Materials and Methods

Mice. *Tgfbr2*$^{fl/fl}$ × CD4-Cre (TGFβR-KO) mice were previously reported (30). Cre negative littermates were used as wild-type controls. DN-TGFβRII (TGFβR-DN) mice were provided by Ronald Gross (National Cancer Institute, NIH) (34). Foxp3^{gfp} and TEa mice were provided by Alexander Y. Rudensky (Sloan-Kettering Institute, New York). LSL-TβRI^{CA} (TGFβR-KO) mice were generated in house as previously described (40). CD3-KO, C57BL/6 (B6), Thy1.1 congenic B6 and Rag2$^{-/-}$ (Rag-KO) mice were purchased from Charles River Laboratories. Mice were housed and bred in a specific pathogen free animal facility, AniCan, at the Cancer Research Center Lyon.

Cell isolation and adoptive cell transfer. Single cell suspensions were prepared from thymus, spleen or peripheral lymph nodes in the case of autoimmunity (pLN; pool of inguinal, brachial, axillary and cervical lymph nodes) or draining lymph nodes (dLN; inguinal) in the case of immunization by manual disruption using glass slides. For cell sorting, cells were first purified with Miltenyi-Biotec beads followed by sorting on a FACSAria (BD Biosciences). For adoptive cell transfer, CD4$^+$ T cells were pre-purified by CD4 negative selection (Miltenyi-Biotec) followed by sorting to high purity before 5 x 10^5 CD4$^+$ T cells were injected into recipient mice. For in vitro T_{FH} differentiation, CD4$^+$ B220$^-$ PD-1$^-$ CXCR5$^-$ CD44^{low} CD62L^{high} Foxp3$^-$ T cells were isolated by a combination of CD4$^+$ Miltenyi beads and cell sorting before culture. For real time qPCR, CD8$^+$ Tregs were purified by CD8 negative selection followed by cell sorting to high purity.

Mouse immunization. Mice were injected subcutaneously in the tail base with 2 x 50µl of keyhole limpet hemoctyanin (KLH; 0.5mg/ml; Calbiochem) emulsified in an equal volume of complete Freund adjuvant (CFA; 1mg/ml; Sigma-Aldrich). Control mice were injected with

CFA alone. Mice were sacrificed and the draining inguinal lymph nodes were examined on day 7 or 21 following immunization.

Antibodies and flow cytometry. Cells were pre-incubated with anti-CD16/32 and stained for 30 minutes at 4°C with the following antibodies. CD4 (RM 4-5), PD-1 (RPMI-30), B220 (RA3-6B2), ICOS (7E.17G9), BTLA (8F4), GL-7 (GL-7), CD8 (53-6.7), Ly49C/I/F/H (14B11), CD122 (TM-b1), CD40L (MR1), Foxp3 (FJK-16s), CD90.1 (Thy1.1), Vα2 (B20.1), Vβ6 (RR4-7), IL-21 (FFA21) and IFNγ (XMG1.2) from eBioscience. CXCR5 (2G8), CD44 (1M7), CD3 (145-2C11), CD19 (1D3), CD95 (J02), Bcl-6 (K112-91), Bcl-2 (3F11) and Ki-67 (B56) from BD Biosciences. Mouse TGF-beta RII biotinylated was from R & D Systems. Steptavidin PeCy7, FITC and APC (BD Biosciences) were also used. Flow cytometric analysis was carried out using a CyAn (Beckman Coulter) or BD Fortessa (BD Biosciences) and analysis of cells was performed using FlowJo software (Tree star Inc.). Fluorescence minus one (FMO) controls were used to set positive staining gates. For apoptosis staining, cells were incubated at 37°C for three hours in RPMI media without fetal calf serum prior to staining with annexin V and 7-AAD (BD Biosciences) in appropriate binding buffer (BD Biosciences) according to manufacturer's instructions. For intracellular cytokine staining, cells were stimulated with 500ng/ml PMA (Sigma-Aldrich) and 500ng/ml ionomycin (Sigma-Aldrich) for three hours in the presence of Golgiplug (BD Pharmingen). Cells were fixed and permeabilized using the BD cytofix/cytoperm kit (BD Pharmingen). For intranuclear staining, the Foxp3 staining kit was used (eBioscience).

Immunofluorescence histology. Kidney tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek) and snap frozen over liquid nitrogen. Sections (6μm) were cut with a Leica X cryostat onto Poly-Prep slides (Sigma-Aldrich). Slides were fixed in cold

acetone/ethanol (3:1) and blocked with 1% BSA (Sigma-Aldrich). Goat anti-mouse IgG alexa fluor 488 (Invitrogen) was used to detect IgG immune complex deposition. Slides were mounted with flouromount (Sigma-Aldrich) and analyzed with a Zeiss 780 Confocal microscope.

Bone marrow chimeras. Bone marrow was isolated and depleted of $CD3^+$ T cells using Miltenyi-Biotec anti-CD3 beads. 1 x 10^6 donor bone marrow-derived cells were injected intravenously into Rag-KO recipient mice that had been irradiated (800 rads). Mice were analyzed 5 - 6 weeks after reconstitution.

ELISA. IgG specific dsDNA was detected using a commercially available ELISA according to the manufacturers instructions and read at a 450 nm wavelength (Alpha Diagnostic International). Values (U/ml) were automatically generated from a standard curve. For antigen-specific ELISA, 96 well plates (Nunc) were coated overnight with 2μg of KLH peptide, blocked with 1% BSA/PBST and incubated with serial serum dilutions. Bound Ig was detected using anti-IgG2a (Invitrogen) directly labeled with streptavidin-HRP conjugate. Color was visualized by the addition of *o*–phenylenediamine dihydrochloride (OPD; Sigma Aldrich). Absorbance at wavelength 490 nm was read using a microplate reader (Tecan) and O.D values were compared.

Real time PCR analysis. Signal joint TREC analysis to detect recent thymic emigrant status of peripheral T cells was performed as described (73). Genomic DNA was isolated from FACS-sorted $CD8^+$ Tregs using the QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. SjTREC and CD45 primers and probes with a FAM reporter dye and TAMRA quencher dye were used as published (74, 75). A CD45 reference gene was used

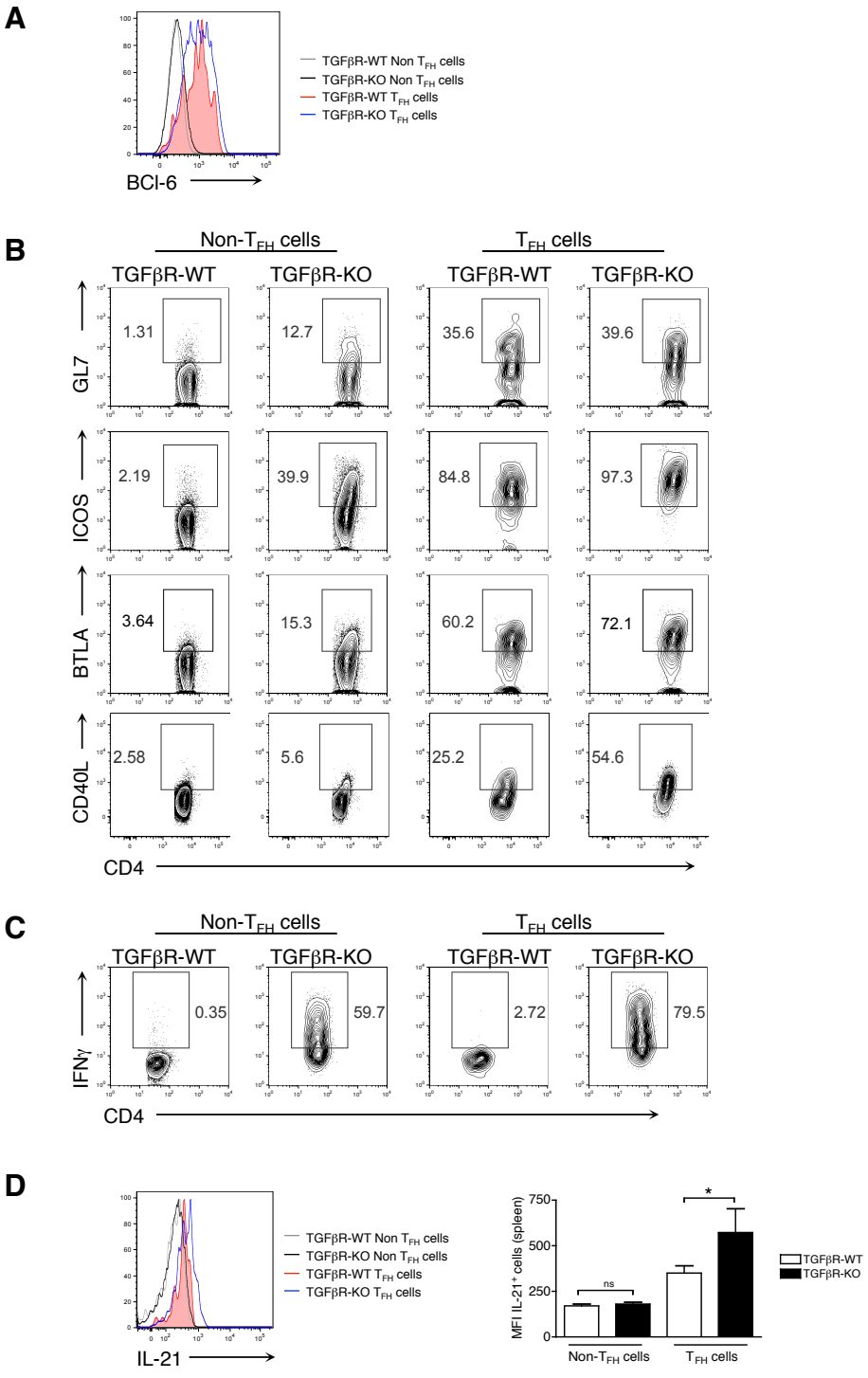
to correct for input genomic DNA of purified lymphocytes. A ROX internal reference dye (Invitrogen) was added to the mixture. The reaction was run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and fold change was calculated using the $2^{-\Delta\Delta ct}$ method.

In vitro T_{FH} differentiation

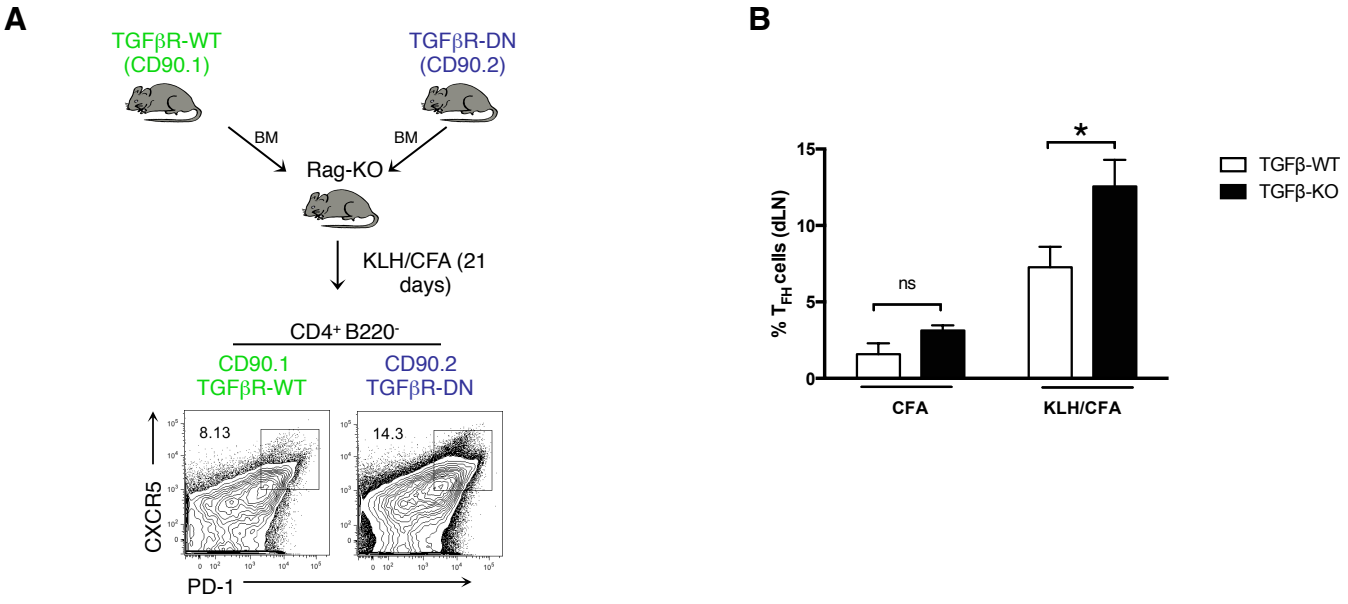
$CD4^+$ $PD\text{-}1^-$ $CXCR5^-$ $CD44^{low}$ $CD62L^{high}$ $Foxp3^-$ T cells were isolated by cell sorting and cultured with irradiated (1500 rad) splenocytes from congenic wild type mice plus soluble CD3 (1 μg/ml) in the presence of polarizing reagents for 4 or 7 days. 5 x 10^5 $CD4^+$ T cells were cultured in complete RPMI media (RPMI, 10% FCS, 2mM L-Glutamine, 100 units/ml penicillin and 100μg/ml streptomycin) with 1 x 10^6 splenocytes in 96 well flat bottom plates. Polarizing reagents for the generation of T_{FH} cells were 45 ng/ml IL-21 and 50 ng/ml IL-6. Cells were supplemented with 45 ng/ml IL-21 on day 2 of culture and IL-2 (200 U) on day 3 and day 6. Recombinant cytokines were purchased from R & D systems.

Statistical Analysis. Mean value and standard error of the mean (SEM) were calculated. An unpaired, two-tailed Student's *t*-test was used as appropriate to calculate significance between two groups. $P \leq 0.05$ was considered significant and illustrated with *. All statistical analysis was carried out with GraphPad Prism Version 4. Comparisons that are non-significant are denoted by 'ns'.

Supplementary Figure 1



Supplementary Figure 2



Supplementary figure legends

Supplementary Figure 1: Accumulating CD4+ T_{FH} cells express characteristic phenotypic and cytokine markers. (**A,B,C,D**) T_{FH} and non-T_{FH} ($CD4^+$ $B220^-$ PD-1^- CXCR5) cells from 18 day old TGFβR-KO and TGFβR-WT mice were compared. Foxp3+ cells were excluded from analysis. (**A**) Representative histogram of intra-nuclear Bcl-6 expression from four independent experiments with a total of eight mice is demonstrated. (**B**) Representative contour plots for intracellular expression of CD40L and surface expression of GL-7, ICOS and BTLA from at least three independent experiments with between four and eight mice are demonstrated. (**C**) IFNγ expression was detected by intracellular flow cytometry from TGFβR-KO compared to TGFβR-WT mice. Data is representative of four independent experiments with eight mice. (**D**) Intracellular IL-21 expression was detected by flow cytometric analysis on cells from TGFβR-KO compared to littermate control (TGFβR-WT) mice after ex vivo stimulation for three hours. Histogram represents IL-21 detected in TFH cells and non-T_{FH} cells in spleen. Graph is MFI of IL-21 expression from T_{FH} and non-TFH cells from TGFβR-WT and -KO mice. Data is representative of three independent experiments with 5 mice.

Supplementary Figure 2: Intrinsic effect of TGFβ signaling on T_{FH} accumulation during immunization (**A and B**) BM from TGFβR-DN mice or control (TGFβR-WT) mice were transferred together in irradiated Rag-KO mice reconstituted with. Reconstituted mice were immunized with KLH in CFA at the tail base and draining lymph nodes were examined 21 days later. (**A**) Representative contour plots with high expression of PD-1 and CXCR5 on $CD4^+$ $B220^-$ T cells representing T_{FH} cells is shown. (**B**) Graph demonstrates mean and SEM of the percentage of T_{FH} cells in immunized reconstituted mice contributed by each BM from two independent experiments with four mice.