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.

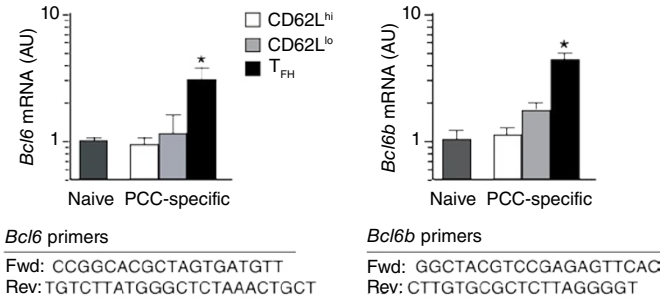
In our more recent study on antigen presentation by plasma cells (*Nat. Immunol.* **11**, 1110–1118 (2010)), we reported that plasma cells negatively regulate the T_{FH} program *in vitro* and *in vivo*. Our conclusions were based on expression of the genes encoding interleukin 21 (IL-21) and Bcl-6, 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*.

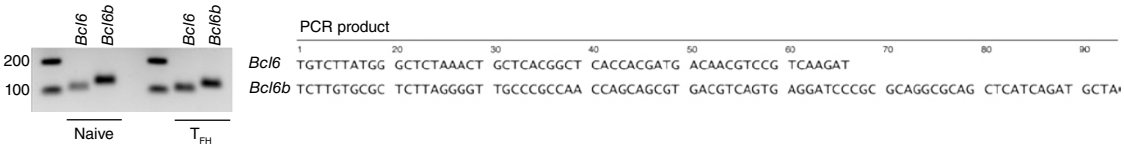
We have also repeated the *in vitro* experiments using dendritic cells (DCs) or plasma cells as antigen-presenting cells to evaluate the expression of *Bcl6* and *Bcl6b* after *in vitro* induction of T_{FH} cell activity (**Addendum Fig. 4**). These studies demonstrated equivalent induction of both *Bcl6* 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)).

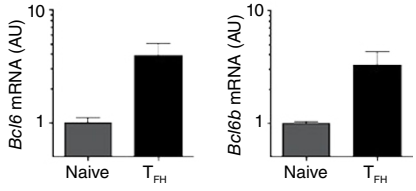
In conclusion, we mixed up primers for *Bcl6* amplification and in parts of our published work inadvertently reported on *Bcl6b*, not *Bcl6*. *Bcl6b* is a distinct gene but remains a structurally and functionally related member of the *Bcl6* gene family. We have now been able to revisit this issue and 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.



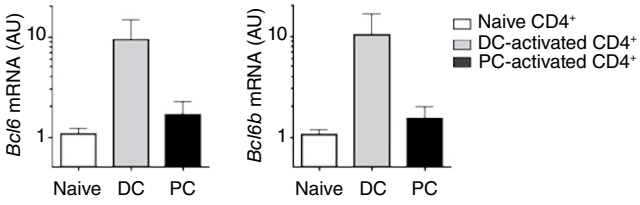
Addendum Figure 1 Upregulation of both *Bcl6* and *Bcl6b* in antigen-specific T_{FH} cells analyzed immediately after isolation. Quantitative PCR analysis of *Bcl6* mRNA and *Bcl6b* mRNA in naive helper T cells ($V_\alpha11^+V_\beta3^+CD44^{lo}CD62L^{hi}$) or PCC-specific T_{FH} cells (PI^-B220^- $CD11b^-CD8^-V_\alpha11^+V_\beta3^+CD44^{hi}CD62L^{lo}CXCR5^{hi}$) 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.