Supporting Information

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SI Materials and Methods

Mice. BALB/c mice (Charles River Laboratories) and GR^{dim} (1), $GR^{LysMCre}$ (2), and GR^{LckCre} (3) were back-crossed for at least four generations to the BALB/c background. $GR^{CD11cCre}$ and $GR^{CD19Cre}$ mice were generated by crossing to CD11cCre transgenic (4) and CD19Cre mice (5), respectively, and were back-crossed for at least four generations to the C57BL/6 background. GR^{dim} mice were also crossed for 12 generations to the DBA/1 background. IL-$17A^{-/-}$ (C57BL/6) and IFN-$\gamma^{-/-}$ (C57BL/6) mice were supplied by Yoichiro Iwakura (Tokyo, Japan) and the Jackson Laboratory, respectively. The mice were kept under specific pathogen-free conditions.

Antigen-Induced Arthritis. In brief, mice were immunized with 100 μg methylated BSA (mBSA; Sigma-Aldrich) emulgated 1:1 with complete Freund's adjuvant (Sigma-Aldrich) supplemented with 1 mg/mL *Mycobacterium tuberculosis* (Difco) and simultaneously treated i.p. with 5×10^8 heat-inactivated *Bordtella pertussis* (Chiron-Behring) at days 21 and 14 before arthritis induction. Arthritis was induced by an intraarticular injection of 100 μg mBSA into the right knee joint cavity (6). This was followed by i.v. treatment with 1.25 mg/kg Micromethason (Novosom AG) or PBS at 4, 24, and 48 h. Knee joint swelling was determined using an Oditest caliper (Kroeplin) and expressed relative to the knee diameter at day 0 (before arthritis induction).

Glucose-6-Phosphate Isomerase-Induced Arthritis. Glucose-6-phosphate isomerase-induced arthritis (G6PI-IA) was established as described elsewhere (7) using 6- to 10-wk-old GR^{dim} mice (DBA/1 background) and monitored for 30 d. A score of 0 indicated no clinical signs of arthritis, 1 slight swelling and redness, 2 strong swelling and redness, and 3 massive swelling and redness. Dexamethasone (Dex; 1 mg/kg; Sigma-Aldrich) or PBS was applied daily i.p. starting from day 9 (onset of the disease) until day 15.

Histology. Knee joints were removed at day 1 after AIA induction, fixed in phosphate-buffered 4% formalin for 24 h, and subsequently decalcified with 15% EDTA, dehydrated, and embedded in paraffin, and 6-μm sections were stained with H&E. Histological scoring was performed blindly according to Tolk and Földi's grading of joint inflammation from 0 to 3 regarding (*i*) cellular infiltration, (*ii*) fibrin exudation into the joint space, (*iii*) lining cell layer, and (*iv*) synovitis of subintimal connective tissue, and the average was calculated (8).

Serum Analysis. Eight hours after AIA induction, several cytokines (IFN-γ, IL-6, IL-17, and TNF-α) were analyzed in serum with a cytometric bead array (BD Biosciences) and subsequent flow cytometry according to the manufacturer's instructions.

Blood was taken from the animals 24 h after AIA induction. Protein levels of IL-6, IL-17, and IFN-γ were measured by a sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

Lymph Node Cell Analysis. Cells from draining lymph nodes (inguinal and popliteal) were isolated 24 h after AIA induction, and 1 mL was cultured (7.5×10^6 cells/mL; 48-well plates; 37 °C, 5% CO_2) in RPMI medium (PAA Laboratories) containing 10% FCS, 1% penicillin/streptomycin, and 50 μM 2-mercapto-ethanol (Gibco) with either 25 μg mBSA or with 5 ng/mL 4α-phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin. After 2 h, brefeldin A (eBioscience) was added, and after a further 4 h, cells were harvested for flow cytometry. Cells were subsequently stained with anti–CD4-PECy7 (L3T3), anti–CD154-APC, anti–IL17A-FITC (eBio17B7), anti–IFN-γ-PE (XMG1.1), and anti–TNFα-eF405 (MP6-XT22).

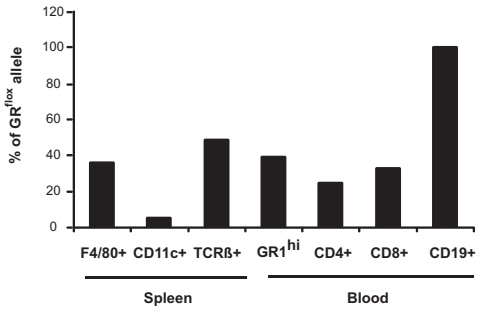
Proliferation and Apoptosis Measurements. BrdU (2 mg) was injected i.p. 4 h after arthritis induction in glucocorticoid (GC)- and PBS-treated mice. Twenty hours after BrdU application, inguinal and popliteal lymph nodes were removed and isolated cells stained for flow cytometry [anti–CD4-PECy7, anti–CD3-APC (17A2)], fixed and permeabilized (BD Cytofix/Cytoperm Buffer), and subsequently stained with anti-BrdU (FITC) antibody (BD Biosciences). Apoptosis was determined by incubating lymph node cells with FITC-labeled annexin V and 50 μg/mL propidium iodide (Molecular Probes). FACS analysis was performed using a FACSCanto (BD Biosciences), and the data were processed with FlowJo software (version 8.7; Tree Star).

T_H17 in Vitro Differentiation. Naïve $CD4^+TCR\beta^+CD62L^{high}CD44^{low}$ cells were purified from spleens and lymph nodes of BALB/c mice by flow cytometry sorting (FACSAria; BD Biosciences) after staining with anti–CD4-PECy7, anti–TCRβ-APC (H57-597), anti–CD62L-FITC (MEL-14), and anti–CD44-PE (IM7) antibodies. Purified T cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies (5 μg/mL of each) and cultured in the presence of 10 μg/mL anti–IL-4 (11B11), 10 μg/mL anti–IFN-γ (XMG.1) (eBioscience), IL-6 (PeproTech), TGF-β (PeproTech), IL-1β (ImmunoTools), and TNF-α (ImmunoTools). After 7 d, cells were restimulated with plate-bound anti-CD3 and anti-CD28 antibodies (5 μg/mL of each) for 6 h, in the presence of brefeldin A for the final 4 h, and treated with 1 μM Dex. Subsequently, cells were analyzed by FACS as described above.

Quantitative Real-Time PCR for Recombination Efficiencies. Leukocytes of $GR^{CD11cCre}$, $GR^{CD19Cre}$, and GR^{LckCre} mice were purified from different hematopoietic organs using flow cytometry sorting (FACSAria; BD Biosciences) after staining with indicated cell surface markers. DNA was isolated from the purified cells, and quantitative real-time PCR amplifying the GR^{flox} allele was performed with an iCycler (Bio-Rad) using a Sensi-Mix DNA Kit containing SYBR Green (Invitrogen). Primer information can be supplied on request.

1. Reichardt HM, et al. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 93:531–541.
2. Tuckermann JP, et al. (2007) Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *J Clin Invest* 117:1381–1390.
3. Baumann S, et al. (2005) Glucocorticoids inhibit activation-induced cell death (AICD) via direct DNA-dependent repression of the CD95 ligand gene by a glucocorticoid receptor dimer. *Blood* 106:617–625.
4. Caton ML, Smith-Raska MR, Reizis B (2007) Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* 204:1653–1664.
5. Rickert RC, Roes J, Rajewsky K (1997) B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res* 25:1317–1318.
6. Brackertz D, Mitchell GF, Mackay IR (1977) Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice. *Arthritis Rheum* 20:841–850.
7. Schubert D, Maier B, Morawietz L, Krenn V, Kamradt T (2004) Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in genetically unaltered mice. *J Immunol* 172:4503–4509.
8. Berry EC, Grundmann E, Kirsten W (1982) *Bone and Joint Disease (Current Topics in Pathology)* (Springer, New York).

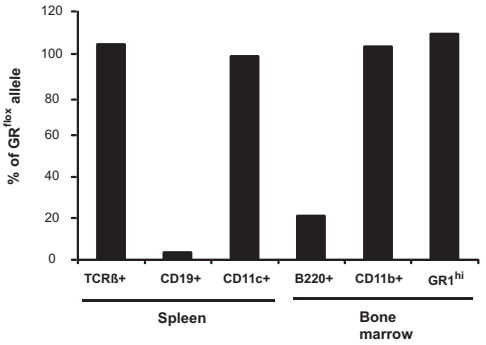
A



B

Cell type	Surface markers	Recombination %
Macrophages (Spleen)	F4/80+/ MHCII+	65.0
DC (Spleen)	CD11c+/ MHCII+	95.3
T cells (Spleen)	TCRß+	52.0
Neutrophile granuloc. (Blood)	GR1hi	62.0
T cells (Blood)	CD4+	76.5
T cells (Blood)	CD8+	67.3
B cells (Blood)	CD19+	0.0

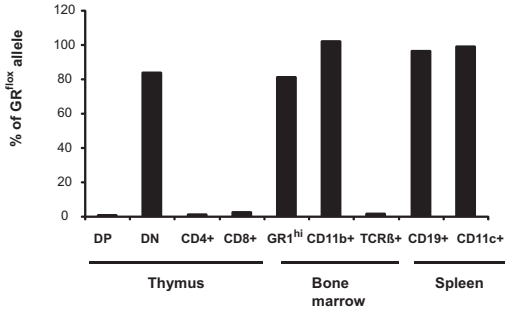
C



D

Cell type	Surface markers	Recombination %
T cells (Spleen)	TCRß+	0.0
B cells (Spleen)	CD19+	96.6
DC (Spleen)	CD11c+	1.7
B cells (BM)	B220+	79.2
Macrophages (BM)	CD11b+	0.0
Neutrophile granulocytes (BM)	GR1hi	0.0

E



F

Cell Type	Surface markers	Recombination %
DP (Thymus)	CD4+CD8+	99.7
DN (Thymus)	CD4-CD8-	16.3
T cells (Thymus)	CD4+	99.1
T cells (Thymus)	CD8+	98.0
Neutrophile granulocytes (BM)	GR1hi	19.8
Macrophages (BM)	CD11b+	0.0
T cells (Spleen)	TCRß+	99.0
B cells (Spleen)	CD19+	3.6
DC (Spleen)	CD11c+	1.0

G

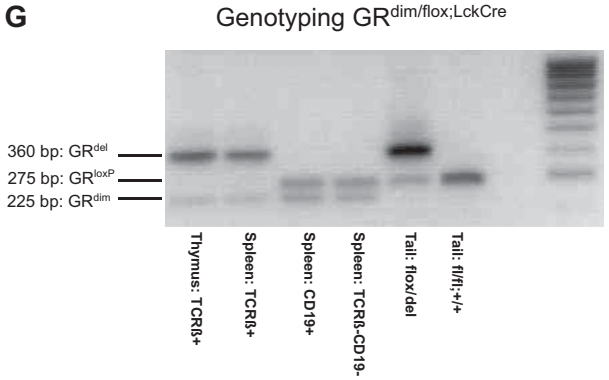


Fig. S1. Recombination efficiency of the GR^{flox} allele in different Cre lines. (*A*, *C*, and *E*) Abundance of the GR^{flox} allele in leukocytes of (*A*) $GR^{CD11cCre}$, (*C*) $GR^{CD19Cre}$, and (*E*) GR^{LckCre} mice determined by quantitative real-time PCR. Cells were sorted by flow cytometry with antibodies against indicated cell surface markers. DP, double-positive ($CD4^+/CD8^+$) cells; DN, double-negative ($CD4^-CD8^-$) cells. (*B*, *D*, and *F*) Summarized recombination efficiencies (%) of the GR^{flox} allele into the deleted GR allele for (*B*) $GR^{CD11cCre}$, (*D*) $GR^{CD19Cre}$, and (*F*) GR^{LckCre} mice, calculated from values in *A*, *C*, and *E*, respectively. (*G*) Determination of the GR^{flox} and the GR^{dim} allele in T cells of $GR^{dim/flox;LckCre}$ mice. Thymocytes and T cells from the indicated organs were sorted by flow cytometry with indicated cell surface markers and the GR^{dim}, the GR^{loxP}, and the deleted GR^{del} allele amplified by PCR.

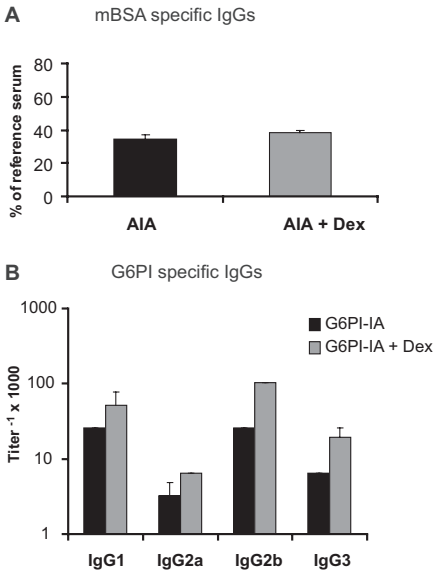


Fig. S2. mBSA-specific IgG levels and G6PI-specific IgG levels in serum of arthritic mice are not changed with GC treatment. (*A*) mBSA-specific IgG levels were determined at day 3 after AIA induction in serum of PBS- and Dex-treated mice by ELISA ($n = 5$). Pooled sera from AIA mice were used as the relative reference for antibodies specific to mBSA. (*B*) G6PI-specific IgG levels were determined at day 12 after G6PI-IA induction in serum of PBS- and Dex-treated mice by ELISA ($n = 3$). The highest serum dilution in which anti-G6PI antibodies were still detectable is shown on the *y* axis.

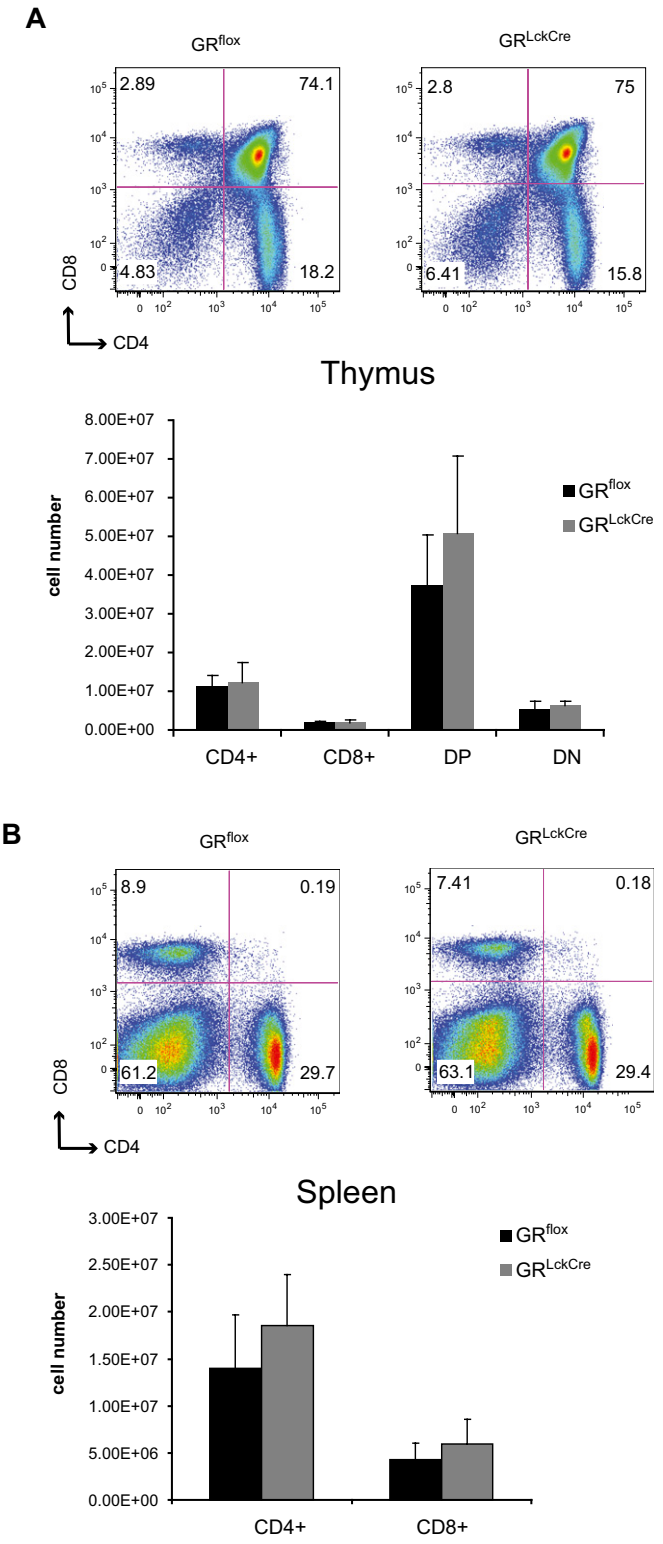


Fig. S3. Cell compartments of GR^{flox} and GR^{LckCre} mice have comparable T cell numbers. (*A*) Thymocytes of GR^{LckCre} mice and their respective littermate control mice GR^{flox} were analyzed by flow cytometry with antibodies against surface expression of CD4 and CD8 (*Upper*). The number of $CD4^+$, $CD8^+$, double-positive (DP) $CD4^+CD8^+$, and double-negative (DN) $CD4^-CD8^-$ cells was determined by calculating the frequencies of the respective cells in the total number of cells (*Lower*; $n = 3$). (*B*) Splenic T cells of GR^{LckCre} and GR^{flox} mice were analyzed by flow cytometry as described in *A*. The number of $CD4^+$ and $CD8^+$ cells was determined by calculating the frequencies of the respective cells in the total number of cells ($n = 3$).

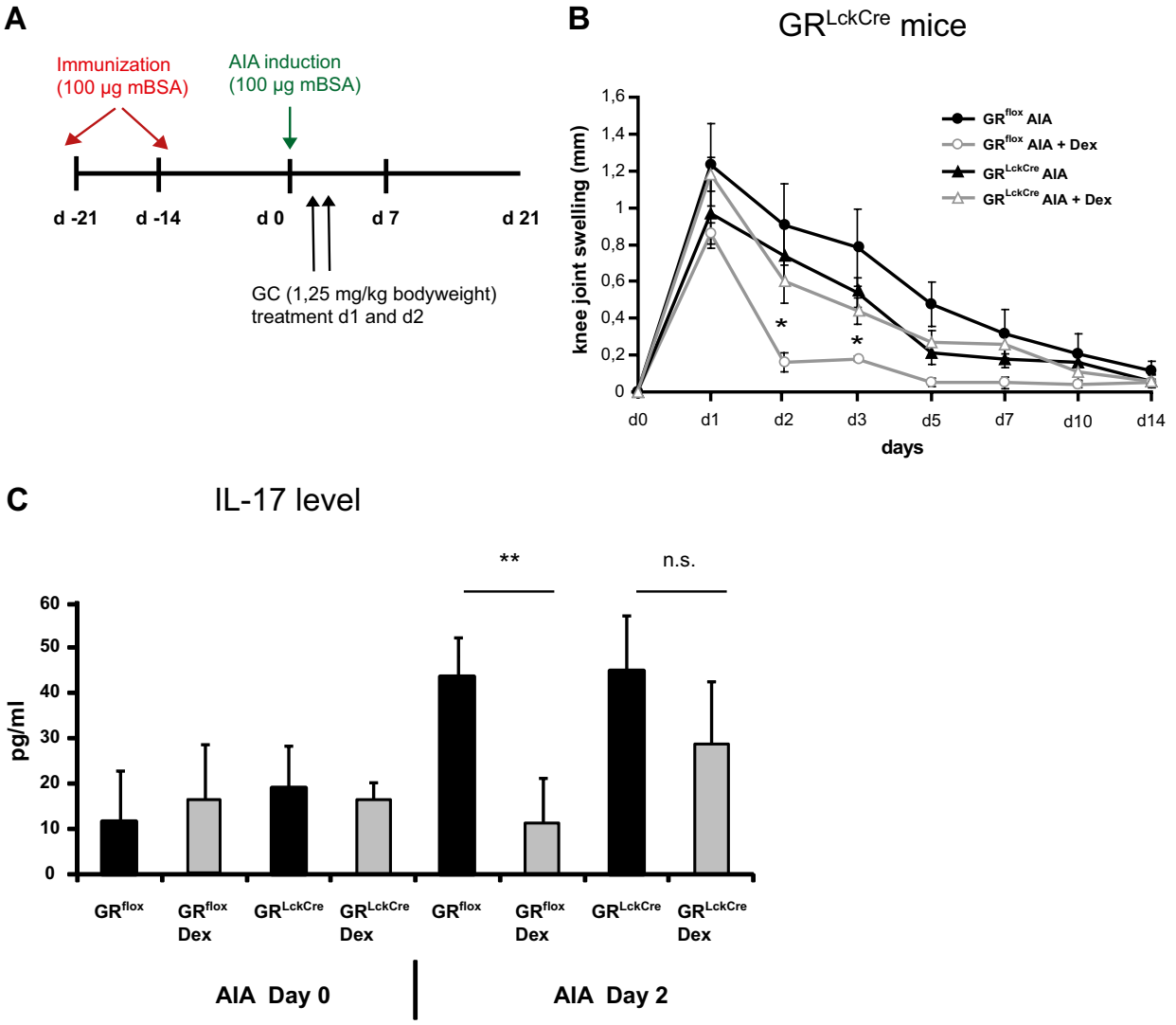


Fig. S4. GC-mediated suppression of AIA and IL-17 in mice with a fully established disease requires the GR in T cells. (*A*) Treatment scheme of AIA induction and Dex application. Mice were immunized s.c. twice at day −21 and −14 with 100 µg mBSA before arthritis induction by intraarticular injection of 100 µg mBSA at day 0. At day 1 (and day 2) after arthritis induction when the disease is already established, mice were treated with either 1.25 mg/kg Dex or PBS i.v. (*B*) GR^{LckCre} mice and their respective littermate control (GR^{flox}) mice were subjected to AIA and treated with PBS or Dex as described in *A*. Knee joint swelling was measured mechanically at indicated time points. (*C*) The serum IL-17 level was measured by ELISA in GR^{flox} and GR^{LckCre} mice before arthritis induction (day 0) and at day 2. Animals were treated as described in *A*. In *B* and *C*, $n = 5–6$; $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

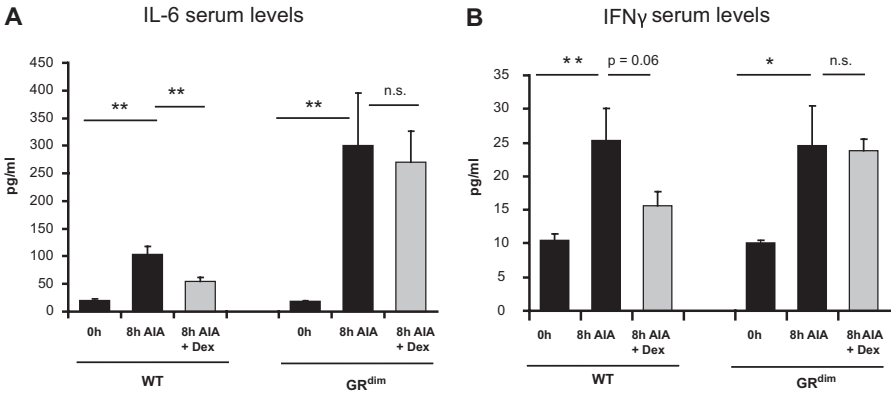


Fig. S5. Serum cytokine levels in PBS- and Dex-treated arthritic WT and GR^{dim} mice 8 h after AIA induction. IL-6 (*A*) and IFN-γ (*B*) were determined in WT and GR^{dim} animals treated as described in Fig. 1*A* 8 h after AIA induction. In *A* and *B*, $n = 5–6$; $*P < 0.05$, $**P < 0.01$; n.s., not significant.

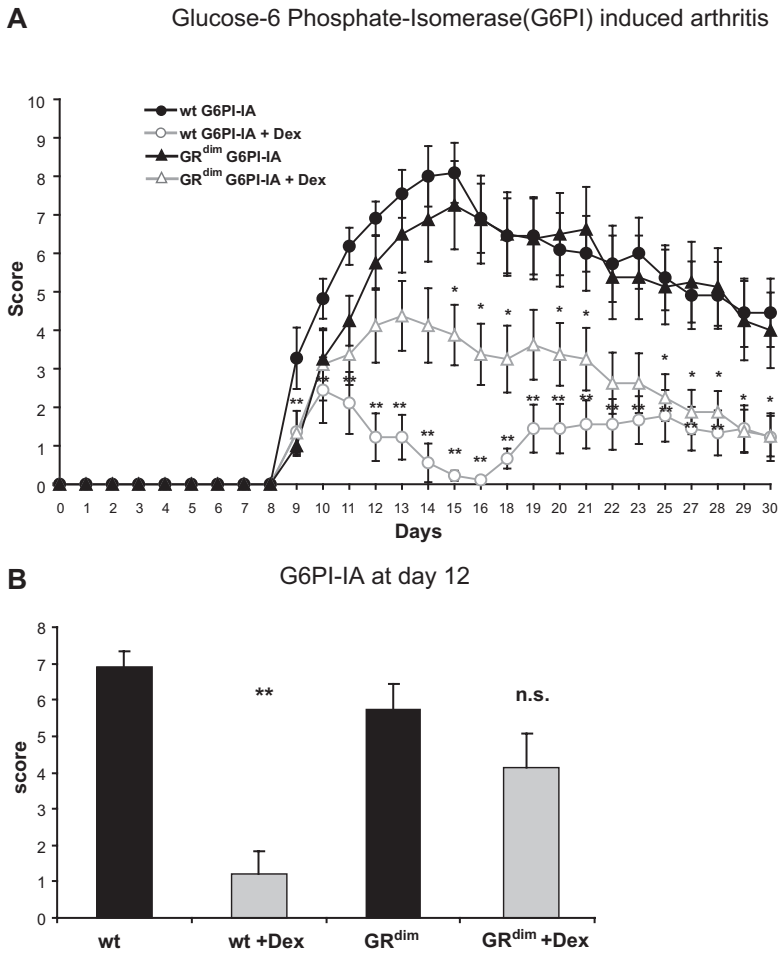


Fig. S6. G6PI-IA in WT and GR^{dim} mice. (*A*) WT and GR^{dim} mice were immunized with G6PI and treated from day 9 until day 15 with PBS or 1 mg/kg Dex. Paw swelling was scored from day 0 to day 30 at indicated time points. (*B*) Severity of G6PI-IA at day 12. In both panels, $n = 5$; $*P < 0.05$; $**P < 0.01$; n.s., not significant.

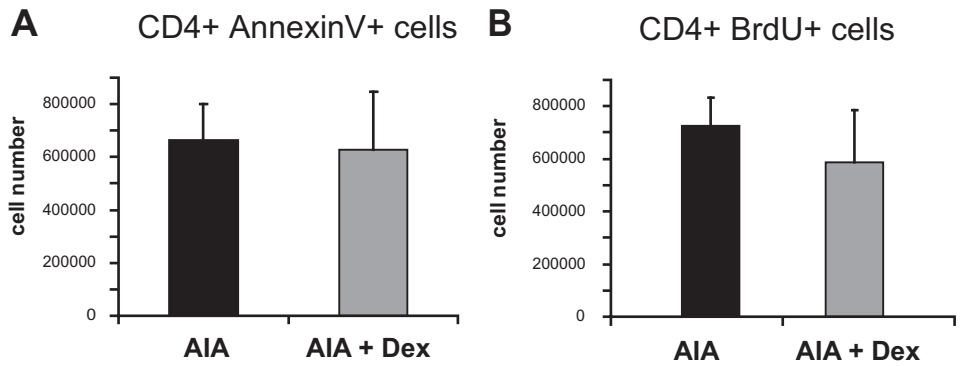


Fig. S7. Apoptosis and proliferation of total lymph node cells from PBS- and Dex-treated WT mice at day 1 after AIA induction. (*A*) Apoptosis was determined by annexin V-positive cells by flow cytometry analysis of $CD4^+$ cells ($n = 5$). (*B*) Proliferation was measured by determination of BrdU incorporation after 24 h. Single cell suspensions of draining lymph nodes were analyzed by flow cytometry for BrdU-positive cells ($n = 5$).

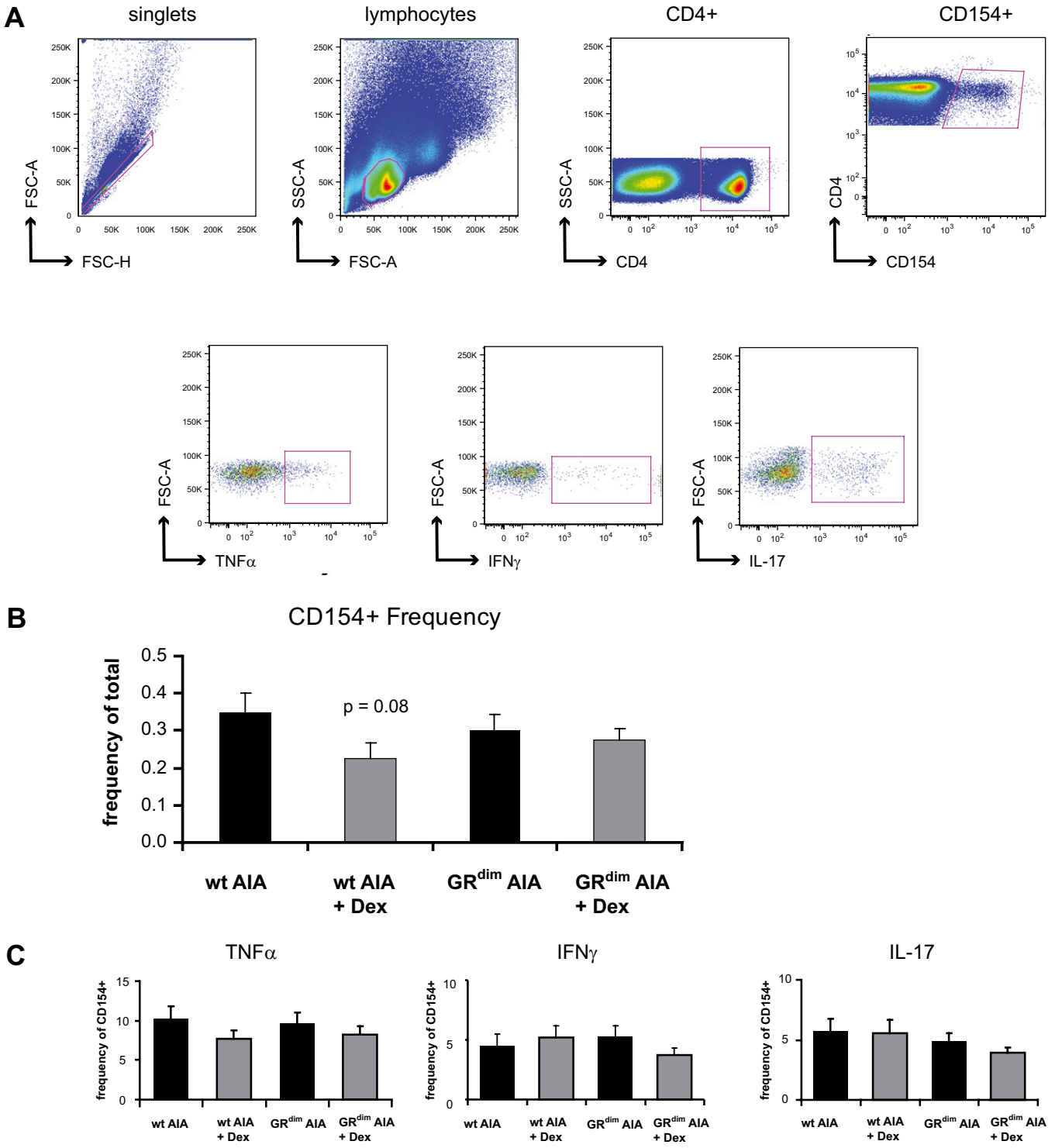


Fig. S8. Gating strategy and FACS analysis of activated CD154$^+$ cells in the draining lymph nodes of mice. (*A*) Cells from draining lymph nodes were isolated at day 1 after AIA induction and restimulated for 6 h with 25 μg mBSA. Cells were subsequently stained for CD4 and intracellularly for CD154, IFN-γ, TNF-α, and IL-17. Cells were gated for singlet cells, followed by a lymphocyte gate. T helper cells were identified through CD4, mBSA-specific T cells through CD154 expression. Cytokine analysis was performed by creating gates for IFN-γ, TNF-α, and IL-17. (*B*) Cells of the draining lymph nodes derived from arthritic PBS- and Dex-treated WT and GR^{dim} mice were restimulated ex vivo for 6 h with mBSA and further analyzed for CD154 expression by intracellular flow cytometry analysis. The frequencies of total for CD154 expression are shown (n = 18–24). (*C*) Intracellular flow cytometry analysis for TNF-α, IFN-γ, and IL-17 in restimulated draining lymph node cells with the gating strategy described in *A*. The bars show the frequencies of the respective cytokines within the CD154$^+$ cells (n = 18–24).

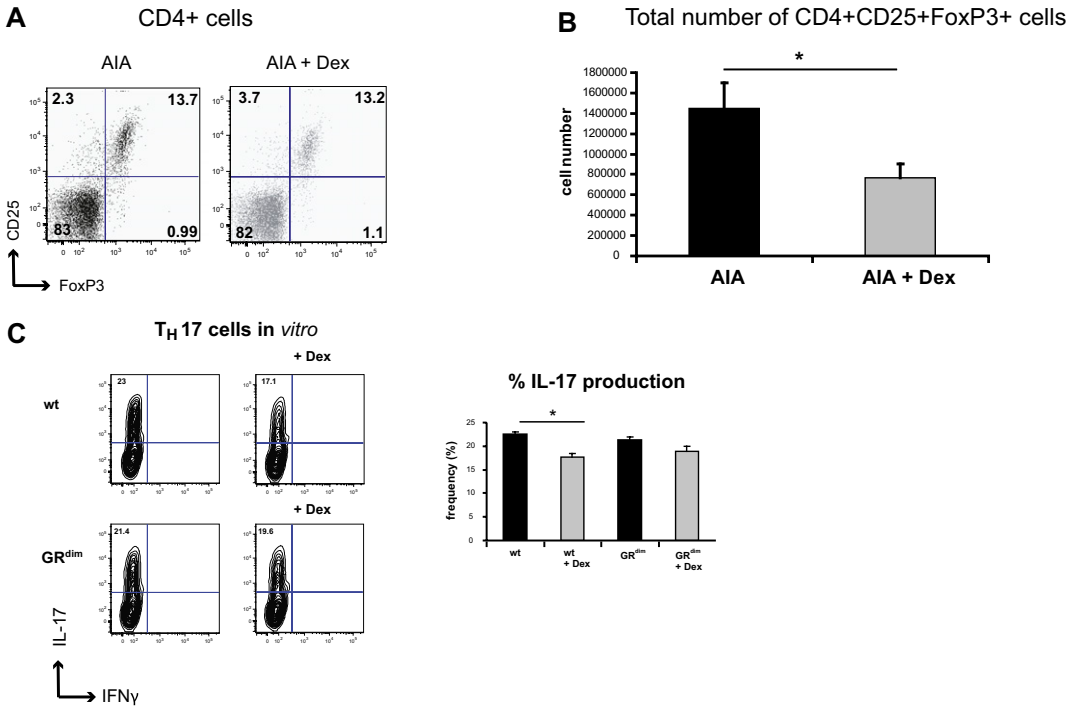


Fig. S9. Regulatory T cells in the draining lymph nodes from PBS- and Dex-treated WT mice at day 1 after AIA induction and influence of GCs on IL-17 production in T_H17 cells. (*A*) FACS analysis of restimulated (6 h with 25 μg mBSA) lymph node cells stained for CD25, CD4, and FoxP3. A representative plot of $CD25^+FoxP3^+$ cells of the $CD4^+$ gate is shown. Numbers indicate the mean of frequencies. (*B*) Total number of regulatory T cells in draining lymph nodes of mice ($n = 5$; $^*P < 0.05$). (*C*) Naïve T cells from WT and GR^{dim} mice were sorted for $CD4^+TCR\beta^+CD62L^{high}CD44^{low}$ cells and differentiated for 7 d under T_H17-promoting conditions. After 7 d, T_H17 cells were restimulated for 6 h and treated with a pharmacological dose (10^{-6} M) of Dex. Representative FACS images with mean values of frequencies are shown. The graph shows the mean and SD of IL-17 frequencies ($n = 3$; $^*P < 0.05$).