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Supplemental Information

The Integrin LFA-1 Controls T Follicular Helper Cell Generation and Maintenance

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SUPPLEMENTARY FIGURES

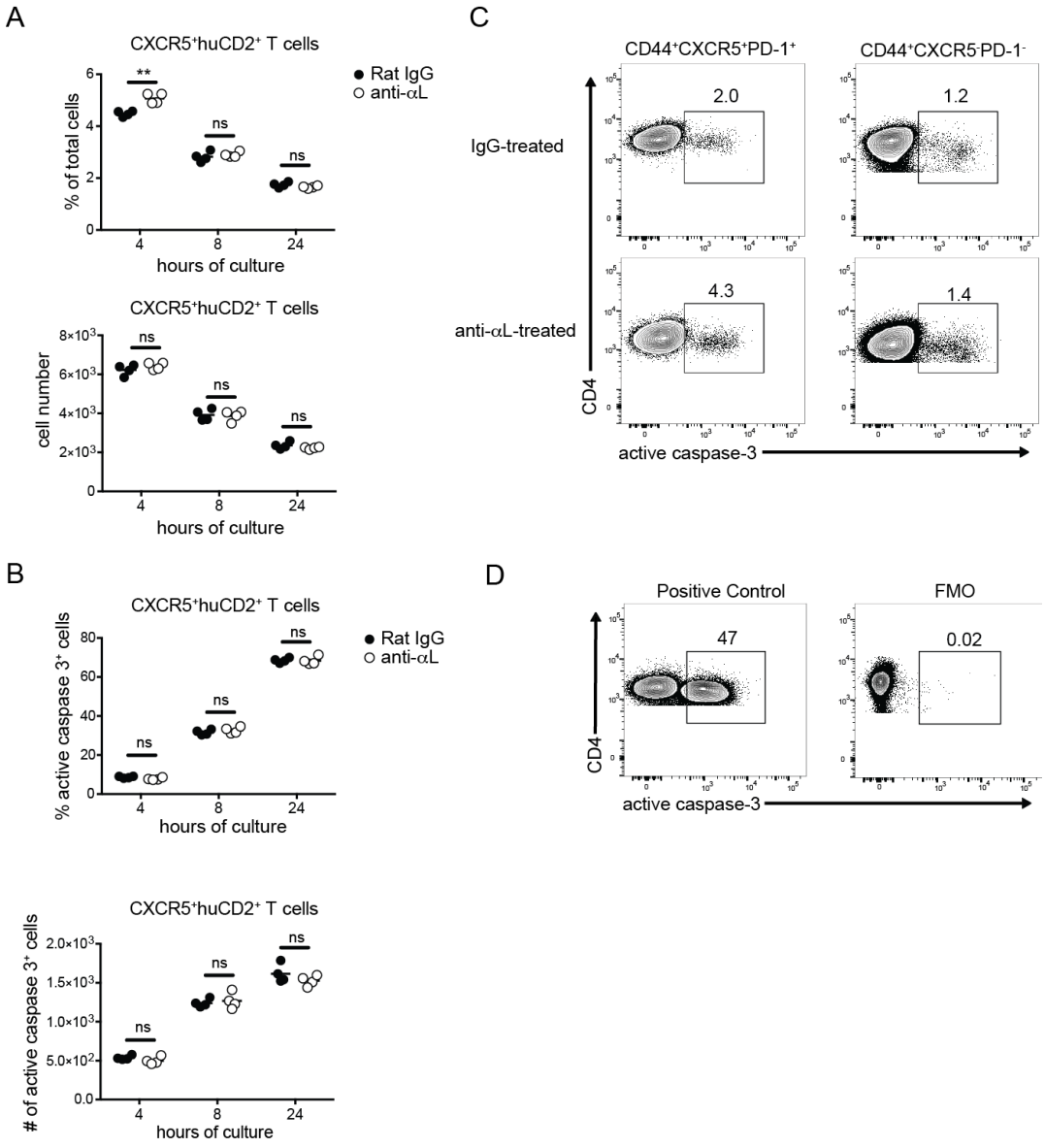

Figure S1 related to Figure 2. Effect of LFA-1 blockade on survival of T cell subsets *in vitro* and *in vivo*. (A, B) mLN cells were harvested from 2 week *Hp* infected mice and cultured with Rat IgG or anti-αL antibodies (10 μg/ml). The data shows the (A) frequency and number of $CXCR5^+huCD2^+$ $CD4^+$ T cells and (B) the frequency and number of active caspase-3^+ $CXCR5^+huCD2^+$ $CD4^+$ T cells after the indicated time of culture. Dots represent experimental replicates. (C) Representative contour plots of active caspase-3^+ cells examined *ex vivo* from *Hp*-infected 4get/KN2 mice 8 hours post treatment with Rat IgG or anti-αL. Contour plots shown are gated on $CD4^+B220^-CD62L^-CD44^+$ cells. (D) Staurosporine-treated (24hr, 5μM) mLN T cells and FMO-stained cells were used as positive and negative staining controls, respectively. Contour plots shown are gated on $CD4^+B220^-$ cells. The numbers in each contour plot represents the frequency of active caspase-3^+ cells. Data is representative of (A, B) one and (C, D) four independent experiments with 3-7 mice per group.

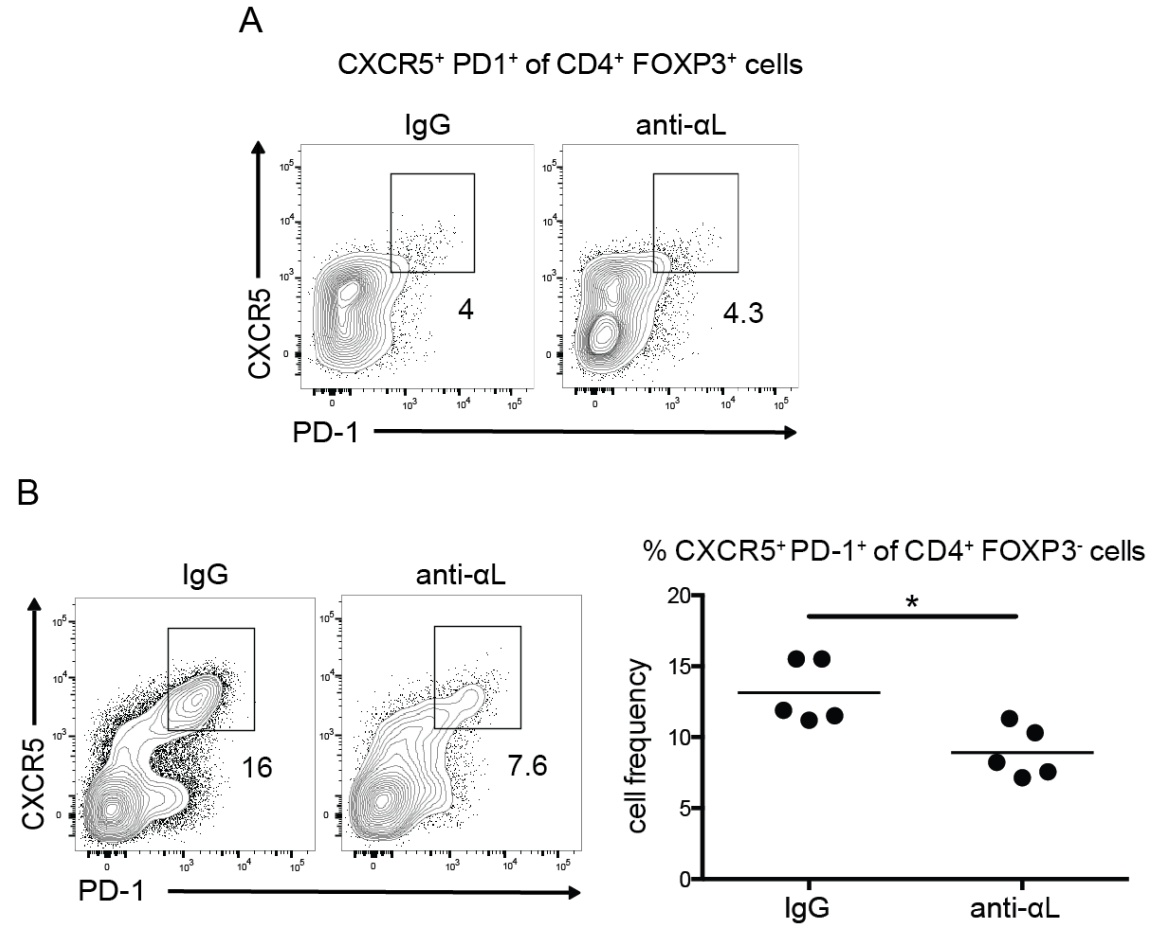

Figure S2 related to Figure 3. Tfh and Tfr cells following LFA-1 blockade. 4get/KN2 mice were infected with 200 L3 *Hp* larvae and mLNs were harvested 7 days post-infection. Mice were treated with 100μg of Rat IgG or anti-αL from the onset of infection. (A) Representative contour plots of $CXCR5^{+}PD\text{-}1^{+}$ mLN cells gated from the $CD4^{+}B220^{-}Foxp3^{+}$ population. (B) Representative contour plots and frequencies of $CXCR5^{+}PD\text{-}1^{+}$ mLN cells gated from $CD4^{+}B220^{-}Foxp3^{-}$ cells. Representative data shown are from at least 2 independent experiments. Dots in graph represent individual mice. $^{*}p<0.05$.

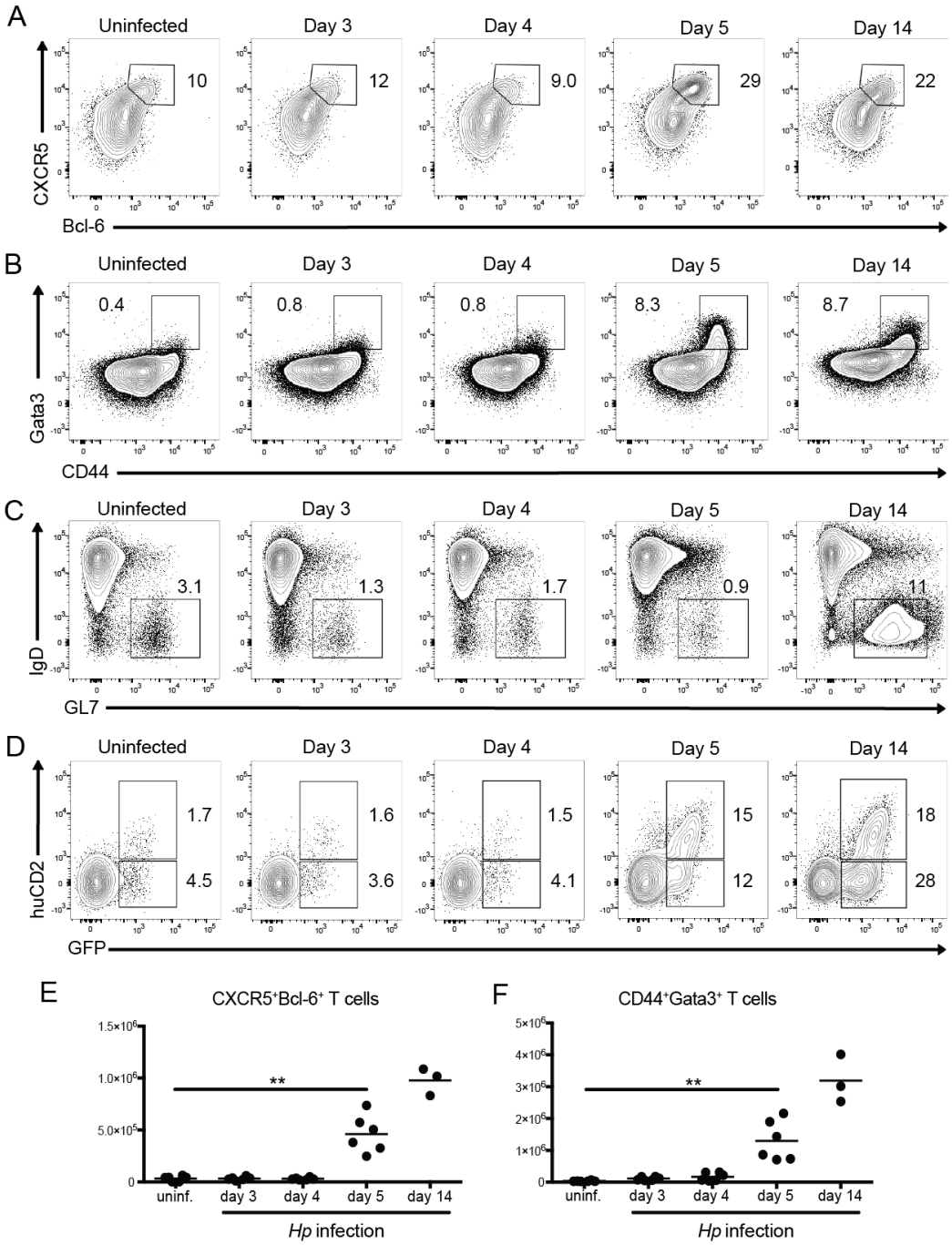

Figure S3 related to Figure 5. Kinetic analysis of the early Th and GC B cell response during *Hp* infection. (A-F) 4get/KN2 mice were infected with 200 L3 *Hp* larvae and mLNs were harvested at the indicated time points post-infection. (A-D) Representative contour plots of (A) $CXCR5^{+}Bcl\text{-}6^{+}Foxp3^{-}$ cells gated from the $CD4^{+}B220^{-}CD44^{+}CD62L^{-}$ population, (B) $CD44^{+}Gata3^{+}$ cells gated from the $CD4^{+}B220^{-}$ population, (C) $IgD^{-}GL7^{+}$ cells from the $B220^{+}CD4^{-}$ population, (D) $CD4^{+}GFP^{+}huCD2^{-}$ and $CD4^{+}GFP^{+}huCD2^{+}$ cells from the $CD4^{+}B220^{-}CD44^{+}$ population. Total cell counts of (E) Tfh ($CD4^{+}B220^{-}CD62L^{-}CD44^{+}Foxp3^{-}CXCR5^{+}Bcl\text{-}6^{+}$) and (F) Th2 ($CD4^{+}B220^{-}CD44^{+}Gata3^{+}$) cells at various time points post-infection. Dots in graphs represent individual mice. The numbers in each contour plot represents cell frequency. Data is representative of at least 2 independent experiments and cell counts are compiled from 2 independent experiments. $^{**}p<0.01$.

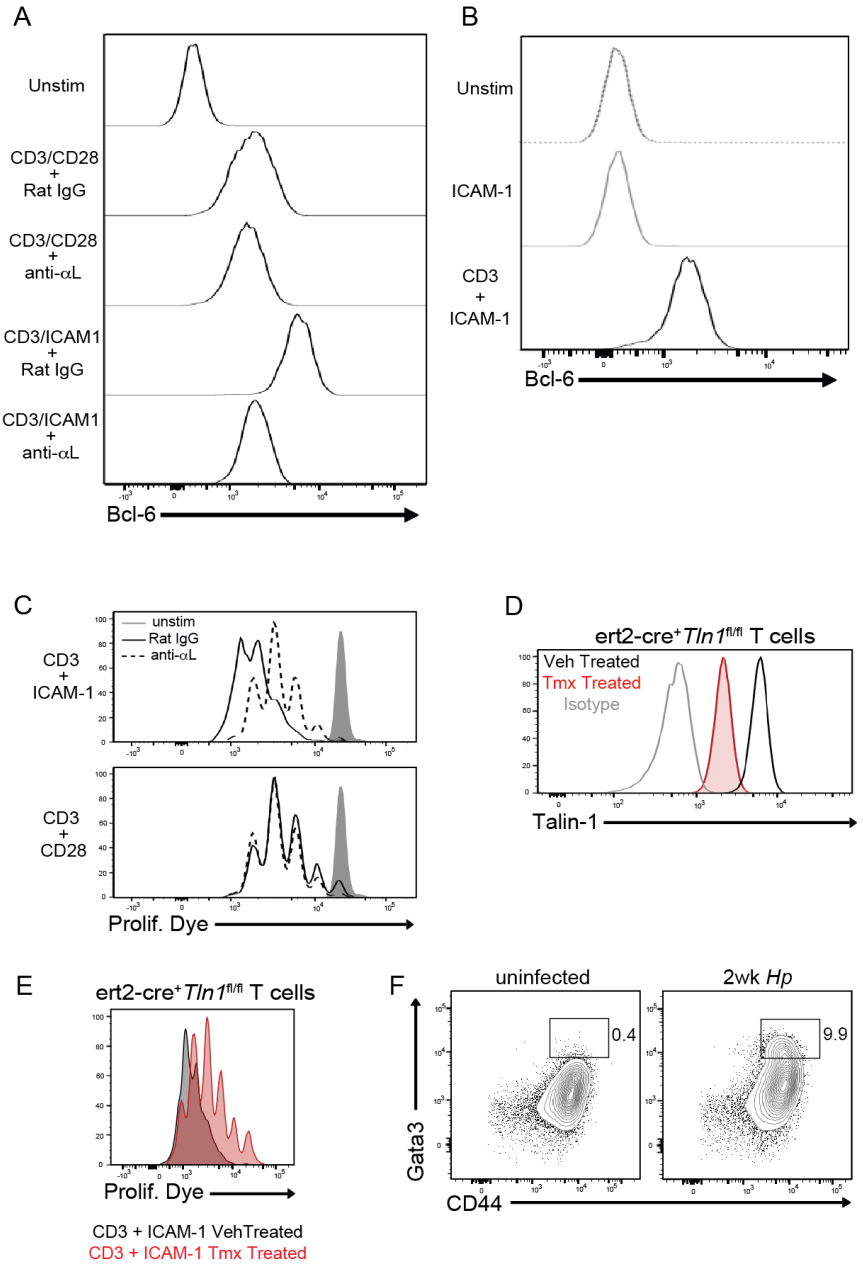

Figure S4 related to Figures 6 and 7. Impact of LFA-1 blockade and Talin-1 deletion on T cell activation and Bcl-6 expression. (A, B) Flow cytometric analysis of Bcl-6 expression in purified $CD62L^{+}CD44^{low}$ $CD4^{+}$ T cells stimulated for 72 hours under the indicated conditions. (C) Proliferation of $CD62L^{+}CD44^{low}$ $CD4^{+}$ T cells from C57BL/6 mice after 72 hours in culture under the indicated conditions. (D) Talin-1 expression in purified $CD62L^{+}CD44^{low}$ $CD4^{+}$ T cells following the indicated treatment and prior to culture. (E) Proliferation of $CD62L^{+}CD44^{low}$ $CD4^{+}$ T cells from tamoxifen or vehicle-treated ert2-cre^{+}*Tln1*$^{fl/fl}$ mice 72 hours post-stimulation with anti-CD3 and recombinant ICAM-1. (F) $2x10^{6}$ purified $CD4^{+}$ T cells from ert2-cre^{+}*Tln1*$^{fl/+}$ mice were transferred to TCRβ-deficient mice. The data shows $Gata3^{+}$ expressing donor $CD44^{hi}$ $CD4^{+}$ T cells from uninfected mice or 2 weeks after *Hp* infection. The numbers in each contour plot represent the frequency of the gated population within the donor $CD4^{+}$ T cell population.

SUPPLEMENTARY MATERIALS AND METHODS

Mice

4get/KN2 mice were obtained from Markus Mohrs (Trudeau Institute), *Tcrb*$^{-/-}$ mice were provided by Ciriaco Piccirillo (McGill University), *Tln1*$^{fl/fl}$ mice were provided by Mark Ginsberg (UC-San Diego), *Itgal*$^{-/-}$ or αL-deficient mice were provided by Dr. Minsoo Kim (University of Rochester Medical Center) and *Rosa26*-ert2-cre mice were purchased from Jackson Laboratories (008463).

Flow Cytometry

Additional murine antibodies used include: GL7 (GL7), CD18 (C71/16), pZap70 (Y319; 17A/P-ZAP70), active Caspase-3 (C92-605; BD Biosciences), IgD (11-26c), huCD2 (RPA-2.10), CD11a (M17/4), CD29 (eBioHMb1-1), CD49b (R1-2), CD51 (RMV-7), CD61 (2C9.G3), TCRβ (H57-597), Nur77 (12.14), CD5 (53-7.3), CD103 (2E7), CD11b (M1/70), CD11c (N418), CD49f (RMV-7), CD62L (MEL-14), CD44 (IM7), ICOS (C398.4A), Gata3 (TWAJ), Foxp3 (FJK-16s), IL-2 (JES6-5H4), Fas (15A7) were purchased from Affymetrix (eBioscience), Anti-Rabbit GFP (FL3;Cedarlane), F(ab')2 Donkey Anti-Rabbit IgG (Affymetrix) and Talin-1 (ab17333; Abcam). Propidium iodide or fixable viability dye (Affymetrix) was used to exclude dead cells. Staining for intracellular proteins was performed using reagants suggested by the manufacturers (Affymetrix or BD Biosciences). Human mononuclear cells were labeled with anti-CD4 (RPA-T4, BD), anti-CD45RA (clone HI100, Affymetrix) and anti-CXCR5 (clone RF8B2, BD) mAbs.

Efferent Lymph Collection

Mice were gavaged with 100 ul of corn oil 1 hour prior to sacrifice. Immediately upon euthanasia, the efferent lymph vessel of the most caudal mLN was cannulated and aspirated using a glass Pasteur pipette coated with 4% BSA. Collected efferent lymph fluid was transferred to a 5 ml conical tube and washed prior to antibody staining.

Confocal Microscopy

Immediately after being isolated from 2 week infected 4get/KN2 mice, mLNs were embedded in clear frozen section compound (VWR) and frozen over liquid nitrogen. Frozen lymph nodes were cut into 8-um sections using a HistoSTAT microtome from Scientific Intruments and incubated for 5 min in a 75% acetone/25% ethanol solution to fix tissues for the detection of surface antigens. Sections were blocked with 2% BSA, 5% mouse serum and CD16/32 Fc block (2.4G2, BioXCell) before surface staining with anti-mouse GL7-FITC (GL7;BD Biosciences), CD21/35-APC (7G6; BD Biosciences) and huCD2-Biotin (RPA-2.10; eBiosciences) primary antibodies followed by Streptavidin-Alexafluor 546 (Life Technologies).

Cell culture

Naive CD62L^{+}CD4^{+} T cells were purified using the negative selection Stem Cell Mouse Naive CD4^{+} T cell Isolation Kit and protocol. Isolation efficiency was equal to 99%. Naïve T cells ($1x10^5$ cells per well) were cultured for the indicated time periods and stimulated as described with various combinations of plate-bound 0.5 μg/ml anti-CD3 (145-2C11; Affymetrix), 1μg/ml anti-CD28 (37.51; Affymetrix), 1μg/ml recombinant ICAM-1 (R&D Systems), 10μg/ml anti-αL (M17/4) or 10μg/ml Rat IgG2a in a 96 well plate suspended in RPMI with the addition of 10% FBS, 1% Penicillin-Streptomycin and 70uM β-mercaptoethanol. In some cases, naïve T cells were labeled with cell proliferation dye eFluor450 (Affymetrix) as per the manufacturer's instructions prior to culture.

Intracellular cytokine detection
$1.5x10^6$ mLN cells were stimulated in RPMI with the addition of 10% FBS, 1% Penicillin-Streptomycin and 70uM β-mercaptoethanol in the absence or presence of 50ng/ml PMA, 1ug/ml ionomycin and 0.67ul/ml BD GolgiStop for 4 hours. After stimulation, cells were surface stained and the BD Cytofix/Cytoperm kit (554714;BD) was used as per manufacturer's instructions for intracellular detection of IL-2 and GFP.

ICAM-1 binding Assay
Single cell suspensions of total mLN cells from *Hp*-infected mice were prepared and resuspended in RPMI containing 0.1% BSA, 1mM EGTA and 5mM Mg^{2+} at a concentration of $1x10^7$ cells per milliliter. Cells suspensions were distributed in triplicate to a 96 well plate and incubated 30 minutes at 37° C in the presence of varying concentrations of soluble recombinant ICAM-1 Fc chimera (R&D Systems) and 10ug/ml anti-αL blocking antibody (M17/4, BioXCell). Samples were subsequently washed, resuspended in FACS Buffer with the addition of propidium iodide and analyzed by flow cytometry.

Protein extraction and immunoblotting
Total lymphocytes or cell-sorted naive $CD4^+$ T cells were extracted in ice-cold cell lysis buffer, then sonicated (25 watts; 30s, on ice), and particulate material removed by centrifugation (10,000 × g; 12 min; 4 °C). The supernatants were collected and quantified using the BCA protein assay kit (Fisher Scientific). Membranes were blocked in 5% nonfat milk in TBS-T (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1h at room temperature.

qPCR
Primers specific for *Gapdh* and *S1p1* were purchased from Biorad. Custom primers were generated for *Bcl6*: forward (5'- TCTTCGGAGGATGAGATTGC-3'), reverse (5'-GATTGAGGCTGTTGAGAACG-3'); *Prdm1*: forward (5'-ACACACAGGAGAGAAGCCACATGA-3'), reverse (5'-TCGAAGGTGGGTCTTGAGATTGCT-3'); *Hprt*: forward (5'-AGGACCTCTCGAAGTGTTGG-3'), reverse (5'- AACTTGCGCTCATCTTAGGC-3') using Primer3 software.

Adoptive transfer experiments
Total $CD4^+$ T cells were purified from pooled peripheral lymph nodes of naive ert2-cre^+ $Tln1^{fl/fl}$ or ert2-cre^+ $Tln1^{fl/+}$ mice or splenocytes from C57BL/6 and $Itgal^{-/-}$ using the Stem Cell Mouse $CD4^+$ T Cell Isolation Kit resulting in 99% purity. Immediately after purification $2x10^6$ $CD4^+$ T cells were transferred by intravenous tail injection into TCRβ deficient mice. Experiments involving inducible Cre recombinase expression, 24 hours after transfer, all mice were administered 5mg of tamoxifen diluted in corn oil by gavage for two consecutive days. 1 week after the final tamoxifen treatment, mice were infected with 200 L3 *Hp* larvae and sacrificed 2 weeks post-infection for analysis. In experiments comparing WT and $Itgal^{-/-}$ T cells, recipient mice were administered SRBCs as decribed above 24 hours after T cell transfer.

Statistics
Data are expressed as mean ± SD. Data were analyzed by 2-tailed Student's *t* test or 1-way ANOVA as appropriate using the GraphPad Prism program (version 6). *P* values of less than 0.05 were considered significant.