Cy7, CD45.1-Pacific Blue, Foxp3-FITC (eBioscience), Foxp3-APC (eBioscience), CD40L-APC (eBioscience), GITR-PE-Cy7 (eBioscience), CTLA4-PE, CD25-PerCP Cy5.5, CD103-FITC, NP-PE (Biosearch Technologies Inc), IL-4-Pe-Cy7 (eBioscience), IFNγ-APC (eBioscience) and Helios-AF488 (BioLegend).

Bone Marrow Chimeras

Recipient C57BL/6 *Rag2*$^{-/-}$ mice were sub-lethally irradiated with 1000 Rad and reconstituted via intravenous injection with $2x10^6$ donor bone marrow-derived hematopoietic stem cells. Chimeric mice were immunized at or after 8 weeks after reconstitution.

Cell isolation, culture and stimulation

Single cell suspensions were prepared from spleens of immunized mice by sieving and gentle pipetting through Falcon 70 μm nylon mesh filters (Becton Dickson). Splenocytes were suspended in RPMI 1640 medium (JRH biosciences) supplemented with 2mM L-Glutamine (Gibco), 100 Units Penicillin Streptomycin (Gibco), 0.1mM Non-Essential amino acids (Gibco), 100mM Hepes (Sigma), $5x10^5$ 2-mercaptoethanol and 10% Fetal Calf serum. Sorted cells were labeled with CFSE by incubating with 1000x dilution of 10μM CFSE, incubated at room temperature for 5 minutes then washed 3 times in media. Then were stimulated with plate bound αCD3 (2μg/mL) and αCD28 (10μg/mL).

Immunohistochemistry

To visualize the responses to SRBC immunization, spleen samples were fixed for 2 hours in 4% paraformaldehyde (PFA) on ice, incubated in six changes of sucrose buffer overnight and embedded in Tissue-Tek OCT compound (Sakura Finetek). Sections were blocked with Streptavidin and Biotin Blocking Kit (Vector Laboratories) before staining. Sections were stained for CD3 using biotinylated hamster anti-mouse CD3 (500A2, BD Pharmingen, Oxford, UK) followed by biotinylated anti-hamster IgG (Vector Laboratories), then streptavidin-HRP conjugates (Zymed) followed by TSA^{TM} kit Alexa Flour@647 Tyramide (Invitrogen).

For Bcl-6, anti-mouse Bcl-6 (N-3, Santa Cruz) was followed by donkey anti-rabbit FITC (Jackson) and then Alexa 488- conjugated goat anti-FITC (Invitrogen). For AID, anti-mouse AID (AID-2, eBioscience) was followed by biotinylated anti-rat IgG, then streptavidin-HRP conjugates followed by TSA^{TM} tetramethylrhodamine (PerkinElmer). Foxp3 was stained with biotinylated anti-mouse Foxp3 (FJK-16s, ebioscience) followed by streptavidin-HRP conjugates and then TSA^{TM} tetramethylrhodamine. For Blimp-1 staining, anti-mouse purified Blimp-1 (6D3, ebioscience) was followed by biotinylated anti-rat IgG (Jackson), then streptavidin-HRP conjugates followed by TSA^{TM} kit Alexa Flour@647 Tyramide. Stained sections were mounted in Fluoromount-g (Southern Biotech) and analyzed with a confocal laser-scanning microscope (model DMRXA2; Leica) using a 40X objective.

Real time PCR analysis

RNA was extracted using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions and cDNA produced from 25ng of RNA using the Ovation Pico WTA System following the manufacturer's instructions (Nugen). Primers specific for *Il21*, *Il4*, *Cxcl13*, *Gzmb*, *Gzma*, *Il10*, *Bcl6*, *Prdm1* and the housekeeping gene *Gapdh* (sequences available on request) were used to amplify the cDNA in the presence of SYBRgreen, on an Applied Biosystems 7900 machine. The gene-specific expression change, normalized to *Gapdh*, was calculated using the $2^{-\Delta\Delta ct}$ method.

Influenza infection

Mice were inoculated intranasally with $10^{4.5}$ p.f.u. of the HKx31 (H3N2) influenza virus. Analysis of the lung-draining mediastinal lymph node and the spleen was performed at d10 post infection, at the peak of the anti-viral response.

Enzyme-Linked Immunosorbent Assay

Anti-NP IgG (Southern Biotechnology Associates) was detected in plasma from blood by enzyme-linked immunosorbent assay (ELISA). 96-well ELISA plates (Nunc) were coated with NP2, NP12 or NP14 (Biosearch Technologies Inc). Serial serum dilutions were applied, immunoglobulin concentration was determined with HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates), and the enzyme bound to plates was developed with Phosphatase Substrate tablets (Sigma

S0942). Plates were read at 405 nm with a Thermomax Microplate Reader (Molecular Devices). The titers for serum samples were calculated as the log serum concentration required to achieve 50% maximum optical density.

Statistical analysis

Single comparisons were analyzed using the non-parametric Mann-Whitney U-test. Multiple comparisons were analyzed using one-way or two-way (where appropriate) ANOVA with Bonferroni's multiple testing correction. All statistical analyses were carried out with GraphPad Prism v5.