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Differential IL-2 Expression Defines Developmental Fates of Follicular versus Non-follicular Helper T cells

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Abstract

In response to infection, naïve CD4⁺ T cells differentiate into two types of helpers: T follicular helper (Tfh) cells, which support B-cell antibody production, and non-Tfh cells, which enhance innate immune cell functions. Although IL-2, the major cytokine produced by naïve T cells, plays an important role in the developmental divergence of these populations, the relationship between IL-2 production and fate determination remains unclear. Using novel reporter mice, we found that differential production of IL-2 by naïve CD4⁺ T cells defined precursors fated for different immune functions. IL-2 producers were fated to become Tfh cells, and delivered IL-2 to non-producers fated to become non-Tfh cells. Because IL-2 production was limited to cells receiving the strongest T-cell receptor signals, a direct link between TCR signal strength, IL-2 production and T-cell fate determination has been established.

Naïve CD4⁺ T cells are multipotent precursors that differentiate into functionally distinct effector subsets to coordinate different aspects of immunity. T helper 1 (Th1), Th2 and Th17 cells are products of developmental pathways induced by different classes of pathogens. They are programmed to egress from T-cell zones of secondary lymphoid tissues soon after induction to orchestrate heightened innate immune cell function at sites of pathogen entry. T follicular helper (Tfh) cells develop concurrently with Th1, Th2 or Th17 cells but are programmed to migrate to B-cell zones within secondary lymphoid tissues. They provide

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help for B cells to support the production of high affinity, class-switched antibodies. Although Tfh and non-Tfh effector cell development diverge early in an evolving adaptive response, the type of immune response (type 1, 2 or 3) is linked such that pathogen clearance mechanisms mediated by innate immune cells are amplified by coordinated help from non-Tfh effectors and the antibodies that result from Tfh-mediated B cell help. Cytokines elicited from innate cells by pathogens appear to be dominant in determining the type of adaptive response (1), whereas the intensity of T-cell antigen receptor (TCR) signaling appears to contribute to Tfh–non-Tfh cell specification (2), by mechanisms that are incompletely understood.

An impediment to understanding the mechanisms controlling Tfh–non-Tfh cell divergence is the absence of reliable early markers to define cells destined for these alternative fates. Unlike effector CD4⁺ T cells, which are distinguished by a diversity of cytokines that define their phenotype and function, naïve CD4⁺ T cells are largely limited to the production of interleukin 2 (IL-2), which is produced rapidly by a subset of antigen-activated cells (3). Through activation of Stat5 and induction of Blimp1 (4, 5), IL-2 suppresses Bcl6—a central Tfh transcription factor—and consequently Tfh development (6). This implies a direct relationship between the production of IL-2 by naïve CD4⁺ T cells and their development into either non-Tfh or Tfh effector cells. Here, we have explored this relationship using transgenic mice engineered to report the expression of IL-2.

IL-2 and Bcl6 expression co-segregate within hours of naïve T-cell activation

IL-2.eGFP reporter mice were generated by the targeted insertion of an IRES-eGFP expression cassette into the fourth exon of the endogenous IL-2 gene (Fig. 1A). Naïve CD4⁺ T cells from IL-2.eGFP mice stimulated under non-polarizing conditions in vitro diverged into CD69⁺IL-2⁺ (GFP⁺) and CD69⁺IL-2[−] (GFP[−]) subpopulations within hours of activation and prior to cell division (Fig. 1B–E). Reporter expression was rapidly detectable and peaked at approximately 24 hours before declining. This decline significantly lagged production of IL-2 due to the relatively long half-life of the reporter. To define genes differentially expressed by IL-2 producers and non-producers, CD69⁺IL-2⁺ and CD69⁺IL-2[−] cells were analyzed by RNA-seq (Fig. 1C). Among 151 genes that were preferentially expressed by IL-2⁺ cells were *Bcl6* and the TNF superfamily member *CD40lg*, which are important in Tfh cell development or function, respectively. Also enriched in IL-2⁺ cells was *Zbtb32*, which, like *Bcl6*, encodes a member of the POK/ZBTB family of transcription factors and has been shown to restrict expression of Th1 and Th2 cytokines (7). In contrast, among the 210 genes preferentially expressed by IL-2[−] cells were multiple genes characteristic of non-Tfh effector cell differentiation, including *Prdm1*, which encodes Blimp1, as well as *S1pr1* and *Klf2*. Similar results were obtained from an analysis of naïve SMARTA TCR transgenic IL-2.eGFP CD4⁺ T cells stimulated with antigen (fig. S1). *S1pr1* is required for the egress of non-Tfh effector CD4⁺ T cells from secondary lymphoid tissues (8), and its expression inhibits Tfh development in vivo (9, 10). *Klf2* was recently shown to suppress Tfh differentiation while promoting non-Tfh effector cell differentiation, at least in part via the induction of Blimp-1 (9). These findings, which were independently validated by

qPCR (Fig. 1D, E), suggested that IL-2 producers may be fated to become Tfh cells, whereas IL-2 non-producers may be fated to become non-Tfh effector cells. Akin to findings in CD4⁺ T cells, differential expression of *Bcl6* and *Blimp1* was found in IL-2⁺ and IL-2⁻ subsets isolated from activated naïve CD8⁺ T cells (fig. S2). This suggests that, despite their lower production of IL-2 relative to CD4⁺ T cells, early divergence of CD8 T cells destined to become *Blimp1*⁺ short-lived effector cells (SLEC) or *Bcl6*⁺ memory precursor effector cells (MPEC) (11) may be similarly linked to differential expression of IL-2.

The differential expression kinetics of *Bcl6* and *Prdm1* by CD4⁺ T cells were discordant (Fig. 1D). *Bcl6* expression tracked with *Il2* expression and decayed to background levels as *Prdm1* expression increased. Indeed, at the peak of differential *Bcl6* expression (8 h), *Prdm1* expression remained at background levels in both IL-2⁺ and IL-2⁻ cells. Thus, although these transcription factors are believed to be directly antagonistic in the specification of Tfh versus non-Tfh effectors (12), the rapid, reciprocal expression of *Bcl6* in IL-2⁺ and IL-2⁻ fractions was not controlled by *Blimp1*. Instead, we found differential expression of the gene encoding *Mxd1*, or *Mad1* (Figs. 1C and S3), which has been shown to directly bind and down-regulate *Bcl6* during the differentiation of germinal center B cells into plasma cells (13). The contemporaneous, reciprocal expression of *Mxd1* and *Bcl6* antecedent to the expression of *Prdm1* suggests that repression of *Bcl6* by *Mxd1*, rather than *Blimp1*, may contribute to the early bifurcation of Tfh and non-Tfh effectors (Figs. 1D and S3).

Although *Bcl6*, like *Blimp1*, often acts as a transcriptional repressor, the parallel kinetics of *Il2* and *Bcl6* expression suggested that *Bcl6* may positively regulate *Il2* expression. Thus, we performed chromatin immunoprecipitation (ChIP) analysis of conserved non-coding sequences in the *Il2* promoter and 35kb upstream that were identified by ATAC-seq analysis as uniquely accessible in IL-2⁺ cells compared with naïve and IL-2⁻ cells (Fig. 2A). *Bcl6* preferentially bound these sites in IL-2⁻producing cells relative to IL-2 non-producers, at a time point (20 h) when expression of *Bcl6* and *Blimp1* overlapped (Fig. 2B). *Blimp1* preferentially bound these sites in IL-2⁻ cells, as did *Foxo1*, which was recently shown to suppress Tfh differentiation (14). The permissive histone modification H3K4me3 was significantly enriched in IL-2⁺ cells at the sites of *Bcl6* binding, whereas repressive H3Kme27 histone marks were reduced in both IL-2⁺ and IL-2⁻ cells relative to naïve cells. Thus, the expression of *Il2* correlates positively with *Bcl6* binding at sites of induced chromatin accessibility in the *Il2* gene locus, and negatively with binding of *Blimp1* (and *Foxo1*) at the same sites. Because expression of *Prdm1* significantly trailed the peak of differential *Il2* expression (Fig. 1D), occupancy of these sites by *Blimp1* did not appear to be required for the repression of *Il2* early in IL-2⁻ cells. Rather, *Blimp1* may act primarily to reinforce the lack of *Il2* expression in the IL-2⁻ fraction of activated naïve T cells at later time points. This is consistent with *Blimp1*'s reported role as a feedback inhibitor of IL-2 (15, 16). In any case, these findings indicate that, in addition to its predictive value in defining early precursors of Tfh and non-Tfh effector cells, expression of IL-2 may be directly regulated by the antagonistic actions of *Bcl6* versus *Blimp1* and *Foxo1* at conserved *cis*-regulatory elements in the *Il2* gene locus.

Because Tfh cell development occurs concurrently with each of the CD4⁺ T effector cell pathways, we determined if the correlation between reciprocal expression of *Bcl6*–*Blimp1*,

and IL-2 occurred under Th1-, Th2-, and Th17-cell polarizing conditions (Fig. 3A), as it did for Th0 cells (Figs. 1 and 3). Under each of these activation conditions, expression of the IL-2.eGFP reporter was limited to a subset of cells expressing the highest CD69 (Fig. 3A). GFP also correlated positively with *Bcl6* expression and negatively with *Prdm1* expression (Fig. 3B). Cells activated under Th17 conditions expressed the highest frequency and single-cell levels of IL-2, despite the reported suppression of Th17 differentiation by IL-2 signaling (17). Thus, *Il2* and *Bcl6* expression mirrored each other under each of the conditions examined (Fig. 3B), with the highest levels of *Il2* and *Bcl6* found in IL-2⁺ cells activated under Th17 conditions. This may reflect the shared requirement for IL-6 in both Th17 and Tfh developmental programs.

IL-2 signaling is predominantly paracrine

Although the foregoing studies suggested a link between *Il2* gene expression and Tfh–non-Tfh fate determination, the differentiation of physiologic Tfh cells *ex vivo* is not yet established. Thus, we examined this relationship *in vivo*, in the context of infection with ActA-deficient *Listeria monocytogenes* (ActA-Lm). This attenuated type 1 bacterial pathogen was engineered to express peptide antigens that enabled the tracking of endogenous antigen-specific CD4⁺ T-cell responses using peptide-loaded MHCII (p:IA^b) tetramers or transferred TCR transgenic T cells (2, 18). Naïve CD4⁺ T cells from CD45.2⁺ IL-2.eGFP-SMARTA TCR-transgenic mice were transferred into WT CD45.1⁺ mice and infected with ActA-Lm expressing ovalbumin (OVA) peptide and the gp66 peptide recognized by the SMARTA TCR (ActA-Lm-OVA-gp66). Antigen-activated SMARTA T cells were recovered near the peak of IL-2 expression and sorted into IL-2⁺ and IL-2[−] fractions for differential gene expression analysis by RNA-seq (Fig. 4A). In agreement with our *in vitro* findings (Fig. 1C), IL-2⁺ cells were significantly enriched for expression of *Il2*, *Bcl6*, *Zbtb32* and *CD40lg*, whereas IL-2[−] cells were significantly enriched for *Prdm1*, *Slpr1*, and *Klf2*. Multiple Th1 cell-associated transcripts (e.g., *Ifng*, *Il12rb2*, *Ltb* and *Gzmb*) were identified in IL-2[−] cells, consistent with the induction of type 1 immunity by ActA-Lm. Gene set enrichment analysis (GSEA) identified enhanced activity of multiple effector signaling pathways in IL-2[−] cells, most significantly among which were interferon and inflammatory signaling gene sets (Fig. 4B). In contrast, the most significantly enhanced gene sets in IL-2⁺ cells were those of Myc and the E2F family of transcription factors (Figs. 4B and S4A,B). Both are involved in cell cycle regulation and are suppressed by Blimp1 in germinal center B cells (19, 20). *Mxd1*, which was again one of the most highly enriched transcripts in IL-2[−] cells, antagonizes Myc by competing for binding to a shared dimerization partner, Max (21). Myc expression by T cells correlates directly with strength of activation (22, 23), implicating a role for Mxd1 in restraining the actions of Myc in less strongly activated T cells and consistent with expression of IL-2 by more strongly activated naïve CD4⁺ T cells.

Notably, *Il2ra*, which encodes the inducible, high-affinity component of the IL-2 receptor (IL-2Rα or CD25) that is up-regulated on activated T cells, was enriched in IL-2[−] cells (Fig. 4A). Accordingly, the hallmark IL-2–Stat5 signaling gene set was significantly enriched in IL-2[−] cells (Fig. 4B). Among a manually curated consensus list of 23 gene sets modulated by IL-2 signaling (fig. S4C), those up-regulated in response to IL-2 were enriched in IL-2[−]

cells, many of which include *Il2ra* (fig. S4D, E), whereas genes down-regulated in response to IL-2 were enriched in IL-2⁺ cells.

Collectively, these findings support a model in which highly activated naïve T cells up-regulate Bcl6, produce IL-2, and are fated to become Tfh effectors. IL-2 producers deliver IL-2 to non-producers, inducing the latter's up-regulation of Blimp1 and differentiation into non-Tfh effectors. To examine the relationship between IL-2 production and utilization in vivo, and directly address the fate of IL-2 producers and non-producers, we generated a second transgenic IL-2 reporter mouse line with features complementary to those of the IL-2.eGFP mice (Fig. 4C). IL-2.BAC-in Thy1.1 (2BiT) reporter mice were engineered to express high levels of the surface molecule Thy1.1 under control of the IL-2 gene locus to facilitate intracellular co-staining by flow cytometry and enable the in vivo deletion of IL-2 producing cells (24). As with T cells from IL-2.eGFP mice, activated (CD69⁺) 2BiT T cells rapidly bifurcate into Thy1.1⁺ (IL-2⁺) and Thy1.1⁻ (IL-2⁻) fractions (Figs. 4D and S5). To determine whether IL-2 production and signaling segregate in antigen-activated naïve CD4⁺ T cells, 2BiT mice were infected with ActA-Lm and analyzed for the expression of IL-2 versus intracellular phospho (p)-Stat5 at the peak of IL-2 expression (Fig. 4E). Reciprocal IL-2 expression and IL-2 signaling were observed; Thy1.1 (IL-2) was almost exclusively expressed by p-Stat5⁻ CD4⁺ T cells (Fig. 4E), whereas p-Stat5 was limited to Thy1.1⁻ cells. Consistent with gene expression results (Figs. 4A), nearly all Thy1.1⁺ cells were CD25⁻ at this time point, while nearly all p-Stat5⁺ cells were CD25⁺. Thus, IL-2 signals predominantly in a paracrine, not autocrine, manner (25). Moreover, IL-2 producers are initially resistant to IL-2 signaling, in accord with their lack of CD25 up-regulation.

The majority of endogenous p-Stat5⁺ CD4⁺ T cells immediately following infection are Foxp3⁺ regulatory T (Treg) cells (25), due to their constitutive expression of CD25 and relative abundance compared to naïve clonal precursors. To examine IL-2-induced Stat5 signaling in naïve pathogen-specific non-Treg cells, naïve CD45.2⁺ 2BiT-SMARTA T cells were transferred into CD45.1⁺ WT mice infected with ActA-Lm-OVA-gp66 (Fig. 4F). Analysis of transferred 2BiT-SMARTA (clonotypic) and endogenous CD4⁺ T cells showed that the majority of endogenous p-Stat5⁺ cells were Foxp3⁺, whereas p-Stat5⁺ clonotypic T cells were Foxp3⁻. Thus, the paracrine model of IL-2 signaling applies to both “bystander” Treg cells as well as naïve CD4⁺ T cells responding to infection.

Tfh cells are derived from IL-2 producers

To examine the fate of antigen-activated IL-2 producing and non-producing T cells in vivo, 2BiT mice were treated with a depleting anti-Thy1.1 or non-depleting control antibody (24) immediately prior to infection with ActA-Lm co-expressing OVA and the antigenic peptides gp66, 2W1S, Cbir1 or FliC (Figs. 5 and S6). MHCII tetramer analysis of endogenous CD4⁺ T cells specific for each of these peptides showed that IL-2 (Thy1.1) expression was restricted to CXCR5⁺ cells and that depletion of IL-2-expressing cells preferentially eliminated Tfh cells and spared non-Tfh (Th1) effectors, as defined by expression of CXCR5 and PD1 (Figs. 5A and S6) or CXCR5 and Bcl6 (Fig. 5B). Notably, the number of non-Tfh effectors was not compromised by the depletion of IL-2 producers (Fig. 5C). This suggested that IL-2 was not required for the clonal expansion of non-Tfh effectors. However,

this likely reflects discordant kinetics of IL-2 secretion relative to reporter expression and antibody-mediated cell depletion, as examination of IL-2 reporter expression and Stat5 phosphorylation were only partially decreased at the peak of IL-2 expression (fig. S7). Thus, Tfh effector cells developed from IL-2-expressing precursors, whereas non-Tfh effectors did not. Accordingly, IL-2 was a reliable marker with which to distinguish precursors fated to become Tfh or non-Tfh effector cells.

Although surface markers define Tfh cells capable of providing B-cell help, Tfh function is predicated on a subset of Tfh cells that localize to the germinal center following productive interactions with cognate B cells. Referred to as germinal center (GC-Tfh) T cells, these cells express high levels of PD-1, Bcl6 and CXCR5, and support the germinal center response and production of high-affinity, class-switched antibodies (12). To examine the effects of depletion of IL-2 expressing precursors on the development and function of this Tfh cell subset, we characterized the effects of anti-Thy1.1 depletion on antibody responses and the generation of GC-Tfh cells. 2BiT mice were immunized with heat-killed Lm (HKLM) (Fig. 5D–F), because infection with live *Listeria monocytogenes* does not induce good antibody responses (26). Anti-Thy1.1 depletion of IL-2 producing T cells reduced the production of Lm-specific IgG by over 90% compared to treatment with an isotype control antibody (Fig. 5D). Similarly, anti-Thy1.1 treatment of 2BiT mice immunized with ovalbumin (OVA) under type 1 conditions markedly impaired the anti-OVA IgG response, in association with the depletion of endogenous OVA-specific Tfh cells and reduction of germinal center B cells (fig. S8).

To examine the effects on GC-Tfh cell differentiation, we transferred equivalent numbers of congenically marked 2BiT and GFP-expressing WT CD4⁺ T cells into T cell-deficient (*Tcrb*^{−/−}) mice, which were immunized with HKLM (Fig. 5 E, F). Anti-Thy1.1 treatment selectively depleted CD45.1⁺ 2BiT PD1⁺/CXCR5⁺ T cells, with no significant effect on CD45.2⁺ GFP⁺ control T cells (Fig. 5E). The magnitude of reduction was highest among PD1^{hi}/CXCR5^{hi} cells. Immunohistology revealed that Thy1.1 depletion dramatically reduced the number of 2BiT CD4⁺ T cells found within germinal centers, with a compensatory increase in the numbers of WT GFP⁺ T cells, resulting in no change in total numbers of germinal center T cells or B cells (Fig. 5F). These data establish that functional Tfh effectors that populate germinal centers and provide help for class-switched antibody responses in response to type 1 pathogens develop from IL-2-producing precursors.

To extend these findings, we determined whether the in vivo depletion of IL-2⁺ cells also selectively targeted Tfh cells under conditions of type 2 (Th2) and type 3 (Th17) immune induction (Fig. 6). Anti-Thy1.1 treatment of 2BiT mice immunized with OVA using the Th2-inducing adjuvant, alum, resulted in specific depletion of OVA-specific Tfh cells and ablation of germinal center B cell and anti-OVA antibody responses, while sparing OVA-specific non-Tfh effectors (Fig. 6A). Similarly, anti-Thy1.1 depletion of 2BiT mice challenged with the Th17-inducing enteric pathogen, *Citrobacter rodentium* (27), resulted in loss of clearance of the bacterium, with kinetics that are characteristic of an impaired anti-*Citrobacter* antibody response (28, 29). This was associated with depletion of Tfh cells specific for the immunodominant *Citrobacter* antigen, intimin, as well as a markedly impaired germinal center B cell response (Fig. 6B). There was no significant decrease in

Th17 and Th1 cells in infected spleens (Fig. 6C)—both of which are characteristic of the T-cell response against *Citrobacter* (27), yet a modest, but significant, decrease in Th17 cells, but not Th1 cells, was observed in the mesenteric lymph nodes of mice depleted of IL-2 producers (Fig. 6D). Although these findings indicate that in the context of type 3 responses there is some overlap in the developmental fate of IL-2-producing precursors of Tfh and Th17 effector cells, they also establish that IL-2⁺ T cells are precursors for Tfh cells in the context of type 1, type 2 and type 3 effector responses.

IL-2 production and Tfh differentiation correlate with aggregate TCR signal strength

The developmental divergence of Tfh and non-Tfh cells is influenced by a combination of cell-intrinsic factors, including TCR affinity, and cell-extrinsic factors, such as antigen availability, strength of costimulation and cytokine milieu. Given its direct correlation with Tfh–non-Tfh fate determination, we examined whether IL-2 expression shared similar mechanistic underpinnings. To examine the relationship between antigen dose, IL-2 expression and Tfh–non-Tfh specification on T cells of uniform TCR affinity, naïve CD45.2⁺ IL-2.eGFP-SMARTA T cells were transferred into WT CD45.1⁺ recipients, which were infected with various doses of ActA-Lm expressing the gp66 peptide (ActA-Lm-OVA-gp66; “Lm-gp66”). Non-specific effects of Lm-induced inflammatory signals were excluded by keeping the total dose of ActA-Lm constant via co-infection with ActA-Lm expressing an irrelevant specificity (ActA-Lm-OVA-2W1S; “Lm-2W1S”) (Fig. 7 A, B). Splenic CD45.2⁺ cells were analyzed for CD69 and GFP (IL-2) expression around the peak of IL-2 expression (Fig. 7A), and frequencies and absolute numbers of endogenous gp66-specific Tfh and non-Tfh cells were quantified near the peak of the effector T-cell response (Fig. 7B). The expression of both CD69 and GFP correlated tightly with pathogen-expressed antigen dose, as did the magnitude of clonal expansion and reciprocal Tfh versus non-Tfh differentiation. There was a similar correlation over a broader dose range of ActA-Lm-OVA-gp66 administered alone (fig. S9). Thus, the frequencies of CD69 and IL-2 positive cells correlated positively with Lm-gp66 dose, as did the generation of Tfh cells. This indicates that antigen dose—and consequently TCR signal strength—is a major determinant of the fraction of clonal precursors that express IL-2 and are fated to become Tfh cells.

The relative development of Tfh and non-Tfh effectors is also influenced by TCR affinity, which is invariant on individual T-cell clones but varies between different clones within the repertoire (2). In agreement with the strong correlation between differential IL-2 expression and Tfh–non-Tfh bifurcation, we found that two clonal populations of the same precursor frequency, but differing TCR specificities (OVA vs. gp66) and affinities, produced significantly different frequencies of IL-2 expressing T cells in response to the same antigen dose despite no difference in the frequency of cells expressing CD69 (Fig. 7C). Similarly, the relative frequencies of endogenous Tfh and non-Tfh cells generated by two TCR specificities of differing TCR affinities diverged in response to the same antigen dose (Fig. 7D). In accord with these results, it was found that Tfh cells were characterized by a significantly greater 2D TCR affinity than non-Tfh cells that developed in a polyclonal T-cell response to the same antigen (gp66) (Fig. 7E). With our antigen dose results, these

findings support a deterministic function of TCR signal strength in driving Tfh versus non-Tfh development (2), with higher TCR signaling favoring Tfh development.

In order to calculate 2D affinity values, normalized adhesion bond measurements must be adjusted to control for TCR density (30). Although equivalent in cell size, gp66-specific Tfh cells expressed more TCR molecules per cell than non-Tfh cells (Fig. 7E). Similar results were found for OVA-specific and total Tfh cells. Accordingly, the difference in normalized adhesion bond was accounted for by differences in both TCR affinity and TCR number per cell. We therefore examined the influence of variation in TCR number on T-cell activation and IL-2 production (fig. S10A). As expected, there was no difference in 2D TCR affinity of CD69⁺IL-2⁺ SMARTA IL-2.eGFP T cells compared to CD69⁺IL-2⁻ cells. However, when naïve SMARTA IL-2.eGFP T cells were stimulated with limiting concentrations of gp66 peptide, only cells expressing the highest levels of Vα2 up-regulated CD69 and IL-2 (Fig. 7F). Moreover, when naïve T cells were sorted on the basis of high or low expression of TCRβ and stimulated with a range of anti-CD3 concentrations, cells with higher TCR numbers showed higher CD69 expression across the full range of anti-CD3 concentrations, and expressed increased IL-2 (fig. S10B). Thus, in addition to intrinsic TCR affinity differences between T-cell clones, variation in TCR number within a clonal population may influence the probability that a given cell will exceed a threshold for activation and expression of IL-2, and therefore Tfh versus non-Tfh differentiation.

The expression of CD69 by activated naïve T cells has been shown to correlate linearly with expression of Nur77 and Myc, providing an indicator of graded TCR signal intensity (22, 31). Yet, based on the observed limitation of IL-2 expression to a subset of the highest CD69 expressors (Figs. 3A and 7A), our findings suggested that only those T cells that exceeded a minimum TCR signaling intensity produced IL-2, linking Tfh–non-Tfh bifurcation to a threshold of TCR signaling. To explore this further, the relative expression of CD69 and IL-2 by naïve T cells was assessed under conditions in vitro where only the intensity of TCR stimulation was varied (Fig. 8A). The percentage of cells expressing CD69 and IL-2 correlated positively with anti-CD3 dose, as did expression levels of CD69 and IL-2—consistent with our in vivo antigen dose experiments (Figs. 7A and S8A). However, although the distribution and mean expression of CD69 varied with the intensity of TCR stimulation, CD69 expression among IL-2⁺ cells was constant, with only cells exceeding an invariant, high magnitude of CD69 expressing IL-2. A similar effect was seen with the induction of ICOS (fig. S11A), expression of which is a functional marker of Tfh differentiation (32). ICOS expression was highest among IL-2⁺ cells across a range of stimulation conditions and, although the mean expression of ICOS increased with the intensity of TCR stimulation among IL-2⁻ cells, its expression by IL-2⁺ cells was constant. The expression of ICOSL by APCs was not required for induction of IL-2 (fig. S11B). Thus, under conditions where only TCR signaling strength is varied, there is a minimum threshold for IL-2 expression and by extension Tfh cell differentiation.

The expression of Myc, which has been directly correlated with the number of cell divisions T cells are fated to undergo (23), was tightly correlated with CD69 expression and thus TCR signal intensity and IL-2 expression (Figs. 8B). The early expression of E2F family members, which are associated with cell cycle entry, was limited to cells that exceeded a

threshold for IL-2 expression. This was consistent with the gene set enrichment data (Figs. 4B and S4), which indicated that E2F family targets are strongly enriched in IL-2⁺ cells compared to IL-2⁻ cells. This suggests that IL-2⁺ cells enter cell cycle more rapidly than IL-2⁻ cells and are likely to undergo a greater number of cell divisions before exiting cell cycle. Accordingly, Tfh effectors demonstrated an increase in the average number of cell divisions relative to non-Tfh effectors in vivo (Fig. 8C). Thus, although IL-2 has traditionally been viewed as a T-cell growth factor, precursors of Tfh cells, which do not respond to IL-2 despite producing it, appear programmed for earlier cell cycle entry and more cell divisions than precursors of non-Tfh effectors that do respond to IL-2.

Our results support a model whereby cell-intrinsic and -extrinsic variables that influence TCR signal strength contribute to a threshold that is tightly associated with IL-2 production and Tfh differentiation. Although TCR affinity and antigen dose clearly contribute to the probability that a cell will attain this threshold, costimulation and cytokine signals can also contribute. It has been shown that TGFβ signaling can attenuate TCR signaling (33) and limit T-cell responses to high affinity antigens (34). Indeed, TGFβ addition significantly reduced the mean expression of CD69 by activated naïve T cells and the proportion of CD69⁺ cells that produced IL-2, as well as the mean expression of IL-2, whereas TGFβ blockade had the opposite effect (fig. S12). However, the percentage of cells that expressed CD69 was unaltered by either intervention. Thus, in addition to modulating TCR-independent signaling cascades that control effector T-cell specification, TGFβ may also influence IL-2 production by limiting TCR signal strength without limiting the frequency of naïve T cells that receive activating TCR signals (35). In this regard, it is notable that the addition of IL-6 overrode the repressive effects of TGFβ (Th17 conditions; Fig. 3A), resulting in significantly higher IL-2 expression—indeed, greater than any other T-effector polarizing conditions. Because the major effects of IL-6 are independent of TCR signal strength, and its actions contribute to both Tfh and Th17 development, clearly TCR-independent factors that modulate IL-2 production may impact Tfh–non-Tfh developmental decisions. Furthermore, in view of the shared requirement for IL-6-induced STAT3 signaling and high production of IL-21 by both Th17 and Tfh cells, these data suggest significant overlap in the developmental programming of these two subsets, and perhaps shared regulation of the tightly syntenic *Il2* and *Il21* loci (Fig. 2A).

DISCUSSION

Findings in this study provide new insights into the mechanics that control the early bifurcation of CD4⁺ T cells into Tfh and non-Tfh effectors, placing reciprocal production and utilization of IL-2 at the center of this key developmental decision. Because divergent IL-2 signaling and Bcl6 expression have been linked to effector versus central memory, respectively (36), the current findings may also have implications for alternate programming of CD4⁺ T-cell memory. Our findings predict that IL-2 non-producers are fated for effector memory, whereas IL-2 producers are fated for central memory.

It has been proposed that asymmetric cell division results in the partitioning of factors that guide the divergent development of progeny of activated naïve T cells (37). Although the observation herein that Tfh–non-Tfh fate determination is initially encoded well prior to cell

division does not preclude a role for asymmetric cell division, it does suggest that signaling between T cells that receive differing activation signals likely plays a dominant role. It has been shown that homotypic T-cell conjugation mediated by LFA-1–ICAM interactions between activated T cells facilitates directional, paracrine delivery of IL-2 via multifocal synapses (38). The expression of *Icam1* was enhanced on IL-2⁺ cells in the current study (Figs. 1C and 4A). Because the kinetics of IL-2 production are within the average dwell time of T cells that form long-lived interactions on an activating dendritic cell (39–41), our findings suggest that T-T cell interactions that result in directional IL-2 signaling between IL-2 producers and non-producers may occur on the same DC, although this will require further study. The role of Treg cells in buffering IL-2 availability to non-IL-2 producers due to their constitutive expression of the high affinity IL-2 receptor and high avidity LFA-1 will also require further study.

Results in this report indicate that naïve CD4⁺ T cells that receive differing strengths of TCR signals by p:MHC complexes—whether the result of intrinsic TCR affinity or expression differences or receipt of contemporaneous non-TCR signals that augment or repress TCR signal strength—stratify into those that exceed a threshold that predisposes to IL-2 production and early Tfh commitment, and those that fail to express IL-2 yet are programmed to receive IL-2 signaling that reinforces non-Tfh effector commitment. However, although the exceedance of this threshold appears necessary, it is not always sufficient, as some cells that express comparable levels of CD69 do not express IL-2, implying the contribution of additional factors yet to be defined. Moreover, although IL-2 expression strongly correlates with Tfh versus non-Tfh fate determination in a primary response, this correlation is not fixed for subsequent responses (fig. S13). Thus, IL-2 expression by Tfh precursors does not insure IL-2 expression by Tfh effectors in a recall response, nor does lack of IL-2 expression by non-Tfh precursors preclude IL-2 expression by non-Tfh effectors. Nevertheless, the utility of IL-2 as an early marker for cells fated to these different effector programs is established herein, and will provide an opportunity for discovery of new factors that determine the bifurcation into Tfh and non-Tfh effectors, as exemplified by the finding of a possible Mxd-Myc-Max axis in controlling the early differential expression of Bcl6. This should provide a basis for novel strategies to modulate the balance of effector T-cell responses for therapeutic ends.

MATERIALS AND METHODS

Mice

B6.Cg-Tg-*IL-2*^{tm1(eGFP)Weav} (IL-2.eGFP) and B6.IL-2.BAC-inThy1.1 (2BiT) were generated using strategies previously described (23) and bred at the University of Alabama at Birmingham (UAB) animal facility. B6N-*Tyr*^{c-Brd}/BrdCrCrI (albino B6) and B6-LY5.2/Cr (congenic B6 CD45.1) were purchased from Frederick Cancer Center and intercrossed to produce albino B6.CD45.1. C57BL/6 (WT B6), *Tcrb*^{-/-} (B6.129P2-*Tcrb*^{tm1Mom}/J), OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice and mice transgenic for constitutive eGFP expression (C57BL/6-Tg(CAG-EGFP)^{1310sb/LeySop}/J) were purchased from The Jackson Laboratory. SMARTA Tg (Tg(TcrLCMV)1Aox) (35) on a B6 background were a generous gift from Dr. A. Zajac (Dept. of Microbiology, UAB). All intercrosses to generate additional strains, such

as SMARTA.IL-2.eGFP, SMARTA.2BiT, SMARTA.IL-2.eGFP Thy1.1⁺, OT-II.IL-2.eGFP, and 2BiT.CD45.1 were generated by crosses in UAB's breeding facility. Animals were bred and maintained under specific pathogen-free conditions in accordance with institutional animal care and use committee regulations.

Tissue processing and flow cytometric analysis

Mice were sacrificed by isoflurane euthanasia before removal of spleen and/or lymph nodes. Secondary lymphoid tissues were disrupted by mashing with a syringe in complete RPMI-1640 over a 70- μ m filter. Surface staining was performed in PBS with 2% FBS and 0.1% sodium azide. T-cells from 2BiT animals were directly stained for surface Thy1.1 (clone HIS5-1) without secondary stimulation. For identification of Tfh, cells were incubated with biotinylated anti-CXCR5 for 1 h at room temperature, then washed and incubated with streptavidin-APC or PE-Cy7 and additional surface markers for 20 m at 4°C. Intracellular staining for transcription factors was performed using either BD Fix/Perm or eBioscience Foxp3 staining kits. For ex-vivo phospho-stat staining, freshly harvested splenocytes were fixed for 10 m at 37°C in 4% PFA in PBS, stained with eFluor450- or PacBlue-conjugated anti-Thy1.1, re-fixed with 4% PFA in PBS and permeabilized in 90% MeOH for 30 m on ice. Following this, cells were stained for phosphorylated Stat5 and additional markers at room temperature for 1 h. Alternatively, cells were fixed in 4% PFA in PBS for 10 m at 37°C, permeabilized in 90% MeOH for 30 m on ice, then stained for 1 h at room temperature.

Absolute T- and B-cell numbers were calculated using PKH reference beads (Sigma-Aldrich) or concentration values (events per microliter) obtained on the Attune NxT. Absolute TCR β numbers were calculated using BD Quantibrite Beads Fluorescence Quantification Kits. Briefly, cells were stained at saturating concentrations of PE-labeled anti-TCR β (clone H57-597). A standard curve generated from PE Quantibrite Beads was then used to transform TCR β MFI measurements into absolute quantifications.

All flow cytometry data were acquired on an Attune NxT (Thermo Fisher Scientific), LSRII, or an Aria II (BD Immunocytometry Systems, San Jose, CA) and analyzed with FlowJo software (Tree Star, Eugene, OR).

T-cell isolation

Naïve T-cell isolation—Polyclonal CD62L^{hi}CD25⁻CD44^{lo}CD4⁺ and CD8⁺ T-cells were purified from single-cell suspensions of secondary lymphoid tissues (spleen with or without axillary, brachial, cervical, mesenteric, inguinal, and medial iliac lymph nodes) in two stages. First, CD8⁺ or CD4⁺ T-cells were isolated using Dynabeads (ThermoFisher 11445D), MACS Cell Separation (Miltenyi Biotec 130-104-454) or prepared by negative selection against CD8 or CD4, MHC class II, CD11b, B220, and CD25 (all Abs labeled with FITC) using anti-FITC BioMag particles (Polysciences, Warrington, PA). Second, cells were purified by sorting on a FACS Aria II, gating on the CD4⁺CD25⁻CD62L^{hi}CD44^{lo} (and in some cases IL-2.Thy1.1⁻/IL-2.eGFP⁻) fraction. Naïve SMARTA TCR Tg cells were sorted directly as above from lymph node and splenic tissue.

Activated T-cell isolation—Cells cultured in vitro were harvested at various time-points, re-suspended in labeling buffer (2% FBS in PBS), and FAC-sorted a second time as CD4⁺ or CD8α⁺ CD69⁺ and either IL-2.eGFP⁺/Thy1.1⁺ or as IL-2.eGFP[−]/Thy1.1[−]. SMARTA IL-2.eGFP T-cells isolated ex vivo from acutely-activated recipient mice were processed from tissue by negative selection with biotinylated antibodies to CD11b, CD11c, and B220, streptavidin-conjugated microbeads, and LS columns (Miltenyi Biotech). Column flow-through fractions were then stained for a congenic marker (Thy1.1, Thy1.2, or CD45.2) prior to sorting as above.

In vitro T-cell activation

Sorted naïve T-cells were activated in complete RPMI-1640 (RPMI medium containing 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 50 µM β-mercaptoethanol and 2 mM l-glutamine) for 4–36 hs with anti-CD3 (2.5 µg mL^{−1}) or 1 µg mL^{−1} LCMV glycoprotein peptide 66–77, anti-CD28 (1 µg mL^{−1}), and irradiated splenocytes at a 5:1 ratio of splenocytes to T-cells under non-polarizing conditions (i.e. without additional cytokines or antibodies). In some experiments, sorted naïve T-cells were activated with a range of plate-bound anti-CD3 concentrations and 0.5 µg/mL anti-CD28 or a range of soluble anti-CD3 concentrations and irradiated feeders at a CD4:feeder ratio of 1:5.

For re-stimulation of splenic Tfh and non-Tfh cells, magnetically enriched splenic CD4⁺ T-cells were stained simultaneously with tetramer and biotin-labeled anti-CXCR5 (see table above) for 1 h at room temperature. Cells were then washed and stained with Fluorophore-labeled streptavidin and PD1 for 20 m at 4°C. Labeled cells were then incubated for 4 h in complete RPMI with 2 µg/mL anti-CD28 on flat-bottom 96-well plates pre-coated with 5 µg/mL anti-CD3. Following re-stimulation, cells were stained for CD69, CD44, CD4, additional surface markers and viability dye for 15–20 m at 4°C.

Adoptive transfer and Ab-mediated in vivo depletion

For adoptive transfer experiments examining IL-2.eGFP or IL-2.Thy1.1 expression at early time points, 1–2.5×10⁶ naïve cells were injected i.v. into congenic recipient mice infected 24 h prior to transfer unless otherwise indicated. For experiments involving co-transfer of Smarta.IL-2.eGFP and OT-IL-2.eGFP donor cells, 5×10⁵ sorted naïve cells of each donor strain were injected retro-orbitally (RO) into mice infected 24 h prior to transfer. For adoptive transfer experiments examining Tfh differentiation 3 or more days following transfer, 5×10⁴ sorted naïve donor cells were injected RO into congenic recipients infected 24 h prior to transfer unless otherwise indicated. For co-transfer into TCRβ KO recipients, 1×10⁶ magnetically-enriched bulk CD4⁺ T-cells from wild-type B6 or transgenic eGFP and CD45.1 2BiT congenic mice were injected RO into TCRβ-deficient mice followed by infection one day after transfer. For depletion of IL-2.Thy1.1 (2BiT) cells in vivo, mice were given a single intra-peritoneal injection of 250 µg anti-Thy-1.1 or isotype monoclonal antibody 24 h prior to infection or immunization.

Infections and protein immunizations

Lm—Mice were immunized i.v. with 200µL PBS containing live (dose as indicated) or heat-killed Actin A-deficient *Listeria monocytogenes* (ActA⁻Lm; 2×10^9 – 2×10^{10}) (42). All Lm strains used were transformed by a plasmid containing OVA_{250–387} and one of 4 different I-A^b-specific ‘foreign’ peptides: a) a mutant epitope of I-Ea (‘2W1S’); b) flagellin peptide 456–475 from *Clostridium* (‘Cbir1’); c) glycoprotein 66–77 peptide of LCMV (‘gp66’); d) flagellin peptide 427–441 from *Salmonella typhimurium* (‘FliC’) all expressed under the control of the *hly* (listeriolysin O) promoter. All Lm strains were produced in the laboratory of Dr. S-S Way as previously described (36). Bacteria were grown in brain-heart infusion (BHI) medium with 15 µg/mL chloramphenicol to an absorbance of >0.1 at 600 nm, and doses varied as indicated. The actual number of live bacteria injected was confirmed by dilution and growth on BHI agar plates containing chloramphenicol.

OVA/CFA—Mice were immunized i.p. with 100µL of a 100µg chicken egg ovalbumin emulsion in CFA.

OVA/Alum—Mice were injected i.p. with 200µL of a 0.5mg/mL emulsion of chicken egg ovalbumin in alum. The emulsion was prepared by mixing 1mg/mL ovalbumin dissolved in water (Invivogen vac-pova) 1:1 with Alum (FisherScientific Imject Alum 77161).

Citrobacter rodentium—Mice were orally gavaged with 1 – 2×10^9 cfu of *Citrobacter rodentium* strain DBS100 (ATCC 51459) or the bioluminescent ICC180 derivative (generously provided by G. Frankel and S. Wiles, Imperial College London). Mice infected with the ICC180 derivative were shaved and imaged with an IVIS 100 Imaging System (Xenogen, Inc.) as previously described (28).

RNA-sequencing and analysis

For sample preparation and hybridization, total RNA was isolated from purified naïve (CD4⁺ or CD8a⁺, CD25⁻ CD69⁻ CD44^{lo} CD62L⁺ IL-2.eGFP⁻) and activated (CD4⁺ or CD8a⁺, CD69⁺ IL-2.eGFP⁺ or IL-2.eGFP⁻) T-cells with Qiazol and miRNeasy micro kits according to manufacturer’s recommendations (Qiagen). Library preparation was performed using Illumina TruSeq technology. Samples were processed at UAB Heflin Center for Genomic Science for Next Generation Sequencing (NGS) or La Jolla Institute (LJI) using the Illumina HiSeq2000 Sequencing System. Reads were mapped to the mm10 genome using TopHat (version 2.0.12) (43). BAM files were sorted using SAMtools (version 0.1.19) (44), and reads were counted for each gene using HTSeq (version 0.6.1) (45) and NCBI *Mus musculus* Annotation Release 106 (GRCm38.p4). RNA expression was normalized using the rlog function from the DESeq2 R package (version 1.8.2) (46). Differential gene expression analysis was performed using DESeq2, and *p*-values were corrected with the Benjamini–Hochburg procedure. Volcano plots were created using the ggrepel R package. To calculate gene set enrichment, a differential expression probability density function (PDF) was determined for each gene using Quantitative Set Analysis for Gene Expression (QuSAGE) (47). PDFs were combined for each gene set to calculate gene set activity after correcting for gene-gene correlation. Gene set PDFs were compared using Welch’s *t*-test and *p*-values were adjusted using the Benjamini–Hochberg procedure.

Statistical analysis

P values were calculated using unpaired Student's *t*-tests, Welch's *t*-tests, and one-way or two-way ANOVA tests with Tukey's post-hoc multiple comparisons analysis. A *p*-value of <0.05 was considered significant. See figure legends for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Differential expression of Bcl6 and Blimp1 by IL-2⁺ and IL-2⁻ T cells.

(A) Gene targeting strategy for the generation of IL-2.eGFP knock-in reporter mice. The loxP-flanked neomycin resistance cassette was deleted by crossing founders to EIIa-Cre transgenic mice. (B) Sorted naïve (GFP⁺CD44⁺CD62L⁺) IL-2.eGFP CD4⁺ T cells were labeled with CellTraceViolet, stimulated in vitro with soluble anti-CD3 (5μg/mL) and irradiated CD4-depleted feeder cells, then examined for expression of CD69 and IL-2.eGFP by flow cytometry at the indicated time points. Data are representative of four experiments with at least three replicates per condition. CTV staining performed in two of four experiments. (C) Total RNA isolated from naïve IL-2.eGFP CD4⁺ T cells stimulated for 18–

24 h as in B and FACS-purified into CD69⁺GFP⁻ (IL-2⁻) or CD69⁺GFP⁺ (IL-2⁺) fractions was analyzed by comparative expression profiling using RNA-seq. Data depict two biological replicates per condition. **(D)** RNA isolated from IL-2.eGFP CD4⁺ T cells stimulated and FACS-purified as in C was analyzed by qPCR for expression of *Il2*, *Bcl6* and *Prdm1* at the indicated time points. Error bars represent SEM of three technical replicates per sample. Data are representative of four experiments. **(E)** Validation of selected transcript expression using RNA isolated from IL-2.eGFP CD4⁺ T cells stimulated and FACS-purified as in C. Three technical replicates per sample shown. Data were analyzed using Student's *t*-tests and are representative of two experiments. For all experiments: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Error bars depict SEM.

Fig. 2. Differential chromatin accessibility and transcription factor binding at the IL-2 locus in IL-2⁺ and IL-2⁻ T cells.

(A) ATAC-seq was performed on nuclei isolated from naïve (GFP⁻CD44⁻CD62L⁺) IL-2.eGFP CD4⁺ T cells and FACS-purified CD69⁺GFP⁺ (IL-2⁺) and CD69⁺GFP⁻ (IL-2⁻) fractions treated as in Fig. 1C. Chromatin accessibility peaks were visualized using IGB browser and are shown aligned against a VISTA plot of syntenic regions of human and mouse chromosomes corresponding to *Il2-Il21/IL2-IL21* gene loci. Data are representative of two experiments. (B) Naïve IL-2.eGFP CD4⁺ T cells were treated as in Fig. 1C, and the IL-2 promoter region (*Il2p*) and conserved non-coding sequence 35kb upstream of the *Il2* transcription start site (CNS-35kb) of CD69⁺GFP⁺ (IL-2⁺) and CD69⁺GFP⁻ (IL-2⁻) fractions were analyzed by quantitative ChIP-PCR for the presence of Bcl6, Blimp1, and Foxo1 binding, or H3K4me3 and H3K427me3 histone modifications, normalized to total DNA input. Three technical replicates per group. Data for each region analyzed separately by one-way ANOVA. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Error bars depict SEM.

Fig. 3. Bcl6 and IL-2 co-segregate early in each T effector cell developmental program.

(A) Naive (GFP⁻CD44⁻CD62L⁺CD69⁻CD25⁻) IL-2.eGFP CD4⁺ T cells were stimulated in vitro under Th0, Th1, Th2 and Th17 conditions for 20 h and examined by flow cytometry for CD69 and IL-2.eGFP expression. Data are representative of 2 experiments. Flow plots depict cell number-controlled concatenated averages of three samples per group. Error bars depict SD. (B) Experiment performed as in A, with CD69⁺ IL-2.eGFP⁺ and IL-2.eGFP⁻ CD4⁺ T cells sorted 20 h after activation. RNA was isolated and analyzed by qPCR for expression of *Il2*, *Bcl6* and *Prdm1*. Three technical replicates per condition are shown. Error bars depict SEM. Data for A and B are representative of two experiments each.

Fig. 4. IL-2⁺ T cells activate IL-2⁻ T cells via paracrine IL-2 signaling to drive differential gene expression in vivo.

(A) Sorted naïve (GFP⁻CD44⁻CD62L⁺) IL-2.eGFP CD45.2⁺ SMARTA CD4⁺ T cells were transferred into CD45.1⁺ WT mice infected with ActA-Lm-gp66 24 h prior to transfer. Total RNA was isolated by FACS-purified CD45.2⁺ CD69⁺GFP⁺ (IL-2⁺) and CD69⁺GFP⁻ (IL-2⁻) CD4⁺ T cells 20–24 h after transfer and analyzed by RNA-seq. Data depict three biological replicates per condition from three separate experiments. (B) Hallmark gene set enrichment analysis of IL-2⁺ and IL-2⁻ T cells from A. For each pathway, mean and 95% confidence

intervals are plotted then color-coded to indicate false discovery rate corrected *p*-values. **(C)** Schematic of targeting strategy to generate IL-2.Thy1.1 Bac-In (2BiT) transgenic reporter mice. **(D)** Sorted naïve (Thy1.1⁻CD44⁻CD62L⁺) 2BiT CD4⁺ T cells were stimulated in vitro with soluble anti-CD3 (5µg/mL) and irradiated CD4-depleted feeder cells for 24 h then examined by flow cytometry for expression of CD69 and Thy1.1. RNA isolated from CD69⁺Thy1.1⁺ (IL-2⁺) and CD69⁺Thy1.1⁻ (IL-2⁻) CD4⁺ T cells was analyzed by qPCR for expression of *Ii2* mRNA. Error bars represent SEM of three technical replicates per sample. Data are representative of two experiments. **(E)** 2BiT mice were infected with ActA-Lm. After 18 h mice were sacrificed and splenic CD4⁺ T cells were analyzed by flow cytometry for the expression of IL-2.Thy1.1, CD25, and tyrosine phosphorylation of Stat5 (p-Stat5). Data are representative of two experiments. **(F)** Congenic CD45.1⁺ WT mice were infected with ActA-Lm-gp66. Twenty-four hours later, naïve CD45.2⁺ SMARTA 2BiT CD4⁺ T cells were transferred into infected CD45.1⁺ recipients. Mice were sacrificed at the indicated times, and splenic CD4⁺ T cells were analyzed for expression of Thy1.1, Foxp3 and p-Stat5. Data are representative of two experiments.

Fig. 5. IL-2 producers are precursors of Tfh cells.

(A-C) 2BiT mice were injected with 250 μ g anti-Thy1.1 or isotype control mAb, then infected 1 day later with ActA-Lm-gp66. Endogenous CD4⁺ T cells specific for IA^b-gp66 were enriched from lymph nodes and spleens 3 d following infection using tetramer-based magnetic sorting and analyzed by flow cytometry for IA^b-gp66 tetramer binding and expression of Ly6C, CXCR5, IL-2.Thy1.1 and PD-1 (A) or Bcl6 (B). Flow plots depict cell-number-controlled concatenated averages of all samples within a group. Data for A and B are representative of two experiments each. (C) Data from the experiments depicted in A and supplemental fig. S6 were analyzed by two-way ANOVA. A total of eight control and eight treatment animals from two separate experiments are shown. (D) 2BiT mice were injected with 250 μ g anti-Thy1.1 or isotype control mAb and immunized with 2 $\times$ 10¹⁰ CFU heat-killed Lm (HKLm). Mice were bled every 6 d for 24 days, and serum anti-LM IgG was

measured by ELISA. $n = 7$ per group. Data are representative of two experiments. (E) Magnetically enriched WT CD45.1⁺ and 2BiT CD45.2⁺ CD4⁺ T cells were transferred into TCR β -deficient mice (*Tcrb*^{-/-}). Twenty-four hours later, mice were immunized with 2×10^{10} CFU HKLm and injected with 250 μ g anti-Thy1.1 or isotype control mAb. Mice were sacrificed 5 d following immunization, and splenic CD4⁺ T cells were analyzed by flow cytometry for expression of CD44, CD45.1, CD45.2, PD-1, and CXCR5. Results were analyzed by two-way ANOVA. $n = 3$ per group. Data are representative of three experiments. (F) CD4⁺ T cells magnetically enriched from WT CAG-eGFP (CD45.2) mice and CD45.1⁺ 2BiT mice were adoptively transferred into TCR β -deficient recipients. Twenty-four hours later, the mice were immunized with 2×10^{10} CFU HKLm and injected with 250 μ g anti-Thy1.1 or isotype control mAb. Two weeks following immunization, spleens were collected and analyzed by confocal microscopy for the expression of GFP (WT), CD45.1 (2BiT), Ki67 and IgD. Quantitation of WT (GFP⁺), 2BiT (CD45.1⁺) and total T-cell numbers in germinal centers was done using computer-assisted counting. Splenic B cells were analyzed by flow cytometry for the expression of IgD, B220, GL7 and Fas in an IgD^{lo} B-cell gate. $n = 3$ per group. Data are representative of three experiments. For all experiments: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Error bars depict SEM.

Fig. 6. IL-2 producers are fated to become Tfh cells in type 2 and type 3 immune responses.

(A) 2BiT mice were injected with 250 μ g anti-Thy1.1 or isotype control. Twenty-four hours later, they were immunized with OVA emulsified in Alum. Mice were bled and sacrificed at day 12. Splenic IA^b-OVA tetramer-specific CD4⁺ T cells were analyzed by flow cytometry for the expression of CD44, PD-1, and CXCR5. Splenic B cells were analyzed for the expression of B220, IgD, GL7 and Fas. Serum anti-OVA IgG was measured by ELISA. $n = 5$ to 6 per group. (B) 2BiT mice were injected with 250 μ g anti-Thy1.1 or isotype control. Twenty-four hours later they were orally gavage with 1–2 $\times 10^9$ CFU *Citrobacter rodentium* strain DBS100 (ATCC 51459) or the bioluminescent ICC180 derivative. Whole body bioluminescence was tracked and quantified after infection. Splenic IA^b-Int884C tetramer-specific CD4⁺ T cells harvested on day 14 were analyzed by flow cytometry for the expression of CD44, PD-1, and CXCR5. Splenic B cells were analyzed for expression of B220, IgD, GL7 and Fas. $n = 5$ to 6 per group. Flow plots depict cell-number-controlled

concatenated averages. Data are representative of two experiments. **(C+D)** Splenic (C) and MLN (D) CD4⁺ T cells harvested from mice treated as in (B) were isolated, re-stimulated with PMA and ionomycin then analyzed by flow cytometry for expression of CD44, Foxp3, IFN γ , and IL-17A. Flow plots and bar graphs are gated on CD4⁺CD44⁺Foxp3⁻ cells. Flow plots depict cell-number-controlled concatenated averages. $n = 5$ to 6 per group. For all experiments: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data were analyzed using Student's t -tests. Error bars depict SEM.

Fig. 7. IL-2 production and Tfh differentiation correlate with TCR signal strength.

(A) WT CD45.1⁺ recipient mice were infected with ActA-Lm-OVA-gp66 and/or ActA-Lm-OVA-2W1S at the indicated doses. After 24 h, 10⁶ naïve (GFP-CD44-CD62L⁺) SMARTA IL-2.eGFP CD4⁺ T cells were adoptively transferred into infected hosts. Splenic CD4⁺ T cells were harvested 15 h following transfer and analyzed for expression of CD69 and IL-2.eGFP by flow cytometry. Values in the larger boxes of flow cytometric plots represent percentages of CD69⁺ cells, and values in the smaller boxes represent percentages IL-2.eGFP⁺ cells within the CD69⁺ fraction. *n* = 4 per group. Data are representative of 2

experiments. **(B)** WT mice were infected with ActA⁻Lm-OVA-gp66 and/or ActA⁻Lm-OVA-2WIS at the indicated doses. Five days later, magnetically enriched endogenous splenic CD4⁺ T cells were analyzed by flow cytometry for binding of IA^b-gp66 tetramer and expression of CD44, CXCR5, and PD-1. $n = 3$ per group. Data are representative of two separate experiments. **(C)** 2D affinity measurements were performed on splenic TCR transgenic CD4⁺ T cells via micropipette adhesion frequency assays with biotinylated pMHC IA^b-gp66- and IA^b-OVA3C monomers. Log-normalized data are shown. WT CD45.1⁺ recipient mice were infected with ActA⁻Lm-OVA-gp66. After 24 h, 0.5×10^6 naïve (GFP⁻CD44⁻CD62L⁺CD69⁻CD25⁻) SMARTA IL-2.eGFP and OTII IL-2.eGFP CD4⁺ T cells were pooled and adoptively transferred into infected hosts. Splenic CD4⁺ T cells were harvested 18 h following transfer and analyzed for expression of CD45.1, CD45.2, Vβ5, CD69 and IL-2.eGFP by flow cytometry. Values in the larger boxes of flow cytometric plots depicting CD69 and IL-2.eGFP represent percentages of CD69⁺ cells, and values in the smaller boxes represent the percentages of IL-2.eGFP⁺ cells within the CD69⁺ fraction. $n = 3$ per group. Data are representative of 2 experiments. **(D)** WT mice were infected with ActA⁻Lm-OVA-gp66. Five days after infection, magnetically enriched endogenous splenic CD4⁺ T cells were co-stained with IA^b-gp66- and IA^b-OVA3C tetramers and analyzed by flow cytometry for expression of CD44, CXCR5, and PD-1. Data are representative of 4 experiments. **(E)** WT mice were infected with 2.5×10^7 CFU ActA⁻Lm-OVA-gp66. Enriched splenic CD4⁺ T cells harvested 5 d following infection were stained for IA^b-gp66, CD44, TCRβ, PD-1 and CXCR5. Log-normalized 2D affinity measurements were performed on FACS-purified IA^b-gp66 tetramer-positive splenic Tfh and non-Tfh cells pooled from 3–5 animals. TCR β quantifications were performed on unsorted aliquots stained separately. Data from 2 experiments are shown. **(F)** Naïve (GFP⁻CD44⁻CD62L⁺) SMARTA IL-2.eGFP CD4⁺ T cells were stimulated for 16 h with irradiated CD4-depleted feeder cells and 1 μg/mL gp66 and analyzed by flow cytometry for expression of CD69, Vα2 and IL-2.eGFP. Data are representative of 2 experiments. For all experiments: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data were analyzed using Student's *t*-tests. Error bars depict SEM.

Fig. 8. IL-2 producers and Tfh exhibit enhanced cell cycle progression.

(A) Naïve (GFP⁺CD44⁺CD62L⁺CD69⁺CD25⁻) IL-2.eGFP CD4⁺ T cells were stimulated for 18 h with indicated concentrations of plate-bound anti-CD3 and 1 μg/mL soluble anti-CD28 then analyzed for expression of CD69 and IL-2.eGFP by flow cytometry. The MFI of CD69 expression within the CD69⁺GFP⁻ and CD69⁺GFP⁺ gates was quantitated for the indicated concentrations of anti-CD3 (right). Three technical replicates per condition. Experiment performed three times. (B) Naïve (GFP⁺CD44⁺CD62L⁺CD69⁺CD25⁻) IL-2.eGFP CD4⁺ T cells were stimulated in vitro with soluble anti-CD3 (2.5 μg/mL), soluble anti-CD28 (0.5 μg/mL) and irradiated CD4-depleted feeder cells. qPCR was performed on CD69⁺ IL-2.eGFP⁺ and IL-2.eGFP⁻ CD4⁺ T cells sorted 20 h after activation. Three technical replicates per condition shown. Data are representative of two experiments and were analyzed by one-way ANOVA. (C) WT CD45.1⁺ recipient mice were infected with ActA-Lm-OVA-gp66. After 24 h, 5 × 10⁴ CTV-labeled naïve (GFP⁺CD44⁺CD62L⁺CD69⁺CD25⁻) SMARTA CD4⁺ T cells were adoptively transferred into infected hosts. Splenic CD4⁺ T cells harvested 3 d following transfer were analyzed for expression of CD44, PD-1 and CXCR5 by flow cytometry. *n* = 4 per experiment. Experiment performed three times. For all experiments: ns, *p* > 0.05; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; ****, *p* < 0.0001. Error bars depict SEM.