Supporting Information

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SI Materials and Methods

Mice. Female C57BL/6 mice were obtained from the National Cancer Institute (CD45.2) or The Jackson Laboratory (B6 Ly5.2-congenic CD45.1). B6 IL-27RαKO mice were obtained from Genentech. STAT1KO mice were obtained from The Jackson Laboratory on the 129 background and back bred 10× to B6. CD45.1$^+$ ova-specific TCR-transgenic T cell (OT-1), IL-27RαKO CD45.1 OT1, CD45.1$^+$ Vβ5, and IL-27RαKO CD45.1$^+$ Vβ5 mice were bred and maintained at the Biological Resource Center at National Jewish Health. STAT3 fl/fl × CD4-Cre mice were provided by Charles Drake (The Johns Hopkins University, Baltimore, MD). STAT1 fl/fl mice were kindly provided by Matt Frieman (University of Maryland, College Park) with permission from Lothar Hennighausen (National Institutes of Health, Frederick, MD) (1) and back bred to STAT3 fl/fl × CD4-Cre mice to generate T-cell conditional STAT1, STAT3 single KOs, and STAT1/3 double knockouts. All mice were age matched within 10 d for experiments and used at greater than 5 wk of age. The Institutional Animal Care and Use Committee at National Jewish Health approved all animal experimental procedures and housing conditions according to guidelines provided by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Bone Marrow Chimeras. Recipient mice received 1,000 rad and rested 4 h before i.v. reconstitution with magnetic antibody depleted (anti-CD3, NK1.1, B220, CD19,CD4, CD8) bone marrow of equal cellularity from congenically disparate donors as designated in Figs. 2–5. Twelve weeks after reconstitution, ratios of CD45.2/CD45.1 were determined before use in experiments. Only mice with relatively equal engraftment were used for experiments. Donor bone marrow was isolated from WT, IL-27RaKO, STAT1KO, and STAT1fl/fl × STA3fl/fl ± CD4cre hosts.

Immunization and Pathogen Challenge. Mice were immunized via tail-vein injections with 2–50 μg (as indicated) of innate receptor agonist in 200 μL of 1× PBS containing 100–150 μg per mouse detoxified (LPS free as determined by limulus assay) whole chicken ovalbumin (Sigma) (2). Where indicated, mice were administered 50 μg per mouse of αCD40 antibody clone FGK-45 (BioXcell). For pathogen exposure, female mice were injected i.v. with either 1×10^7 pfu per mouse Vaccinia Virus Western Reserve Strain (Vv) or the same strain recombinantly expressing chicken ovalbumin (Vv-ova), or 2,000 cfu per mouse *Listeria monocytogenes* (Lm) expressing either whole ovalbumin (Lm-ova) or the vaccinia virus epitope B8R (Lm-B8R). For secondary Lm-ova challenges, 250,000 cfu were delivered i.v. Innate immune receptor agonists were obtained from the following sources and used in the following doses: Pam3-Cys (Invivogen), 25 μg per mouse ; α-Gal Cer (306-027-M001; Alexis Biochemicals), 2 μg per mouse; Flagellin was prepared from homogenate of *Salmonella minnesota* used at 15 μg per mouse (3); and Poly I:C (GE HealthCare), 50 μg per mouse. The following doses of adjuvants were used: flagellin (15 μg), Pam3Cys (25 μg), Poly I:C (50 μg), αCD40 (50 μg), or αGalcer (2 μg).

Lm-Ova Protection Assay. Congenically disparate WT and IL-27RαKO Vβ5 TCRβ transgenic mice were directly immunized with Poly I:C/αCD40/ova and allowed to rest for more than 35 d. Spleens were harvested, and CD8 T cells were enriched by magnetic negative selection (Miltenyi) to a purity of greater than 90%. The percent tetramer-positive was used to calculate the numbers of antigen-specific T cells transferred to congenically disparate naive WT hosts. The following day, animals were challenged with a lethal dose (250,000 cfu) of erythromycin-resistant Lm-ova. Then, 5 d later, spleens were harvested, and supernatants were plated out on erythromycin (5 μg/mL) containing brain–heart infusion (BHI) agar plates incubated for 36 h at 37 °C to count splenic bacterial burden.

OT-1 Adoptive Transfer. WT and IL-27RαKO OT-1 T cells were purified from spleen by magnetic negative selection for CD8 (Miltenyi). Populations were always greater than 90% pure. WT and IL-27RαKO OT-1s were mixed 1:1 and transferred i.v. The following day, mice were immunized as described in Fig. 4. For carboxyfluorescein succinimidyl ester (CFSE) labeling, cells were incubated at 2×10^7/mL with 10 μM CFSE in PBS for 10 min at 37 °C and then quenched with an equal volume of heat-inactivated FBS.

Flow-Cytometric Staining and Determination of Antigen-Specific T Cells. Animals were euthanized at day 7 post immunization [days postimmunization (dpi)] for all subunit vaccinations and 8 dpi for vaccinia and *Listeria* primary infection. Antigen-specific T-cell responses in spleen and blood were determined by tetramer staining as previously described (4). Analysis of tetramer staining was performed as previously described (5, 6). The black line in Fig. 2 denotes the limit of detection for tetramer in all tetramer experiments (0.07%) established by tetramer staining of unimmunized animals and use of irrelevant tetramers in immunized mice. Flow cytometry data were obtained using a DakoCytomation CyAN ADP flow cytometer, and analysis was performed using FlowJo Software (Treestar, PC version 7.6.5). The following cell-surface antibodies and clones were used: Brilliant Violet anti-mouse B220 (clone RA3-6B2; Biolegend), antigen-presenting cell (APC)-AF750 anti-mouse CD8a (clone 53-6.7; eBioscience), PerCP-Cy5.5 anti-mouse /human CD44 (clone IM7; Biolegend), and APC anti-mouse CD3e (clone 145-2c11, Biolegend). For bone marrow chimera (BMC) experiments, the congenic CD45 marker was determined by using APC labeled anti-mouse CD45.1 (clone A20; Biolegend). Kb SIINFEKL tetramers coupled to fluorochromes BV421 (Biolegend) and PE (Prozyme) were generated from baculovirus as previously described (5). Peptides were synthesized at the University of Colorado Denver Anschutz Medical Campus Peptide Core facility. I-Ab 2W1S tetramer was kindly provided by John Kappler and Philippa Marrack (National Jewish Health, Denver, CO). For transcription factors, cells were stained as described above and fixed with 1% paraformaldehyde-3% (wt/vol) sucrose-PBS (fixation buffer) for 10 min and then washed with 1× eBioscience Foxp3 perm buffer. Cells were then resuspended in eBioscience Foxp3 perm buffer, and transcription factor antibodies were added for 2 h, washed, fixed, and run on a flow cytometer. Intracellular cytokine-staining procedures were followed as described previously (5). Briefly, aliquots of splenic suspensions were restimulated with 1 μg/mL SIINFEKL peptide in the presence of 3 μg/mL Brefeldin A for more than 4 h. Surface stains were then added. Cells were washed, fixed, and stained for intracellular cytokine using the eBioscience Foxp3 perm buffer followed by washing and fixation. gMFI on graphs of flow-acquired data indicates the geometric mean fluorescence intensity of the indicated cellular subset for the stated parameter as determine by Flow Jo analysis software.

Calculation of Total Tetramer Plus Cell Number. Calculation of total antigen-specific cell numbers was performed by determining the relative percentage of tetramer-positive cells in a splenocyte sample multiplied by the total splenocyte count as determined by a Vi-Cell automated cell counter (Beckman Coulter).

Recombinant IL-27. Plasmid for generation of recIL-27 was generously provided by Xiao-Qing Wei (Cardiff University, Cardiff, UK). The plasmid encodes for the expression of the dimeric components of IL-27 covalently linked to one another and attached to an Fc backbone (7). The plasmid was transfected by use of Lipofectamine Ltx (Invitrogen) into HEK293T cells, and supernatants were collected. RecIL-27 was isolated through affinity chromatography across a protein G column. RecIL-27 free of Fc was generated through enzymatic digestion by overnight 37 °C incubation with papain 0.05 mg/mL. Purified recIL-27 free of Fc was separated from papain-digested reagents by dialysis with a 30-kDa cutoff. Recombinant IL-27 (250 μg per injection) was administered i.v.

IL-10R Blockade. Antibody (clone 1B1.3) recognizing the unique IL-10R (CD210) was purchased from Bioexcell and used at 250 μg i.p. per injection. Antibody was administered at day 3 and day 4 after Poly I:C/αCD40 immunization so as not to interfere with innate IL-10 signals and to blockade T cell-generated IL-10. This administration schedule preceded the alteration of WT/IL-27RaKO OT-1 observed in Fig. 4*D*.

Tetramer Dissociation Assay. WT Vβ5 and IL-27RαKO Vβ5 cohorts were used for the elevated naive precursor frequency of ova antigen-specific CD8 T cells and immunized as described in Fig. 4*B*. At day 7 peak of primary response, splenic suspensions were stained first with K^b-SIINFEKL-PE tetramer and then with antibodies for immune subsetting. Samples were thoroughly washed from unbound antibodies and tetramer. Aliquots for a 0 time point were then removed and fixed with 1% PCHO-3% sucrose. The 25D1.16 antibody (eBioscience) against K^b-SIINFEKL was added at a final concentration of 20 μg/mL. Aliquots were removed and fixed at time points of 0.5 min, 2 min, 4 min, 6 min, 8 min, 10 min, and 20 min. Geometric MFI (gMFI) of tetramer-positive cells was obtained by flow cytometry and plotted for each mouse. Data were plotted using Microsoft Excel and fit to a second-order polynomial resulting in the displayed R^2 line fit values. From the equations of the line, the time for each time 0 sample to have half of the tetramer competed off by 25D1 was determined and plotted by Prism (Graph Pad), reflecting the average TCR affinity for ova for each sample. Statistical significance was determined by Student *t* test.

Statistical Analyses. Data displayed include paired (bone marrow chimeras) and unpaired statistical analyses, which were made between experimental populations or groups using the Student *t* test or across time or dose by two-way ANOVA with GraphPad Prism Software (version 5). All experiments were performed independently at least twice with a minimum of three mice per group. Figures and figure legends detail replicates of experiments and numbers within each experiment. For figures, *P* values are coded as follows: $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. All error bars depict the SEM.

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2. Anis MM, Fulton SA, Reba SM, Harding CV, Boom WH (2007) Modulation of naive CD4+ T-cell responses to an airway antigen during pulmonary mycobacterial infection. *Infect Immun* 75(5):2260–2268.
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5. Kurche JS, Burchill MA, Sanchez PJ, Haluszczak C, Kedl RM (2010) Comparison of OX40 ligand and CD70 in the promotion of CD4+ T cell responses. *J Immunol* 185(4):2106–2115.
6. Sanchez PJ, Kedl RM (2012) An alternative signal 3: $CD8^+$ T cell memory independent of IL-12 and type I IFN is dependent on CD27/OX40 signaling. *Vaccine* 30(6):1154–1161.
7. Niedbala W, et al. (2008) Interleukin 27 attenuates collagen-induced arthritis. *Ann Rheum Dis* 67(10):1474–1479.

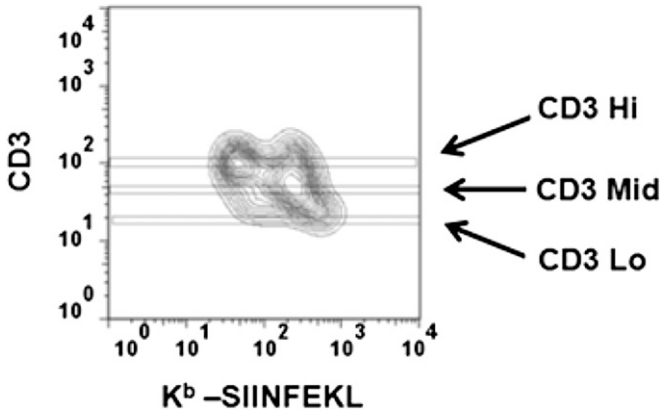


Fig. S1. Thin CD3 Slices. Splenocytes from mice immunized with Poly I:C/αCD40 adjuvant were analyzed by flow cytometry for antigen-specific tetramer-positive cells. Populations of tetramer-positive cells were subgated based upon differing expression of CD3 (*y* axis).

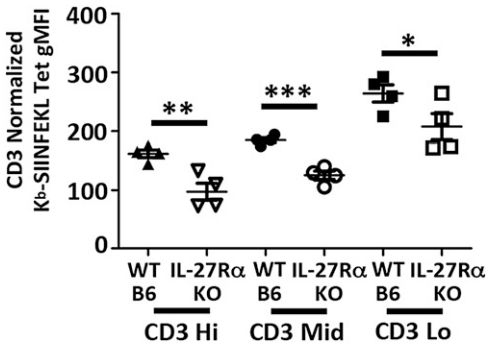
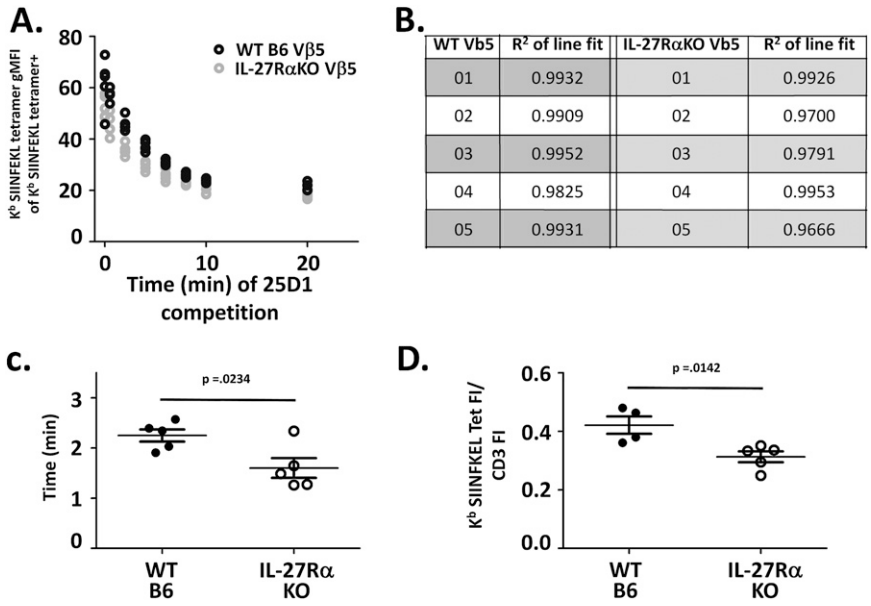


Fig. S2. Tetramer gMFI of thin CD3 slices. The geometric tetramer mean fluorescence intensity (MFI) was determined for the tetramer-positive population from within the narrow regions of CD3 staining described in Fig. S1 for both WT and IL-27RαKO mice immunized with Poly I:C/αCD40.



WT Vb5	R^2 of line fit	IL-27RαKO Vb5	R^2 of line fit
01	0.9932	01	0.9926
02	0.9909	02	0.9700
03	0.9952	03	0.9791
04	0.9825	04	0.9953
05	0.9931	05	0.9666

Fig. S3. Tetramer dissociation assay. WT Vβ5 and IL-27RαKO Vβ5 cohorts were immunized as described in Fig. 4*B*. At day 7 peak of primary response, splenic suspensions were stained first with K^b-SIINFEKL-PE tetramer and then with antibodies for immune subsets. Unbound tetramer and antibody were washed free from samples, and aliquots for a 0 time point were then removed and fixed with 1% PCHO-3% sucrose. Then 25D1 antibody against K^b-SIINFEKL was added at a final concentration of 20 μg/mL to the remaining bulk of tetramer-stained cells. Aliquots were removed and fixed at 0.5 min, 2 min, 4 min, 6 min, 8 min, 10 min, and 20 min. (*A*) Geometric MFI (gMFI) of tetramer-positive cells was obtained by flow cytometry and plotted for each mouse. (*B*) Data were fit to a second-order polynomial resulting in the displayed R^2 line fit values. (*C*) From the equations of the line, the time for each time 0 sample to have half of the tetramer competed off by 25D1 was determined and plotted, reflecting the average TCR affinity for ova for each sample. (*D*) The same samples were treated to the Tet FI/CD3 FI analysis described in Fig. 3 and plotted.

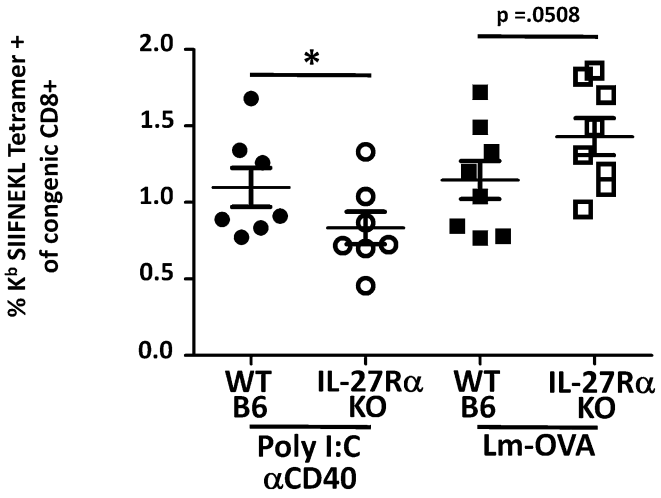


Fig. S4. Resting memory of IL-27RαKO T cells. WT/IL-27RαKO mixed bone marrow chimeras generated for Fig. 4*A* were assessed for their resting memory as a percentage of their congenic CD8 compartment 50 d after primary immunization with Poly I:C/αCD40/ova or Lm-Ova. A paired Student *t* test was performed for determination of statistical significance.

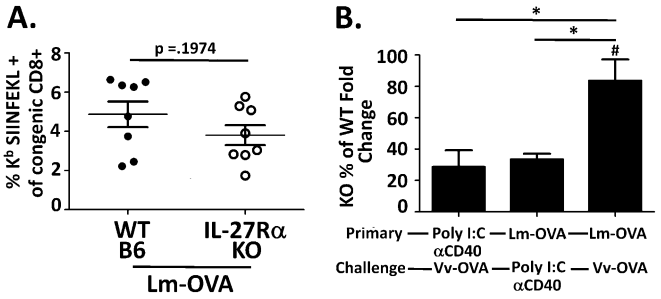


Fig. S5. Mixed bone marrow chimera Lm-Ova primary and Vv-Ova challenge. Mixed bone marrow chimeras from Fig. 4*A* were (*A*) analyzed for their primary response to Lm-Ova as a percentage of congenic CD8 T cells binding Kb-SIINFEKL tetramer. (*B*) The KO percentage of WT fold change was calculated for each mouse, by calculating the fold change from rest for WT and IL-27RαKO T cells after challenge with 1 x 10^7 pfu vaccinia virus expressing chicken ovalbumin and then dividing the fold change in antigen-specific IL-27RαKO T cells by the fold change of WT T cells, and is expressed as a percentage. #No statistical difference, $P = 0.2554$.

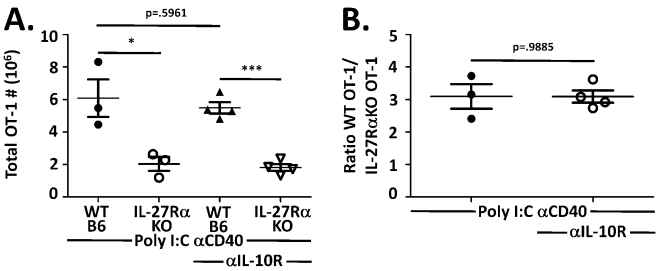


Fig. S6. IL-10R blockade does not alter T-cell expansion or WT/IL-27RαKO antigen-specific T-cell ratio. Three thousand WT and IL-27RαKO OT-1 T cells of different congenic background were purified by CD8 PE-mixture negative selection to greater than 90% purity and then transferred 1:1 into congenically distinct WT host. The following day, mice were immunized with Poly I:C/αCD40/ova (50 μg/50 μg /150 μg). To prevent interference with early innate IL-10 signaling, we chose to block IL-10R starting 72 h after immunization immediately before when T cells should begin to produce effector cytokines. Then, 72 h and 96 h postimmunization, half of the mice were given 250 μg per mouse anti–IL-10R antibody. Mice were killed at peak of OT-1 primary response (day 5), and (*A*) total numbers of OT-1 T cells were enumerated, and the (*B*) the ratio of WT/IL-27RαKO OT-1 T cells was determined. Student's paired *t* test determined the statistical significance between WT and IL-27RαKO responses for a given treatment whereas an unpaired Student *t* test was used for comparing between treatment groups.

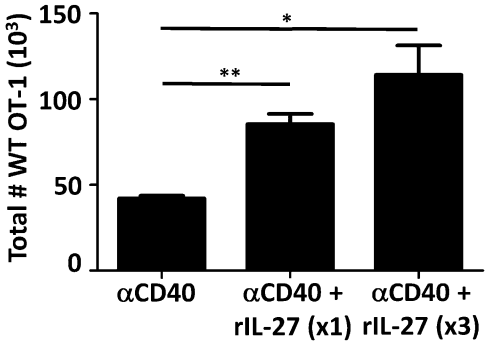


Fig. S7. Recombinant IL-27 enhances T-cell responses to αCD40 immunization. Recombinant IL-27 (IL-27p28/EBI3) was produced and purified as Fc-fusion proteins as described in detail in *SI Materials and Methods*. Three thousand purified WT OT-1s were adoptively transferred into congenically disparate host. Mice were then immunized i.v. with αCD40/Ova (50 μg/150 μg per mouse). Half of the recIL-27 was papain digested to liberate functional IL-27 from the Fc portion and then repurified by dialysis to separate from papain digestion reagents. When mice received recIL-27, they were immunized with 250 μg of rec IL-27 per treatment (125 μg of papain digested and 125 μg of Fc-conjugated recIL-27). One cohort of mice was immunized (1x) with rec IL-27, 2 h after Poly αCD40 immunization, and another cohort received an additional 250 μg of recIL-27 injections on day 3 and day 4 (×3). At peak of OT-1 response (day 5), mice were killed, and the numbers of OT-1s were enumerated. Statistical differences were determined by Student *t* test between cohorts.