Immunity, Volume *33*

Supplemental Information

γδ T Cells Enhance Autoimmunity by Restraining Regulatory T Cell Responses via an Interleukin-23-Dependent Mechanism

Franziska Petermann, Veit Rothhammer, Malte C. Claussen, Jan D. Haas, Lorena Riol Blanco, Sylvia Heink, Immo Prinz, Bernhard Hemmer, Vijay K. Kuchroo, Mohamed Oukka, and Thomas Korn

Figure S1

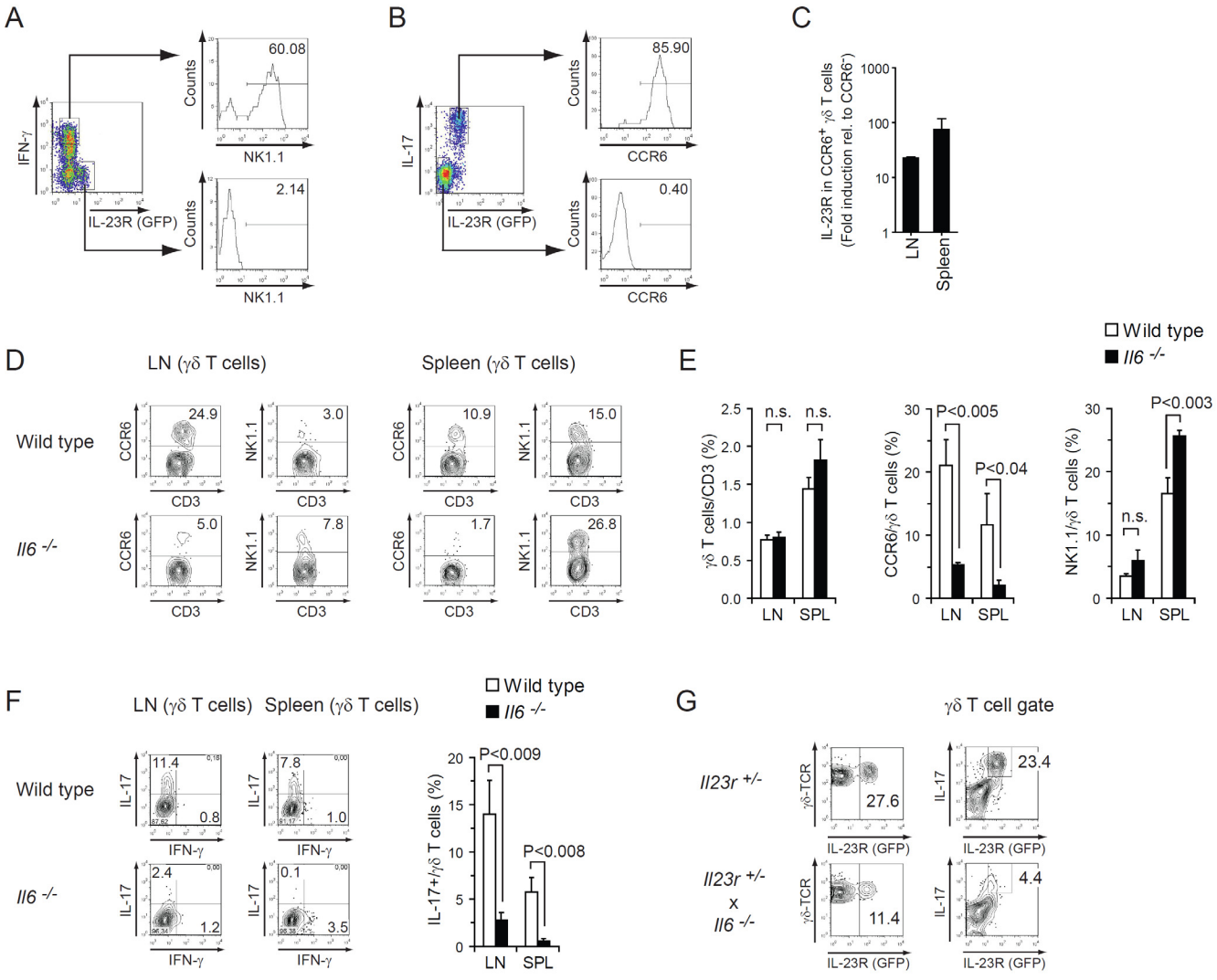

Figure S1, related to Figure 1. The precursor frequency of IL-23R$^+$ γδ T cells is reduced in the absence of IL-6. (A, B) γδ T cells were isolated from lymph nodes and spleen of naïve *Il23r*$^{+/-}$ mice and *ex vivo* stimulated with PMA plus ionomycin prior to staining for NK1.1 and intracellular IFN-γ (A) or CCR6 and intracellular IL-17 (B). (C) CCR6$^+$ γδ T cells were purified from lymph nodes and spleens of naïve wild type mice by flow cytometry and analyzed for *Il23r* mRNA expression by quantitative PCR directly *ex vivo*. Fold induction of *Il23r* mRNA in CCR6$^+$ γδ T cells as related to CCR6$^-$ γδ T cells. All results are representative of at least two independent experiments. (D) Lymph node cells or splenocytes were isolated from wild type mice or IL-6 deficient animals (n=4 per group). The frequencies of CCR6$^+$ or NK1.1$^+$ cells were determined in the γδ-TCR (GL-3)$^+$ T cell population by flow cytometry. Mean frequencies + SD. (E) While IL-6 deficient mice had slightly increased total fractions of γδ T cells and NK1.1$^+$ γδ T cells in lymph nodes and spleen, the percentage of CCR6$^+$ cells within the γδ T cell population was significantly reduced in *Il6*$^{-/-}$ mice. (F) Lymph node cells and splenocytes from naïve wild type or IL-6 deficient mice were *ex vivo* stimulated with PMA plus ionomycin and stained for intracellular IL-17 and IFN-γ. Percentages of IL-17$^+$ and IFN-γ$^+$ cells within the gate of γδ-TCR$^+$ T cells are displayed. Mean frequencies + SD. (G) IL-6 deficiency was crossed into the *Il23r*$^{+/-}$ reporter background. The frequency of IL-23R (GFP)$^+$ cells and – following stimulation with PMA plus ionomycin – the frequency of IL-23R$^+$IL-17$^+$ cells were determined within the γδ-TCR$^+$ T cell population by flow cytometry.

Figure S2

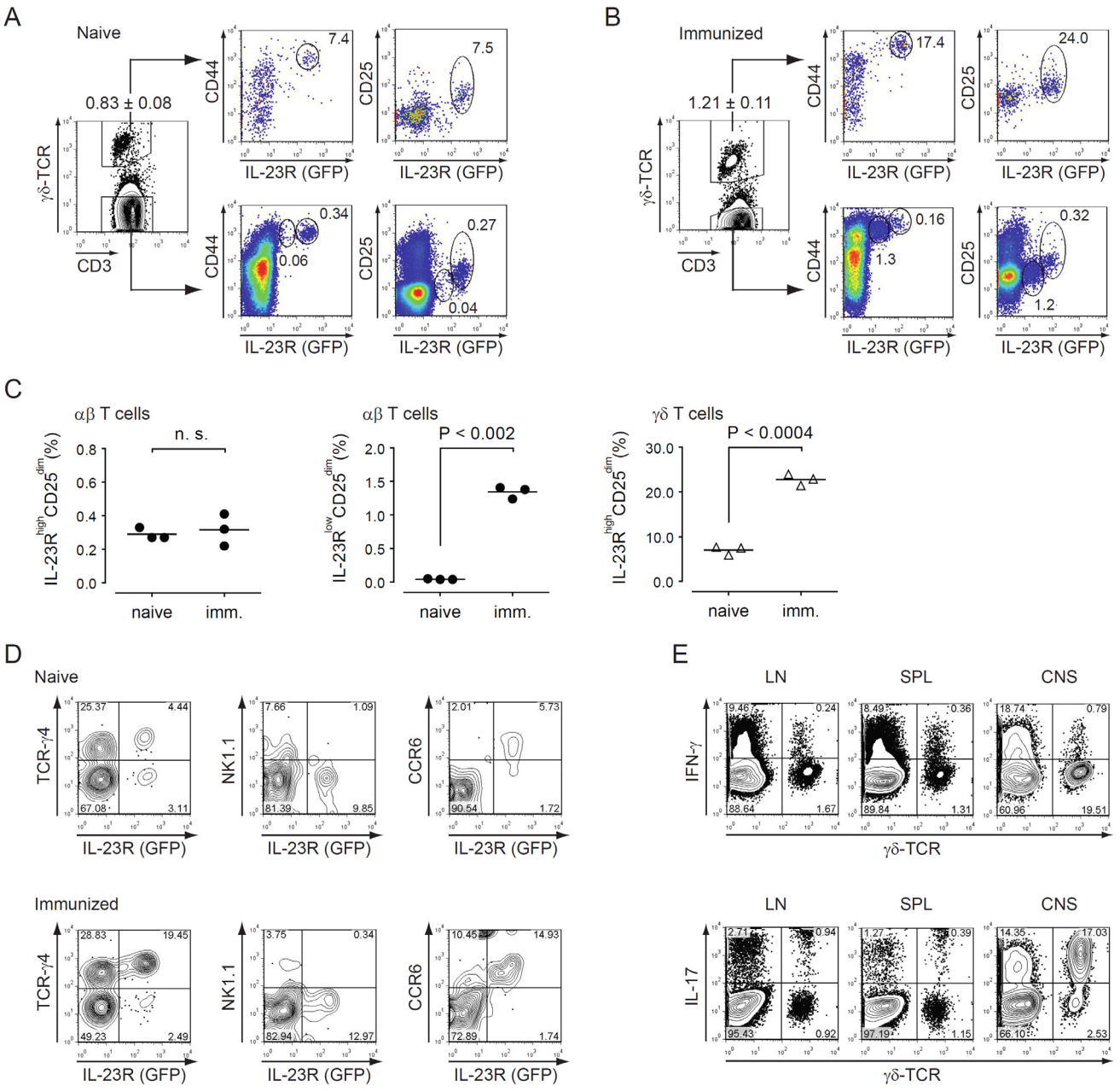

Figure S2, related to Figure 2. IL-23R$^+$ γδ T cells expand *in vivo* upon immunization with MOG$_{35-55}$ plus CFA and produce IL-17. Lymph node cells were isolated from naïve *Il23r*$^{+/-}$ mice (A) or from *Il23r*$^{+/-}$ mice that had been *in vivo*-sensitized with MOG$_{35-55}$ plus CFA (B). αβ T cells (CD3$^+$γδ-TCR$^-$) and γδ T cells (CD3$^+$γδ-TCR$^+$) were analyzed for expression of IL-23R (GFP), CD44, and CD25 by flow cytometry. (C) Percentages of IL-23R^{high} (CD4$^-$CD8$^-$) and IL-23R^{low} (CD4$^+$) cells within the αβ T cell compartment (filled circles) and of IL-23R$^+$ cells within the γδ T cell compartment (open triangles) of individual mice before and after immunization. (D) Surface staining of lymph node cells from naïve or MOG$_{35-55}$ plus CFA immunized *Il23r*$^{+/-}$ mice. The gate is set on γδ-TCR (GL-3)$^+$ cells. Numbers in the quadrants indicate percentages. (E) At the peak of disease, lymph node cells, splenocytes, and mononuclear cells from the CNS were recovered and stained for intracellular IFN-γ and IL-17 after *ex vivo* stimulation with PMA plus ionomycin. The gate was set on CD3$^+$ T cells. Numbers in the quadrants represent percentages.

Figure S3

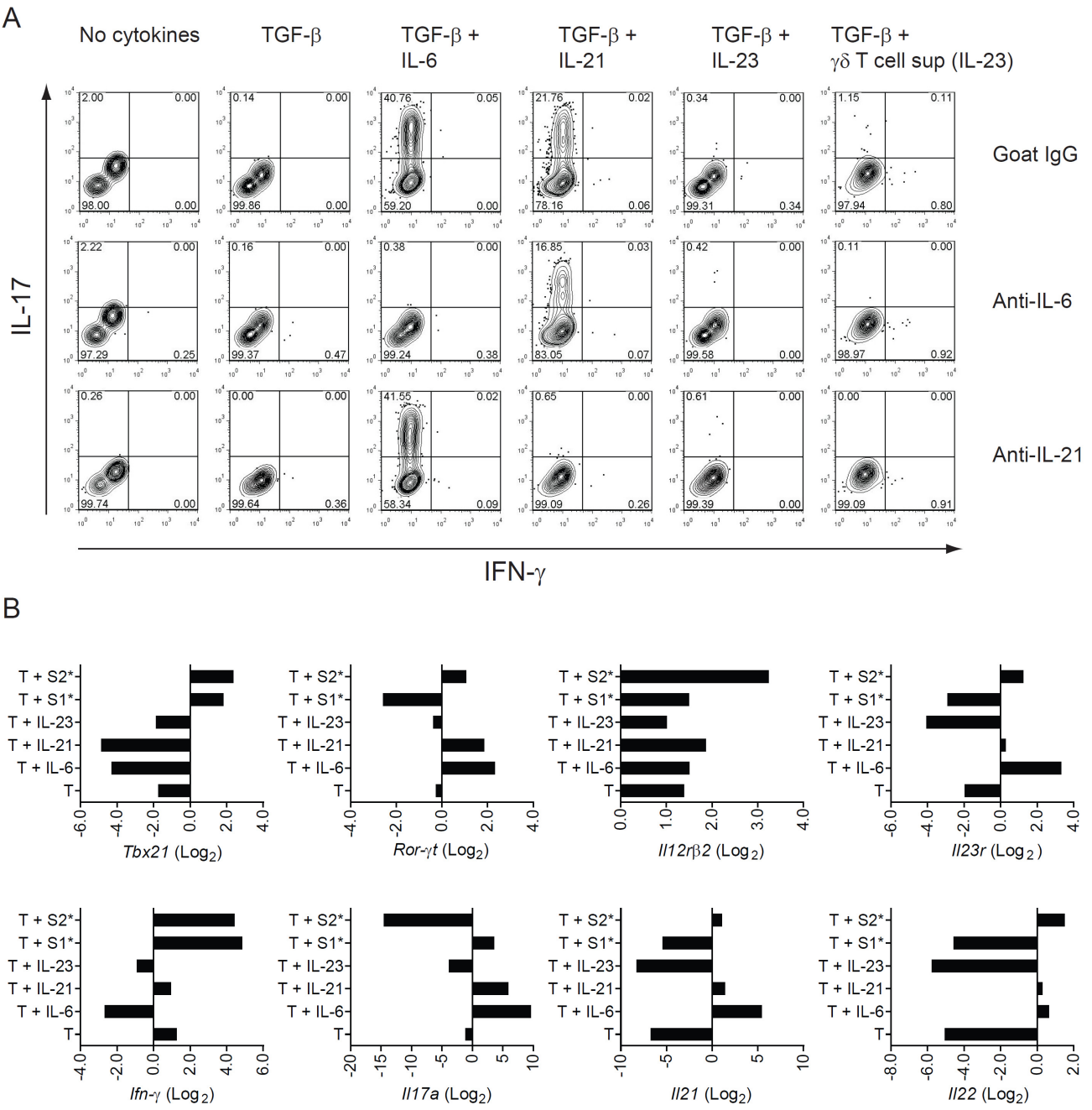

Figure S3, related to Figure 3. Expression profiling of conventional αβ T cells upon exposure with IL-23-activated γδ T cell supernatant. (A) As described in Figure 3, naïve T cells ($CD4^+CD44^-Foxp3^-$) were purified from *Foxp3gfp*.KI mice by FACS and stimulated with plate-bound antibodies to CD3 and CD28 in the presence of the indicated cytokines or supernatant from γδ T cells and in the presence of goat IgG or blocking antibodies to IL-6 or IL-21. After 3 days, intracellular IL-17 and IFN-γ (A) were determined by flow cytometry. (B) mRNA was isolated from naïve conventional $CD4^+$ T cells that had been stimulated for 3 days under the indicated cytokine conditions. (T=TGF-β; S1*=supernatant from γδ T cells; S2*=supernatant from IL-23-activated γδ T cells). Relative expression of the indicated genes in αβ T cells responder cells in relation to the expression level in αβ T cells cells stimulated under neutral conditions (no cytokine condition), which was arbitrarily set to 1.0. Representative out of 3 independent experiments.

Figure S4

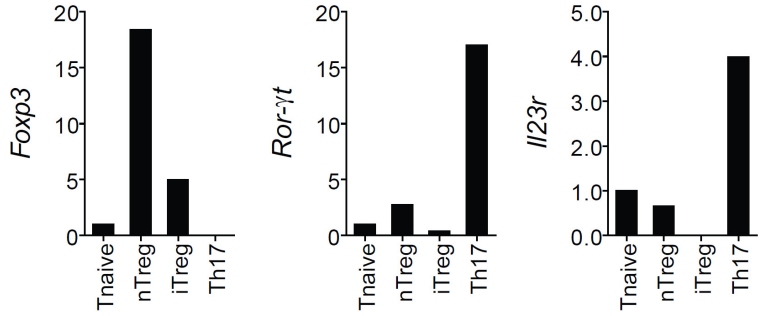

Figure S4, related to Figure 5. Foxp3$^+$ Treg cells do not express IL-23R. Naïve conventional T cells or Foxp3 (GFP)$^+$ natural Treg cells (nTregs) were isolated by flow cytometric cell sorting from *Foxp3gfp*.KI mice. Induced Treg cells (iTregs) and Th17 cells were differentiated *in vitro* from naïve T cells (CD4$^+$CD44$^-$Foxp3$^-$) by culture in the presence of TGF-β (5 ng/ml) or the combination of TGF-β (2 ng/ml) plus IL-6 (50 ng/ml), respectively. RNA was isolated from the various T cell populations and tested for relative abundance of *Foxp3* mRNA, *Ror-γt* mRNA, or *Il23r* mRNA by Taqman analysis as described in Supplemental Experimental Procedures. Representative analysis out of three independent experiments.

Supplemental experimental procedures

Preparation of CNS mononuclear cells and antibody staining

After perfusion through the left cardiac ventricle with cold PBS, forebrain and cerebellum were dissected and spinal cords flushed out with PBS by hydrostatic pressure followed by digestion with collagenase D (2.5 mg/ml, Roche Diagnostics, Indianapolis, IN) and DNAseI (1 mg/ml, Sigma, Saint Louis, MO) at 37 °C for 45 min. Mononuclear cells were isolated by passing the tissue through a cell strainer (70 μm) and percoll gradient (37% over 70%) centrifugation. Mononuclear cells were removed from the interphase, washed and resuspended in culture medium for further analysis. Surface staining of T cells was carried out with antibodies to TCR-β chain (H57-597), γδ-TCR (GL3), TCR-γ4 chain (49.2-1), CD4 (RM4-5), CD3 (145-2C11), CD25 (PC61 or 7D4), CD44 (IM7), CD103 (M290), NK1.1 (PK136), and CCR6 (140706).

Intracellular cytokine staining

Cells were stimulated in culture medium containing phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma), and monensin (GolgiStop 1 μl/ml, BD Biosciences) at 37 °C and 10% CO_2 for 4hr. After staining of surface markers, cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash buffer, BD Biosciences) followed by staining with monoclonal antibodies to mouse IL-1, IL-6, IL-10, IL-17, IFN-γ, and TNF (BD Biosciences) and flow cytometric analysis (CYAN, Beckmann/Coulter).

Measurement of cytokines

Cell culture supernatants were collected after 72 hr and the secreted cytokines were determined by enzyme-linked sorbent assay (R & D systems) according to the manufacturers' instructions. For quantitative PCR, RNA was extracted from magnetic bead-purified or flow cytometry-sorted cells after *in vitro* stimulation as indicated in the figure legends using the RNeasy columns (Qiagen, Valencia, CA). Complementary DNA was transcribed as recommended (Applied Biosystems, Foster City, CA) and used as template for quantitative PCR. Primer plus probe mixtures were obtained from Applied Biosystems. The Taqman analysis was performed on a StepOne system from Applied Biosystems. The gene expression was normalized to the expression of β-actin.