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The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding

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How follicular helper T cells (T_{FH} cells) differentiate to regulate B cell immunity is critical for effective protein vaccination. Here we define three transcription factor T-bet–expressing antigen-specific effector helper T cell subsets with distinguishable function, migratory properties and developmental programming *in vivo*. Expression of the transcriptional repressor Blimp-1 distinguished T zone 'lymphoid' effector helper T cells ($CD62L^{hi}CCR7^{hi}$) from $CXCR5^{lo}$ 'emigrant' effector helper T cells and $CXCR5^{hi}$ 'resident' T_{FH} cells expressing the transcriptional repressor Bcl-6 ($CD62L^{lo}CCR7^{lo}$). We then show by adoptive transfer and intact polyclonal responses that helper T cells with the highest specific binding of peptide–major histocompatibility complex class II and the most restricted T cell antigen receptor junctional diversity 'preferentially' developed into the antigen-specific effector T_{FH} compartment. Our studies demonstrate a central function for differences in the binding strength of the T cell antigen receptor in the antigen-specific mechanisms that 'program' specialized effector T_{FH} function *in vivo*.

Vaccination with protein induces high-affinity B cell memory for long-term protection against infectious disease. Antigen-specific helper T cells with many effector functions are the central cognate regulators of this B cell immune response *in vivo*[1,2]. Follicular helper T cells (T_{FH} cells) have emerged as another lineage of helper T cells functionally subspecialized to regulate many facets of the development of effector and memory B cells[3,4]. Understanding the antigen-specific mechanisms that control the differentiation of effector T_{FH} cells is critical for the design of future vaccines but remains poorly resolved *in vivo*.

The appropriate placement of antigen-specific T_{FH} cells during immune responses is important for the cognate delivery of effector function to antigen-primed B cells[5,6]. Loss of the chemokine receptor CCR7 and expression of the chemokine receptor CXCR5 relocate effector T_{FH} cells to the B cell areas of secondary lymphoid tissue[7,8]. In these locations, expression of the costimulatory molecules CD40L[9], ICOS[10,11], PD-1 (ref. 12) and OX40 (ref. 13) helps modify antigen-specific contact with B cells expressing peptide–major histocompatibility complex (MHC) class II (pMHCII). The cytokines interleukin 4 (IL-4; A001262), IL-10 (ref. 6) and interferon-γ (IFN-γ)[12] can also serve an important regulatory function at this cognate interface to affect subsequent development and function of antigen-specific B cells. Early studies of the differentiation program of T_{FH} cells indicated involvement of expression of the transcriptional repressor Bcl-6 (A000369)[13], local lymphoid priming[14], expression of the ICOS ligand on B cells, IL-6, IL-21 (A001258), the transcription factor STAT3 (ref. 15), the strength of T cell antigen receptor (TCR) signaling through greater abundance of the Vav1 guanine nucleotide–exchange factor[16] and the duration of stable T cell–B cell contacts controlled by signaling lymphocytic activation molecule–associated proteins[17]. To delineate the cognate mechanisms of effector T_{FH} differentiation, it remains necessary to define the dynamics of the development of antigen-specific effector T_{FH} cells and distinguish those cells from the many other antigen-specific effector helper T cells that may arise after local protein vaccination.

The strength of TCR signaling affects lineage commitment during thymic development, and in the periphery, it is important for the establishment of selection thresholds for fitness and survival *in vitro*[18,19] and clonal expansion after antigen priming *in vivo*[20–22]. It has also been shown that the strength of the pMHCII signal to the TCR influences helper T cell function differently both *in vitro*[23] and *in vivo* with TCR-transgenic helper T cells[24,25]. Helper T cells with strong pMHCII binding 'preferentially' emerge in the draining lymph nodes and persist locally as a unique compartment of antigen-specific memory T_{FH} cells[14]. However, it remains unclear whether the distribution of TCRs with substantial pMHCII binding strength is a consequence of local lymphoid priming or whether intrinsic TCR signal strength causally directs the effector T_{FH} developmental program.

To address those issues, it is important to connect the functional development of antigen-specific helper T cells with TCR binding strength and repertoire diversity. The I-E^k-restricted helper T cell response of B10.BR mice to pigeon cytochrome *c* (PCC) is the only tractable protein vaccination model available at present with this capacity[26,27]. Antigen-specific helper T cells can be detected with antibodies to expressed variable (V) regions such as $V_\alpha 11$ and $V_\beta 3$, as well as pMHCII tetramers presenting the dominant peptide epitope. Responding pMHCII-specific helper T cells express a restricted TCR

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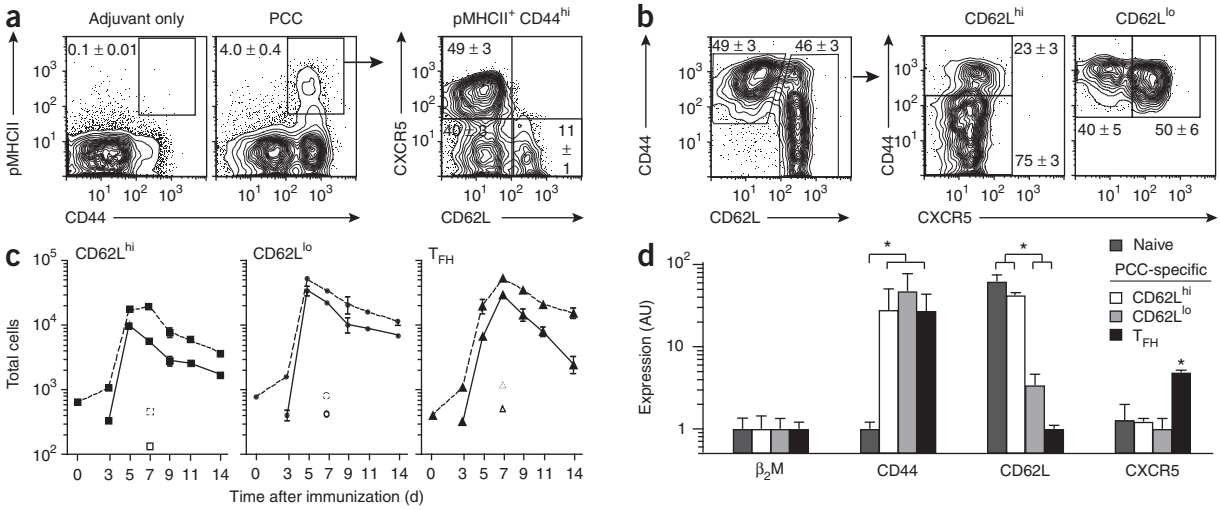

Figure 1 Three subsets of antigen-specific effector helper T cells emerge in draining lymph nodes. (**a**,**b**) Expression of CD62L and CXCR5 (**a**) or CD44 and CXCR5 (**b**) on the surface of cells from draining lymph nodes (inguinal and periaortic) of B10.BR mice ($n \geq 6$) 7 d after priming with PCC (**a**,**b**) or adjuvant only (**a**). (**a**) PCC-specific effector helper T cells (propidium iodide–negative (PI^-), $B220^-CD8^-CD11b^-V_\alpha11^+$pMHCII$^+$CD44^{hi}). (**b**) Helper T cells ($PI^-B220^-CD8^-CD11b^-V_\alpha11^+V_\beta3^+$, and either CD62L^{hi} or CD62L^{lo}). Numbers adjacent to outlined areas or in quadrants indicate percent cells in each (mean ± s.e.m.). (**c**) Total PCC-specific $V_\alpha11^+V_\beta3^+$CD44^{hi} (dashed lines) or $V_\alpha11^+$pMHCII$^+$CD44^{hi} (solid lines) effector helper T cells over time in draining lymph nodes after PCC priming ($n \geq 3$ mice for each time point). Open symbols indicate estimate of total cells from B10.BR mice 7 d after adjuvant-only priming. (**d**) Expression of mRNA immediately after the isolation of 2×10^3 naive helper T cells ($V_\alpha11^+V_\beta3^+$CD44^{lo}CD62L^{hi}) or PCC-specific helper T cells ($V_\alpha11^+V_\beta3^+$CD44^{hi}) from lymph nodes on day 7 after subcutaneous priming ($n = 3$ mice). Estimates are presented in arbitrary units (AU) relative to β_2-microglobulin mRNA (β_2M), set as 1. PCC-specific: CD62L^{hi}, CD62L^{hi}CXCR5^{lo}; CD62L^{lo}, CD62L^{lo}CXCR5^{lo}; T_{FH}, CD62L^{lo}CXCR5^{hi}. *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of at least three experiments.

repertoire with demonstrable clonal diversity[26,27] and a range of TCR-binding properties[28,29]. In this model, antigen-specific effector helper T cells have many effector functions[30] and changes in TCR triggering associated with the response to antigen[31]. After vaccination with protein, intrinsic TCR affinity thresholds regulate clonal selection and the composition of pMHCII-specific clones in the local draining lymphoid tissues[28,29]. Hence, it is critical to determine whether similar TCR affinity–based mechanisms exist for the regulation of the function of antigen-specific T_{FH} cells.

Here we subcategorized the total antigen-specific helper T cell compartment into three subsets of CD44^{hi}ICOS^{hi} effector helper T cells. All antigen-specific subsets expressed similar amounts of IL-2, IL-10, IFN-γ and the transcription factor T-bet. Antigen-specific 'lymphoid' effector helper T cells expressing Blimp-1 (A003268) retained expression of L-selectin (CD62L) and CCR7, confining their effector activity to the T cell zones of responding lymph nodes. In contrast, most antigen-specific helper T cells that developed locally exited the lymph nodes as CD62L^{lo}CCR7^{lo} 'emigrant' effector helper T cells. There were also many Bcl-6-expressing CXCR5^{hi} T_{FH} cells that remained 'resident' in lymph nodes with the highest expression of IL-4, IL-21, PD-1 and unique expression of CD69 and OX40 in the B cell zones to regulate antigen-primed B cells. Naive antigen-specific helper T cells with TCR of higher affinity 'preferentially' skewed into the CXCR5^{hi} 'resident' T_{FH} compartment. Increasing doses of agonist peptide over 3 d *in vitro* induced maximum IL-21 expression and lower Blimp-1 expression without affecting Bcl-6 expression. In the polyclonal model, TCRs with the strongest pMHCII binding and more restricted junctional diversity 'preferentially' skewed into the antigen-specific effector T_{FH} compartment. Furthermore, vaccine adjuvants that promoted responders of higher affinity also induced the largest number of antigen-specific T_{FH} cells *in vivo*. Thus, we demonstrate that antigen-specific mechanisms for 'programming' the differentiation of effector T_{FH} cells *in vivo* are causally related to the strength of TCR binding and the diversity of the responding helper T cell population. Manipulating these mechanisms *in vivo* will underpin the formulation of the next generation of protein vaccines.

RESULTS

Three subsets of antigen-specific effector helper T cells

We immunized mice subcutaneously at the base of tail with whole protein antigen in the monophosphoryl lipid A–based adjuvant Ribi. Maximum accumulation of PCC-specific effector helper T cells in draining lymph nodes was reached by the end of the first week after subcutaneous vaccination of B10.BR mice with protein[26]. Using pMHCII binding (**Fig. 1a**) and upregulation of CD44 on $V_\alpha11^+V_\beta3^+$ helper T cells (**Fig. 1b**), we separated PCC-specific helper T cells into three distinct subsets on the basis of their expression of CXCR5 and CD62L. Two CXCR5^{lo} subsets reached peak numbers by day 5 (**Fig. 1c**) and could be distinguished by their abundance of CD62L protein and mRNA (**Fig. 1d**). CXCR5 expression was present in the third major subset of antigen-specific helper T cells that had lost CD62L expression and reached peak numbers by day 7. Quantitative evaluation of mRNA expression *in vivo* correlated well with protein expression for CD44, CD62L and CXCR5 (**Fig. 1d**). Thus, after protein vaccination, CXCR5^{hi} T_{FH} cells constitute only half of the antigen-specific helper T cells that accumulate in the draining lymph node.

Blimp-1-expressing 'lymphoid' effector helper T cells

All three antigen-specific helper T cells had the qualities of effector cells *in vivo* by day 7. All subsets had higher *in vivo* surface expression of CD28 protein and mRNA (**Fig. 2a**) and ICOS protein and mRNA (**Fig. 2b**). There was much higher expression of IL-2, IFN-γ and IL-10 mRNA without *in vitro* restimulation (**Fig. 2c**), which indicated similar effector cell potential for all subsets *in vivo*. The CD62L^{hi}

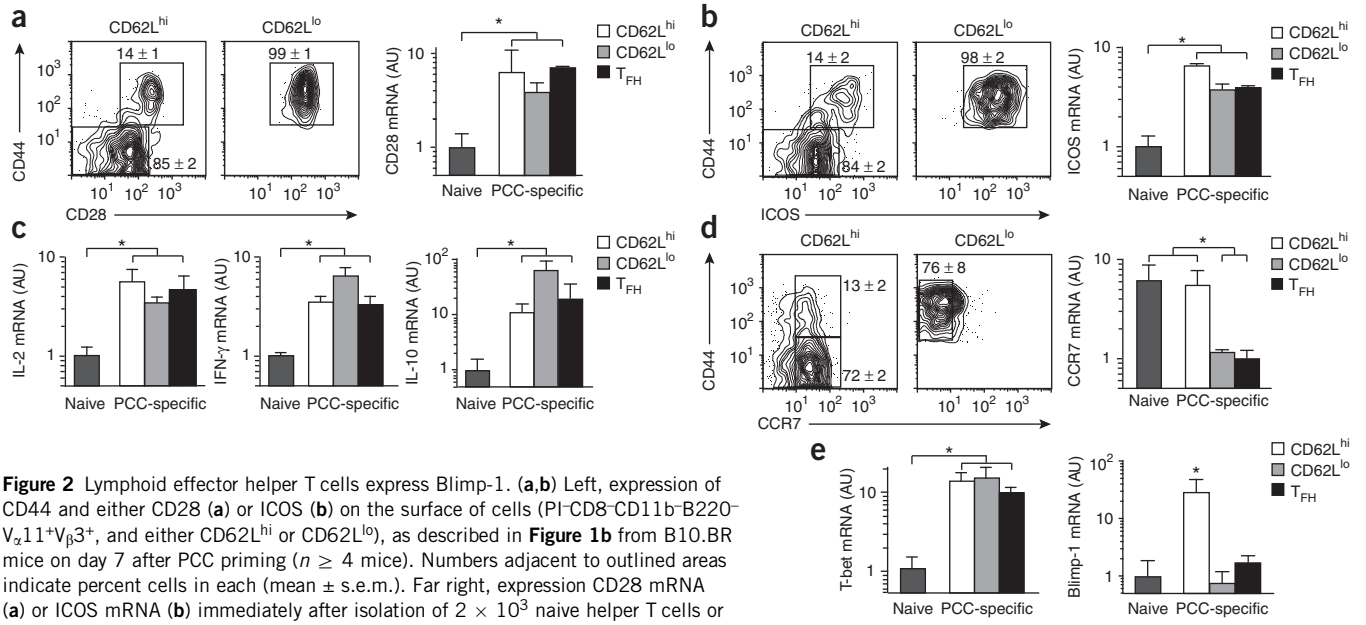

Figure 2 Lymphoid effector helper T cells express Blimp-1. (**a**,**b**) Left, expression of CD44 and either CD28 (**a**) or ICOS (**b**) on the surface of cells ($PI^-CD8^-CD11b^-B220^-V_\alpha11^+V_\beta3^+$, and either $CD62L^{hi}$ or $CD62L^{lo}$), as described in **Figure 1b** from B10.BR mice on day 7 after PCC priming ($n \geq 4$ mice). Numbers adjacent to outlined areas indicate percent cells in each (mean ± s.e.m.). Far right, expression CD28 mRNA (**a**) or ICOS mRNA (**b**) immediately after isolation of 2×10^3 naive helper T cells or PCC-specific helper T cell subsets (as described in **Fig. 1d**) from lymph nodes on day 7 after priming. (**c**) Expression of IL-2, IFN-γ and IL-10 mRNA, analyzed as described in **Figure 1d**. (**d**) Expression of CD44 and CCR7, analyzed as described in **Figure 1d**. (**e**) Expression of T-bet and Blimp-1 mRNA, analyzed as described in **Figure 1d**. Estimates for mRNA are presented in arbitrary units (mean and s.e.m.) relative to results obtained for naive cells, set as 1 ($n = 3$ mice). *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of three experiments.

subset also retained expression of CCR7 similar to that of naive helper T cells (**Fig. 2d**), which indicated a capacity for lymphoid recirculation and placement in the T cell zones, even during an active immune response. As for the transcriptional regulation of effector cell differentiation, all subsets had similarly higher expression of T-bet than that of naive helper T cells, but only the $CD62L^{hi}CXCR7^{hi}$ subset expressed Blimp-1 at this stage of the immune response (**Fig. 2e**). On the basis of the cell surface phenotype, we refer to the Blimp-1-expressing subset as 'lymphoid' effector helper T cells.

Most helper T cells are 'emigrant' effector helper T cells

Sphingosine 1-phosphate receptor agonists such as FTY720 and its chiral analog AAL-R (2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol) inhibit the migration of and promote the lymph node retention of lymphocytes[32,33]. We found many more antigen-specific helper T cells in draining lymph nodes after 7 d of treatment with AAL-R than after treatment with vehicle only (**Fig. 3a** and **Supplementary Fig. 1** online). There were no changes in the number of antigen-specific T_{FH} cells, which indicated the 'resident' nature of this effector helper T cell compartment. The 'lymphoid' effector helper T cells also remained mostly unchanged, which suggests that this T zone–localized effector compartment delivers its function locally during this acute phase of the immune response. In contrast, there were over ten times more $CD62L^{lo}CCR7^{lo}CXCR5^{lo}$ effector helper T cells after AAL-R treatment (**Fig. 3a**), which suggested that most of these effector helper T cells developed locally but exited the draining lymph node during this first week after priming. Furthermore, there were considerably fewer antigen-specific helper T cells in the spleen after AAL-R treatment, which could be accounted for mostly by the lower number of $CD62L^{lo}CXCR5^{lo}$ effector helper T cells than in untreated mice (**Supplementary Fig. 2** online). Thus, we refer to this $CD62L^{lo}CXCR5^{lo}$ antigen-specific compartment as 'emigrant' effector helper T cells to emphasize their most distinguishing cellular attribute *in vivo*.

Bcl-6-expressing 'resident' effector T_{FH} cells

In addition to CXCR5, the negative regulator of T cell activation PD-1 has been reported to be present on a germinal center subset of T_{FH} cells[34]. We found that similar fractions of $CD44^{hi}CD62L^{lo}$ antigen-specific helper T cells and cells of the $CXCR5^+$ T_{FH} subset expressed PD-1 protein and mRNA (**Fig. 3b**). Although all three populations of effector helper T cells expressed more PD-1 mRNA than did naive helper T cells, the effector T_{FH} compartment had significantly higher expression than did the other two subsets. Cytokines IL-4 and IL-21, which are also associated with the generation and function of effector T_{FH} cells[1–4], were expressed by all three effector helper T cell subsets, but the effector T_{FH} compartment had significantly higher expression (**Fig. 3c**). In contrast, high expression of CD69 (**Fig. 3d**) and OX40 (**Fig. 3e**) 'assorted' uniquely into the effector T_{FH} compartment. As for the transcriptional control of effector compartments, Bcl-6 was also uniquely expressed by the effector T_{FH} compartment and was not present in the other two subsets at this stage of the acute response (**Fig. 3f**). Therefore, higher expression of PD-1, IL-4 and IL-21 and unique expression of CD69, OX40 and Bcl-6 distinguish the 'resident' effector T_{FH} compartment that is 'placed' in the B cell zones of lymph nodes to deliver these activities to antigen-primed B cells.

High-affinity precursors develop into 'resident' T_{FH} cells

To begin to assess the involvement of TCR affinity in the regulation of effector T_{FH} function, we used the 5C.C7 (higher affinity) and 2B4 (lower affinity) PCC-specific TCRαβ-transgenic helper T cell populations in adoptive-transfer experiments[22,28]. We transferred small numbers of CD90.2-expressing transgenic cells into otherwise replete unmanipulated recipient mice, which allowed the emergence of endogenous CD90.1-expressing PCC-specific responses (**Supplementary Fig. 3** online). The frequency of recruited 5C.C7 helper T cells differed from that of the 2B4 cells with no indication of the differences in the dynamics of clonal expansion or contraction expected in these vaccination conditions[28,29]. Nevertheless, a significantly larger fraction

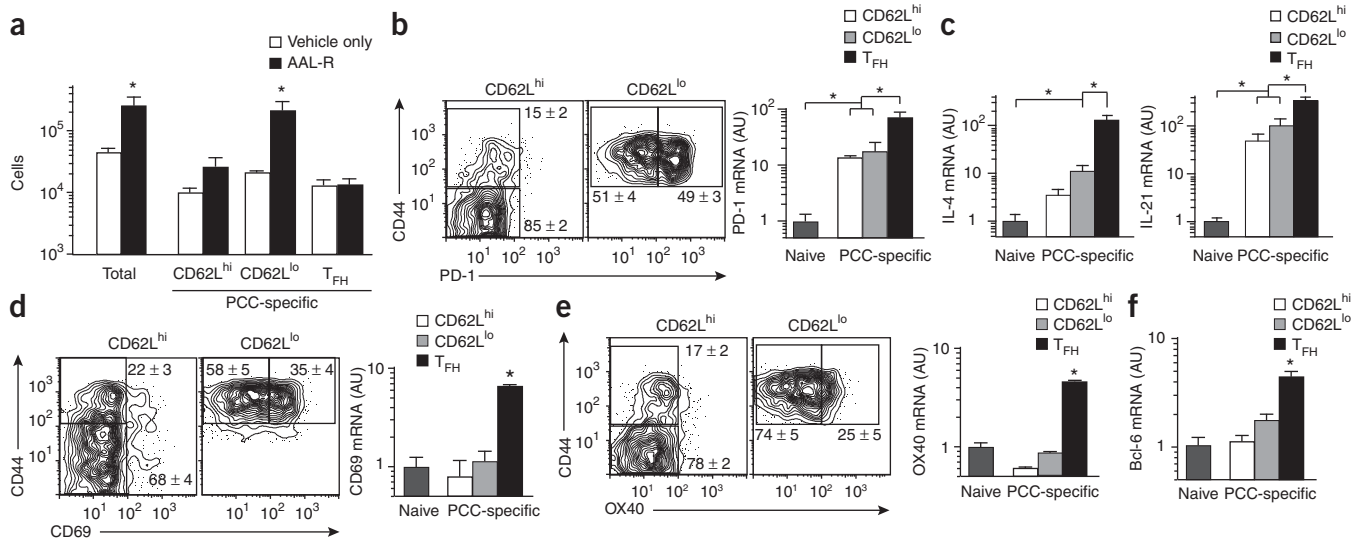

Figure 3 'Resident' effector T_{FH} cells express Bcl-6. (**a**) Total PCC-specific effector helper T cells ($V_\alpha11^+V_\beta3^+CD44^{hi}$) and PCC-specific effector helper T cell subsets (horizontal axis, as described for **Fig. 1d** key) from lymph nodes on day 7 after priming of mice ($n = 3$) treated with AAL-R or vehicle only (mean and s.e.m.). (**b**) Left, expression of CD44 and PD-1 on the surface of lymph node cells ($CD8^-CD11b^-B220^-PI^-V_\alpha11^+V_\beta3^+$ and either $CD62L^{hi}$ or $CD62L^{lo}$) from B10.BR mice on day 7 after PCC priming ($n \geq 4$ mice). Far right, expression PD-1 mRNA immediately after isolation of 2×10^3 naive helper T cells or PCC-specific helper T cell subsets (as described in **Fig. 1d**) from lymph nodes on day 7 after priming. (**c**) Expression of IL-4 and IL-21 mRNA, analyzed as described in **Figure 1d**. (**d**,**e**) Expression of CD44 and either CD69 (**d**) or OX40 (**e**) on the surface of lymph node cells (left), and CD69 mRNA (**d**, right) or OX40 mRNA (**e**, right), analyzed as described in **Figure 1d**. (**f**) Expression of Bcl-6 mRNA, analyzed as described in **Figure 1d**. Numbers adjacent to outlined areas (**b**,**d**,**e**) indicate percent cells in each (mean ± s.e.m.). Results for mRNA (**b**,**d**,**e**, far right; **c**,**f**) are presented in arbitrary units (mean and s.e.m.) relative to results obtained for naive cells, set as 1 ($n \geq 3$ mice). *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of at least three experiments.

of high-affinity responders than low-affinity responders developed into $CXCR5^+$ 'resident' T_{FH} cells (**Fig. 4a**). Even after 7 d of activation and effector cell differentiation *in vivo*, the differences in mean fluorescence intensity (MFI) for pMHCII tetramer binding correlated with the preimmune differences in TCR affinity for the helper T cell populations (**Fig. 4b**). All subsets had equivalent expression of $V_\alpha11$ (data not shown). With both transgenic populations together in a cotransfer experimental design, significantly more higher affinity 5C.C7 helper T cells than lower affinity 2B4 helper T cells developed into $CXCR5^+$ 'resident' T_{FH} cells (**Fig. 4c**). These data show that monoclonal TCR-expressing helper T cells can develop into all three subsets, yet precursors with a high-affinity TCR 'preferentially' develop into $CXCR5^+$ 'resident' T_{FH} cells *in vivo*.

In the cotransfer experiment, we detected more binding of pMHCII in the effector T_{FH} compartment than in the other effector compartments, consistent with the higher ratio of 5C.C7 helper T cells to 2B4 helper T cells in the T_{FH} compartment. We obtained similar results using cytochalasin D (**Fig. 4d**), which disrupts cytoskeletal activity needed for aggregation of TCRs at the cell surface. These results suggest that the greater intensity of pMHCII binding in the T_{FH} compartment was due more to TCR repertoire differences than to physiological changes associated with effector cell differentiation *in vivo*. The endogenous PCC-specific response in this cotransfer experiment showed trends for pMHCII binding similar to those obtained with the transgenic helper T cells and also remained in the presence of cytochalasin D (**Supplementary Fig. 4** online). Consistent with those findings, adoptive transfer of 5C.C7 TCRβ chain–transgenic helper T cells into unmanipulated mice followed by immunization with PCC also produced a $CXCR5^+$ T_{FH} compartment with the highest pMHCII tetramer binding of the three phenotypically defined helper T cell subsets that emerged (**Supplementary Fig. 5** online). These data collectively indicate a causal connection between the TCR affinity of antigen-specific precursors and their differentiation into various effector helper T cell compartments.

Greater TCR signal strength induces IL-21 *in vitro*

We next addressed the function of TCR signal strength with peptide stimulation of transgenic helper T cells *in vitro*. All doses of moth cytochrome *c* (MCC) agonist peptide induced maximum clonal expansion of naive 5C.C7 helper T cells, as assessed by the resultant abundance of β_2-microglobulin mRNA after the use of various stimuli (**Supplementary Fig. 5a**). In contrast, expression of IL-21 mRNA required tenfold higher concentrations of MCC peptide, with 10 μM MCC inducing maximum amounts of IL-21 *in vitro* (**Fig. 4e**). Furthermore, all peptide doses induced similar Bcl-6 expression, but Blimp-1 expression was inversely correlated with IL-21 induction. The lower affinity 2B4 clonotype induced less maximum clonal accumulation at all doses of MCC peptide (**Supplementary Fig. 6a** online) but failed to induce substantial expression of IL-21 mRNA above the baseline expression detected *in vitro* (**Fig. 4f**). Unlike the results obtained with 5C.C7 cells, expression of both Bcl-6 and Blimp-1 remained unchanged in 2B4 cells at all peptide doses tested (**Fig. 4f**). We used the peptide MCC(102S)[22] to create a ligand of lower affinity for the 5C.C7 αβ TCR and again found less total clonal expansion at all peptide concentrations *in vitro* (**Supplementary Fig. 6a**). In these conditions, we noted higher IL-21 expression only at the highest peptide concentration; this also correlated with higher Bcl-6 expression. However, in these conditions, Blimp-1 expression was much higher at all doses of peptide (**Supplementary Fig. 6b**). Thus, it is clear that maximum IL-21 expression can be induced by stronger TCR

signals *in vitro* with an effect on the relative balance in the expression of Bcl-6 and Blimp-1, even in the absence of B cells.

'Resident' T_{FH} cells have stronger pMHCII binding

Although adoptive transfer provides direct access to antigen-specific precursors, it was important to address this issue in B10.BR mice with a polyclonal TCR repertoire in which precursor frequencies are more physiologically relevant. We detected considerable differences in pMHCII binding intensity for the three antigen-specific effector helper T cell compartments (**Fig. 5a**). All subsets expressed similar amounts of TCR, on the basis of $V_\alpha 11$ expression, but had progressively higher MFI values of pMHCII binding (**Fig. 5b**). 'Resident' T_{FH} cells had the greatest pMHCII binding intensity for each mouse tested. At all concentrations of pMHCII, the 'resident' T_{FH} compartment bound significantly more pMHCII complex than did the 'lymphoid' or 'emigrant' effector helper T cell compartment (**Fig. 5c** and **Supplementary Fig. 7** online). Although the 'emigrant' effector helper T cells present at day 7 in draining lymph nodes represented a small fraction of this compartment (**Fig. 3a** and **Supplementary Fig. 2**), the splenic 'effector' helper T cells present from this time point on are considered part of the emigrant subset and have also been shown to have less pMHCII binding[14]. At optimal concentrations of pMHCII, the 'resident' T_{FH} compartment bound more pMHCII more quickly than did the other effector compartments, reaching the maximum tetramer binding detected for the $CD62L^{hi}$ subset in only 30 min (**Fig. 5d** and **Supplementary Fig. 8** online). These trends in the polyclonal repertoire also indicate a skewing of antigen-specific precursors with stronger pMHCII binding of TCR into the 'resident' T_{FH} compartment.

'Resident' T_{FH} cells express a restricted TCR repertoire

As found in the adoptive-transfer experiments, we suspected that differences in pMHCII binding *in vivo* reflected differences in the expressed TCR. To test our idea more directly in the polyclonal model, we sorted single antigen-specific helper T cells from each effector helper T subset for TCR repertoire analysis. There are eight canonical features in the complementarity-determining region (CDR3) of PCC-specific TCR receptors, which for the TCRα chain consists of glutamic acid and serine at positions 93 and 95, respectively, a CDR3 length of eight amino acids and $J_\alpha 16$, $J_\alpha 17$, $J_\alpha 22$ or $J_\alpha 34$, and for TCRβ, asparagine at position 100 and alanine or glycine at position 102, a CDR3 length of nine amino acids and $J_\beta 1.2$ or $J_\beta 2.5$. The presence of six or more of these features defines clonal dominance in this polyclonal TCR repertoire[26,27]. Most antigen-specific helper T cells

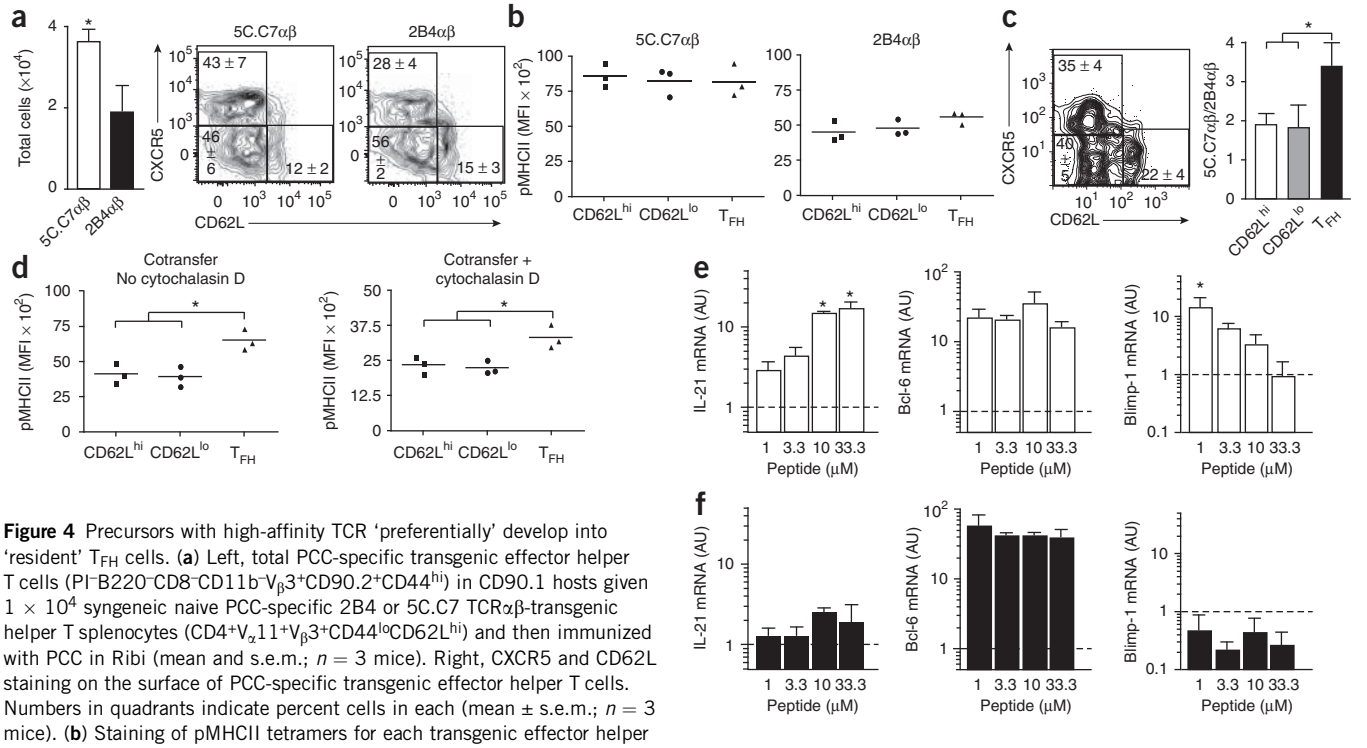

Figure 4 Precursors with high-affinity TCR 'preferentially' develop into 'resident' T_{FH} cells. (**a**) Left, total PCC-specific transgenic effector helper T cells ($PI^-B220^-CD8^-CD11b^-V_\beta 3^+CD90.2^+CD44^{hi}$) in CD90.1 hosts given 1×10^4 syngeneic naive PCC-specific 2B4 or 5C.C7 TCRαβ-transgenic helper T splenocytes ($CD4^+V_\alpha 11^+V_\beta 3^+CD44^{lo}CD62L^{hi}$) and then immunized with PCC in Ribi (mean and s.e.m.; $n = 3$ mice). Right, CXCR5 and CD62L staining on the surface of PCC-specific transgenic effector helper T cells. Numbers in quadrants indicate percent cells in each (mean ± s.e.m.; $n = 3$ mice). (**b**) Staining of pMHCII tetramers for each transgenic effector helper T cell subset in **a** (all $CD90.1^-V_\alpha 11^+$pMHCII$^+CD44^{hi}$; horizontal axis, as described for **Fig. 1d** key). Each symbol represents an individual mouse; small horizontal bars indicate the mean. (**c**) Left, CXCR5 and CD62L staining on the surface of PCC-specific transgenic effector helper T cells ($PI^-B220^-CD8^-CD11b^-V_\beta 3^+CD90.22^+CD44^{hi}$) from CD90.1 hosts given a mixture of 1×10^4 syngeneic naive PCC-specific 2B4 and 5C.C7 TCRαβ-transgenic helper T splenocytes and then immunized with PCC in Ribi; cells are from lymph nodes obtained on day 7 after immunization. Numbers in quadrants indicate percent cells in each (mean ± s.e.m.; $n = 3$ mice). Right, single-cell RT-PCR analysis of the cell origin of 2B4αβ and 5C.C7αβ helper T cells among antigen-experienced PCC-specific transgenic helper T cells (all $V_\beta 3^+CD44^{hi}$ $CD90.2^+$; horizontal axis, as described for **Fig. 1d** key), assessed with $J_\beta 2.5$- and $J_\beta 1.2$-specific primers and presented as the ratio of total 5C.C7αβ-transgenic cells participating in the PCC response to 2B4αβ-transgenic cells in each effector helper T cell subset (mean and s.e.m.; $n = 3$). (**d**) Staining of pMHCII tetramers for each transgenic effector helper T cell subset (as described in **b**), assessed in the presence or absence of cytochalasin D. (**e**,**f**) Quantitative PCR analysis of IL-21, Bcl-6 and Blimp-1 mRNA from 5×10^4 naive PCC-specific, 5C.C7 (**e**) or 2B4 (**f**) TCRαβ-transgenic helper T splenocytes ($CD4^+V_\alpha 11^+V_\beta 3^+CD44^{lo}CD62L^{hi}$) cultured for 3 d *in vitro* in the presence of 5×10^3 $CD11c^+$ antigen-presenting cells loaded with various concentrations of MCC(88–103) (Peptide; horizontal axis). Results are presented in arbitrary units (mean and s.e.m.) relative to results obtained for naive cells (dashed lines), set as 1 ($n = 3$ mice for each peptide concentration). *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of three experiments.

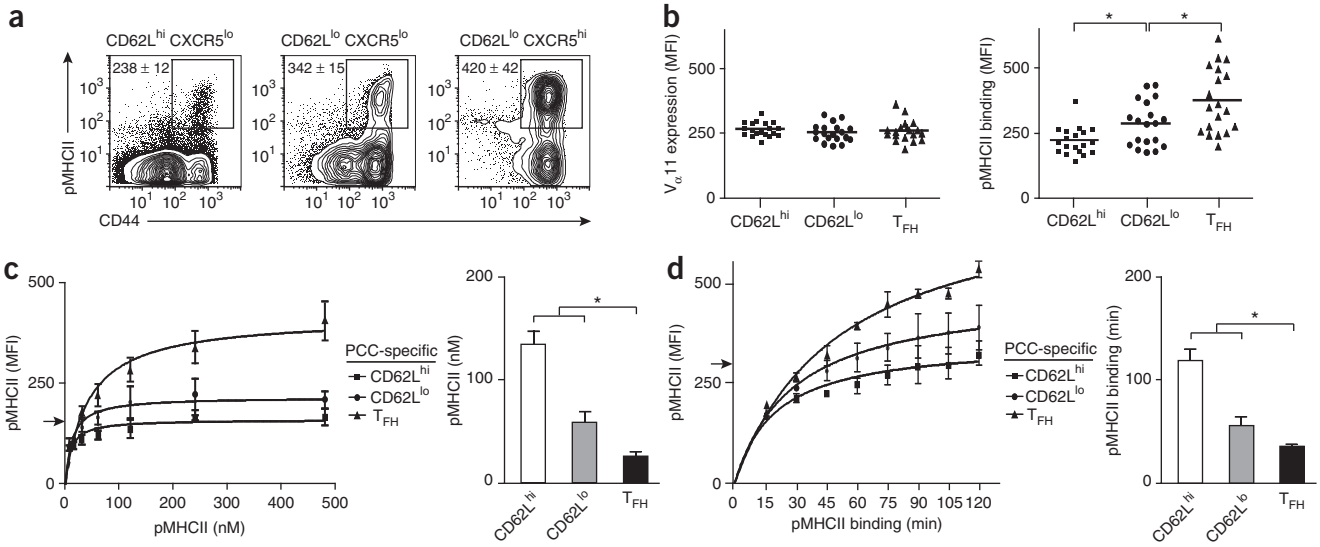

Figure 5 'Resident' T_{FH} cells have stronger pMHCII binding than do other effector helper T cells. (**a**) Expression of pMHCII and CD44 on the surface of helper T cell subsets (CD8$^-$CD11b$^-$B220$^-$PI$^-$V$_\alpha$11$^+$ and CD62L^{hi}CXCR5^{lo}, CD62L^{lo}CXCR5^{lo} or CD62L^{lo}CXCR5^{hi}) from lymph nodes on 7 d after PCC priming of mice. Numbers adjacent to outlined areas indicate MFI of each (mean ± s.e.m.; $n \geq 4$ mice). (**b**) V$_\alpha$11 expression and pMHCII tetramer staining for each PCC-specific effector helper T cell subset (all V$_\alpha$11$^+$pMHCII$^+$CD44^h; horizontal axis, as described for **Fig. 1d** key). Each symbol represents an individual mouse; small horizontal bars indicate the mean. *, $P \leq 0.01$ (one-tail paired Student's *t*-test). (**c**,**d**) Left, pMHCII tetramer staining on the surface of PCC-specific helper T cell subsets (as in **b**; day 7 after priming) after staining with varying amounts of pMHCII tetramer (**c**) or with optimal pMHCII tetramer concentration but varying times of pMHCII tetramer binding (**d**). Right, pMHCII tetramer concentration required for each subset to reach an MFI of 150 (arrow at left; **c**) and time of pMHCII tetramer binding required for each subset to reach an MFI of 300 (arrow at left; **d**). Results presented as mean and s.e.m. ($n = 3$ mice for each condition). *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of at least three experiments.

in all functional subsets had six or more of these attributes (**Fig. 6a**). However, consistent with the pMHCII binding, there was a progressive increase in the proportion of restricted TCR use for the CD62L^{hi}CXCR5^{lo} 'lymphoid', CD62L^{lo} CXCR5^{lo} 'emigrant' and CD62L^{lo}CXCR5^{hi} 'resident' effector helper T cell compartments. The differences in repertoire diversity were noticeable in the TCRα chain use of the lymphoid compartments (**Fig. 6b** and **Supplementary Fig. 9** online) and with differences in the TCRβ chains for all three effector subsets (**Fig. 6c** and **Supplementary Fig. 10** online). The TCR use of T_{FH} cells resembled that of only 36% of the 'emigrant' helper T cells and 17% of the 'lymphoid' helper T cells in this response (**Supplementary Fig. 11** online). Thus, the 'resident' T_{FH} subset of antigen-specific effector cells expressed TCRs with stronger pMHCII binding and a much more restricted repertoire.

Vaccine adjuvants affect T_{FH} development *in vivo*

To further address the mechanism underlying functional 'programming' *in vivo*, we sought ways to alter the responder clonal composition in the polyclonal vaccine model. Published studies have shown that decreasing the antigen dose over a two-log range does not affect clonal composition in this model[28,29]. Using a dose of protein that was tenfold higher or one-tenth as high, we found no effect on clonal expansion (**Supplementary Fig. 12a** online), overall pMHCII tetramer binding (**Supplementary Fig. 12b**) or the proportion or number of antigen-specific T_{FH} cells (**Supplementary Fig. 12c**). With these very different doses of antigen stimuli, we also detected similar numbers of antigen-specific helper T cells from all three subsets (**Supplementary Fig. 12d**), which indicated there was no effect on functional differentiation.

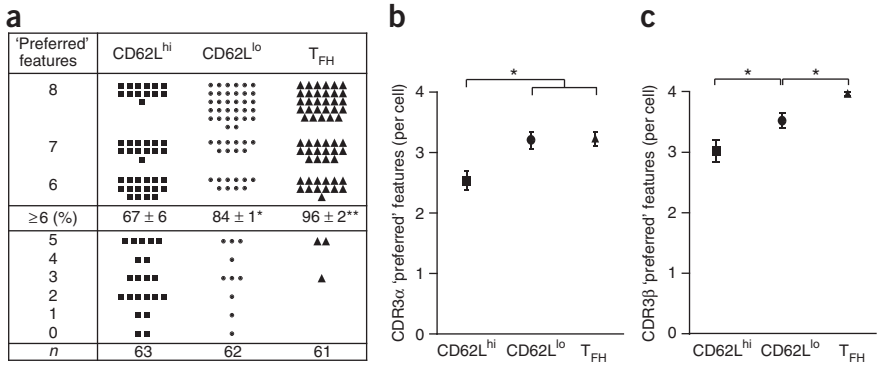

Figure 6 'Resident' T_{FH} cells express a more restricted TCR repertoire than do other effector helper T cells. Single-cell repertoire analysis of individual PCC-specific effector helper T cells (V$_\alpha$11$^+$V$_\beta$3$^+$CD44^{hi}) for each subset (as described in **Fig. 1d**). (**a**) Each filled symbol represents the number of the following 'preferred' CDR3 features known to be selected in the PCC response for a single cell: CDR3α, glutamic acid at α93, serine at α95, eight amino acids, Jα16, Jα17, Jα22 and Jα34; CDR3β, asparagine at β100, alanine or glycine at β102, nine amino acids, Jβ1.2 and Jβ2.5. Middle row: percent cells (± s.e.m.) with six or more 'preferred' features, expressing a restricted TCR of the dominant clonotype (among n = number of single cells used in the analysis (bottom row); three individual mice). *, $P \leq 0.05$, and **, $P \leq 0.01$ (unpaired Student's *t*-test). (**b**,**c**) 'Preferred' CDR3 features for one single cell of each subset for the TCRα chain (**b**) or TCRβ chain (**c**), presented as the mean ± s.e.m. *, $P \leq 0.05$ (unpaired Student's *t*-test). Data are representative of three experiments.

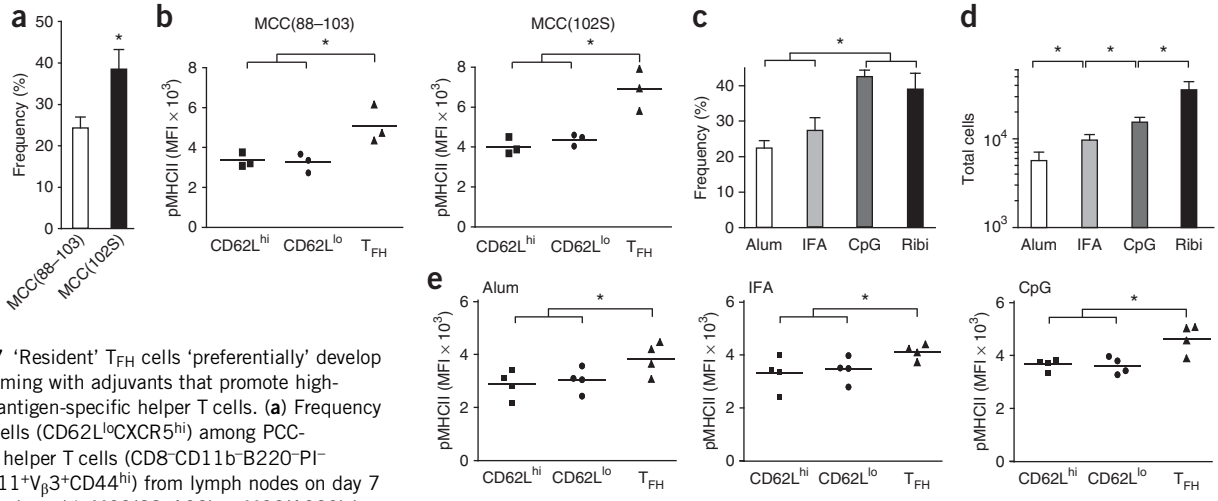

Figure 7 'Resident' T_{FH} cells 'preferentially' develop after priming with adjuvants that promote high-affinity antigen-specific helper T cells. (**a**) Frequency of T_{FH} cells ($CD62L^{lo}CXCR5^{hi}$) among PCC-specific helper T cells ($CD8^-CD11b^-B220^-PI^-CD4^+V_\alpha11^+V_\beta3^+CD44^{hi}$) from lymph nodes on day 7 after priming with MCC(88–103) or MCC(102S) in Ribi (mean ± s.e.m.; $n = 3$ mice). *, $P \leq 0.05$ (unpaired Student's *t*-test). (**b**) Staining of pMHCII tetramers for each PCC-specific effector helper T cell subset (as in **Fig. 5b**) from the mice in **a**. *, $P \leq 0.05$ (one-tail paired Student's *t*-test). (**c**,**d**) Frequency of T_{FH} cells among PCC-specific helper T cells (**c**) or total PCC-specific T_{FH} cells (**d**) from lymph nodes on day 7 after priming with PCC in alum, IFA, CpG or Ribi (mean and s.e.m.; $n = 4$ mice). *, $P \leq 0.05$ (unpaired Student's *t*-test). (**e**) Staining of pMHCII tetramers for each PCC-specific effector helper T cell subset (as in **Fig. 5b**) from lymph nodes on day 7 after priming with PCC in alum, IFA or CpG. *, $P \leq 0.05$ (one-tail paired Student's *t*-test). Data are representative of at least three experiments.

Next we used the agonist MCC peptide consisting of amino acids 88–103 (MCC(88–103)) and the altered peptide MCC(102S) as immunogens to vaccinate B10.BR mice. Vaccination with MCC(102S) induced substantial clonal expansion but less than that obtained with the MCC agonist peptide (**Supplementary Fig. 13a** online). However, the helper T cells selected into this response had an overall greater pMHCII binding intensity than that of the total responding population obtained by vaccination with the agonist MCC peptide (**Supplementary Fig. 13b**). These trends suggested the imposition of a higher selection threshold at the initiation of the immune response. In these conditions of more stringent clonal selection, there were significantly larger proportions of antigen-specific T_{FH} cells with stronger pMHCII binding with MCC(102S) than with the agonist MCC peptide (**Fig. 7a**,**b**).

Different vaccine adjuvants alter the selection threshold and clonal composition of antigen-responsive helper T cells[29]. Aluminum hydroxide (alum) and incomplete Freund's adjuvant (IFA) allowed clonotypes of lower affinity to emerge and persist, whereas the oligodinucleotide CpG adjuvant promoted a high-affinity clonal composition similar to that obtained with the monophosphoryl lipid A–based adjuvant Ribi (**Supplementary Fig. 14a**,**b** online). Here, we noted a significantly smaller proportion of antigen-specific T_{FH} cells (**Fig. 7c**) and fewer total antigen-specific T_{FH} cells (**Fig. 7d**) when we used alum or IFA than when we used CpG or Ribi. The T_{FH} composition of the responding population correlated with the different effects of these adjuvants on clonal expansion *in vivo*. With each adjuvant, the antigen-specific T_{FH} cell subset expressed TCRs with the strongest pMHCII binding relative to that of the other two antigen-specific effector compartments (**Fig. 7e**). These data further support the idea of a causal relationship between the strength of TCR signals delivered during clonal selection and the functional program subsequently adopted by the antigen-specific effector T_{FH} cell compartment *in vivo* (**Supplementary Fig. 15** online).

DISCUSSION

The capacity for lymphoid recirculation, regional 'placement' in lymphoid tissue and migratory activity can be used to categorize cells of the antigen-responsive effector helper T cell compartment. The $CD62L^{hi}$ 'lymphoid' effector helper T cells retain the ability to reenter lymph nodes but remain $CCR7^{hi}$ with negligible exit from lymph nodes during the first week of priming. Hence, these cytokine-producing $CD44^{hi}ICOS^{hi}$ effector cells may provide additional cognate help for antigen-primed B cells already in the extrafollicular regions[2]. These specialized effectors could also maintain[35] or reestablish contact with pMHCII-expressing dendritic cells for their own survival and development[36]. Alternatively, this effector helper T subset may be needed to 'license' dendritic cells for help to CD8 T cells in cross-presentation functions or perhaps an extrafollicular function for helper T cells to promote isotype switching without somatic diversification[37]. As there are $CD62L^{hi}$ memory helper T cells with this cell surface phenotype[38], it is plausible that this 'lymphoid' effector program may also give rise to a recirculating lymphoid 'central memory' helper T cell compartment.

$CXCR5^{hi}$ effector helper T cells had all lost CCR7 expression, ensuring their 'placement' in the follicular B cell zones of lymphoid tissues[7,8]. These cells had lost CD62L expression, which indicated that if they left the lymph node, reentry would be a rare event. The results obtained with AAL-R treatment[32,33] strongly supported the idea of the 'resident' status of these follicular helper T cells[14]. In contrast, the expression of ICOS, PD-1, IL-4, IL-10 and IL-21 associated with T_{FH} function[1,3,4] was not exclusive to the $CXCR5^{hi}$ effector T_{FH} compartment. ICOS expression and the regulation of its amount *in vivo*[11] are important for effective B cell immunity and are required for T_{FH} maintenance *in vivo*. Furthermore, ICOS ligand[10,15] expressed by B cells is required for T_{FH} development and effective B cell immunity *in vivo*. Similarly, IL-21 is critical to T_{FH} development and can act in an autocrine way in the generation of T_{FH} cells and can costimulate initial TCR-triggering events[16]. The absence of IL-21 or its receptor on helper T cells leads to much lower B cell responses *in vivo*[16]. Our findings presented here are consistent with an early function for ICOS and IL-21 in effector helper T cell function that requires chemokine receptor 'reprogramming' for the delivery of cognate effector T_{FH} functions to antigen-primed B cells.

The transcription factor T-bet is required for the differentiation of T helper type 1 cells[39]. In our study here, all subsets had equivalent expression of T-bet mRNA that would account for the presence of IFN-γ in all effector compartments. However, the cognate delivery of IFN-γ to different target cells would induce separate immune functions *in vivo*, such as immunoglobulin G2a isotype switching in antigen-primed B cells[12,39]. Such a pattern would suggest the presence of T helper type 1–like cells in the broad class of effector T_{FH} cells. The presence of IL-4 in the antigen-specific T_{FH} compartment supports the same idea, with evidence of the presence of T helper type 2 cells as well in the T_{FH} compartment. Published data indicate that T_{FH} cells are able to produce IL-17 (ref. 40). Such trends suggest that effector T_{FH} cells have heterogeneous patterns of production of cytokines all known to be involved in the regulation of B cell immunity.

The larger amounts of transcriptional repressor Blimp-1 only in the 'lymphoid' effector compartment suggested different programs of development. Blimp-1 controls the plasma cell program of B cells[41,42] and is involved in regulatory helper T cells[43] and T helper type 1 differentiation[44]. Bcl-6 is also involved in antigen-driven B cell development and is required for germinal center formation[45]. Notably, Bcl-6 is a direct target for Blimp-1 repression, and the mutual repression of these two factors is important for maintaining the separate B cell fates[44]. In the $CD4^+$ compartment, Blimp-1-deficient helper T cells have higher Bcl-6 expression[46]. Thus, Blimp-1 and Bcl-6 may regulate mutually exclusive 'lymphoid' effector helper T cell and 'resident' effector T_{FH} cell developmental programs in the response to vaccination with protein.

TCR recognition of pMHCII complexes on antigen-presenting cells defines the first main checkpoint in the development of antigen-specific helper T cells *in vivo*[2]. Most models favor the idea that TCR affinity[21,22,29,47] or the strength and duration[48,49] of the binding of pMHCII to antigen-specific helper T cells controls these initial recognition events. Unlike $CD8^+$ cells, the helper T cell compartment requires continued[35] or multiple[36] contacts with antigen-presenting cells for maximum clonal expansion *in vivo*. These sequential cognate interactions provide opportunities for the antigen-specific helper T cells to deliver effector functions and receive new developmental programming signals at later phases of the immune response. The costimulatory context of pMHCII expression and the cytokines produced by the antigen-presenting cell and by the responding helper T cells can affect the differentiation of helper T cells. IL-21 is important in the costimulation of antigen-specific T_{FH} differentiation[15,16]. Hence, soluble and cell-associated microenvironmental factors also influence the differentiation of antigen-specific effector helper T cells during the acute phase of an immune response.

T_{FH} cells represent a class of effector helper T cells responsible for the antigen-specific regulation of B cell immunity[1,3,4]. Published studies have further identified a function for IL-6 and STAT3 in the IL-21-dependent genetic program that initiates T_{FH} development[15]. Notably, these studies have also demonstrated that expression of the ICOS ligand on B cells is needed to promote this specialized effector T_{FH} compartment *in vivo*. Other studies have also indicated that ICOS expression on helper T cells is needed for the production of IL-4 and IL-21 but not for CXCR5 expression[37]. The costimulation of TCR signals has been identified as an important factor in IL-21 production, thereby promoting effector T_{FH} development *in vivo*[16]. Our analyses here have added involvement of the strength of TCR signal itself in the early programming events involved in the development of antigen-specific T_{FH} cells. It remains unclear whether the initial selection events between naive helper T cells and antigen-primed dendritic cells impose the critical selection events that affect the functional program. Alternatively, it may be the secondary encounter with pMHCII-expressing antigen-primed B cells that is critical for the 'imprinting' of effector T_{FH} function, as suggested before[17].

Vaccine adjuvants affect clonal-selection mechanisms, changing the distribution of the 'preferred' TCR affinity for responding clonotypes and altering the clonal composition of the effector helper T cell compartment. Our studies here indicate that the initial strength of TCR binding also regulates the function of antigen-specific effector helper T cells. TCR with stronger pMHCII binding 'preferentially' drive antigen-specific precursors into the 'resident' effector T_{FH} compartment with the ability to control B cell immunity. Thus, manipulation of the clonal selection in helper T cells and regulation of the balance of effector helper T cell differentiation will provide a powerful new strategy for the future design of protein vaccines.

METHODS

Mice. B10.BR, B10.BR–Thy-1.1^+ congenic and TCR-transgenic mice (2B4 TCRαβ, 5C.C7 TCRαβ, 5C.C7 TCRβ) were maintained in pathogen-free conditions at The Scripps Research Institute. Experiments were approved by the Institutional Animal Care and Use Committee and The Scripps Research Institute.

Immunization, adoptive transfer and AAL-R treatment. Mice were immunized subcutaneously with 400 μg whole PCC (Sigma), 60 μg MCC(88–103) (ANERADLIAYLKQATK; Anaspec) or 60 μg MCC(102S) (ANERADLIAYLK QASK; Anaspec) in Ribi (lab formulation based on ref. 29), IFA (Sigma), alum (Sigma) or CpG (ODN1826; Coley). For adoptive transfer, sorted naive Thy-1.2^+ PCC-specific helper T splenocytes from 5C.C7 TCRαβ and 2B4 TCRαβ mice (1×10^4 of each) or total splenocytes from 5C.C7β mice (2×10^6) were transferred intravenously into B10.BR–Thy-1.1 mice. Recipient mice were then immunized as described above. For AAL-R treatment, mice received intraperitoneal administration (1 mg AAL-R per kg body weight) daily from the day before PCC immunization to day 6 after immunization.

Flow cytometry. For population analysis, draining lymph nodes (inguinal and periaortic) were removed from immunized mice and were prepared and pelleted, then were resuspended in PBS with 5% (vol/vol) FCS and stained for flow cytometry at a density of 2.0×10^8 cells per ml. For tetramer staining, cells were first stained for 2 h at 20 °C with the optimal concentration of pMHCII tetramer (240 nM, prepared as described[28]). Where required, cytochalasin D was used at a final concentration of 50 μM (Sigma). Then, cells were incubated for 45 min at 4 °C with predetermined optimal concentrations of the following fluorophore-labeled (or biotin-labeled) monoclonal antibodies: fluorescein isothiocyanate–conjugated anti-V_α11 (RR8.1) or anti-Thy-1.2 (53-2.1), phycoerythrin-conjugated anti-CD4 (L3T4), allophycocyanin-conjugated anti-CD44 (Pgp1) or anti-V_β3 (KJ25), indodicarbocyanine-phycoerythrin–conjugated anti-B220 (6B2) or anti-CD8 (53-6.7), and biotin-conjugated anti-CXCR5 (2G8) or anti-CD28 (37.51; all from BD Pharmingen); indodicarbocyanine-phycoerythrin–conjugated anti-Thy-1.1 (HIS51), indotricarbocyanine-phycoerythrin–conjugated anti-CD62L (Mel14), indotricarbocyanine-allophycocyanin–conjugated or allophycocyanin–Alexa Fluor 750–conjugated anti-CD44 (IM7), and biotin-conjugated anti-CCR7 (4B12), anti-CD69 (H1.2F3), anti-PD-1 (J43), anti-ICOS (7E.17G9) or anti-OX40 (OX86; all from eBioscience); and indodicarbocyanine-phycoerythrin–conjugated anti-CD11b (M1/70.15) or anti-CD4 (L3T4), and Pacific blue–conjugated anti-B220 (6B2; all from Biolegend). Streptavidin-phycoerythrin (BD Pharmingen) or streptavidin–indotricarbocyanine-allophycocyanin (BD Pharmingen) was used as a second-step visualization reagent. Cells were washed, were resuspended in propidium iodide (2 μg/ml in 5% (vol/vol) FCS in PBS) for exclusion of dead cells and were analyzed with a FACSVantage SE (BD Biosciences) with CellQuest or FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Profiles are presented as 5% probability contours with outliers.

Single-cell repertoire analysis. Single cells with the appropriate surface phenotype were sorted for repertoire analysis with a FACSVantage SE and

CloneCyt software (BD Biosciences). Synthesis of cDNA and amplification of the TCR $V_\alpha 11$ and $V_\beta 3$ regions were done as described[26]. The purified PCR products were sequenced directly with a $V_\alpha 11$-specific or $V_\beta 3$-specific primer and the BigDye Terminator cycle sequencing kit (Applied Biosystems). Sequences were analyzed on an ABI 373A DNA sequencer (Applied Biosystems).

Quantification of J_β-gene-segment use. Single-cell cDNA was amplified with primers specific for the $V_\beta 3$ region and constant region, then 4% of the PCR product was used for J_β-specific amplification with nested primers specific for the $V_\beta 3$ region and antisense primers specific for the $J_\beta 1.2$ or $J_\beta 2.5$ gene segment as described[29].

Quantitative PCR. Naive and PCC-specific helper T cells (2×10^3) were sorted by flow cytometry and were suspended in lysis buffer (Qiagen). RNA was purified and cDNA was generated as described[14]. Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and a RotorGene RG-3000 (Corbett Research) or StepOne Real-Time PCR system (Applied Biosystems) were used for PCR of 10% of the cDNA (primers, **Supplementary Table 1** online). The results of quantitative PCR were analyzed with Rotor-Gene 6.0 software (Corbett Research) or StepOne software v2.0 (Applied Biosystems).

***In vitro* stimulation.** Sorted naive transgenic helper T splenocytes were cultured for 3 d in RPMI medium supplemented with 10% (vol/vol) FCS, 1 mM sodium pyruvate, nonessential amino acids, 50 μM 2-mercaptoethanol (Gibco, Invitrogen) in the presence of 5×10^3 $CD11c^+$ APCs loaded with MCC(88–103) or MCC(102S) at various concentrations.

Statistical analysis Mean values, standard error of the mean and Student's *t*-test (paired and unpaired) were calculated with GraphPad Prism (GraphPad Software).

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001262, A000369, A001258, and A003268.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

N.F., L.J.M.-W. and M.G.M.-W. designed experiments; N.F. did experiments and analyzed data; N.F., L.J.M.-W. and M.G.M.-W. wrote the manuscript; and H.R. consulted on AAL-R experiments and provided necessary reagents.

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Addendum: The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding

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Plasma cells negatively regulate the follicular helper T cell program

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It has been called to our attention that in a series of studies on the development of follicular helper T cells (T_{FH} cells), the primers we used to amplify *Bcl6* were specific for *Bcl6b*, not *Bcl6*. We have now traced back the results and have redone experiments that demonstrate that both *Bcl6* and *Bcl6b* are modified in the same way and to a similar extent in antigen-specific and non–antigen-specific T_{FH} cells induced both *in vivo* and *in vitro*.

In the original studies we inadvertently mixed up primers specific for *Bcl6b* (but not for *Bcl6*) with those specific for *Bcl6*. After closer inspection, we found that this error was made when the wrong set of primers was ordered and labeled "Bcl6 #2" in our laboratory but the primers were in fact specific for *Bcl6b*. We used both sets of primers in the original studies of gene expression on antigen-specific T_{FH} cells analyzed immediately after isolation (**Fig. 3f** in *Nat. Immunol.* **10**, 375–384 (2009), and **Addendum Fig. 1**). Hence, the expression of both *Bcl6* and *Bcl6b* seemed to be three- to fourfold higher in pigeon cytochrome *c* (PCC)-specific T_{FH} cells than in naive $CD4^+$ helper T cells.

As these genes are structurally related, we were concerned that the primers for *Bcl6b* may have sufficiently cross-reacted with *Bcl6* and perhaps amplified the latter gene in T cells. Thus, we sorted antigen-specific T_{FH} cells immediately after isolation and amplified random hexamer–primed cDNA with both sets of primers (**Addendum Fig. 2**). Both gene products were amplified and produced PCR products of different sizes (the predicted sizes for the different gene products). We then sequenced these products to demonstrate they belonged to the different genes as presented. Thus, both primers amplified the expected PCR products in both naive helper T cells and antigen-specific T_{FH} cells.

To explore this issue further, we sorted non–antigen-specific T_{FH} cells on the basis of cell surface phenotype and amplified DNA from these cells by RT-PCR without further treatment (**Addendum Fig. 3**). These experiments also showed that both *Bcl6* and *Bcl6b* had similar basal expression in naive helper T cells and a similar change of expression in $CXCR5^+$ T_{FH} cells.

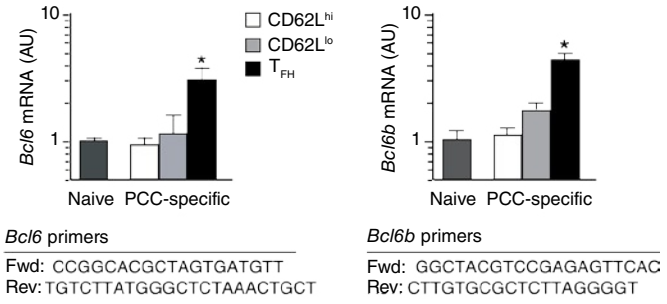
In our more recent study on antigen presentation by plasma cells (*Nat. Immunol.* **11**, 1110–1118 (2010)), we reported that plasma cells negatively regulate the T_{FH} program *in vitro* and *in vivo*. Our conclusions were based on expression of the genes encoding interleukin 21 (IL-21) and Bcl-6, as measured by reverse transcription and quantitative PCR. The original primer mix-up continued in the early phase of these studies. However, the error was sporadic and these more recent studies reported on *Bcl6* and not *Bcl6b*, as outlined below (**Addendum Table 1**).

Hence, we concluded that antigen presentation by plasma cells inhibits the expression of mRNA for IL-21 and Bcl-6 by *in vivo*–derived, endogenous, antigen-specific T_{FH} cells (**Fig. 5d**, second panel, in *Nat. Immunol.* **11**, 1110–1118 (2010)). Furthermore, the adoptive transfer of antigen-pulsed plasma cells significantly inhibited the expression of mRNA for both IL-21 and Bcl-6 on antigen-specific T_{FH} cells (**Fig. 7i**, in *Nat. Immunol.* **11**, 1110–1118 (2010)) without affecting the expression of mRNA for IL-4 or the transcription factor GATA-3 *in vivo*. Therefore, this provided evidence of antigen-specific negative regulation of the T_{FH} program by plasma cells *in vivo*.

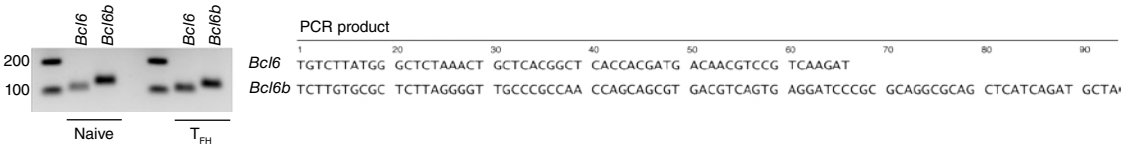
We have also repeated the *in vitro* experiments using dendritic cells (DCs) or plasma cells as antigen-presenting cells to evaluate the expression of *Bcl6* and *Bcl6b* after *in vitro* induction of T_{FH} cell activity (**Addendum Fig. 4**). These studies demonstrated equivalent induction of both *Bcl6* and *Bcl6b* *in vitro* with DCs as antigen-presenting cells and naive helper T cells with transgenic expression of the 5C.C7 αβ T cell antigen receptor (αβTCR) as antigen-specific helper T cells in these cultures. Consistent with the overall conclusions of our earlier study (*Nat. Immunol.* **11**, 1110–1118 (2010)) plasma cells with antigen did not induce *Bcl6* or *Bcl6b* in 5C.C7 αβTCR–transgenic helper T cells *in vitro*.

We did these new experiments without secondary *in vitro* transcription or specific synthesis of target cDNA and preamplification by PCR to evaluate the extent of signal to be expected for *Bcl6* after *in vitro* activation of T_{FH} cells. The tenfold induction of both *Bcl6* and *Bcl6b* in these studies is more in line with the expected results and the results of other studies using standard culturing conditions to induce T_{FH} cell activity. These data are also more in line with the expression of *Bcl6* and *Bcl6b* on antigen-specific T_{FH} cells derived *in vivo* we have reported in all our studies. We believe that the starting cell population, in particular the primary culture stimulus, atypical secondary culture conditions and secondary amplification of the assay all contributed to the more enhanced signals we reported before for *Bcl6b* (**Fig. 5b**,**c** in *Nat. Immunol.* **11**, 1110–1118 (2010)).

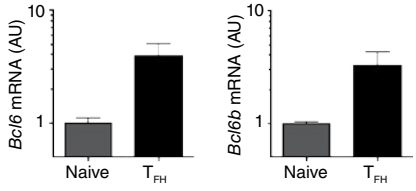
In conclusion, we mixed up primers for *Bcl6* amplification and in parts of our published work inadvertently reported on *Bcl6b*, not *Bcl6*. *Bcl6b* is a distinct gene but remains a structurally and functionally related member of the *Bcl6* gene family. We have now been able to revisit this issue and have demonstrated that both *Bcl6* and *Bcl6b* seemed to be coexpressed to a similar extent in naive helper T cells. Both gene products were similarly enhanced in T_{FH} cells that emerged *in vivo* or were derived *in vitro*. Furthermore, both genes seemed to be inhibited similarly by antigen presentation by plasma cells. Hence, we believe that this addendum will rectify the mistake made in the initial studies and clarify the coexpression of *Bcl6* and *Bcl6b* in antigen-specific T_{FH} cells.



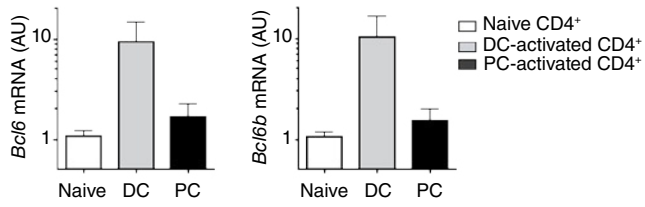
Addendum Figure 1 Upregulation of both *Bcl6* and *Bcl6b* in antigen-specific T_{FH} cells analyzed immediately after isolation. Quantitative PCR analysis of *Bcl6* mRNA and *Bcl6b* mRNA in naive helper T cells ($V_\alpha11^+V_\beta3^+CD44^{lo}CD62L^{hi}$) or PCC-specific T_{FH} cells (PI^-B220^- $CD11b^-CD8^-V_\alpha11^+V_\beta3^+CD44^{hi}CD62L^{lo}CXCR5^{hi}$) sorted from draining lymph nodes on day 7 after subcutaneous priming with PCC in Ribi adjuvant (2×10^3 cells), followed by cDNA synthesis and amplification with Platinum SYBR Green (details as for **Figs. 1–3** in the original study (*Nat. Immunol.* **10**, 375–384 (2009))); results are presented in arbitrary units (AU) relative to the expression of β_2-microglobulin mRNA, set as 1. Other antigen-specific helper T cell subsets are sorted on the basis of differences in the expression of CD62L and CXCR5 as described in detail in the original study (*Nat. Immunol.* **10**, 375–384 (2009)).



Addendum Figure 2 Primers for *Bcl6* and *Bcl6b* amplify the expected PCR products from naive helper T cells and T_{FH} cells. PCR analysis of *Bcl6* and *Bcl6b* in naive helper T cells or antigen-specific T_{FH} cells (5×10^3) sorted (as described in **Addendum Fig. 1**) on day 8 of the memory response to PCC; cDNA synthesized from RNA by random-hexamer priming was analyzed by 40 cycles of PCR with both sets of primers (**Addendum Fig. 1**), then primers were removed and PCR products were analyzed directly with a BigDye Terminator Cycle Sequencing kit on a 3130 Genetic Analyzer.



Addendum Figure 3 Equivalent upregulation of *Bcl6* and *Bcl6b* in $CXCR5^+$ T_{FH} cells assessed immediately after isolation. Reverse transcription and quantitative PCR analysis of *Bcl6* and *Bcl6b* in naive helper T cells ($PI^-CD8^-CD11b^-CD4^+CD44^{lo}CD62L^{hi}CXCR5^-$) or T_{FH} cells ($PI^-CD8^-CD11b^-CD4^+CD44^{hi}CD62L^{lo}CXCR5^+$) from the draining lymph nodes of mice immunized with Ribi adjuvant only and sorted, results are presented in arbitrary units relative to the expression of β-actin mRNA.



Addendum Figure 4 Selective upregulation of *Bcl6* and *Bcl6b* in antigen-specific T_{FH} cells with the use of DCs but not PCs for antigen presentation *in vitro*. Quantitative PCR analysis of *Bcl6* and *Bcl6b* in cells derived from $CD11c^+$ DCs or IgM^-CD138^+ plasma cells (PC) sorted *ex vivo* with protein antigen and cultured for 4 d *in vitro* together with cytosolic dye CFSE–labeled naive $CD4^+$ PCC-specific 5C.C7 αβTCR–transgenic helper T cells, followed by sorting of $CD44^{hi}$ 5C.C7 αβTCR–transgenic helper T cells that diluted CFSE, for RNA extraction, cDNA synthesis and SYBR Green–based quantitative PCR amplification of *Bcl6* and *Bcl6b* (details as for **Fig. 4** in the original study (*Nat. Immunol.* **11**, 1110–1118 (2010))); results are presented in arbitrary units relative to β_2-microglobulin mRNA expression.

Addendum Table 1 Induction and inhibition of *Bcl6* or *Bcl6b* in T_{FH} cells

Figure	Amplification method	T_m	Gene amplified
4c	IVT	86.88–87.32	*Bcl6b*
5b	None	87.02–87.31	*Bcl6b*
5c	STA	87.03–87.47	*Bcl6b*
5d	STA	82.75–83.06	*Bcl6*
7i	STA	82.99–83.56	*Bcl6*

Induction of *Bcl6* or *Bcl6b* in T_{FH} cells selectively by DC antigen presentation and inhibition of *Bcl6* by PC antigen presentation, assessed by two rounds of *in vitro* amplification (IVT) of RNA with a MessengerAmp II aRNA Amplification kit (Ambion), similar to what is done before microarray analysis, followed by the generation of cDNA from 10 μg amplified RNA as the starting material for SYBR Green–based quantitative PCR analysis; by specific target amplification (STA) with specific primers to generate cDNA, followed by 14 cycles of PCR preamplification and dilution of the product 1:5 for use as the starting material for SYBR Green–based quantitative PCR analysis; or the melting curve (T_m) peak signal after SYBR Green–based quantitative PCR analysis.