Immunity

Supplemental Information

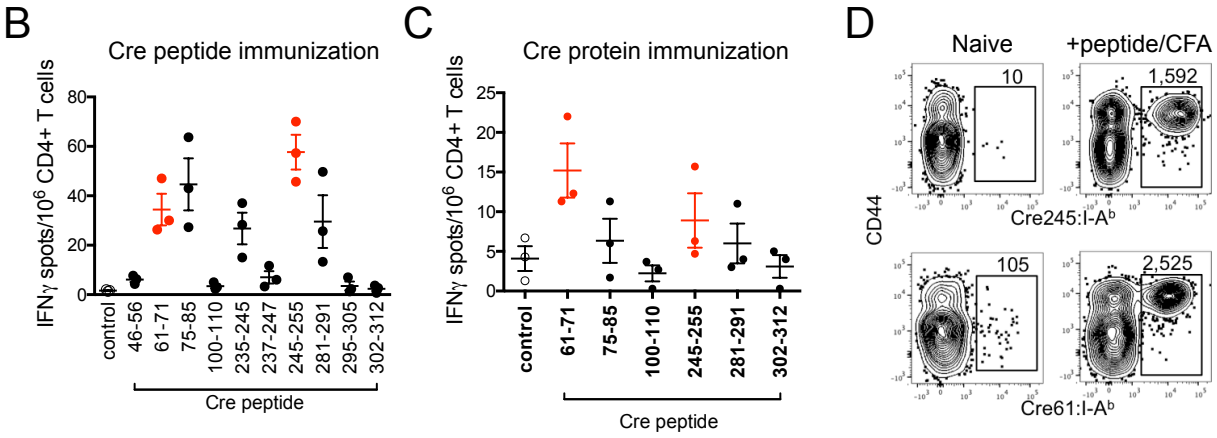
$CD4^+$ T Cell Tolerance to Tissue-Restricted Self Antigens Is Mediated by Antigen-Specific Regulatory T Cells Rather Than Deletion

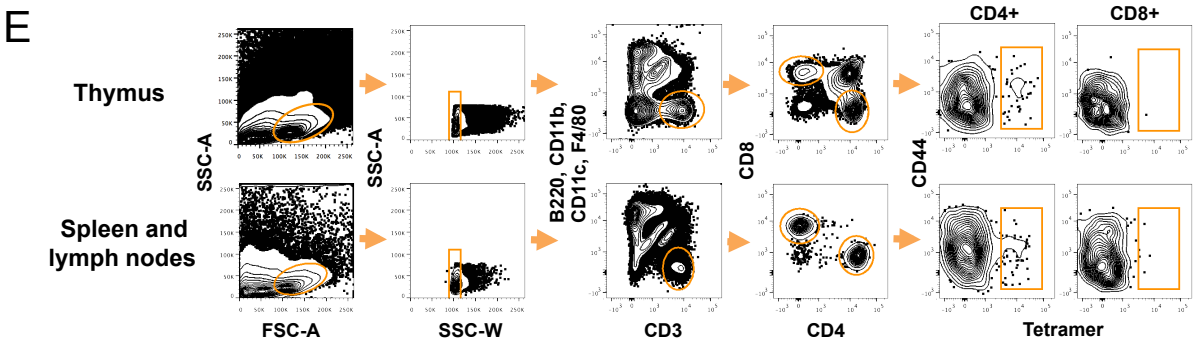
Francois P. Legoux, Jong-Baeck Lim, Andrew W. Cauley, Stanislav Dikiy, James Ertelt, Thomas J. Mariani, Tim Sparwasser, Sing Sing Way, and James J. Moon

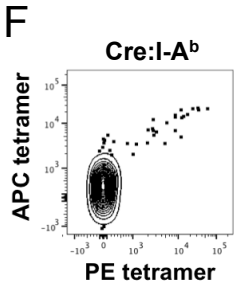
Figure S1 related to Fig. 1

A

Cre peptide	Amino acid sequence
46-56	LSVCRSWAAWC
61-71	**RKWFPAEPEDV**
75-85	LLYLQARGLAV
100-110	RRSGLPRPSDS
235-245	NNYLFCRVRKN
237-247	YLFCRVRKNGV
245-255	NGVAAPSATSQ
281-291	QRYLAWSGHSA
295-305	AARDMARAGVS
302-312	AGVSIPEIMQA







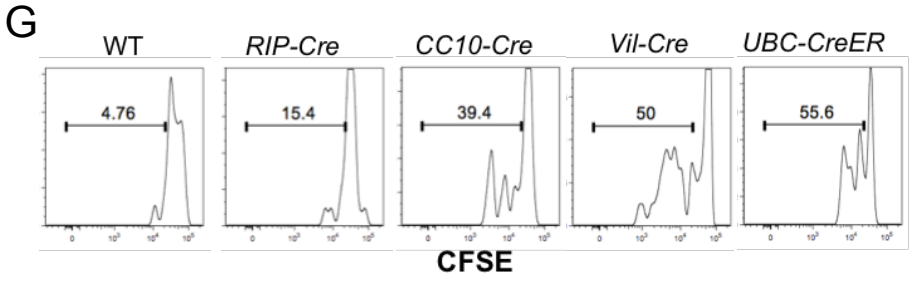


Figure S1 related to Figure 1. Cre epitope mapping and flow cytometric analysis of Cre:I-A^b-specific $CD4^+$ T cells

(A) List of candidate Cre peptide epitopes with top I-A^b-binding scores from an automated epitope prediction algorithm. (B-C) ELISpot analysis of CD4+ T cell cytokine responses to candidate peptide epitopes. B6 mice were immunized with either a mix of all 10 candidate peptides + CFA (B) or splenocytes from *Rosa26-CreER* mice (C) and 10 days later, CD4+ T cells were isolated and analyzed for frequencies of IFNγ-producing responders after *in vitro* restimulation with each individual peptide. Mean values ±SEM are shown. Epitopes selected for further analysis with peptide:MHCII tetramers are highlighted in red. (D) Representative flow cytometry of CD4+ T cells following $Cre_{61\text{-}71}$:I-A^b or $Cre_{245\text{-}255}$:I-A^b tetramer-based enrichment of total spleen and lymph node cells from either naive or peptide+CFA immunized B6 mice. Events were gated as indicated in E. Numbers indicate total tetramer positive cells calculated for the entire mouse. $Cre_{61\text{-}71}$:I-A^b was chosen for use throughout this study and is subsequently referred to as Cre:I-A^b. (E) Sequential gating strategy used for analysis of $CD4^+$ and $CD8^+$ T cells following tetramer-based enrichment of the thymus (top) or pooled spleen and lymph nodes (bottom). (F) Representative flow cytometry of Cre:I-A^b-specific $CD4^+$ T cells following simultaneous enrichment of pooled spleen and lymph node cells of naive WT mice with both PE- and APC-labeled versions of the tetramer. (G) ~2 x 10^7 total $CD4^+$ T cells were isolated from WT mice immunized 8 days earlier with Cre peptide + CFA and then CFSE-labeled and adoptively transferred into indicated recipient mice. 6 days later, transferred Cre:I-A^b-specific $CD4^+$ T cells were analyzed for CFSE dilution following tetramer-based enrichment from pooled spleen and lymph node cells. Histograms depict representative data from at least two mice from two independent experiments.

Figure S2 related to Fig. 2

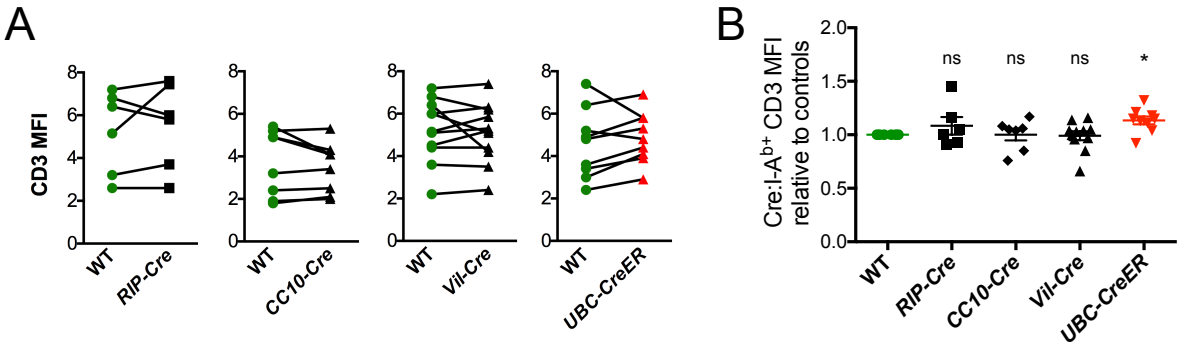


Figure S2 related to Figure 2. CD3 expression levels
(A) CD3 mean fluorescence intensity (MFI) in arbitrary units for Cre:I-A^b-specific $CD4^+$ T cells in indicated mice 7 days after immunization with peptide+CFA. Lines connect data for pairs of WT and *Cre* mice analyzed within the same experiments. (B) CD3 antibody MFI for the same Cre:I-A^b-specific T cells. Values were normalized to those of WT mice analyzed in the same experiments. Datapoints represent individual mice. Mean values ±SEM are shown. ns not significant, * $p<0.05$ for unpaired *t* tests between WT and each *Cre* mice.

Figure S3 related to Fig. 3

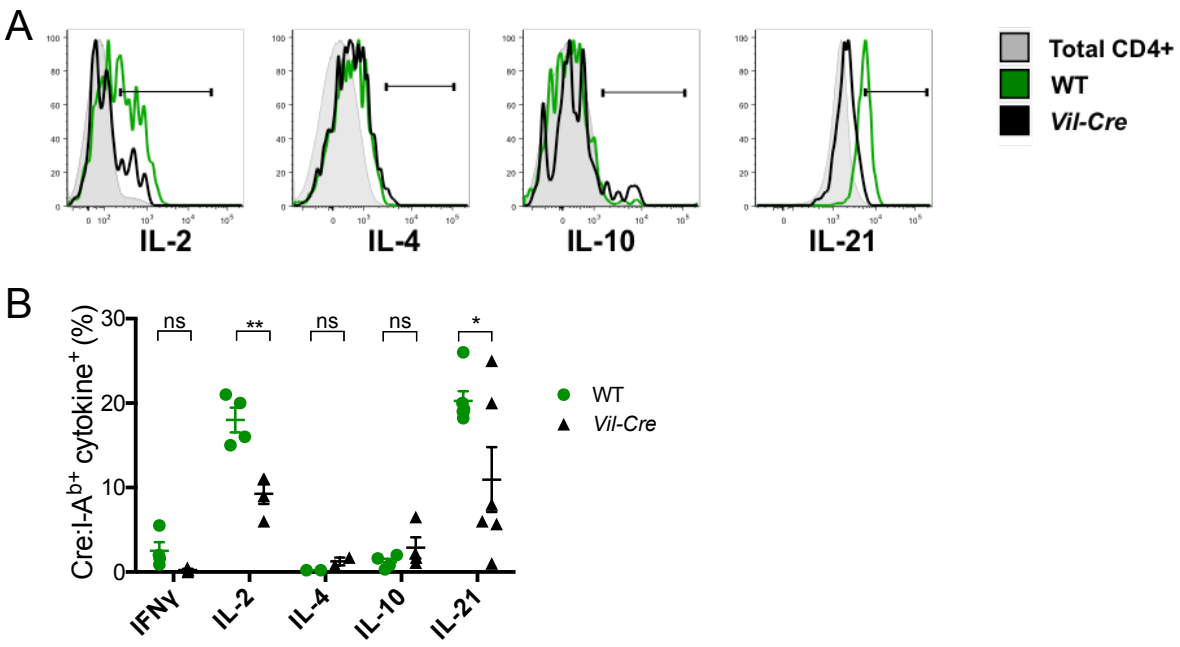


Figure S3 related to Figure 3. Cytokine production by self antigen-specific CD4+ T cells. WT and *Vil-Cre* mice were immunized with Cre peptide + CFA and analyzed 7 days later. (A) Representative intracellular flow cytometry of IL-2, IL-4, IL-10, and IL-21 cytokine expression by total CD4+ T cells (shaded) or by Cre:I-A^b-specific T cells from the pooled spleen and lymph nodes of WT (green) or by Cre:I-A^b-specific T cells from the pooled spleen and lymph nodes of *Vil-Cre* (black) mice following *in vitro* restimulation with PMA + ionomycin. (B) Quantitative summary of the proportion of Cre:I-A^b-specific T cells expressing the indicated cytokines in WT and *Vil-Cre* mice. Mean values ±SEM are shown. ns not significant, * $p<0.05$, ** $p<0.01$ for unpaired *t* tests between WT and *Vil-Cre* mice.

Figure S4 related to Fig. 4

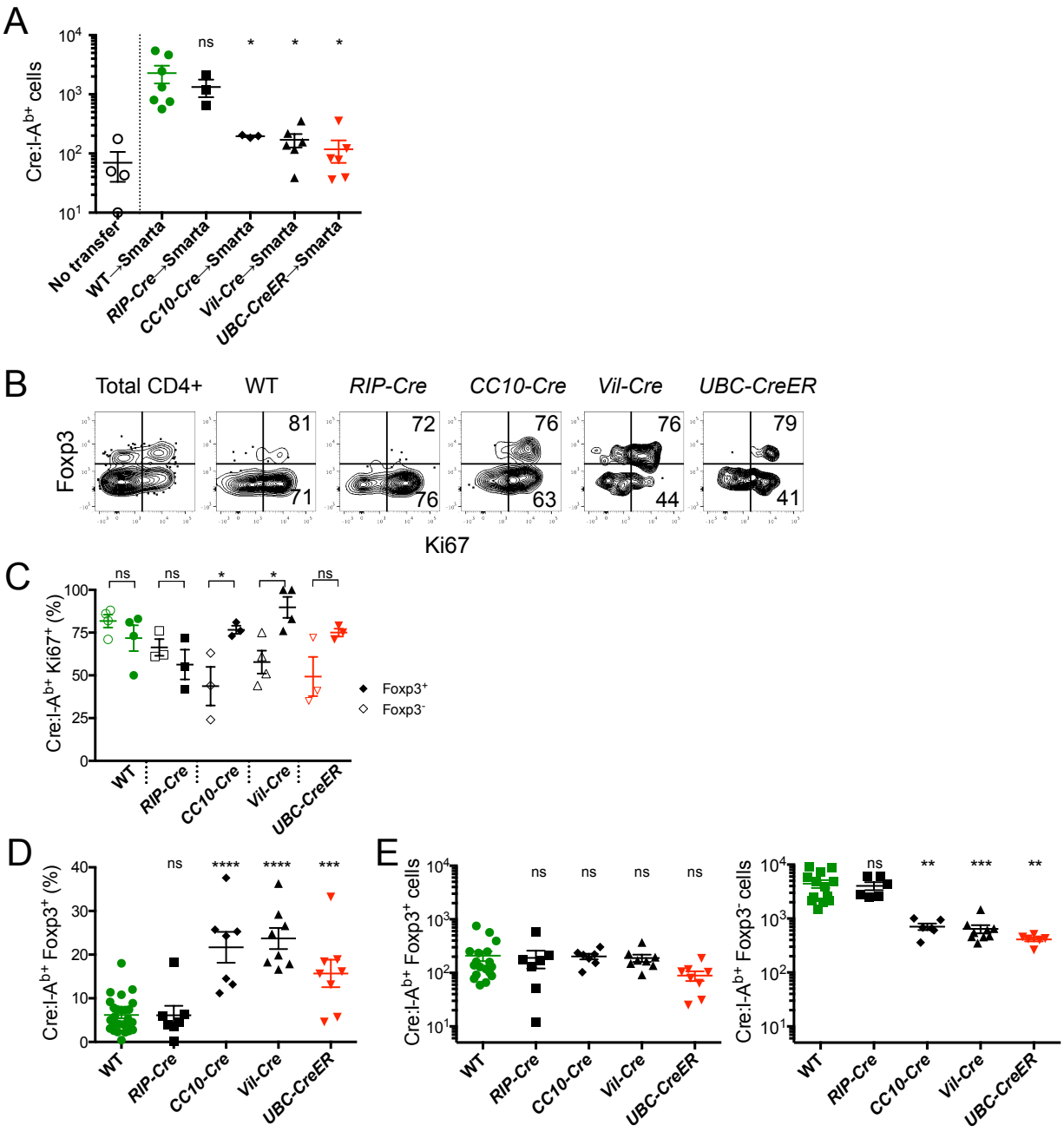


Figure S4 related to Figure 4. Self antigen-specific tolerance is intrinsic to the CD4$^+$ T cell compartment

(A) 2 x 10^7 purified CD4$^+$ T cells from indicated WT or *Cre* mice were adoptively transferred into Smarta TCR transgenic mice, which were then immunized with Cre peptide + CFA. Cre:I-A^b-specific T cell numbers were then quantified 7 days later by tetramer-based cell enrichment of pooled spleen and lymph nodes. (B) Representative flow cytometry of Foxp3 and Ki67 expression in total CD4$^+$ T cells or Cre:I-A^b-specific T cells following tetramer-based cell enrichment of pooled spleen and lymph node cells from indicated mice 7d after immunization with Cre peptide + CFA. Numbers indicate proportions of Ki67$^+$ cells in Foxp3$^+$ and Foxp3$^-$ compartments. (C) Proportions of Foxp3$^-$ (open symbols) or Foxp3$^+$ (filled symbols) Cre:I-A^b-specific T cells expressing Ki67 in indicated mice. (D) Quantitative summary of the proportion of Cre:I-A^b-specific T cells expressing Foxp3 in each indicated mouse strain 7d after immunization with Cre peptide + CFA. (E) Quantitative summary of the total numbers of Foxp3$^+$ and Foxp3$^-$ Cre:I-A^b-specific T cells in each indicated mouse strain 7d after immunization with Cre peptide + CFA. (A, C-E) Mean values ±SEM are shown. ns not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ for unpaired *t* tests between WT and each *Cre* mouse (A, D-E) or between Foxp3$^+$ and Foxp3$^-$ T cells (C).

Figure S5 related to Fig. 5

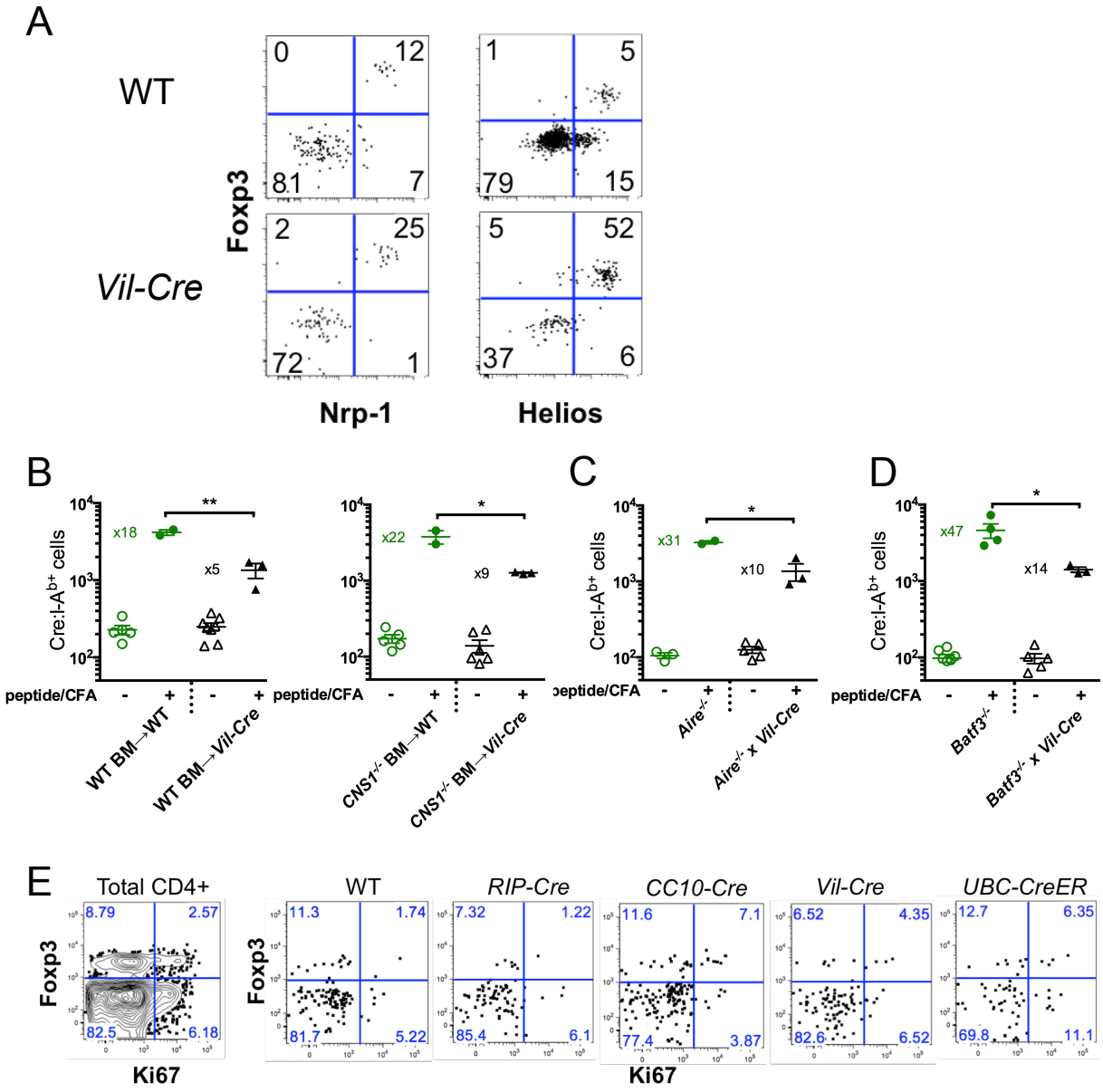


Figure S5 related to Figure 5. Cre:I-A^{b}-specific Foxp3^{+} Tregs
(A) Neuropilin-1 (Nrp-1) and Helios expression on Cre:I-A^{b}-specific CD4^{+} T cells following tetramer-based enrichment of pooled spleen and lymph node cells in WT and *Vil-Cre* mice. Nrp-1 staining was performed on cells from unimmunized mice, while Helios staining was performed on mice immunized 7 days earlier with peptide+CFA. Numbers indicate proportions of cells in each quadrant. (B) Cre:I-A^{b}-specific T cell numbers in irradiation bone marrow chimeras harvested either naive (open symbols) or 7 days after immunization with peptide+CFA (filled symbols). (C-D) Cre:I-A^{b}-specific T cell numbers in indicated mice harvested either naïve (open symbols) or 7 days after immunization with peptide+CFA (filled symbols). (B-D) Each datapoint represents an individual mouse. Fold of expansion upon immunization and mean values ±SEM are shown. *$p<0.05$, ** $p<0.01$ for *t* tests between WT and each *Cre* mouse. (E) Representative flow cytometry of Foxp3 and Ki67 expression in total CD4^{+} T cells or Cre:I-A^{b}-specific CD4^{+} T cells following tetramer-based cell enrichment of pooled spleen and lymph node cells. Numbers indicate proportions of cells in each quadrant.

Figure S6 related to Fig. 6

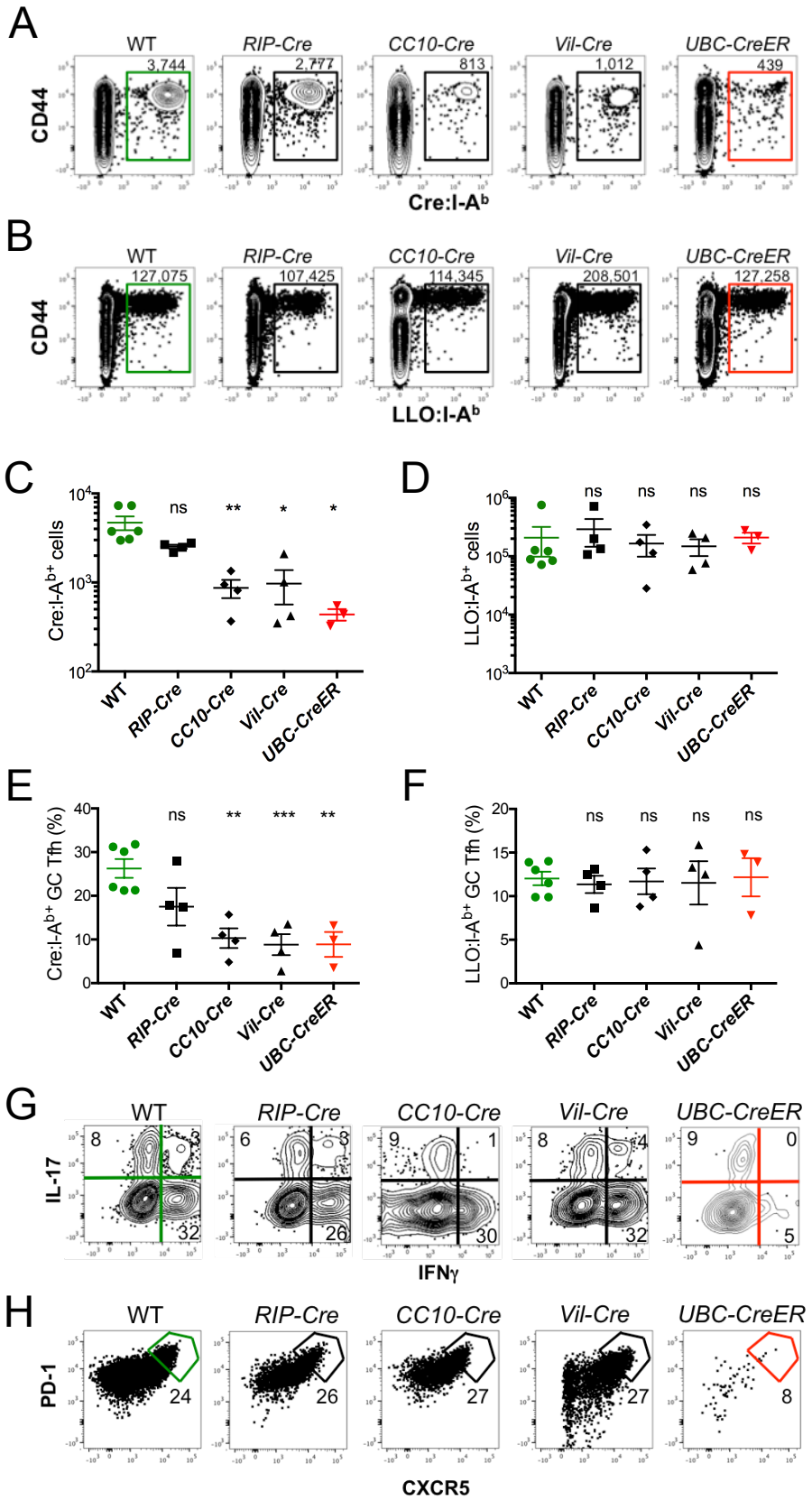


Figure S6 related to Fig. 6 (cont.)

I

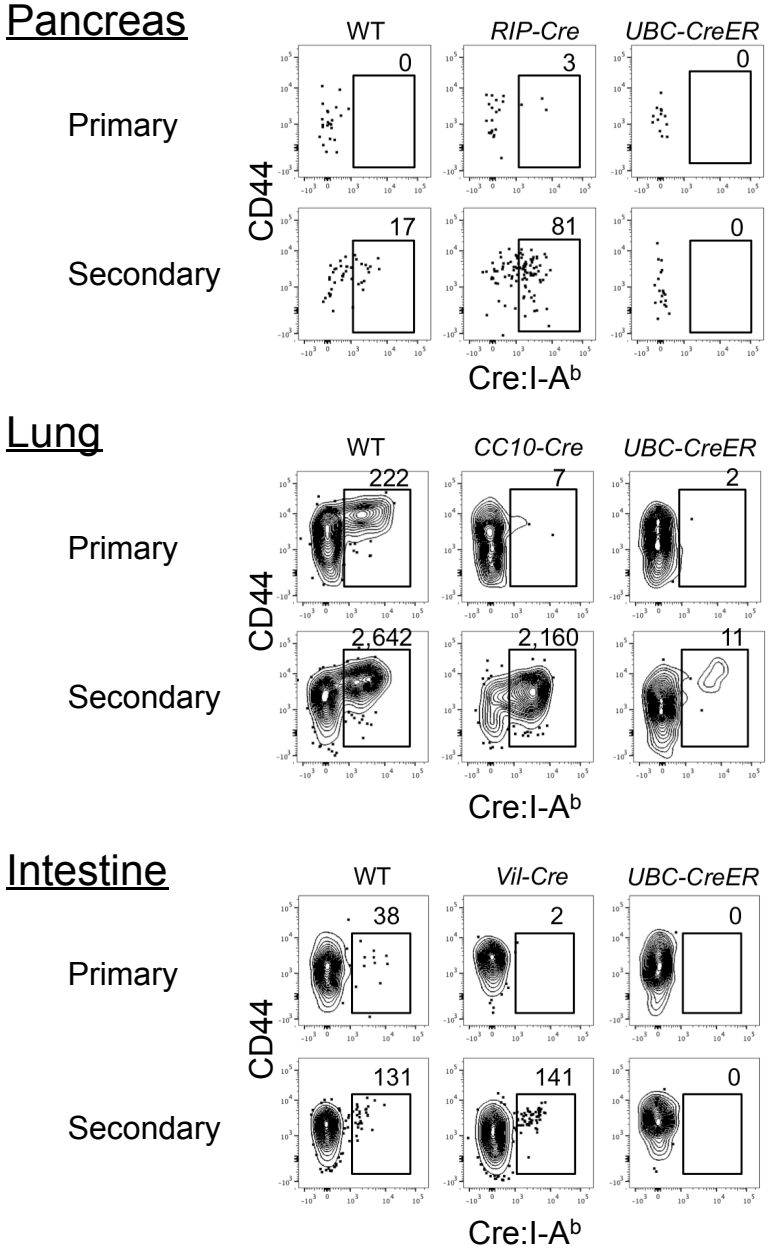


Figure S6 related to Figure 6. Cre:I-A^b-specific CD4$^+$ T cell effector functions during secondary immune response

(A-D) Representative flow cytometry and quantification of CD4$^+$ T cells following (A, C) Cre:I-A^b or (B, D) LLO:I-A^b tetramer-based cell enrichment of pooled spleen and lymph nodes from indicated mice infected 7 days earlier with Lm-Cre. Numbers indicate total tetramer$^+$ cells calculated for the whole mouse and are summarized in scatter plots. (E-F) Proportions of (E) Cre:I-A^b- or (F) LLO:I-A^b-specific T cells exhibiting a PD-1^{hi}CXCR5^{hi} GC-Tfh phenotype 7 days after infection with Lm-Cre. (G-H) Mice were infected with Lm-Cre, rechallenged 4 weeks later with Cre peptide+CFA, and then harvested 7 days later for Cre:I-A^b-specific T cell analysis. (G) Representative flow cytometry of intracellular IL-17 and IFNγ expression in Cre:I-A^b-specific CD4$^+$ T cells following *in vitro* restimulation with PMA+ionomycin. Numbers indicate proportions of cells in each quadrant. (H) Representative flow cytometry of PD-1 and CXCR5 expression on Cre:I-A^b-specific CD4$^+$ T cells. Numbers indicate proportions of cells in the PD-1^{hi}CXCR5^{hi} GC-Tfh gate. (I) Representative flow cytometry of CD4+ T cells following Cre:I-A^b tetramer-based enrichment of cells isolated from the pancreas, lung and intestine of indicated mice immunized with Cre peptide + CFA (Primary) or infected with Lm-Cre and then rechallenged 4 weeks later with Cre peptide + CFA (Secondary). Mice were harvested 7-10 days after Cre peptide + CFA immunization. Numbers indicate total tetramer$^+$ cells calculated for the whole mouse. (C-F) Mean values ±SEM are shown. ns not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for *t* tests between WT and each *Cre* mouse.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse Strains

CC10-Cre (Simon et al., 2006), and *Foxp3-DEREG* (Lahl et al., 2007) mice were provided by their originating labs. C57BL/6 (B6), B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (CD45.1), *Foxp3*^{EGFP} (Haribhai et al., 2007), *RIP-Cre* (Postic et al., 1999), *Vil-Cre* (Madison et al., 2002), *UBC-CreER* (Ruzankina et al., 2007), *Rosa26-CreER* (Ventura et al., 2007), *Aire*$^{-/-}$ (Anderson et al., 2002), and *Batf3*$^{-/-}$ (Hildner et al., 2008) mice were purchased from the Jackson Laboratory. Smarta TCR transgenic mice (Oxenius et al., 1998) were a gift from Dr. Michael Bevan (University of Washington). All of these strains and combinations thereof were bred and maintained in specific pathogen-free (SPF) conditions at Massachusetts General Hospital. *Foxp3*$^{\Delta CNS1}$ mice (Zheng et al., 2010) were bred and maintained in SPF conditions at the Memorial Sloan-Kettering Cancer Center, and harvested bones were shipped to Massachusetts General Hospital.

Quantitative RT-PCR

Whole tissue samples were homogenized with a GentleMACS dissociator (Miltenyi), and subjected to Trizol RNA extraction (Life Technologies). For the pancreas, the tissue was flash-frozen with liquid nitrogen and homogenized with a mortar and pestal. For TEC analysis, thymi from 3 mice were pooled and enzymatically digested as described (Xing and Hogquist, 2014). EpCAM^{+} cells were magnetically enriched using EpCAM-APC antibody and anti-APC microbeads (Miltenyi). 500,000 EpCAM^{+}CD45^{-} cells (TECs) were sorted by FACS and total RNA was extracted using the RNeasy Plus Microkit (Qiagen). All cDNA samples were synthesized with Superscript III reverse transcriptase (Life Technologies), and subsequent quantitative real-time PCR was run on a LightCycler 480 II thermocycler (Roche) using the SYBR Green Master I kit (Roche) with primers specific for *Cre, Ins2, Gapdh*, *or B2m*. mRNA expression levels were normalized to *B2m* or *Gapdh*.

Epitope Mapping

Candidate I-A^{b}-restricted CD4^{+} T cell epitopes within Cre were first identified using a peptide:I-A^{b} binding prediction algorithm based on a position-specific scoring matrix developed from the crystal structure of CLIP:I-A^{b} complexes (Lee et al., 2012). Mice were immunized subcutaneously with CFA containing a mix of the top 10 scoring peptides, and one week later, CD4^{+} T cells were isolated (Miltenyi) from peripheral lymphoid organs and restimulated *in vitro* with irradiated naive splenocytes plus each of the peptides at a dose of 20 μg/ml. After overnight incubation, the frequencies of IFNγ-producing cells were quantified by ELISpot assay. The 6 most active peptides were then each tested for immunogenicity in mice immunized with splenocytes expressing whole Cre protein from *Rosa26-CreER* mice. Tetramers were generated for the 2 most immunodominant peptide epitopes, and these were used to quantify epitope-specific CD4^{+} T cell frequencies in naive and peptide-immunized mice. The Cre$_{61\text{-}71}$ (RKWFPAEPEDV) epitope was recognized by the largest number of CD4^{+} T cells in both naive and immunized mice and was subsequently chosen as our model Cre epitope for the entire study.

Tetramer Generation

The generation of peptide:MHCII tetramers has been described in detail (Moon et al., 2007; 2011). In brief, soluble I-A^{b} molecules covalently linked to Cre$_{61\text{-}71}$ (RKWFPAEPEDV) or LLO$_{190\text{-}201}$ (NEKYAQAYPNVS) (Pepper et al., 2011) peptide epitopes were expressed and

biotinylated in stably transfected *Drosophila* S2 cells. Following immunoaffinity purification, these biotinylated peptide:MHCII complexes were titrated and tetramerized to PE or APC fluorochrome-conjugated streptavidin (Prozyme).

***In Vivo* Cell Division Analysis**

For *in vivo* analysis of Cre antigen presentation, total $CD4^+$ T cells were isolated (Miltenyi) from the tail draining lymph nodes of WT mice immunized 8 days earlier with Cre peptide + CFA. Cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies) and ~2 x 10^7 were adoptively transferred into congenic WT or *Cre* mice. 6 days later, pooled spleen and lymph node cells were harvested, subjected to Cre tetramer-based cell enrichment, and diluted levels of CFSE fluorescence on Cre:I-A^b-specific T cells, indicative of cell division, were analyzed by flow cytometry.

Blood Glucose Measurement

Blood glucose levels were read using a Contour blood glucose reader (Bayer).

Statistical Analysis

All statistical calculations were performed using Prism software (GraphPad).

SUPPLEMENTAL REFERENCES

Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S., et al. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science *322*, 1097–1100.

Lee, S.-J., Mclachlan, J.B., Kurtz, J.R., Fan, D., Winter, S.E., Baumler, A.J., Jenkins, M.K., and McSorley, S.J. (2012). Temporal Expression of Bacterial Proteins Instructs Host CD4 T Cell Expansion and Th17 Development. PLoS Pathog *8*, e1002499.

Madison, B.B., Dunbar, L., Qiao, X.T., Braunstein, K., Braunstein, E., and Gumucio, D.L. (2002). Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem *277*, 33275–33283.

Oxenius, A., Bachmann, M.F., Zinkernagel, R.M., and Hengartner, H. (1998). Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. Eur J Immunol *28*, 390–400.

Pepper, M., Pagán, A.J., Igyártó, B.Z., Taylor, J.J., and Jenkins, M.K. (2011). Opposing Signals from the Bcl6 Transcription Factor and the Interleukin-2 Receptor Generate T Helper 1 Central and Effector Memory Cells. Immunity *35*, 583–595.

Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem *274*, 305–315.

Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cotsarelis, G., Zediak, V.P., Velez, M., Bhandoola, A., and Brown, E.J. (2007). Deletion of the Developmentally Essential Gene ATR in Adult Mice Leads to Age-Related Phenotypes and Stem Cell Loss. Cell Stem Cell *1*, 113–126.

Simon, D.M., Arikan, M.C., Srisuma, S., Bhattacharya, S., Tsai, L.W., Ingenito, E.P., Gonzalez, F., Shapiro, S.D., and Mariani, T.J. (2006). Epithelial cell PPAR[gamma] contributes to normal lung maturation. The FASEB Journal *20*, 1507–1509.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. Nature *445*, 661–665.

Xing, Y., and Hogquist, K.A. (2014). Isolation, identification, and purification of murine thymic epithelial cells. JoVE e51780.