Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection

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Interleukin 2 (IL-2) promotes Foxp3+ regulatory T (Treg) cell responses, but inhibits T follicular helper (TFH) cell development. However, it is not clear how IL-2 affects T follicular regulatory (TFR) cells, a cell type with properties of both Treg and TFH cells. Using an influenza infection model, we found that high IL-2 concentrations at the peak of the infection prevented TFH cell development by a Blimp-1-dependent mechanism. However, once the immune response resolved, some Treg cells downregulated CD25, upregulated Bcl-6 and differentiated into TFH cells, which then migrated into the B cell follicles to prevent the expansion of self-reactive B cell clones. Thus, unlike its effects on conventional Treg cells, IL-2 inhibits TFH cell responses.

IL-2 is essential for the development and maintenance of Foxp3-CD4+ Treg cells, which prevent autoimmune disease development1. The principal mechanism by which IL-2 promotes Treg cell development is by activating the transcription factor STAT5, which binds to the Foxp3 locus and promotes Foxp3 expression2-4. IL-2 signaling is also required to maintain the competitive fitness of Treg cells in secondary lymphoid organs5,6 and for reinforcing their suppressive activity7,8. Thus, mice lacking IL-2 or IL-2Rα (CD25) fail to maintain peripheral tolerance, and they develop autoimmune disease6.

Treg cells express high amounts of CD25, the α chain of the high-affinity IL-2 receptor, allowing them to effectively compete with other cells for available IL-2 (refs. 10–12). Indeed, IL-2 consumption by Treg cells is one of the primary mechanisms by which they prevent effector T cell (Teff) responses13. Conversely, IL-2 consumption by Treg cells facilitates CD4+ Treg cell development14, given that IL-2 signaling inhibits Treg cell differentiation13-16. Notably, some activated Treg cells downregulate CD25 and do not require IL-2 for their homeostatic maintenance17. Instead, their survival is dependent on interactions between the co-stimulatory receptors ICOS and ICOS-L17. Similarly, antigen-experienced Treg cells in the skin18 and in aged mice19 express less CD25, and depend on interleukins 7 and 15 (IL-7 and IL-15) rather than IL-2 for their maintenance, suggesting that IL-2 might be dispensable for the homeostasis of some Treg cell subsets.

Notably, some Foxp3-expressing Treg cells upregulate Bcl-6 and CXCXR5, molecules that are normally expressed by T FH cells20,21. These Foxp3+Bcl-6+CXCXR5+CD4+ cells are known as TFH cells20-22, which migrate to B cell follicles, where they suppress B cell responses20-25. The ability of TFH cells to coexpress Foxp3 and Bcl-6 is somewhat surprising, as IL-2 signaling is important for Foxp3 expression, but inhibits Bcl-6 (refs. 14,15,26). Thus, it is unclear how IL-2 might be involved in the differentiation or maintenance of TFH cells.

We investigated the role of IL-2 in TFH cell responses to influenza. We found that high concentrations of IL-2 at the peak of the infection promoted the expression of the transcriptional repressor Blimp-1 in TFH cells, which suppressed Bcl-6 expression and thereby precluded TFH cell development. As a consequence, TFH cells failed to accumulate at the peak of the influenza infection. However, once the virus was eliminated and IL-2 concentrations declined, some CD25hi Treg cells downregulated CD25, upregulated Bcl-6 and differentiated into TFH cells, which migrated into the B cell follicles to prevent the accumulation of self-reactive B cell clones. Collectively, our data indicate that IL-2 signaling differentially controls conventional Treg and TFH cell responses to influenza virus, and reveal an important role for TFH cells in maintaining B cell tolerance after influenza infection.

RESULTS
Kinetics of TFH cell expansion following influenza infection
To evaluate whether TFH cells could be detected after influenza infection, we intranasally (i.n.) infected C57BL/6 (B6) mice with influenza A/PR8/34 (PR8) and characterized Foxp3+CD4+ T cells in the lung-draining mediastinal lymph node (mLN) 30 d later (Fig. 1a-c). Foxp3+CD69hiCD4+ cells expressed low amounts of Bcl-6 and the chemokine receptor CXCR5 (Fig. 1a). In contrast, Foxp3+CD69loCD4+ T cells could be separated into Bcl-6loCXCR5lo cells, which showed low expression of the co-stimulatory molecule PD-1 and the germinal center marker GL-7, and Bcl-6hiCXCR5hi cells, which showed high expression of PD-1 and GL-7 (Fig. 1a-c). Thus, we designated the Bcl-6loCXCR5loFoxp3+CD4+ T cells as conventional Treg cells and...

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Bcl-6hiCXCR5hiFoxp3+CD4+ T cells as TFr cells. TFr cell development requires SLAM-associated protein (SAP)-mediated interaction with B cells21. As such, the frequency and number of Bcl-6hiCXCR5hi TFr cells were decreased in SAP-deficient (B6/Sh2d1a−/−) mice relative to B6 mice (Fig. 1d,e). Finally, to determine whether Foxp3+ cells home to germinal centers (GCs) following influenza infection, we examined the placement of Foxp3+ cells relative to B cell follicles and GCs in sections of mLN s obtained from mice infected with influenza for 30 d (Supplementary Fig. 1a). As expected, we identified CD4+Foxp3+ cells in the B cell follicles, the interfollicular area and inside the GCs (Supplementary Fig. 1a,b). These results indicate that bona fide TFr cells develop following influenza virus infection.

We next infected B6 mice with influenza and enumerated TFr cells and conventional Treg cells in the mLN at different times after infection (Fig. 1f–h). Bcl-6hiCXCR5hi TFr cells were barely detectable at the peak of the infection (day 7–15), but largely accumulated during the late phase of the primary response (day 30–60) (Fig. 1f,g). In contrast, conventional Treg cells rapidly expanded between days 3 and 7 (Fig. 1h). Notably, the paucity of TFr cells at the peak of the infection was not a result of a lack of GC B cells, as GC B cells were easily detected at day 10, continued to expand through day 15 and declined thereafter (Fig. 1i,j). We also observed that Bcl-6hiCXCR5hi Tfh cells (Fig. 1k,l) peaked between days 7 and 15 after infection, and subsequently contracted between days 15 and 30. Thus, in contrast with GC cells, Tfh cells and conventional Treg cells, TFr cells fail to accumulate at the peak of the infection.

To further confirm this conclusion, we evaluated the presence of Foxp3+ cells in the GCs at days 10 and 30 after infection by immunohistochemistry (Supplementary Fig. 1c). Foxp3+ cells were easily detected in the B cell follicles, interfollicular area and GCs on day

Figure 1 Kinetics of the TFr cell response to influenza. (a–c) B6 mice were infected with PR8 and cells from the mLN were analyzed by flow cytometry 30 d after infection. (a) Expression of Bcl-6 and CXCR5 in Foxp3+CD69hi and Foxp3+CD69lo CD4+ T cells. Expression of PD-1 (b) and GL-7 (c) on Bcl-6hiCXCR5hi and Bcl-6loCXCR5lo Foxp3+CD69hi CD4+ T cells. Data are representative of five independent experiments (3–5 mice per experiment). (d,e) B6 and B6/Sh2d1a−/− mice were infected with PR8 and the frequency (d) and number (e) of Foxp3+CD69hi CD4+ T cells with a Bcl-6hiCXCR5hi TFr cell phenotype were evaluated in the mLN 30 d after infection. Data are representative of three independent experiments (mean ± s.d. of 3–5 mice per group). ***P < 0.001. P values were determined using a two-tailed Student’s t test. (f–i) B6 mice were infected with PR8 and cells from the mLN were analyzed by flow cytometry at the indicated time points. Frequency (f) and number (g) of Bcl-6hiCXCR5hi TFr cells. Representative plots were gated on Foxp3+CD69hiCD19−CD4+ T cells. (h) Number of Foxp3+CD69hi Treg cells with a Bcl-6hiCXCR5lo phenotype. Frequency (i) and number (j) of CD19+CD138−PNAhiCD95hi GC B cells. Frequency (k) and number (l) of Bcl-6hiCXCR5hi Tfh cells. Representative plots were gated on CD4+Foxp3+CD19− T cells. Data are shown as the mean ± s.d. (n = 4–5 mice per time point). Data are representative of three independent experiments.
Supplementary Fig. 1f) FR

Previous studies have shown that FR cells develop quickly (days 7–14) following immunization with soluble antigens. To determine whether the delayed appearance of FR cells is unique to influenza, we enumerated FR cells in B6 mice that were either immunized with influenza hemagglutinin (HA) adsorbed to alum (Supplementary Fig. 1d,e) or infected with LCMV-Armstrong (Supplementary Fig. 1f,g). Consistent with the published studies, we found that FR cells were readily detected at day 9 after HA immunization and were maintained for at least 30 d (Supplementary Fig. 1d,e). By contrast, FR cells were virtually undetectable at day 9 following LCMV-Armstrong infection, but accumulated in large numbers at day 30 after infection (Supplementary Fig. 1f,g). Collectively, these results suggest that, although FR cell responses quickly develop following

Figure 2 Treg cells are CD25+. (a–d) B6 mice were infected with PR8 and cells from the mLN were analyzed by flow cytometry 30 d later. (a) Frequency of CD25hi and CD25lo Foxp3+CD69hiCD4+ T cells with a Bcl-6hiCXCR5hi phenotype. Expression of PD-1 (b) and GL-7 (c) in CD25hi and CD25lo Bcl-6hiCXCR5hi cells. Data are representative of five independent experiments. Data are shown as the mean ± s.d. (n = 4 mice). P values were determined using a two-tailed Student’s t test. (d) B6 mice were infected with PR8 and STAT5 phosphorylation in CD4+ B220+ CD25hiBcl-6hiCXCR5hiFoxp3+ Treg cells, CD4+B220+CD25hiBcl-6hiCXCR5hiFoxp3+ conventional Treg cells and CD4+B220+Bcl-6hiCXCR5hiFoxp3+ Treg cells was determined by flow cytometry on day 15. Cells were stimulated with 100 ng ml−1 of IL-2 for 15 min before analyzing staining. Data are representative of two independent experiments. Data are shown as the mean ± s.d. (n = 4 mice). *P < 0.05, **P < 0.01, ***P < 0.001. P values were determined using a two-tailed Student’s t test. (e,f) Conventional Treg cells (CD19−CD4+Foxp3+CD69hiPD−1hiCXCR5hiCD25hi) and TFR cells (CD19−CD4+Foxp3+CD69hiPD−1hiCXCR5hiCD25hi) were sorted from the mLN of Foxp3-DTR-GFP 30 d after infection and RNA-seq was performed. (e) GSEA (Broad Institute) examining differentially expressed genes between Treg cells and TFR cells (adjusted P value < 0.05, log2-fold change ≥ 1). The NES and the number of upregulated genes for each of the top ten hallmark signaling pathways resulting from the GSEA analysis are shown. (f) Heat map displaying the expression of the IL-2-STAT5 hallmark signaling pathway genes that are differentially expressed in conventional Treg cells relative to TFR cells. Three replicates for each cell type were obtained from three independent experiments. (g,h) Expression of CD25 (g) and CD122 (h) in Bcl-6hiCXCR5hiFoxp3+CD69hiCD4+ T cells, Bcl-6hiCXCR5hi TFR cells and CD44hiCD69hiFoxp3+CD4+ T cells (naive). Data are representative of two independent experiments. Data are shown as the mean ± s.d. (n = 5 mice). ***P < 0.001. P values were determined using a two-tailed Student’s t test.
soluble protein immunization, T_{FR} cells fail to differentiate at the peak of acute viral infections.

**T_{FR} cells exhibit low expression of CD25**

T_{FR} cells depend on Bcl-6 (refs. 20–22), whose expression is inhibited by IL-2 (refs. 10,14,16). To examine the relationship between T_{FR} cells and IL-2 signaling, we divided the CD69^{hi}Foxp3^{CD4^{+}} T cell population into CD25^{hi} and CD25^{lo} cells and analyzed the expression of T_{FR} cell markers in these subpopulations (Fig. 2a–c). We found that CD25^{hi}Foxp3^{+} cells, but not CD25^{lo}Foxp3^{+} cells, upregulated T_{FR} markers following infection (Fig. 2a–c). Notably, although CD25^{hi}Foxp3^{+} cells were present at the peak of the infection (day 10), they expressed low amounts of CXCR5, Bcl-6 and PD-1 relative to later time points, suggesting that a lack of T_{FR} cells at the peak of the infection was not a result of a lack of CD25^{hi}Foxp3^{+} cells (Supplementary Fig. 2a–c). Similar results were obtained in LCMV-infected mice or HA-Alum-immunized mice (Supplementary Fig. 2d,e). Thus, unlike conventional T_{reg} cells, which are CD25^{hi}, T_{FR} cells are CD25^{lo}.

We next evaluated STAT5 phosphorylation in T_{FR} and conventional T_{reg} cells and found that phosphorylation of STAT5 was reduced in T_{FR} cells compared with conventional T_{reg} cells (Fig. 2d), suggesting that T_{FR} and conventional T_{reg} cells respond differently to IL-2. To confirm this conclusion, we sorted conventional T_{reg} cells (Foxp3^{CD69^{hi}}PD-1^{CXCR5^{hi}}CD25^{hi}) and T_{FR} cells (Foxp3^{CD69^{hi}}PD-1^{CXCR5^{hi}}CD25^{lo}) from the mLN of B6.Foxp3-DTR-GFP mice 30 d after infection and performed RNA sequencing (RNA-seq) and gene set enrichment analysis (GSEA) to identify hallmark signaling pathways that were differentially enriched in these populations (Fig. 2f). Approximately 2,000 genes were differentially expressed between conventional T_{reg} and T_{FR} cells (Supplementary Table 1). Notably, the IL-2-STAT5 signaling pathway had the highest normalized enrichment score (NES = 3.54, FDR < 0.001) and contained the largest number of genes upregulated in T_{reg} cells relative to T_{FR} cells (Fig. 2e). Of the 200 genes
in the hallmark IL-2-STAT5 signaling pathway, 72 were significantly downregulated in T_{FR} versus T_{reg} cells (Fig. 2i and Supplementary Tables 2 and 3). Despite their low expression of CD25 (Fig. 2g), T_{FR} cells expressed high amounts of IL-2Rβ (CD122; Fig. 2h), suggesting that T_{FR} cells may have some remaining capacity to respond to IL-2, particularly when its physiological concentration is sufficiently high. Altogether, our results indicate that the IL-2-STAT5 signaling pathway is downregulated in T_{FR} cells compared with conventional T_{reg} cells.

Figure 4  IL-2 inhibits T_{FR} cell differentiation. (a,b) B6 mice were infected with PR8 and treated daily with 30,000 U of rIL-2 or PBS starting on day 20. Cells from the mLNs were analyzed by flow cytometry on day 30. As a control, cells from the mLNs of mice infected for 10 d are also shown. The frequency (a) and number (b) of CD25^{hi}FoxP3^{hi} T cells with a Bcl-6^{CXCR5}^{hi} phenotype. Data are representative of three independent experiments. Data are shown as the mean ± s.d. (n = 3–5 mice per group). ***P < 0.001. P values were determined using a two-tailed Student’s t test. (c) IL21-mCherry-Il2-emGFP mice were infected with PR8 and cells from the mLN were analyzed at the indicated time points. Representative plots are gated on CD4^{+} T cells. Data are shown as the mean ± s.d. (n = 3–5 mice per group). Data are representative of three independent experiments. (d–h) B6 mice were infected with PR8 and treated daily with 500 μg of a mix of anti-IL-2 neutralizing antibodies (UE56-1A12 + S4B6-1) or control antibody (2A3) starting 3 d after infection. The frequency (d) and number (e) of Bcl-6^{CXCR5}^{hi} T_{FR} cells were calculated on day 10 after infection. Plots are gated on FoxP3^{hi}CD69^{hi}CD4^{+} T cells. (f) Number of Bcl-6^{CXCR5}^{hi} T_{FR} cells. Frequency (g) and number (h) of CD25^{hi}FoxP3^{hi} T cells. Plots are gated on CD4^{+} T cells. Data are representative of three independent experiments. Data are shown as the mean ± s.d. (n = 4–5 mice per group). **P < 0.01. P values were determined using a two-tailed Student’s t test. (i–o) B6.FoxP3-DTR-GFP mice were infected with PR8 and CD25^{hi}FoxP3^{hi} CD4^{+} T cells were sorted from the mLN and spleens at 7 days after infection. Sorted cells were then activated with anti-CD3-CD28 beads in the presence of high (200 U ml^{-1}) or low (5 U ml^{-1}) IL-2 concentrations. The expression of CD25 (i), Blimp-1 (j), T-bet (k), Bcl-6 (l) and CXCR5 (m) were assessed 72 h later by flow cytometry. The frequency (n) and number (o) of FoxP3^{hi}CD4^{+} T cells with a Bcl-6^{CXCR5}^{hi} phenotype are shown. Data are representative of three independent experiments. All values were obtained in triplicate and the data are shown as the mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001. P values were determined using a two-tailed Student’s t test.
CD25+ T_{reg} cells are the precursors of T_{FR} cells

Previous studies have shown that T_{FR} cells differentiate from pre-existing Foxp3+ T_{reg} precursors. Given that T_{FR} cells were CD25+ (WT), we used adoptive-transfer experiments to test whether T_{FR} cells are derived from pre-existing CD25+Foxp3+ or CD25- Foxp3+ T cells. Thus, we sorted CD25+Foxp3+ or CD25- Foxp3+ CD4+ T cells from

Figure 5 IL-2 inhibits T_{FR} cell differentiation by a Blimp-1 mechanism. (a,b) B6.Blimp-1/YFP reporter mice were infected with PR8 and cells from the mLNs were analyzed on day 30 by flow cytometry. (a) Expression of Bcl-6 and Blimp-1/YFP in Foxp3+CD69+CD4+ T cells. (b) Expression of CD25 and Blimp-1/YFP in Foxp3+CD69+CD4+ T cells. Data are representative of three independent experiments (mean ± s.d. of 3–5 mice per group). (c) B6 and Tbx21-/- mice were infected with PR8 and the expression of T-bet in CD25+ Blimp-1 and CD25+ Blimp-1 Foxp3+CD69+CD4+ T cells was analyzed at days 10 and 30 by intracellular staining. Data are representative of two independent experiments (mean ± s.d. of 3–5 mice per group). **P < 0.001. P values were determined using a two-tailed Student’s t test. (d) Tcrd+ Tcrd- mice were irradiated and reconstituted with a 50:50 mix of BM from CD45.1+ WT and CD45.2+ Tbx21-/- donors. We reconstituted mice 8 weeks later with PR8 and analyzed cells from the mLNs on day 10. The frequency of CD25+ Foxp3+ and CD25- Foxp3- cells with a Bcl-6+CXCR5+ phenotype was determined in the CD45.1+ and CD45.2+ compartments. Representative plots are shown. Data in the graph are shown as the mean ± s.d. (n = 4 mice). Data are representative of two independent experiments. (e) Tcrd+ Tcrd- mice were irradiated and reconstituted with a 50:50 mix of BM from CD45.1+ WT and CD45.2+ Tbx21-/- donors. We reconstituted mice 8 weeks later with PR8 and analyzed cells from the mLNs on day 10. The frequency of CD25+ Foxp3+ and CD25- Foxp3+ cells with a Bcl-6+CXCR5+ phenotype were calculated in the CD45.1+ and CD45.2+ compartments. Representative plots are shown. Data in the graph are shown as the mean ± s.d. (n = 5 mice). Data are representative of three independent experiments. **P < 0.001. P values were determined using a two-tailed Student’s t test. (f) WT-Pdmd1+/B6-Lckcre+/- chimeric mice were infected with PR8 and treated daily with 30,000 U of rIL-2 or PBS (control) starting 20 days after infection. Cells from the mLNs were analyzed by flow cytometry on day 30. (f) Frequency of CD45.1+ and CD45.2+ CD25+ Foxp3+ CD4+ T cells with a Bcl-6+CXCR5+ T_{reg} cell phenotype. Data in the graph are shown as the mean ± s.d. (n = 5–7 mice per group). Representative plots are shown. (g) Ratio of Pdmd1+/ to WT T_{FR} cell was calculated in control and rIL-2-treated mice. Data are shown as the mean ± s.d. (n = 5–7 mice per group). Data are representative of two independent experiments. **P < 0.01, ***P < 0.001. P values were determined using a two-tailed Student’s t test.
the spleens of naive B6.Foxp3-DTR-GFP (CD45.2+) mice, which express the diphtheria-toxin receptor and the eGFP genes under the control of the Foxp3 promoter, and adoptively transferred equivalent numbers of these cells into naive Tcrb−/−Tcdn−/− mice, which lack T cells. We also transferred total CD8+ and CD4+ T cells from B6,CD45.1 mice to provide a competent T cell environment. We infected mice 1 d later with influenza, and characterized the donor-derived Foxp3+cells 30 d later. The frequencies and numbers of activated Foxp3+ cells derived from the CD4.2+ donors were increased in recipients of CD25hiFoxp3+ cells compared with recipients of CD25loFoxp3+ cells (Fig. 3a,b). We also found that nearly 6% of the progeny derived from CD25hiFoxp3+ cells upregulated Bcl-6 and CXCR5, whereas few of the progeny derived from CD25loFoxp3+ cells upregulated Bcl-6 and CXCR5 (Fig. 3c). As a result, the numbers of CD4.2+ TFR cells were significantly higher in recipients of CD25hiFoxp3+ cells than in recipients of CD25loFoxp3+ cells (Fig. 3d). Notably, the number of CD45.1+Foxp3+ cells, and the number of CD45.1+TFR cells were similar in the two groups (Fig. 3e,f). These data indicate that pre-existing CD25hiFoxp3+ cells, but not pre-existing CD25loFoxp3+ cells, differentiate into TFR cells following influenza infection.

Recent data suggest that TFR cells can also be derived from CD4+Foxp3−precursoress. Thus, we performed adoptive-transfer experiments to compare the capacity of CD4+Foxp3− and CD25loFoxp3−cells to differentiate into TFR cells following infection. Although some CD4+Foxp3− cells upregulated Bcl-6 after infection (data not shown), CD4.2+ TFR cells were only generated from CD25hiFoxp3−cell precursors (Fig. 3g,h). Collectively, these data indicate that CD25loFoxp3−cells are the precursors of TFR cells after influenza infection.

We next tested whether the Foxp3+ cells derived from the CD25hiFoxp3− donors maintained CD25 expression (Fig. 3i). We found that, although the majority of Foxp3+ progeny derived from the CD25hiFoxp3− donors were CD25hi, some cells lost expression of CD25 and only the CD25lo cells upregulated TFR markers (Fig. 3i). Collectively, these results suggest that a fraction of the activated CD25hiFoxp3−CD4+ T cells downregulate CD25 and upregulate Bcl-6, PD-1 and CXCR5 late after infection.

IL-2 signaling precludes TFR cell development

Given that IL-2 signaling inhibits Bcl-6 expression, we next tested whether TFR cells can develop in a high-IL-2 environment. To do this, we infected B6 mice with influenza, treated them with either 30,000 units (U) of recombinant IL-2 (rIL-2) or phosphate-buffered saline (PBS) for nine consecutive days starting on day 20 and enumerated TFR cells in the mLN on day 30. As a control, we analyzed mice that had been infected for 10 d. As expected, the frequencies and numbers of TFR cells were higher in mice infected for 30 d relative to mice infected for 10 d (Fig. 4a,b). By contrast, TFR cells failed to accumulate in rIL-2-treated mice (Fig. 4a,b), suggesting that a high-IL-2 environment prevents the accumulation of TFR cells.

We next determined the kinetics of IL-2 production during influenza infection using IL21-mCherry-IL2-emGFP dual reporter transgenic mice (Fig. 4c). We found that the frequency of IL-2-producing CD4+ T cells peaked at day 10 and declined thereafter (Fig. 4c). We also found that the frequency of IL-2-producing CD4+ T cells was significantly increased in mice that had been infected with influenza for 10 d relative to mice that had been immunized with HA for 10 d (Supplementary Fig. 3). Given that IL-2 is highly produced at the peak of the infection, we hypothesized that strong IL-2 signals prevented the differentiation of TFR cells at this time. To address this possibility, we infected B6 mice with influenza, treated them daily from day 3 to day 9 with neutralizing IL-2 antibodies (JES6-1A12 + S4B6-1) or control antibody (2A3), and analyzed the Foxp3 compartment in the mLN on day 10 (Fig. 4d,e). As expected, we detected very few TFR cells in control-treated mice. The frequency and number of TFR cells was, however, significantly increased in the anti-IL-2-treated mice (Fig. 4d,e). These results suggest that an elevated concentration of IL-2 at the peak of the infection prevents Foxp3-expressing cells from differentiating into TFR cells. We also found more TFR cells, but less conventional CD25+Treg cells, in the anti-IL-2-treated mice compared
with control mice (Fig. 4f–h), which is consistent with previous studies showing that IL-2 signaling is required for CD25hiFoxp3+ Treg cell expansion27,30–32, but prevents Treg cell differentiation14–16,26.

To confirm that IL-2 signaling inhibits Treg cell differentiation, we sorted CD25hiFoxp3+ cells from B6.Foxp3-DTR-GFP mice infected with influenza for 7 d, activated them in vitro with anti-CD3 and anti-CD28 beads in the presence of high (200 U ml−1) or low (5 U ml−1) rIL-2 concentrations, and assessed the phenotype of Foxp3+ cells 3 d later (Fig. 4i–o). Foxp3+ cells activated in the presence of high-IL-2 concentrations expressed high levels of CD25, Bcl-2 and the transcription factor T-bet relative to Foxp3+ cells cultured in low-IL-2 conditions (Fig. 4i–k). In contrast, Bcl-6 and CXCR5 were upregulated in Foxp3+ cells cultured in low-IL-2 concentrations (Fig. 4l,m). As a result, Bcl-6hiCXCR5hi cells differentiated in the low, but not high, IL-2 cultures (Fig. 4n,o). Collectively, our data indicate that, although elevated IL-2 signaling at the peak of the infection promotes CD25hi Treg cell responses, it simultaneously prevents Foxp3-expressing cells from upregulating Bcl-6 and CXCR5 and differentiating into Treg cells.

IL-2 prevents Treg cell responses by promoting Blimp-1
IL-2 inhibits Bcl-6 expression by upregulating Blimp-1 (refs. 14,15) and by favoring the formation of T-bet-Bcl-6 complexes, which mask the Bcl-6 DNA-binding domain and prevent it from binding to its target genes26. Thus, we next assessed the expression of these transcription factors in Foxp3+ cells following influenza infection (Fig. 5a–c). To do this, we infected B6.Blimp-1-YFP reporter mice and examined the expression of the Blimp-1-YFP reporter in CD69+Foxp3+CD4+ T cells (Fig. 5a,b). We found that nearly 12% of these cells were Blimp-1-YFP+ (Fig. 5a). As predicted, Blimp-1-YFP+ cells were Bcl-6hi (Fig. 5a). We also found that, although some Blimp-1-YFP+ cells were CD25int/low, the majority of Blimp-1-YFP+ cells expressed high amounts of CD25 (Fig. 5b). We next analyzed T-bet expression and found that it was highly expressed in CD25hiBlimp-1+ cells relative to CD25intBlimp-1− cells (Fig. 5c). Thus, consistent with previous studies33–35, Treg cells express T-bet and Blimp-1 following influenza infection.

To investigate the role of these transcription factors in Treg cell development, we made mixed bone marrow (BM) chimeras in which irradiated Tcrb−/−/Tcrd−/− recipient mice were reconstituted with a 50:50 mixture of BM obtained from CD45.1+ wild-type and CD45.2+ Tbx21−/− (T-bet deficient) B6 donor mice. We infected the chimeras 2 months later with influenza and determined the frequency of Treg cells derived from each donor 10 d later. As expected, we failed to detect Bcl-6hiCXCR5hi cells in the CD25hiFoxp3+ compartment of either donor (Fig. 5d). Although we did observe approximately 5% Bcl-6hiCXCR5hi cells in the CD25hiFoxp3+ compartment, there was no difference between the wild-type and Tbx21−/− donor cells
(Fig. 5d). These results suggest that T-bet does not prevent T_{Fr} cell differentiation at the peak of the infection.

To address a potential role for Blimp-1 in preventing T_{Fr} differentiation, we generated mixed BM chimeras using wild-type (CD45.1\(^++\)) and Prdm1\(^{fl/fl}\), Lck\(^{cre/+}\) (CD45.2\(^++\)) donors (WT-Prdm1\(^{−/−}\) chimeras). After reconstitution, chimeras were infected with PR8 and analyzed on day 10. As expected, very few wild-type CD25\(^+\)Foxp3\(^+\) cells expressed T\(_{Fr}\) markers on day 10 post-infection. However, 20% of the Prdm1\(^{−/−}\)-CD25\(^+\)Foxp3\(^+\) cells were T_{Fr} cells (Fig. 5e). These results suggest that Blimp-1 prevents the development of T_{Fr} cells at the peak of the influenza infection.

Finally, we examined whether IL-2 prevents T_{Fr} cell differentiation via a Blimp-1-dependent mechanism. Thus, we infected WT-Prdm1\(^{−/−}\) chimeras with influenza, treated them daily with rIL-2 or control PBS starting at day 20, and assessed the T_{Fr} cell response in the wild-type and Prdm1\(^{−/−}\) compartments on day 30 after infection (Fig. 5f). Similar to our prior experiment, we found more CD25\(^+\)Foxp3\(^+\) cells with a T_{Fr} cell phenotype in the Prdm1\(^{−/−}\) compartment relative to the wild-type compartment in the PBS-treated mice (Fig. 5f). We also found diminished frequencies of T_{Fr} cells in the wild-type compartment of the rIL-2-treated mice relative to PBS-treated mice (Fig. 5f). However, we observed similar frequencies of Prdm1\(^{−/−}\) T_{Fr} cells in both PBS control and IL-2-treated mice (Fig. 5f). As a consequence, the ratio of Prdm1\(^{−/−}\) to wild-type T_{Fr} cells was increased in the rIL-2-treated mice compared with PBS control counterparts (Fig. 5g). These results indicate that high IL-2 signaling directly prevents T_{Fr} cell differentiation by an intrinsic IL-2/Blimp-1-dependent mechanism.
**T FR cells maintain B cell tolerance after infection**

We first used three independent approaches to test the effect of T FR cells on the B cell response to influenza. First, we crossed Bcl-6 fl/fl mice to Foxp3-YFP/Cre mice to generate Bcl-6 fl/fl Foxp3 YFP/Cre mice, in which the zinc finger domains of Bcl-6 are conditionally deleted in Foxp3-expressing T cells. Bcl-6 fl/fl Foxp3 YFP/Cre mice were infected with influenza and analyzed on day 30. As expected, the number of T FR cells was reduced in Bcl-6 fl/fl Foxp3 YFP/Cre mice compared with control mice (Fig. 6a,b). In contrast, T FH cells normally accumulated in Bcl-6 fl/fl Foxp3 YFP/Cre mice (Fig. 6c,d). Similar results were obtained when we used fluorochrome-labeled MHC class II tetramers to identify influenza nucleoprotein (NP)–specific T FH cells (Supplementary Fig. 4a,b). We also found that GC B cells accumulated normally in control and Bcl-6 fl/fl Foxp3 YFP/Cre mice (Fig. 6e,f). Notably, however, the frequency and number of CD138 + antibody-secreting cells (ASCs) were increased in Bcl-6 fl/fl Foxp3 YFP/Cre mice relative to control mice (Fig. 6g,h). These results suggest that lack of T FR cells do not change T FH cell or the GC B cell responses, but instead promote the accumulation of CD138 + ASCs.

To confirm this observation, we generated BM chimeras in which T FR cells were selectively depleted after diphtheria toxin (DT) administration (Fig. 7a–f). Thus, we reconstituted irradiated Tcrb–/– Tcrd–/– recipient mice with a 50:50 mix of Cxcr5 +/– and Foxp3-DTR BM (Foxp3-Cxcr5 +/– chimeras), or wild-type BM and Foxp3-DTR BM (Foxp3-WT chimeras). Because T FR cells cannot be produced from Cxcr5 +/– precursors, all of the T FR cells in the influenza-infected Foxp3-Cxcr5 +/– chimeras developed from the Foxp3-DTR donors (Fig. 7a). In contrast, T FH cells in the Foxp3-WT chimeras developed equally from the WT and Foxp3-DTR donors (Fig. 7a). As a consequence, T FR cells were depleted following DT administration in the Foxp3-Cxcr5 +/– mice, but not in the Foxp3-WT influenza-infected chimeras (Fig. 7b,c). The T FR cell response was, however, similar in the two groups (Fig. 7d). Thus, we infected the chimeric mice with influenza, treated them with DT every 4 days starting on day 15, and enumerated T FH cells, GC B cells and CD138 + ASCs on day 50 in the mLNs. We found similar frequencies and numbers of T FH cells and GC B cells (Supplementary Fig. 5a–d) in the DT-treated Foxp3-Cxcr5 +/– and Foxp3-WT chimeras. However, CD138 + ASCs accumulated at a greater frequency in Foxp3-Cxcr5 +/– chimeras than in Foxp3-WT controls (Fig. 7e,f). Notably, no differences were detected between PBS-treated Foxp3-Cxcr5 +/– and Foxp3-WT chimeras (Supplementary Fig. 5e). These findings indicate that a lack of T FR cells promotes the expansion of CD138 + ASCs.

To confirm our observations, we treated influenza-infected B6 mice daily with 15,000 U of rIL-2 starting on day 20 to deplete T FR cells, and evaluated the B cell response in the mLN on day 30. T FR cells were significantly depleted in rIL-2-treated mice (Supplementary Fig. 5f). In contrast, rIL-2 treatment did not affect the accumulation of total or NP-specific T FH cells at this time and dosage (Supplementary Fig. 5g–j). Similarly, the GC B cell response was similar in PBS and rIL-2-treated mice (Supplementary Fig. 5k,l). In contrast, the frequencies and numbers of CD138 + ASCs were increased in rIL-2-treated mice (Fig. 7g,h). Collectively, our data indicate that absence of T FR cells late after infection promotes the expansion of CD138 + ASCs.

Lack of T FR cells promotes the outgrowth of self-reactive B cell clones following immunization with T-dependent antigens. To characterize the role of T FR cells in controlling the influenza-specific B cell response, we used fluorochrome-labeled recombinant NP-tetramers to identify NP-specific B cells in IL-2-treated and Bcl-6 fl/fl Foxp3 YFP/Cre mice (Fig. 8a–h). The frequencies and numbers of NP-specific GC B cells were similar in control and rIL-2-treated mice (Fig. 8a,b). In contrast, although 30% of the CD138 + ASCs were NP specific in control mice, only 8% of the CD138 + ASCs were NP specific in the rIL-2-treated mice (Fig. 8c). As a consequence, the number of NP-specific CD138 + ASCs was similar in control and rIL-2-treated mice (Fig. 8d). Similar results were obtained when comparing Bcl-6 fl/fl Foxp3 YFP/Cre and control counterparts (Fig. 8e,f). Finally, the titers of influenza-specific serum IgG were similar in PBS and rIL-2-treated mice (Fig. 8g) or when we compared the serum from Bcl-6 fl/fl Foxp3 YFP/Cre and control mice (Fig. 8h). These results suggest that a lack of T FR cells does not significantly affect the influenza-specific B cell response, but instead promotes the accumulation of non-influenza-specific CD138 + ASCs.

We next hypothesized that these ASCs represent the differentiated daughter cells of self-reactive B cells that are known to be generated in the GCs during infections. To test this possibility, we first enumerated histone-specific, IgG-secreting cells by ELISPOT in the mLNs of day-30-infected PBS- and rIL-2-treated mice. As expected, we found very few histone-specific ASCs in the mLN of PBS-treated mice (Fig. 8i). In contrast, the frequency of anti-histone, IgG ASCs was increased in rIL-2–treated mice, suggesting that a lack of T FR cells results in the development of anti-nuclear antibody (ANA) responses (Fig. 8j). Thus, we evaluated the serum samples for ANA reactivity. As expected, PBS-treated control mice lacked ANAs, whereas IL-2–treated mice were ANA positive (Fig. 8j). Finally, we evaluated the presence of ANAs in the sera of control mice. We found that, although the sera from B6-infected mice (Fig. 8k) or naïve Bcl-6 fl/fl Foxp3 YFP/Cre mice (Supplementary Fig. 6) were negative for ANA staining, the sera obtained from day-30-infected Bcl-6 fl/fl Foxp3 YFP/Cre mice were positive (Fig. 8k). Thus, our data indicate that T FR cells prevent the expansion of self-reactive ASCs after influenza infection.

**DISCUSSION**

We found that T FR cells are characterized by low expression of CD25 and that IL-2 signaling inhibits, rather than promotes, the development of T FR cells. Correspondingly, T FR cells fail to accumulate at the peak of the influenza infection, a time during which IL-2 is highly produced. However, after IL-2 withdrawal, some Foxp3 + cells down-regulate CD25, upregulate Bcl-6, express CXCR5 and differentiate into T FR cells, which migrate into the B-cell follicles to prevent self-reactive B-cell responses. Thus, unlike conventional T FR cells, high IL-2 signaling precludes T FR cell development. Notably, IL-2 consumption by CD25 + T FR cells is required for the initial development of virus-specific T FH cells. Thus, IL-2 consumption acts as a rheostat that, although facilitating virus-specific T FH cell responses, selectively prevents T FR cell development at the peak of the infection.

T FR cells prevent self-reactive ASCs, but had no substantial effect on the influenza-specific B cell response, suggesting that T FR cells may be self specific rather than influenza specific. This idea is consistent with data showing that the TCR repertoire of T FR cells is skewed toward self-antigens, as is the TCR repertoire of thymic Treg cells, which we found were the likely precursors of T FR cells after influenza infection. However, T FR cells prevent antigen-specific B cell responses following immunization with soluble antibodies, suggesting that antigen-specific T FR cells can develop under some circumstances. In this regard, a recent study suggests that a fraction of the T FR cells differentiate from naive T cell precursors following immunization and are specific for the immunizing Ag. Thus, depending on the specific nature of the immune response, T FR cells can prevent foreign and self-reactive B cell responses based on their origin and TCR specificity. In any case, the capacity of T FR cells to prevent influenza-specific effector B responses is overcome...
Follicular regulatory T cells can be specific for the virus.

We found that Blimp-1, but not T-bet, suppressed Treg cell development. However, we failed to detect Bcl-6 upregulation in CD25hi Treg cells, even in Blimp-1-deficient cells, suggesting that Bcl-6 expression in CD25hi cells is prevented by additional Blimp-1-independent mechanisms. Given that STAT5 binds to the Bcl-6 promoter and directly represses Bcl-6 expression in response to high IL-2 signaling, it is likely that strong IL-2 signaling through CD25 prevents Bcl-6 expression by a direct STAT5-dependent mechanism.

Our data showing that IL-2 prevents Treg cell responses conflict with the notion that IL-2–STAT5 signaling is required for maintaining Foxp3 expression. However, Treg cells express high amounts of CD122. Thus, although insufficient for inducing sustained Blimp-1 expression in low-IL-2 environments, basal IL-2–STAT5 signaling through the intermediate-affinity IL-2R may be sufficient to prevent Foxp3 downregulation in Treg cells. Alternatively, Treg cell homeostasis may be partially independent of IL-2, but may require signals from other common γ-chain cytokines and co-stimulatory molecules, such as IL-7, IL-15 or ICOS, which can contribute toward the maintenance of Foxp3-expressing cells in the absence of IL-2 (refs. 18,19,47,48).

In any case, it is likely that conventional CD25reg and Treg cells use different cellular and molecular pathways for their homeostatic maintenance.

In summary, our data demonstrate that IL-2 signaling temporarily inhibits Treg cell responses during influenza infection. However, once the immune response is resolved, Treg cells differentiate and home to B cell follicles, where they are required for maintaining B cell tolerance after infection. Thus, the same mechanism that promotes conventional Treg cell responses, namely IL-2 signaling, also prevents Treg cell formation. Collectively, our data provide a new perspective into how IL-2 dynamically regulates Treg cell homeostasis and function along the course of a relevant pathogen infection.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.B.-T. designed and performed the experiments with help from B.L., H.B., M.J.F., I.E.B. and D.B. A.B.-T., D.B., and B.L. analyzed the data. T.T.M.L. and A.S.W. analyzed the RNAseq data. A.B.-T. wrote the manuscript. D.B. and B.L. contributed to data interpretation and manuscript editing. A.I.J. performed LCMV infections. T.D.R. and F.E.L. contributed to manuscript editing and discussion, and provided reagents that were critical to this work. All of the authors reviewed the manuscript before submission.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 (B6), B6.SJL-Ptprca Pepcb/BoyJ (B6.CD45.1), B6.129S6-Tbx21tmGlmi/J, (B6. Tbx21+/-), B6.129P2-Tcrdfl/fl, B6.129Prdm1tm1Mom (Tcrd-/-, Tcrb-/-), B6.129S6-Sh2d1atm1Pls (Sh2d1a-/-), B6.129-Pdmd1tm1Cimel/J (Pdmd1fl/fl), and B6.129-Cg-Tg(Tcr-cre)3779Nik/J, B6.129S6(FVB)-Bcl-6fl/fl,1Dentf/J (Bcl-6fl/fl, B6.129-Cg)-Foxp3tm1C551Cre/+/C57BL/6J (Foxp3Cre/+) were originally obtained from Jackson Laboratories. Il21r−/−Il2-emGFp dual-reporter transgenic mice were obtained from W. Leonard (NHLBI). Blimp-1 reporter mice were obtained from E. Meffre (Yale University). B6.129S6-Foxp3tm1DTR;Foxp3-Δ32-GFP mice were originally obtained from A. Rudensky (Memorial Sloan-Kettering Cancer Center). Pdmd1tm1Cimel/J mice were crossed to B6.129-Cg-Tg(Tcr-cre)3779Nik/J fl/fl mice to generate B6.129P2-Tcrdfl/fl, Pdmd1tm1Cimel/J, B6.129S6-Sh2d1atm1Pls (Sh2d1a-/-), and B6.129-Cg-Tg(Tcr-cre)3779Nik/J fl/fl mice to generate B6.129S6-Tbx21tmGlmi/J, B6.129P2-Tcrdfl/fl, Pdmd1tm1Cimel/J, and B6.129S6-Sh2d1atm1Pls (Sh2d1a-/-) mice. Bl6-/-, B6-/-, and B6.129-Cg-Tg(Tcr-cre)3779Nik/J fl/fl mice were used for adoptive transfer experiments.

Cell purification and adoptive transfer. CD45.1+ CD4+ and CD45.1+ CD8+ T cells were purified from the spleens of naïve B6.CD45.1+ mice by positive selection with anti-CD4 and anti-CD8 MACs beads (Miltenyi Biotech). CD4+Foxp3+CD25hi, CD4+Foxp3+CD25lo and CD4+Foxp3− T cells were sorted from spleens of Foxp3-DTR/GFP mice using a FACSaria (BD Biosciences) after positive selection with anti-CD4 MACs beads. Sorted cells (5 × 10^5) were transferred intravenously into naïve Tcrd-/- Tcrb-/- mice. All sorted T cell subsets were more than 95% pure.

In vitro stimulation of influenza-induced Treg cells. CD4+Foxp3+CD25hi cells were sorted from spleens and MLNs of influenza-infected B6.129S6-Foxp3tm1DTR mice using a FACSaria (BD Biosciences) after positive selection with anti-CD4 MACs beads. Sorted cells were activated using pre-load anti-CD3/CD28 MACsBeadParticles (Treg Expansion Kit, Miltenyi Biotech) at a bead-to-cell ratio of 3:1 in the presence of the indicated concentration of rIL-2 (Peprotech). Cells were cultured for 72 h at 37 °C in 125 µl in round-bottomed 96-well plates in RPMI-1640 supplemented with sodium pyruvate, L-glutamine (10 mM, pH 7.2–7.6 range), nonessential amino acids, penicillin, streptomycin, 2-mercaptoethanol and 10% heat-inactivated FCS (all from Gibco).

ELISAs. 96-well plate wells were coated overnight with purified PR8 proteins at 1 µg/ml in 0.05 M Na2CO3 pH 9.6. Coated plates were then blocked for 1 h with 1% BSA in PBS. Serum from PR8-infected mice was collected and serially diluted (threefold) in PBS with 10 mg/ml BSA and 0.1% Tween 20 before incubation on coated plates. After washing, bound antibody was detected with HRP-conjugated goat Anti-Mouse, IgG (1:5,000, g heavy chain specific) Ab (Southern Biotech) and quantified by spectrophotometry at 405 nm (OD).

ELISPOT. Multiscreen cellulosel filter plates (Millipore) were coated overnight with 10 µg of histone from calf thymus (Sigma-Aldrich). Cells from the mLN and RNA-sequencing (RNA-seq). Conventional Treg cells (CD19+ CD4+ Foxp3+CD69+PD-1+CXCR5+CD25hi) and T cells TFr cells (CD19+ CD4+ Foxp3+CD69+PD-1+CXCR5+CD25lo) were sorted from the mLN of Foxp3-DTR/GFP mice at day 30 after influenza infection using a FACSaria (BD Biosciences) after positive selection with anti-CD4 MACs beads (Miltenyi Biotech). RNA was isolated from the sorted cells using the Single cell RNA purification kit (Norgen Biotek Corp). Three replicates from three independent experiments for each condition were analyzed with RNA-seq. Library preparation and RNA sequencing was conducted through Geneviz. Libraries were sequenced using a 1× 50-bp single end rapid run on the HiSeq2500 platform. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality (error rate < 0.05) at the end. After trimming, sequence reads shorter than 30 nucleotides were discarded. Remaining sequence reads were mapped to the Mus musculus mm10 reference genome using CLC genomics workbench v. 9.0.1. Differential gene expression was determined using DESeq2. Genes with an adjusted p value < 0.05 and an
absolute log₂-fold change > 1 were considered significantly differentially expressed genes between conventional T_{reg} cells and T_{FR} cells.

GSEA, hierarchical clustering and visualization of RNA sequencing results. GSEA was performed using the Molecular Signatures Database on the publicly available MIT BROAD Institute server. We ranked the 2002 genes obtained from RNA sequencing (Genewiz) according to a logarithmic transformation of each gene’s P-value multiplied by the sign of the corresponding logarithmic fold change, and subsequently used these rank lists to perform a gene set enrichment analysis (Broad Institute’s GSEA Java app, version 2.2.4). Given gene sets can include both activated and repressed genes, we additionally performed an extended assessment of gene enrichment of the two complementary gene sets against N ranked genes\(^{51,52}\), to confirm the original GSEA results. Separately, we performed hierarchical clustering analysis\(^{53,54}\) of IL-2-induced genes that are differentially expressed in T_{reg} cells versus T_{FR} cells, using Matlab (version R2016b). Differential clusters are presented in the form of an annotated heatmap based on standardized expression values, along with the resulting hierarchical clustering dendogram. We additionally plotted in Matlab (version R2016b) the number and proportion of genes expressed within hallmark sets (GSEA), and their corresponding P values.

Statistical analysis. GraphPad Prism software (Version 5.0a) was used for data analysis. The statistical significance of differences in mean values was determined using a two-tailed Student’s t test. P values of less than 0.05 were considered statistically significant.

A Life Sciences Reporting Summary for this paper is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. RNA-seq data are available from GEO under accession code GSE101016.

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54. Eisen, M.B., Spellman, P.T., Brown, P.O. & Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863–14868 (1998).
Experimental design

1. Sample size

Describe how sample size was determined. Sample size were calculated based on preliminary studies. 3 to 10 mice/group/timepoint was sufficient to detect differences between groups with at least 80% power and a 5% significance level in most of the experiments. In addition, this number of mice was sufficient to generate enough cells for analysis.

2. Data exclusions

Describe any data exclusions. No data were excluded from the analysis.

3. Replication

Describe whether the experimental findings were reliably reproduced. All attempts for replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups. Allocation into experimental groups was random.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Blinding was not relevant to the study. All conclusions were made based on quantitative parameters and statistical significance.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Describe the software used to analyze the data in this study.

GraphPad Prism 7 for Mac OS X, Excell version 14.5.7, FlowJo version 9.9, CTL-immunoSpot S5 analyze, DESeq2, Broad Institute’s GSEA Java app, version 2.2.4 and Matlab (version R2016b).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available from commercial sources (listed in the manuscript).

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All the antibodies are from commercial sources and have been validated by the vendors. Were applicable, we carried out additional functional assays to test the specificity and efficacy of these reagents. The clone, specific dilution and commercial source for each specific antibody are listed in the methods section of the manuscript.

BD Biosciences.
- anti-CD45.1 (clone A20, dilution 1/400, cat #560578, 565278)
- anti-CD45.2 (clone 104, dilution 1/400, cat #552950, 558702, 562129)
- anti-CD19 (clone 1D3, dilution 1/200, cat #561739)
- anti-CD138 (clone 281.2, dilution 1/500, cat #563192)
- Anti-Bcl6 (clone K112.91, dilution 1/50, cat #561522)
- anti-CXCR5 (clone 2G-8, dilution 1/50 cat #551960)
- anti-CD4 (clone RM4-5, dilution 1/200, cat #563726, 550954, 552775)
- anti-CD9S (clone Jo2 dilution 1/500, cat #554257)
- anti-CD69 (clone H1.2F3, dilution 1/200, cat #560689, 553236)
- anti-GL7 (clone GL7, dilution 1/500, cat #562967)
- anti-CD2S (clone PC61, dilution 1/200, cat #557192, 561257, 553072, 561780)
- anti-Blimp-1 (clone SE7, dilution 1/100, cat #564270)
- anti-pSTAT5 (PY694, clone 47-BD, dilution 1/50, cat #612598)
- anti-IdDb (clone 2170-170, dilution 1/500 cat #553510)
- anti-CD35 (clone 8C-12, dilution 1/200 cat #553816)
- Anti-B220 (clone RA3-6B2 dilution 1/200 cat #553088, 557683)

eBioscience.
- Anti-PD-1 (clone J43, dilution 1/100, cat #11-9985-85, 12-9985-83)
- anti-FoxP3 (clone FJK-16s, dilution 1/200, cat #11-5773-82, 25-5773-82)

Biolegend
- Anti-T-bet (clone 4B10, dilution 1/200, cat #644814, 644823)
- anti-FoxP3 (clone MF-14, dilution 1/200 cat #126408)
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. No cell lines were used
   b. Describe the method of cell line authentication used. No cell lines were used
   c. Report whether the cell lines were tested for mycoplasma contamination. No cell lines were used
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

• Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

C57BL/6 (B6), B6.SJL-Ptprca Pepcb/BoyJ (B6.CD45.1), B6.129S6-Tbx21tm1Glm/J, (B6. Tbx21-/-) B6.129P2-TcrbtmlMomTcrf1tm1Mom (Tcrbd-/-), B6.129S6-Sh2d1atm1Pls/J (Sh2d1a-/-), B6.129-Prdm1tm1Clme/J (Prdm1flox/flox) and B6.Cg-Tg(Lck-cre)3779Nik/J, B.129S(FVB)-Bcl6tm1.1Dent/J (Bcl6flox/flox), B6.129S(Cg)- Foxp3tm1DTR (FoxP3-DTR/GFP) mice were originally obtained from Jackson Laboratories. Il21-mCherry-Ii2-emGFP dual-reporter transgenic mice were obtained from Dr. Warren J. Leonard (NHLBI). Blimp-1 reporter mice were obtained from Dr. Meffre (Yale University). B6.129S6-Foxp3tm1DTR (FoxP3-DTR/GFP) mice were originally obtained from Dr. Alexander Rudensky (Memorial Sloan-Kettering Cancer Center). Prdm1flox/flox mice were crossed to B6.Cg-Tg(Lck-cre)3779Nik/J mice to generate B6.Prdm1flox/flox-Lckcre/+ mice. Bcl6flox/flox mice were crossed to FoxP3YFP-/Cre mice to generate Bcl6fl/flFoxp3YFP/Cre mice. All mice were bred in the University of Alabama at Birmingham (UAB) animal facility. All experimental procedures involving animals were approved by the UAB Institutional Animal Care and Use Committee and were performed according to guidelines outlined by the National Research Council. In all the experiments mice were 8 weeks old at the time of the infection.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

no human samples
Flow Cytometry Reporting Summary

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.
   Cell suspensions from mLNs were prepared and filtered through a 70 mm nylon cell strainer (BD Biosciences). Cells were washed and resuspended in PBS with 2% donor calf serum and 10 mg/ml FcBlock (2.4G2 -BioXCell) for 10 min on ice before staining with fluorochrome-conjugated antibodies.

6. Identify the instrument used for data collection.
   Flow cytometry was performed using a FACSCanto II (BD Biosciences) and an Attune NxT Flow Cytometer (ThermoFischer Scientific).

7. Describe the software used to collect and analyze the flow cytometry data.
   BD FACS DIVA, Attune NxT software, FlowJo 9.9

8. Describe the abundance of the relevant cell populations within post-sort fractions.
   Purity was higher than 95%. Purity was determined by flow cytometry after sorting.

9. Describe the gating strategy used.
   Gating strategies are provided in the manuscript.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.