Supporting Information

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SI Materials and Methods

Cell Purification and Flow Cytometry. B cells (from CD45.1 congenic mice) and $CD4^+$ Th cells (from CD45.1 congenic or from *Il-21*$^{-/-}$ mice) were prepared by magnetic cell sorting from spleens and lymph nodes (LNs) using the $CD4^+$CD62L T-cell Isolation Kit II (for $CD4^+$ cells) or the anti-FITC-Multi Sort Kit (for B cells) (both from Miltenyi Biotec). Cell purity was always >97%. Cells (8×10^6 per mouse) were adoptively transferred into *Irf4*$^{-/-}$ mice along with infection of the mice with *Leishmania major*. For flow-cytometric analyses and sorting, cell suspensions were stained in PBS/1% (vol/vol) FCS with the following antibodies: anti-mouse B220-FITC, anti-mouse CXC-chemokine receptor 5 (CXCR5)-biotin, both from BD; anti-mouse inducible costimulator (ICOS)-PE, anti-mouse CD4-APC, anti-mouse ICOS-biotin, anti-mouse ICOS ligand (ICOSL)-PE, anti-mouse CD45.1-PerCP-Cy5.1, anti-mouse programmed death-1 (PD1)-PE, all from eBioscience. Anti–CXCR5-biotin and anti–ICOS-biotin antibodies were visualized by secondary staining with streptavidin-APC (Biolegend). For the "CXCR5 enhancer strategy" (1), cells were stained with anti-mouse CXCR5, followed by anti-rat IgG2a-biotin (both from BD) and streptavidin-APC. All anti-mouse CXCR5 stainings were controlled using true isotype control antibodies instead of the respective anti-CXCR5 reagents. In some experiments, T and B-cell conjugates within LN cell suspensions were mechanically separated by syringe-assisted pressure through a needle before analysis of CXCR5 expression. For intracellular B-cell lymphoma 6 (BCL-6) staining, cells were fixed with the FOXP3/Transcription factor staining Kit (eBioscience) for 25 min, washed, permeabilized with 0.3% (wt/vol) saponin/2% (vol/vol) FCS in PBS, and stained with anti-mouse BCL-6 Alexa Fluor-647 (BD). For intracellular IL-2 and IL-21 measurements, cells were restimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (750 ng/mL) for 4 h, fixed with 2% paraformaldehyde, permeabilized, and stained with anti-mouse IL-2–PE or anti-mouse IL-21–PE (eBioscience). In some experiments, cells were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CSFE; Sigma), stimulated for 72 h with 10 ng/mL PMA and 350 ng/mL ionomycin, and stained with anti–CD4-APC. Flow-cytometric analysis was performed on a FACS-Calibur (BD) machine by the FlowJo-software (TriStar). Cell-sorting was performed on a MoFlo machine (Beckman-Coulter) using the Summit-software (DakoCytomation). In some experiments, $CD4^+ICOS^{hi}CXCR5^+$ T_{FH} cells from WT mice infected for 2 wk with *L. major* were sequentially purified by the magnetic mouse $CD4^+$ CD62L T-cell Isolation Kit II (Miltenyi Biotec) and FACS sorting, before injecting the cells into *Irf4*$^{-/-}$ mice (2×10^5 cells per mouse) that by themselves were infected with *L. major* at the day of cell transfer.

Histology. Tissue samples were snap frozen in OCT Tissue-Tek (Sakura Finetek). Cryosections of about 5 μm were made with a Frigocut 2800E (Leica), air dried, and incubated for 20 min in ice-cold acetone and stored at −20 °C or washed three times with PBS for immunostaining. Following incubation in blocking buffer (goat serum), the samples were incubated with labeled antibody for 2 h at room temperature followed by PBS-washing three times. The following primary antibodies were used: anti-mouse CD4-Alexa Fluor 647 (Biolegend), anti-mouse B220-FITC (BD), anti-mouse GL7-FITC (BD), anti-mouse CD45.1-biotin (eBiosience). After washing, anti-CD45.1 was detected for 1 h with Streptavidin-Alexa Fluor 546 (Molecular Probes); anti-GL7-FITC was followed by anti-FITC Alexa Fluor 488 (Molecular Probes) to increase signal strength. Confocal images of cryosections were acquired by fluorescence microscopy on a Leica TCS SP2 microscope (Leica Microsystems) essentially as described previously (2). H&E stainings were performed according to standard procedures and examined with a BX51 microscope (Olympus).

Quantitative Real-Time PCR. Total RNA was isolated from cell pellets using the RNeasy Micro Kit (Qiagen) or the High Pure RNA Isolation Kit (Roche). Synthesis of cDNA was performed using the Maxima First strand cDNA synthesis Kit (Fermentas) according to the manufacturer's instructions. Quantitative real-time RT PCR (qPCR) to determine IL-21-mRNA expression was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using the TaqMan 2× Universal PCR Master Mix Kit (Roche). The PCR conditions are described in ref. 3. The levels of IL-21 expression were normalized to HPRT (hypoxanthine-guanine phosphoribosyl transferase) using the ΔΔCt-method as specified by Applied Biosystems. PCR primers are described in below.

Sequences of the PCR Primers and Probes (Metabion).

IL-21 for: 5′ CCA AAC TCA AGC CAT CAA ACC CTG 3′
IL-21 rev: 5′ TCT GTT TCT TTC CTC CCC TCC TGG 3′
HPRT for: 5′ CTG GTG AAA AGG ACC TCT CG 3′
HPRT rev: 5′ TGA AGT ACT CAT TAT AGT CAA GGG CA 3′
IL-21 probe: 5′-Fam-GAC ATT CAT CAT TGA CCT CGT GGC-Tamra-3′
HPRT probe: 5′-Fam-GTG TTG GAT ACA GGC CAG ACT TTG TTG GAT-Tamra-3′

1. Choi YS, et al. (2011) ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34:932–946.
2. Delacour D, et al. (2006) Requirement for galectin-3 in apical protein sorting. *Curr Biol* 16:408–414.
3. Brüstle A, et al. (2007) The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958–966.

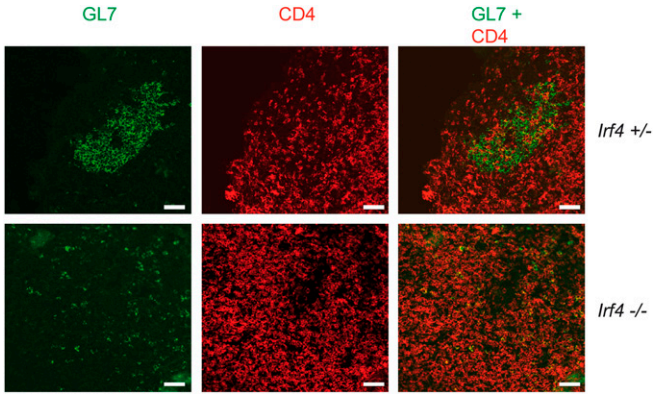

Fig. S1. Lack of germinal center formation in *Irf4*$^{-/-}$ mice after immunization. Mice of the indicated genotypes were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide and complete Freund's adjuvant (CFA). On day 13 after immunization, inguinal LNs were prepared and tissue sections were stained by antibodies to CD4 (red) as well as GL7 (green) and analyzed by fluorescence-microscopy (20× magnification). (Scale bars, 50 μm.) Data are representative of three different experiments, each with three mice per group.

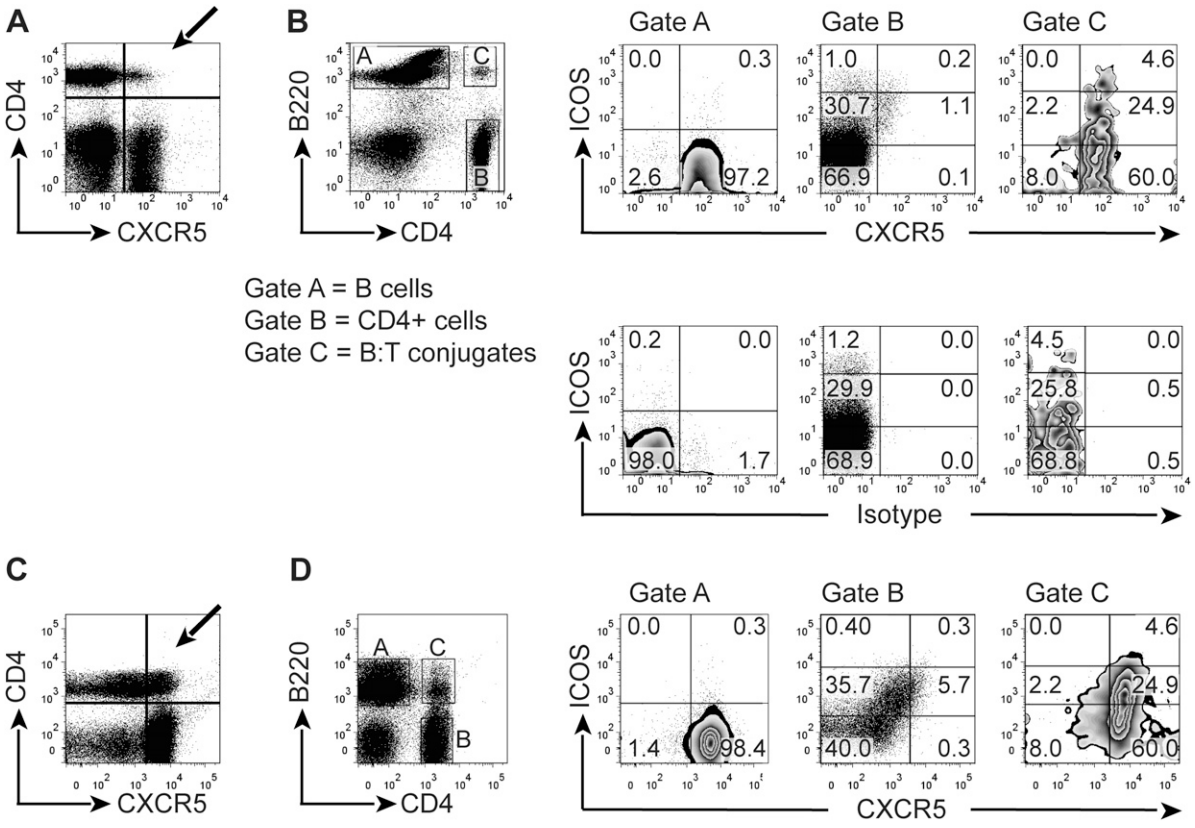

Fig. S2. B- and T-cell conjugates complicating follicular T-helper cell (T_{FH}) analysis. (*A* and *B*) WT mice were infected with *L. major* and the popliteal LN cells were analyzed 2 wk later. (*C* and *D*) Peyer's patches (PP) of naive WT mice. (*A* and *C*) Live gate of all cells stained for CD4 and CXCR5. The arrow indicates CD4$^+$CXCR5$^+$ events. (*B* and *D*) Cells stained for CD4, B220, ICOS, CXCR5 (*B*, *Upper* panels) or CD4, B220, ICOS, isotype (*B*, *Lower* panels). (*B* and *D*, *Left*) Total live cell gate. All other panels: cells gated according to the designations "A" to "C" given in the respective *Left* panel and explained above that panel. Numbers indicate percentages of positive cells in the respective quadrants. The experiments were performed three times with similar outcomes.

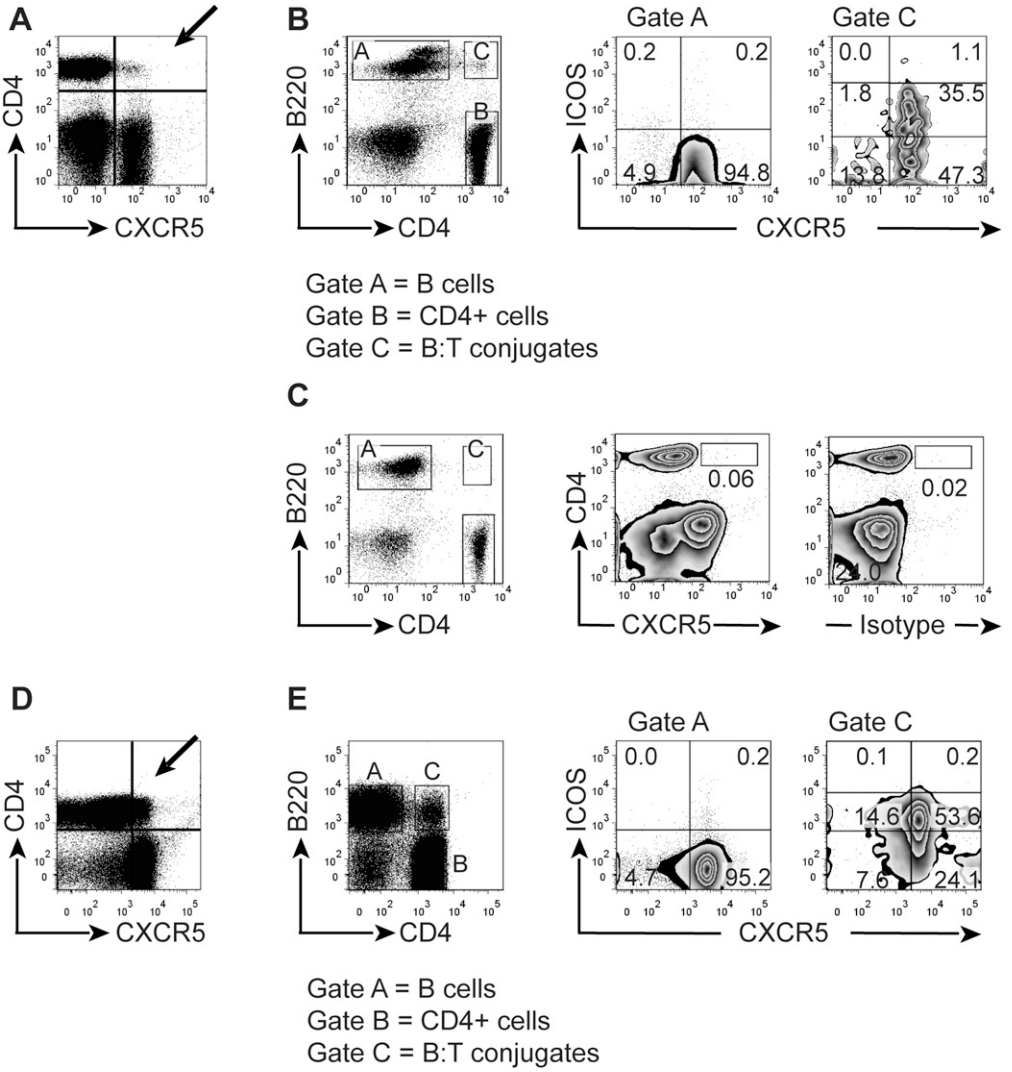

Fig. S3. B- and T-cell conjugates in LN and PP cell preparations of *Irf4*$^{-/-}$ mice. (*A*) The *Irf4*$^{-/-}$ LN cells depicted in Fig. 2 were analyzed as described in that figure. The arrow indicates erroneous CD4^{+}CXCR5^{+} events. Although gate B (single CD4^{+} cells) is shown in Fig. 2*D*, gates A and C of this experiment are displayed here. (*B*) After acquiring the data as shown in *A* and in Fig. 2*D*, the cells were pressed through a syringe for mechanical disruption of the B–T conjugates and reaquired without further out-gating of any cells. (*D* and *E*) The PP cells depicted in Fig. 3 were analyzed as described in that figure. Numbers indicate percentages of cells located within the respective rectangles or quadrants (*B* and *E*) or boxes (*C*). Three independent experiments with similar outcome.

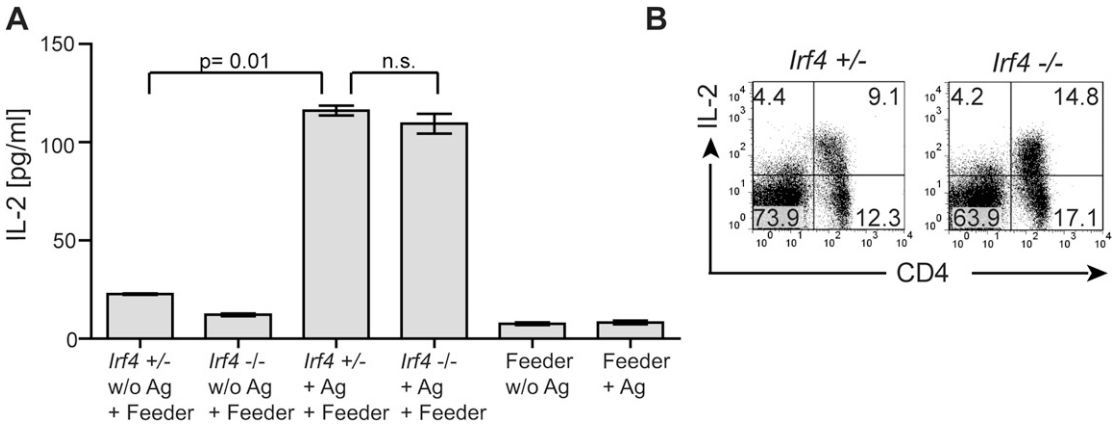

Fig. S4. Normal triggering of *Irf4*$^{-/-}$ CD4^{+} T cells by leishmania antigens. *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ mice were infected with *L. major* and suspensions of their popliteal LN cells analyzed 2 wk after infection. (*A*) LN cells were cultured with syngeneic spleen cells ("Feeder") and leishmania antigens ("Ag"), where indicated. IL-2 production was measured in 48-h culture supernatants by ELISA. Columns represent the means of cytokine amounts according to duplicate determinations and a standard curve. Bars denote the SD. (*B*) After preparation, LN cells were stimulated for 4 h with PMA and ionomycin, processed for staining of extracellular CD4 and intracellular IL-2, and analyzed by flow cytometry (CD4^{+} gate). Numbers indicate percentages of positive cells in the respective rectangles. Data from one representative mouse per group are depicted. Two experiments with similar outcome.

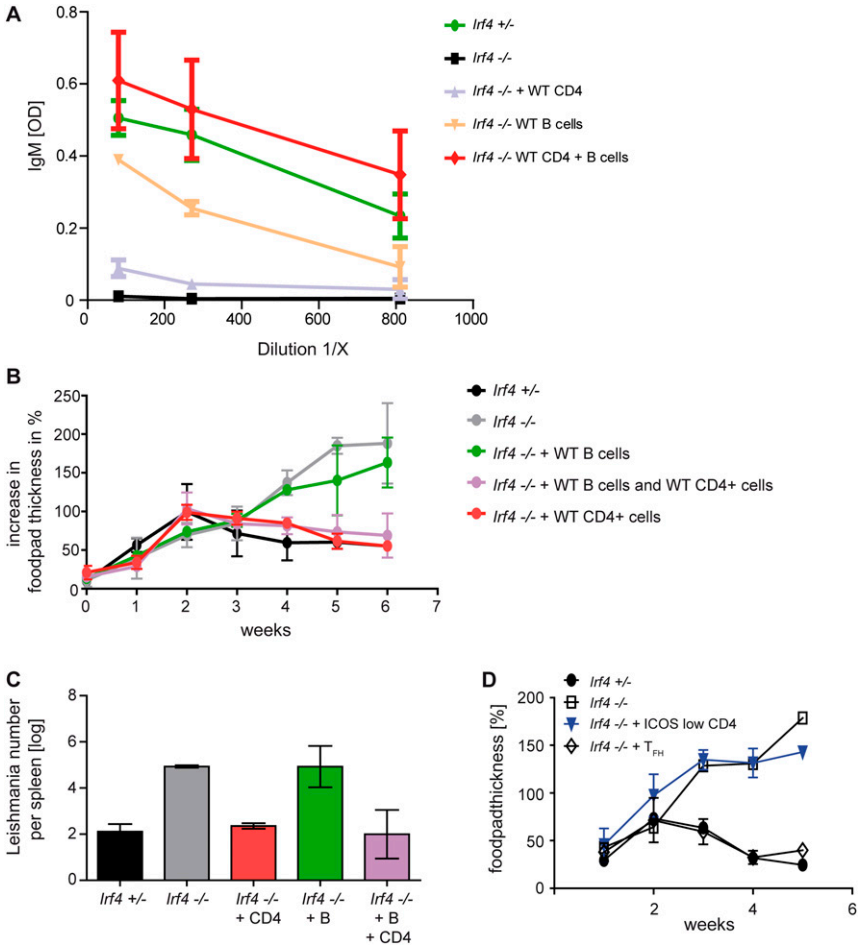

Fig. S5. Course of leishmaniasis in *Irf4*$^{-/-}$ mice depending on cell transfer. Mice of the indicated genotypes were infected with *L. major*. Where indicated, *Irf4* $^{-/-}$ mice received 8×10^{6} Ly5.1 congenic B or CD4^{+} cells or both by intraperitoneal adoptive transfer on the day of infection. (*A*) As a positive control for successful B-cell transfer, sera of the mice were tested 6 wk after infection for the presence of total IgM by ELISA. The blot shows dilution dependent means of ELISA OD values ± SD from sera of the different mice within the respective group. (*B*) Footpad thickness was monitored and the relative increase in lesion size (%) compared with the uninfected footpad was calculated at the indicated timepoints. The data are means ± SD of all individual mice per group. (*C*) Parasite burdens in the spleens 6 wk after infection. The number of parasites was calculated from their growth in limiting dilutions of single spleen cell suspensions from individual mice and from the total cell numbers in these spleens. Results are given as the log of the leishmania number per total spleen (means ± SD of three mice per group). All graphs are representative of two (*A*) or three (*B* and *C*) independent experiments. (*D*) Course of disease of the mice described in Fig. 6 *D* and *E*.

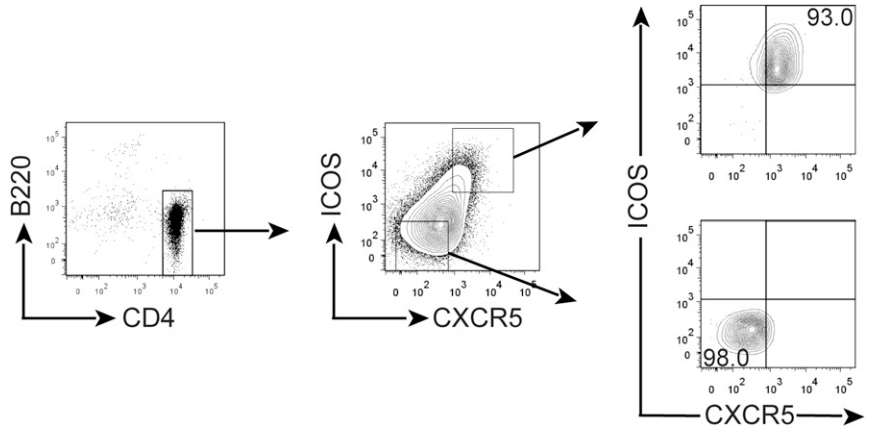

Fig. S6. Sorting of ICOS^{hi}/CXCR5^{+} T_{FH} cells from infected Ly5.1 congenic WT mice. Ly5.1 congenic WT mice were infected with *L. major*. Two weeks later, the draining LNs and spleens were prepared and pooled cells were stained for CD4, B220, ICOS, and CXCR5. Thereafter, CD4^{+}ICOS^{hi}CXCR5^{+} T_{FH} cells or CD4^{+} ICOS^{-}CXCR5^{-} control cells were sorted by MACS followed by FACS. The panels depict either a live cell gate of MACS sorted cells (*Left*) or a CD4^{+} gate of these cells (*Center*) or a live cell gate after MACS and FACS cell sorting (*Right*). Numbers indicate the frequency of ICOS^{hi}/CXCR5^{+} T_{FH} cells or ICOS^{-}CXCR5^{-} cells among all cells. After FACS sorting, the cells characterized in the right panels were injected into *Irf4*$^{-/-}$ mice, which were subsequently infected with *L. major*. Two experiments with similar outcome.