Leddon\_Plosone\_2012\_protocol

**Ethics Statement**

All experiments for this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the approval of Animal Care and Use Committees at the University of Rochester (protocol 2008-023).

**Immunizations**

Two to four month old BALB/c mice (National Cancer Institute, Frederick, MD) were immunized in the pinna of both ears with 10 mL of an IFA/PBS emulsion containing 5 mg of protein (10 mg/mouse) and 0.6 mg/mL LPS (Sigma-Aldrich). For experiments evaluating kinetic stability peptide variants, the portion of the pinna containing the emulsion was excised three days post-immunization. For cell sorting experiments, unless otherwise indicated, for each immunizing protein and replicate experiment, 50–70 mice were sacrificed 8 to 9 days postimmunization, and draining cervical lymph nodes were harvested and pooled as the source of T cells for assays.

**Antibodies and Peptides**

Purified anti-IL-2 (JES6-1A12), anti-IL-2-biotin (JES6-5H4), purified anti-IFNg (AN-18), anti-IFNg-biotin (XMG1.2), purified anti-IL-4 (11B11), anti-IL-4-biotin (BVD6-24G2), Fc Block (2.4G2), anti-CD4-PE-Cy7 (RM4-5), anti-CD4-V450 (RM4-5), anti-B220- PE-Cy5 (RA3-6B2), anti-CD44-APC-Cy7 (IM7), anti-CXCR5- biotin (2G8), and anti-BCL6-Alexa647 (K112-91) antibodies were obtained from BD Biosciences (San Jose, CA). Anti-ICOS-Alexa488 (C398.4A), anti-CD69-Alexa488 (H1.2F3), anti-CD62L-Alexa488 (MEL-14), and anti-CCR7-Alexa488 (4B12) antibodies were obtained from BioLegend (San Diego, CA). Anti-PD1-FITC (J43), anti-PD1-PE-Cy7 (J43), and IL-21 ELISPOT capture and detection (components of the Mouse IL-21 ELISPOT Ready-SET-Go! reagent set) antibodies were obtained from eBioscience. All synthetic peptides were purchased from BioPeptide (San Diego, CA) or produced in-house.

**Analytical Cell Staining and Flow Cytometry**

Single cell suspensions were prepared and depleted of red blood cells (RBC) by treatment with ACK lysis buffer (0.15 M NH4Cl/ 1 mM KHCO3/ 0.1 mM Na2EDTA in H2O, pH 7.2). Cells were then stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies, Carlsbad, CA). After vital staining, cells were pre-incubated with Fc Block (BD Biosciences) before the addition of surface staining antibodies. After incubation with mAbs at 4oC for 30 minutes, cells were washed and incubated with PE-streptavidin to detect anti- CXCR5 mAb binding. Prior to BCL6 staining, cells were fixed and permeabilized with BD Pharmingen Transcription Factor Buffer Set (BD Biosciences). Data were collected on a FACSCanto flow cytometer (BD Biosciences), and analyzed with FlowJo 8.6 software (Tree Star, Inc., Ashland, OR). All events shown in Figure 1 were pre-gated on B220-CD4+Live/Dead negative (live), with doublets excluded.

**Preparative Staining and Cell Purification**

Single cell suspensions from the cervical lymph nodes of immunized mice were prepared and depleted of RBC by treatment with ACK lysis buffer (0.15 M NH4Cl/ 1 mM KHCO3/ 0.1 mM Na2EDTA in H2O, pH 7.2). Cell suspensions were then depleted of non-CD4 T cells by use of negative paramagnetic bead selection (CD4 T Cell Isolation Kit II, Miltenyi Biotec, Auburn, CA). For Treg depletion, CD25+ cells were depleted through the use of negative paramagnetic bead selection (CD4+CD25+ Regulatory T cell Isolation Kit, Miltenyi Biotec). For Tfh sorting, CD4-enriched cells were incubated with Fc Block (BD Biosciences) before the addition of anti-CD4, -CD44, -CXCR5, and -PD1 mAbs. After surface staining (4oC for 30 minutes), cells were washed and incubated with streptavidin-PE to detect anti-CXCR5 mAb staining. Cell sorting was performed using a FACSAria (BD Biosciences), to isolate CD4+CD44highCXCR5highPD1high Tfh cells and CD4+CD44high cells that did not express high levels of CXCR5 or PD1 (non-Tfh cells). The purity of sorted populations was typically 90–95%. Splenocytes from naive mice were depleted of RBC by treatment with ACK lysis buffer and were used as a source of APC in ELISPOT assays.

**Isolation of Cells from Ear Dermis**

The ear pinnae of 5 mice were removed, cut into pieces, and then digested with 1 mg/mL collagenase/dispase for 1 h at 37 °C. The tissue was mashed through a metal strainer, releasing a single cell suspension, which was washed 2 times with media.

**Cytokine-specific CD4 T cell ELISPOT Assay**

Cytokine-secreting T cells were quantified at 18 h (IL-2, IL-4, and IFNg) or at 40 h (IL-21) with cytokine-specific ELISPOT assay. In all cases 10 mM of peptide in the presence of syngeneic APC were used to stimulate T cells, which were added at densities that allowed readable spot counts in each well. Cytokine-specific spots were enumerated with an Immunospot Reader Series 2A (Immunospot Software V.3.2, CTL, Shaker Heights, OH). To determine the frequency of peptide-specific spots, spot counts from background wells (spots measured in the absence of peptide) were subtracted from wells with peptide and normalized to peptide-specific spots per 1,000,000 input CD4 T cells. In all cases, the number of peptide-specific cells was at least 2-fold over background. The frequencies of peptide-specific spots were summed to determine total cytokine-specific spots. The fractional response of each peptide was determined by calculating the percent of the total peptide-specific spots represented by each peptide epitope.

**Transcriptional Analysis**

Total RNA was purified from sorted cells was extracted with TRIzol (Life Technologies, Carlsbad, CA) and used to synthesize cDNA (Ovation PicoSL WTA Kit, NuGEN Technologies, San Carlos, CA). TaqMan Universal PCR Master Mix, No AmpErase and the following TaqMan Gene Expression FAM dye-labeled, non-primer limited assays were obtained from Life Technologies: Bact (Mm00607939\_s1), Bcl6 (mm00477633\_m1), B2m (Mm00437762\_m1), Ccr7 (Mm01301785\_m1), Cxcr5 (MM00432086\_m1), Foxp3 (Mm00475162\_m1), Gata3 (mm00484683\_m1), Gapdh (Mm99999915\_g1), Icos (Mm00497600\_m1), Ifng (Mm01168134), Il2 (Mm00434256\_m1), Il4 (Mm00445259\_m1), Il17a (Mm00439618\_m1), Il21 (Mm00517640\_m1), Pdcd1 (MM00435532\_m1), Prdm1 (Mm01187285\_m1), Rorc (Mm01261022\_m1), Tbx21 (MM00450960\_m1), Tnfrsf4 (Mm00442039\_m1). Real-time PCR reactions were run in triplicate with an Applied Biosystems 7900HT Sequence Detection System (Life Technologies). Data were analyzed with SDS v2.3 software (Life Technologies), and normalized to the average of Bact, B2m, Gapdh expression.