Supporting Information

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SI Materials and Methods

Skin Transplantation. Skin transplantation was performed as previously described (1). Briefly, full-thickness donor flank skin pieces (0.5–1 cm^2) were positioned on a graft bed prepared on the flank of the recipient. The time point of rejection was defined as the complete necrosis of the graft.

IL-17 ELISpot. ELISpot assays were performed as described (1). Briefly, ImmunoSpot M200 plates (Cellular Technology) were coated overnight with 1/250 of anti–IL-17A (clone TC11-18H10; BD Biosciences) capture antibodies in sterile PBS. The plates were blocked for 1 h with sterile PBS 10% (vol/vol) FBS and washed three times with sterile PBS. One million splenocytes from C57BL/6, CARMA1-KO, or IκBαΔ*N*-Tg mice, immunized with $MOG_{35–55}$ peptide 14–20 d before, were plated in complete DMEM with 10 μg/mL of $MOG_{35–55}$ or $OVA_{323–339}$ peptide. Subsequently plates were incubated at 37 °C, 7% (vol/vol) CO_2 for 16 h. After washing with PBS, 1/500 of biotin-labeled anti–IL-17A detection antibody (clone TC11-8H4.1; BD Biosciences) was added for 2 h at 22 °C. The plate-bound secondary antibody was then visualized by adding 1/2,000 streptavidin–horseradish peroxidase (Bio-Rad) and 3-amino-9-ethylcarbazole substrate (Sigma). Image analysis of ELISpot assays was performed with the ImmunoSpot Analysis software after scanning the plates with an ImmunoSpot analyzer (Cellular Technologies).

IFNγ ELISpots. One million splenocytes from WT or CARMA1-KO mice transplanted 32 d prior with BALB/c skin, were stimulated for 18 h with irradiated B6 (syngeneic) or BALB/c (allogeneic) splenocytes (4×10^5 per well). Alternatively, one million splenocytes from C57BL/6 or IκBαΔ*N*-Tg mice immunized with $MOG_{35–55}$ peptide 14–20 d before were plated in complete DMEM with 10 μg/mL of $MOG_{35–55}$ or $OVA_{323–339}$ peptide. The ELISpot assay was conducted according to manufacturer instructions (BD Biosciences), and the numbers of IFN-γ–producing spots per well were calculated using the ImmunoSpot analyzer (CTL Analyzers).

Dendritic Cell Purification and Activation. Purification of splenic $CD11c^+$ cells was performed with EasySep Mouse CD11c Positive Selection kit (StemCell) from splenocytes of WT and CARMA1-KO mice following manufacturer instructions. Purity was routinely >95%. Cells were stimulated with 1 μg/mL lipopolysaccharides from *Escherichia coli* 026:B6 (Sigma) for 24 h, washed, pulsed for 2 h with 2 μg/mL of ovalbumin peptide $OVA_{323–339}$, washed, and then cultured in the presence of purified OT-II CD4 T cells.

Flow Cytometry. To assess intracellular cytokine expression, differentiated T cells were restimulated for 4 h with 150 ng/mL PMA and 500 ng/mL ionomycin (Sigma), in the presence of 10 μg/mL brefeldin A (Biolegend). Cells were stained with the fixable viability dye Live Aqua (Invitrogen) for 20 min at room temperature, before fixation with 1% (vol/vol) paraformaldehyde in FACS buffer [0.1% (wt/vol) BSA, 0.1% (wt/vol) NaN_3 in PBS] and permeabilization in intracellular staining (IC) buffer [0.1% (wt/vol) saponin in FACS buffer], and then stained in IC buffer with fluorochrome-conjugated antibodies to Foxp3, IFNγ, IL-17A, and IL-17F, or their respective isotype controls. For the assessment of phosphoproteins, cells were stimulated for 30 min with the indicated cytokines, fixed with 1% (wt/vol) formaldehyde at 37 °C for 10 min, permeabilized with ice-cold 90% (vol/vol) methanol for 30 min on ice, washed in FACS buffer, and stained with fluorochrome-conjugated antibodies to CD4 and CD44, phospho-STAT3, or phopsho-STAT5 in FACS buffer. Alternatively, viability was assessed by staining cells with allophycocyanin-coupled Annexin V and Live Aqua, following the manufacturer's recommendation (BD Biosciences). Proliferation was assessed in CD4 T cells labeled with 2 μM of carboxyfluorescein succinimidyl ester (CFSE; Sigma) or 5 μM Cell Proliferation dye eFluor 670 (eBioscience). Dendritic cells were analyzed using CD11c, CD80, CD86, and MHC II antibodies. All experiments were analyzed in BD LSR Fortessa and LSR II flow cytometers (BD Biosciences). Antibodies were purchased from eBioscience except for all phospho-specific antibodies (BD Biosciences).

Cytokine ELISAs. Cells stimulated for 72 h in Th0, Th1, Th2, or Th17 conditions were washed twice with complete medium, and 5×10^5 viable cells were restimulated with 50 ng/mL of PMA and 0.5 μg/mL of ionomycin for 24 h. Cytokine production was assessed in supernatants using ELISA kits for IFNγ, IL-17A, and IL-4 ELISAs (BD Biosciences), according to the manufacturer's instructions. Plates were read in a 96-well μQuant spectrophotometer (Bio-Tek Instruments) and data were analyzed using KC4 software (Bio-Tek Instruments) by comparison against a standard curve generated using recombinant cytokines at known concentrations.

Reverse Transcription and Quantitative Real-Time PCR. Total RNA was prepared from T cells with the use of RNEasy Plus Mini kit (Qiagen). cDNA was synthesized with iScript cDNA Synthesis kit (Bio-Rad) and the samples diluted in water (1:10). A total volume of 25 μL containing 5 μL cDNA template, 0.3 μM of each primer, and SYBR Green PCRMaster mix (Applied Biosystems) was analyzed in triplicate. Gene expression was analyzed with an ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection software version 1.9.1 (Applied Biosystems). Reactions were prepared in triplicates and results were normalized by division of the value for the tested gene by that obtained for *Actb* using the standard curve method. Primers used are described in Table S1.

Generation of MIGR1-CARMA1-ΔID. Constitutively active CARMA1 in pcDNA3 (provided by Joel Pomerantz, Johns Hopkins University, Baltimore) was cut with XbaI, site filled with Klenow polymerase, and cut again with NotI. The retroviral vector MIGR1-poly, coding for eGFP after an internal ribosome entry site (IRES) region (provided by Malay Mandal, University of Chicago), was cut with HpaI and NotI and subsequently linked to the CARMA1-ΔID fragment. Cells were transformed into *E. coli* competent cells. The fidelity of the construct was verified by DNA sequencing.

Retrovirus Production and Transduction. Retrovirus packaging PlatE cells were transfected with either MIGR1-eGFP, MIGR1-CARMA1-ΔID-eGFP, MIGR1-hCD2, or MIGR1-RORα-hCD2 (the latter two constructs provided by Chen Dong, University of Texas MD Anderson Cancer Center, Houston) using a calcium chloride transfection protocol (Invitrogen) according to manufacturer instructions. For retroviral transduction, WT and CARMA1-KO $CD4^+$ naïve T cells were stimulated for 24 h in the presence of rhIL-2 (10 units/mL) and plate bound anti-CD3 (5 μg/mL) and anti-CD28 (1 μg/mL), transduced by spin infection (670 × *g* for 99 min at 37 °C) with supernatant of 48 h

transfected-PlatE cells in the presence of 8 μg/mL of polybrene and subsequently stimulated in Th17 culture conditions for 72 h.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described (2). Before the addition of biotin-labeled double-stranded DNA probe, 1.5 μg of nuclear extract was incubated for 20 min on ice in 20 μL of reaction buffer containing 1× binding buffer, 1 μg double-stranded poly(dI:dC), 2.5% (vol/vol) glycerol, 0.05% (vol/vol) Nonidet P-40, and 1 μg BSA. Samples were incubated for 20 min at 22 °C with 20 fmol of biotin-labeled double strand oligonucleotide probe for IL-17–RORE-S GAAAGTTTTCTGACCCACTTTAAATCAATTT (3) or IL-2–NF-κB ACCAAGAGGGATTTCACCTAAATC. Protein–DNA complexes were separated by electrophoresis through 6% nondenaturing Tris-borate-EDTA (TBE) buffer agarose gels (Invitrogen) and were visualized with a LightShift Chemiluminescent EMSA kit according to the manufacturer's procedure (Pierce).

Western Blot. Whole-cell extracts (5–10 μg of proteins) were analyzed by SDS/PAGE, transferred to PVDF membranes, and probed with rabbit polyclonal Ab against acetylated STAT3 (Cell Signaling Technologies), STAT3 (Santa Cruz Biotechnologies), or cyclophilin B (Abcam). Bound antibodies were detected using peroxidase-labeled anti-rabbit IgG (Bio-Rad) and ECL Plus chemiluminescent reagent (GE Healthcare) on CL-Xposure films (Thermo Scientific).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP was performed using the ChIP assay kit from Millipore/Upstate according to the manufacturer's instructions. Briefly, wild-type or CARMA1-deficient $CD4^+$ naïve cells were stimulated in Th17 conditions for 72 h and restimulated the last 2–4 h with 50 ng/mL PMA, 0.5 μg/mL ionomycin, and 20 ng/mL IL-6 (Peprotech). DNA binding proteins were crosslinked to DNA by adding formaldehyde (1% final concentration) and incubation for 10 min at 37 °C and then quenched with 125 mM glycine for 5 min. Cells were lysed and DNA in the supernatant was sheared by a Branson digital sonifier (Branson Ultrasonics). After removing an aliquot of sonicated samples as input material, the remainder was used for immunoprecipitation. Five micrograms of anti–acetyl-H3 (AcH3; Millipore/Upstate), anti-STAT3, and anti-STAT5 (Santa Cruz Biotechnologies; H-190 and sc-836, respectively), anti-H3K4me3 (Millipore), anti-H3K27me3 (Millipore), or normal rabbit IgG (Santa Cruz Biotechnologies) antibodies and protein A magnetic beads (Millipore) were added to the supernatant fraction and incubated overnight at 4 °C with rotation. To reverse histone–DNA cross-links, 20 μL of 5 M NaCl and 1 μL of 20 mg/mL proteinase K were added to each sample before heating at 65 °C for 2 h. DNA was purified using the QIAquick PCR Purification kit (Qiagen) and qPCR was performed using SYBR Green premixed reagents (Applied Biosystems) in ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection software version 1.9.1 (Applied Biosystems). Reactions were prepared in triplicates and results were normalized respective to IgG and input. Primers used are described in Table S1.

Isolation of CNS-Infiltrating Mononuclear Cells for Intracellular Cytokine Staining. Mononuclear cells were isolated following the protocol by Wei et al. (4). Briefly mice were anesthetized with pentobarbital, perfused with PBS, and brains and spinal cords were harvested. Spinal cord tissue was flushed out with cDMEM [DMEM with 10% FBS, 1% (vol/vol) NEAA, 1% (vol/vol) L-glutamine, 50 μM beta-mercaptoethanol, 1% (vol/vol) penicillin/streptomycin, 10 mM Hepes] and an 18-gauge needle. The CNS tissue (brain and spinal cord) was minced and ground against a 70-μM filter using the back of a syringe. After washing with cDMEM, the pellet was resuspended in 4 mL of 70% Percoll (Sigma-Aldrich) and overlaid with 37% Percoll and 30% Percoll. The cell suspensions were then centrifuged for 20 min at 460 g at 20 °C. The leukocytes were harvested at the 37%/70% interface and washed again with cRMPI. The cells were then analyzed by flow cytometry.

1. Molinero LL, et al. (2008) Epidermal Langerhans cells promote skin allograft rejection in mice with NF-kappa B-impaired T cells. *Am J Transplant* 8(1):21–31.
2. Molinero LL, Miller ML, Evaristo C, Alegre ML (2011) High TCR stimuli prevent induced regulatory T cell differentiation in a NF-κB-dependent manner. *J Immunol* 186(8):4609–4617.
3. Yang XO, et al. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28(1):29–39.
4. Wei J, Loke P, Zang X, Allison JP (2011) Tissue-specific expression of B7x protects from CD4 T cell-mediated autoimmunity. *J Exp Med* 208(8):1683–1694.

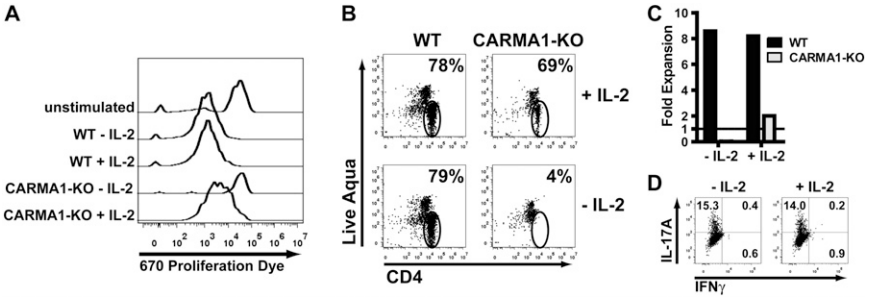

Fig. S1. Exogenous IL-2 corrects survival and partially restores proliferation in CARMA1-deficient T cells. Proliferation Dye 670-labeled WT and CARMA1-KO $CD4^+CD25^-CD44^{lo}$ cells were stimulated for 72 h under Th17 conditions (1 μg/mL anti-CD28, 5 μg/mL anti-CD3, 1 ng/mL TGFβ. and 20 ng/mL IL-6) in the presence or absence of 10 units/mL of IL-2. (*A*) Proliferation was assessed by flow cytometry measuring dilution of the Proliferation Dye 670. (*B*) Cell survival was determined as the percentage of $CD4^+$Live $Aqua^-$ events. (*C*) Fold expansion [(number of cells at the end of culture)/(number of cells at the beginning of culture)] of WT and CARMA1-KO cells described in *B*. Values of >1 = cell expansion; 1 = no change; and <1 = cell loss. (*D*) Intracellular expression of IL-17A and IFNγ was assessed in WT CD4 cells stimulated under Th17 conditions in the presence or absence of 10 units/mL of IL-2.

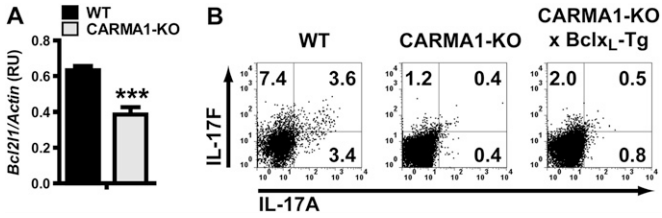

Fig. S2. CARMA1 is required for Bcl-x_L up-regulation, but overexpression of Bcl-x_L does not restore IL-17 production. (*A*) *Bcl2l1* mRNA levels were assessed by RT-qPCR in WT and CARMA1-KO $CD4^+CD25^-CD44^{lo}$ cells stimulated under Th17 conditions for 72 h and normalized to levels of *Actb* gene expression. RU, relative units. (*B*) IL-17A and IL-17F expression was assessed by intracellular flow cytometry in WT, CARMA1-KO, and CARMA1-KO × Bclx$_L$-Tg $CD4^+CD25^-CD44^{lo}$ cells stimulated under Th17 conditions for 72 h. Experiments were performed at least twice.

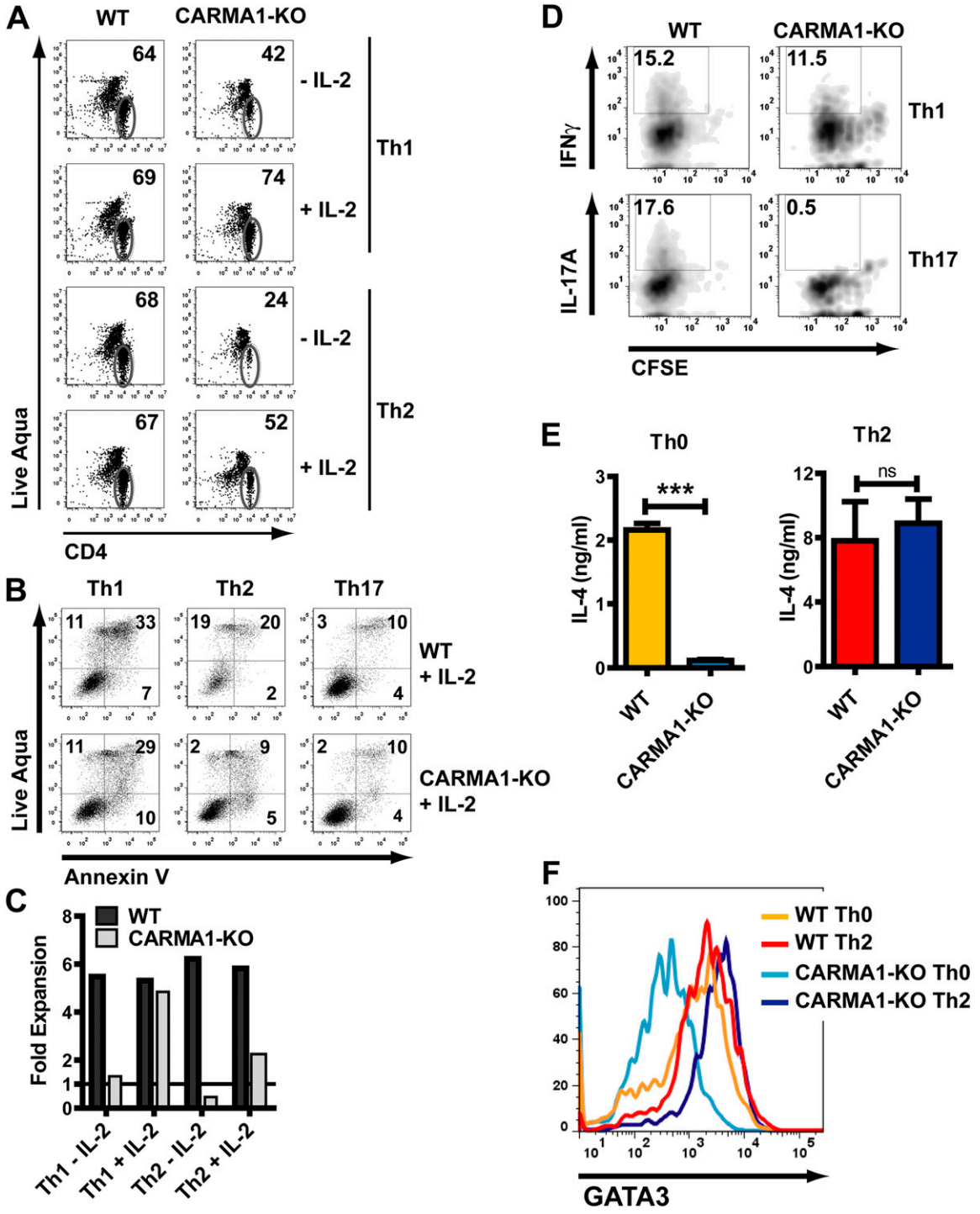

Fig. S3. Cell survival and proliferation of CARMA1-KO CD4 cells stimulated under Th1 and Th2 polarization conditions. (*A*) Live cells ($CD4^+$Live $Aqua^-$) were analyzed by flow cytometry in WT and CARMA1-KO $CD4^+CD25^-CD44^{lo}$ cells stimulated for 72 h in Th1 or Th2 conditions with or without 10 units/mL of IL-2. (*B*) Apoptosis of WT and CARMA1-KO cells (stimulated for 72 h in Th1, Th2, and Th17 conditions in the presence of IL-2) were assessed by flow cytometry in CD4-gated events. (*C*) Fold expansion [(number of cells at the end of culture)/(number of cells at the beginning of culture)] of WT and CARMA1-KO cells described in *A*. Values of >1 = cell expansion; 1 = no change; and <1 = cell loss. (*D*) IFNγ, IL-17A, and proliferation were assessed by flow cytometry in CFSE-labeled WT and CARMA1-KO CD4 naïve cells stimulated under Th1 + IL-2 (*Upper*) or Th17 + IL-2 (*Lower*) conditions for 72 h and restimulated for 4 h with PMA and ionomycin in the presence of brefeldin A. Results are representative of at least four independent experiments. (*E*) Detection of secreted IL-4 by ELISA from WT or CARMA1-KO CD4 naïve cells stimulated in Th0 or Th2 conditions for 72 h. Cells were extensively washed and the same cell numbers were restimulated for 24 h with PMA and ionomycin (mean ± SD). ***$P < 0.001$. (*F*) Detection of intracellular GATA3 in WT and CARMA1-KO CD4 cells stimulated for 72 h under Th0 or Th2 conditions, as assessed by intracellular flow cytometry.

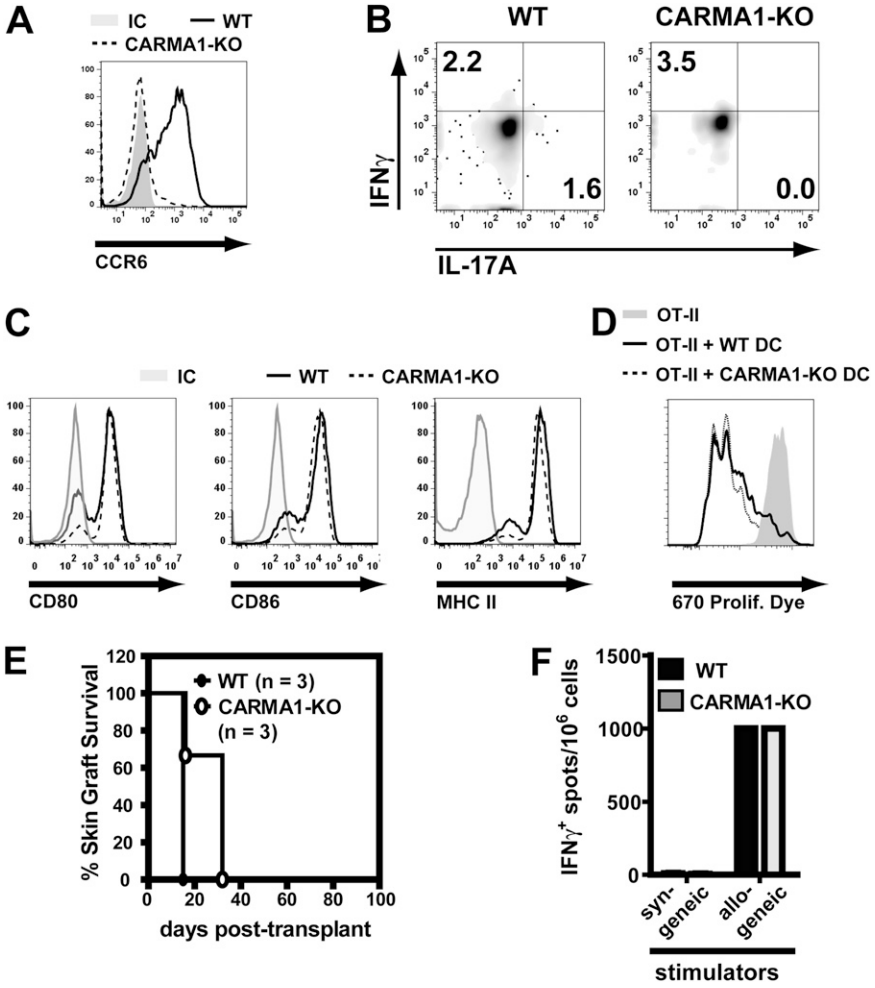

Fig. S4. T cells need CARMA1 for experimental autoimmune encephalomyelitis but not skin allograft rejection. (*A*) Surface expression of CCR6 in WT (solid line) and CARMA1-KO (dashed line) cells stimulated for 72 h under Th17 conditions as assessed by flow cytometry. IC, isotype control (gray-filled histogram). (*B*) Detection of IL-17A and IFNγ in $CD4^+$ CNS-infiltrating cells by intracellular staining. (*C*) Expression of CD80, CD86, and MHC class II in 24 h LPS-stimulated WT and CARMA1-KO $CD11c^+$ dendritic cells (DCs). (*D*) Proliferation of Proliferation Dye 670-labeled OT-II CD4 T cells after 72 h stimulation with LPS-activated WT or CARMA1-KO DCs. (*E*) BALB/c ($H\text{-}2^d$) skin allografts were transplanted into fully mismatched C57BL/6 ($H\text{-}2^b$) WT or CARMA1-KO mice and graft survival was assessed over time. (*F*) Splenocytes from mice that had rejected their skin grafts were prepared and 10^6 responder cells were cocultured with 4×10^5 γ-irradiated syngeneic (C57BL/6) or donor (BALB/c) stimulators. Results represent the mean and SD of triplicates of three different samples.

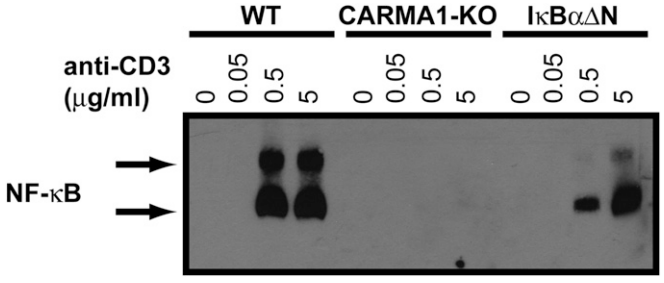

Fig. S5. NF-κB activation in WT, CARMA1-KO, and IκBαΔN-Tg CD4 T cells. Nuclear extracts of WT, CARMA1-KO, and IκBαΔN-Tg $CD4^+CD44^{lo}$ T cells stimulated for 24 h with plate-bound anti-CD28 (1 μg/mL) and increasing doses of anti-CD3 (0, 0.05, 0.5, and 5 μg/mL) in the presence of IL-2 were subjected to EMSA for detection of NF-κB binding to a biotin-labeled DNA probe containing the NF-κB binding site of the *IL-2* gene.

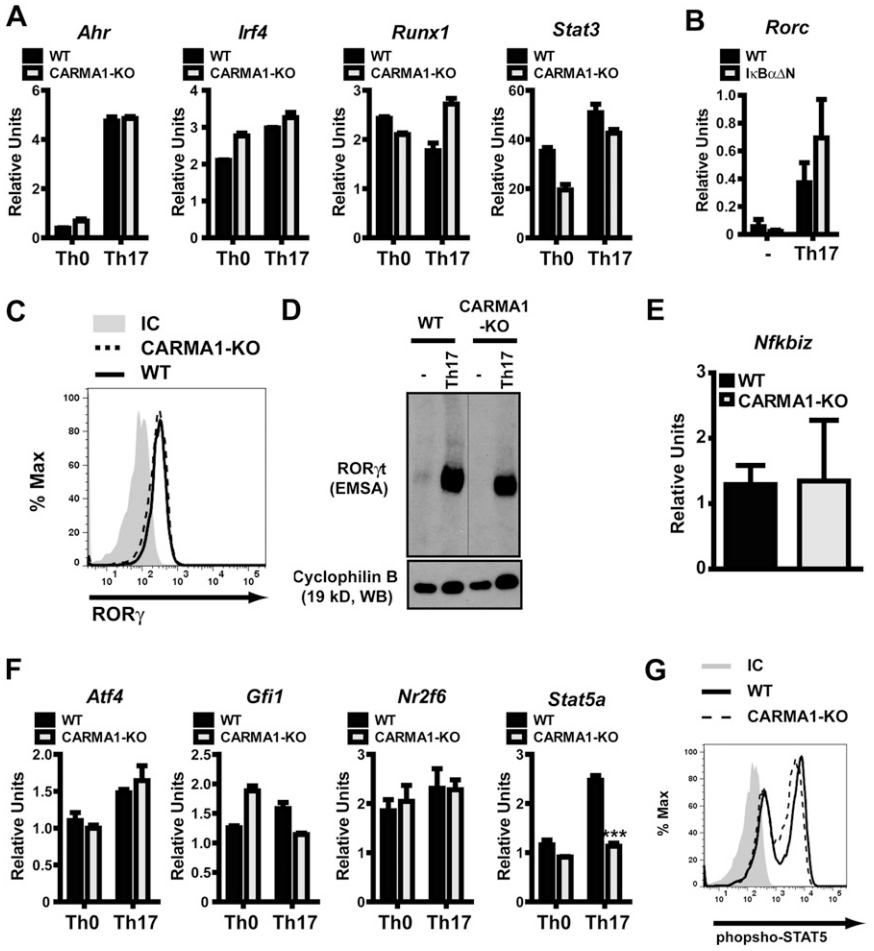

Fig. S6. CARMA1 and transcription factors controlling Th17 differentiation. (*A*) WT and CARMA1-KO $CD4^+CD25^-CD44^{lo}$ cells were stimulated in Th0 or Th17 conditions for 72 h, processed, and levels of *Ahr*, *Irf4*, *Runx1*, and *Stat3* mRNA were assessed by RT-qPCR and normalized to levels of *Actb* (relative units). Values displayed correspond to mean ± SD of triplicates, and data are representative of at least three independent experiments. (*B*) WT and IκBαΔN-Tg $CD4^+CD25^-CD44^{lo}$ cells were left unstimulated or stimulated for 72 h in Th17 conditions. Levels of *Rorc* mRNA were assessed by RT-qPCR as in *A*. (*C*) $CD4^+CD25^-CD44^{lo}$ WT (solid line) and CARMA1-KO (dashed line) cells were stimulated in Th17 conditions for 72 h and levels of RORγt protein were assessed by intracellular flow cytometry. IC, isotype control (gray-filled histogram). Data are representative of at least three independent experiments. (*D*) Nuclear extracts were prepared from $CD4^+CD25^-CD44^{lo}$ WT and CARMA1-KO cells either unstimulated (–) or incubated in Th17 conditions for 72 h. RORγt binding to the IL-17A promoter was analyzed by EMSA. Nuclear protein levels of cyclophilin B, detected by immunoblot, were used for normalization of nuclear protein content. (*E*) *Nfkbiz* mRNA was assessed in 72 h Th17-stimulated WT and CARMA1-KO cells as in *A*. (*F*) Cells were stimulated and processed as in *A*, and expression of *Atf4*, *Gfi1*, *Nr2f6*, and *Stat5a* mRNA was assessed by RT-qPCR as described in *A*. (*G*) STAT5 phosphorylation was assessed in WT and CARMA1-KO CD4 naïve cells stimulated in Th17 conditions for 18 h. ***$P < 0.001$.

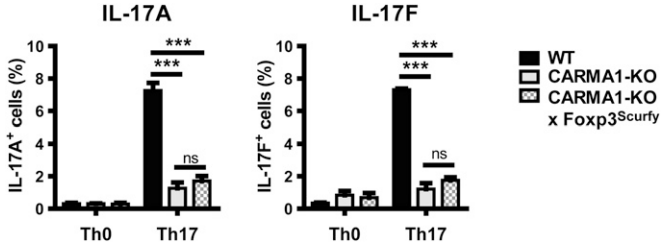

Fig. S7. Genetic ablation of Foxp3 expression does not restore Th17 differentiation in CARMA1-KO T cells. IL-17A and IL-17F expression was assessed by intracellular flow cytometry in WT, CARMA1-KO, and CARMA1-KO × $Foxp3^{Scurfy}$ naïve CD4 T cells stimulated for 72 h under Th0 or Th17 conditions. ***$P <$ 0.001. Values displayed correspond to mean ± SD of triplicates, and data are representative of at least three independent experiments.

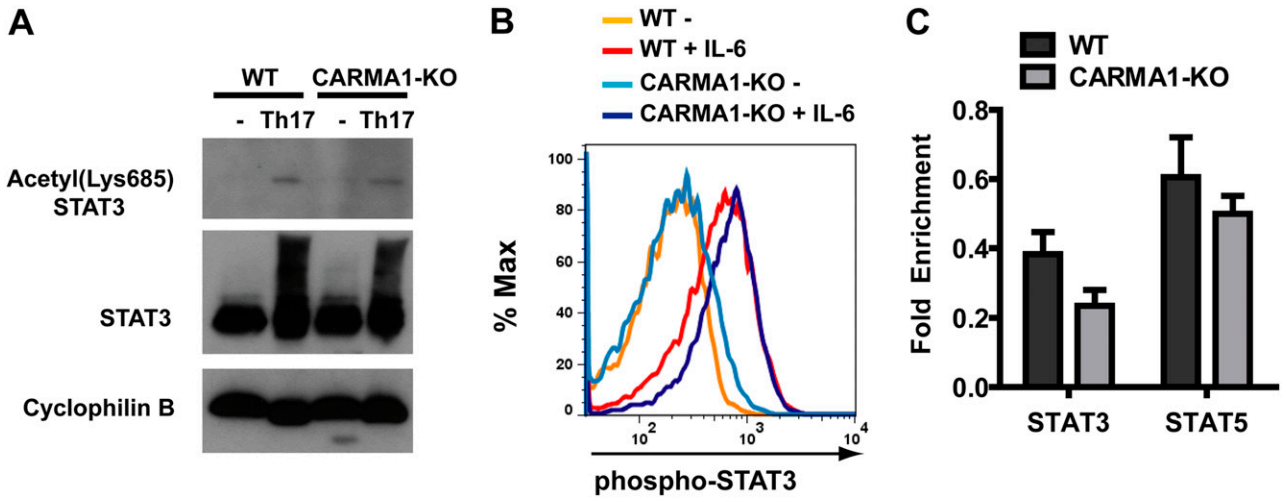

Fig. S8. STAT3 activation is CARMA1 independent. (*A*) Wild-type and CARMA1-KO $CD4^+CD25^-CD44^{lo}$ cells were left untreated (–) or stimulated in Th17 conditions for 72 h. Levels of STAT3 acetylation (lysine 685, *Top*) and total STAT3 (*Middle*) were assessed by Western blot. Cyclophilin B immunodetection (*Bottom*) was used as loading control. (*B*) STAT3 phosphorylation (tyrosine 705) was assessed by flow cytometry in wild-type or CARMA1-deficient $CD4^+CD25^-CD44^{lo}$ cells left unstimulated (–) or stimulated with IL-6 for 30 min. (*C*) WT and CARMA1-KO cells treated as in *A* were processed for chromatin immunoprecipitation using anti-STAT3 and anti-STAT5 Ab, with subsequent amplification of the *Il17a* promoter region. Data represent fold enrichment to isotype control IgG and are normalized to input DNA (mean ± SD of triplicates). All data are representative of at least three independent experiments.

Table S1. PCR oligonucleotides used in this study

5′ oligo	Sequence	3′ oligo	Sequence
RT-qPCR			
Actin-F	TGGAATCCTGTGGCATCCATGAAAG	Actin-R	TAAAACGCAGCCTCAGTAACAGTCCG
AHR-F	GGCCAAGAGCTTCTTTGATG	AHR-R	AAGACCAAGGCATCTGCTGT
ATF4-F	GAAACCTCATGGGTTCTCCA	ATF4-R	AGAGCTCATCTGGCATGGTT
BCL-XL-F	CAAGAGCCATTGAGTGAGGTGCT	BCL-XL-R	CAAGAGCCATTGAGTGAGGTGCT
CCR6-F	GGATAACCACTGAGGCAGGA	CCR6-R	AGTCCGAAAGACCCAGGATT
Foxp3-F	TCTTCGAGGAGCCAGAAGAG	Foxp3-R	TACTGGTGGCTACGATGCAG
GFI1-F1	CCCCGACTCTCAGCTTACC	GFI1-R1	AGAGAGCGGCACAGTGACTT
IFNg-F	GCGTCATTGAATCACACCTG	IFNg-R	ATCAGCAGCGACTCCTTTTC
IL-17A-F	TCCAGAAGGCCCTCAGACTA	IL-17A-R	AGCATCTTCTCGACCCTGAA
IL-17F-R	AAGAAGCAGCCATTGGAGAA	IL-17F-F	ACAGAAATGCCCTGGTTTTG
IL-21-F	AGGAGGGGAGGAAAGAAACA	IL-21-R	GGGAATCTTCTCGGATCCTC
IL-22-F	CAACTTCCAGCAGCCATACA	IL-22-R	GTTGAGCACCTGCTTCATCA
IL-23R-F	AAGGCTTTTCGGAACCTCAT	IL-23R-R	TTCCAGGTGCATGTCATGTT
IL-4-F	CCTCACAGCAACGAAGAACA	IL-4-R	ATCGAAAAGCCCGAAAGAGT
IRF4-F	CAATGTCCTGTGACGTTTGG	IRF4-R	GGCTTCAGCAGACCTTATGC
Nr2f6-F	GGACAAGTCCAGTGGAAAGC	Nr2f6-R	GCACTTCTTGAGCCGACAGT
RORa-F	TCTCCCTGCGCTCTCCGCAC	RORa-R	TCCACAGATCTTGCATGGA
RORgT-F	TGCAAGACTCATCGACAAGG	RORgT-R	GCACTGATGTTGAATCCCCT
Runx1-F	CCAGCAAGCTGAGGAGCGGCG	Runx1-R	TGACGGTGACCAGAGTG
STAT3-F1	AGATCATGGATGCGACCAAC	STAT3-R1	GGCAGGTCAATGGTATTGCT
STAT5a-F	GTTGTATGGGCAGCATTTCC	STAT5a-R	TCAGCAAAAACCCATCTTCC
ChIP			
IL-17-STAT-p2-F	CACCTCACACGAGGCACAAG	IL-17-STAT-p2-R	ATGTTTGCGCGTCCTGATC
IL23r-AcH3-F	CACATTCTGTAAGGAGACTCATCAA	IL23r-AcH3-R	GGACAATAGAACAGGAGGAACTACA
RORg-AcH3-F	GGGAGCCAAGTTCTCAGTCA	RORg-AcH3-R	GAGGCACGGTTTCCAAGATA