B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells

Sau K. Lee,1 Robert J. Rigby,1 Dimitra Zotos,2 Louis M. Tsai,3 Shimpei Kawamoto,4 Jennifer L. Marshall,5 Roybel R. Ramiscal,1 Tyani D. Chan,6,7 Dominique Gatto,6,7 Robert Brink,6,7 Di Yu,3 Sidonia Fagarasan,4 David M. Tarlinton,2 Adam F. Cunningham,5 and Carola G. Vinuesa1

T follicular helper cells (Tfh cells) localize to follicles where they provide growth and selection signals to mutated germinal center (GC) B cells, thus promoting their differentiation into high affinity long-lived plasma cells and memory B cells. T-dependent B cell differentiation also occurs extrafollicularly, giving rise to unmutated plasma cells that are important for early protection against microbial infections. Bcl-6 expression in T cells has been shown to be essential for the formation of Tfh cells and GC B cells, but little is known about its requirement in physiological extrafollicular antibody responses. We use several mouse models in which extrafollicular plasma cells can be unequivocally distinguished from those of GC origin, combined with antigen-specific T and B cells, to show that the absence of T cell–expressed Bcl-6 significantly reduces T-dependent extrafollicular antibody responses. Bcl-6+ T cells appear at the T–B border soon after T cell priming and before GC formation, and these cells express low amounts of PD-1. Their appearance precedes that of Bcl-6+PD-1hi T cells, which are found within the GC. IL–21 acts early to promote both follicular and extrafollicular antibody responses. In conclusion, Bcl-6+ T cells are necessary at B cell priming to form extrafollicular antibody responses, and these pre–GC Tfh cells can be distinguished phenotypically from GC Tfh cells.

© 2011 Lee et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
unmutated and generally of modest affinity, can be critical for protection against infection (Luther et al., 1997). Bcl-6 expression in T cells is required for the formation of T follicular helper cells (Tfh cells), which are essential to support GC reactions (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Whether Bcl-6 expression in T cells is required for B cell differentiation along the extrafollicular pathway is still unknown.

Bcl-6 and Blimp-1 are important transcriptional regulators of terminal differentiation of T and B cells; they are mutually repressive, and their reciprocal appearance appears to specify one or other cell fate when two differentiation pathways are possible. In B cells, Bcl-6 is essential for the development of GC B cells (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997), whereas Blimp-1 is required for extrafollicular plasma cell formation (Shapiro-Shelley et al., 2003). T cells require Bcl-6 expression for up-regulation of CXCR5, the receptor for the chemokine CXCL13, which is produced by follicular DCs in B cell follicles and GC T cells themselves (Cyster et al., 2000; Kim et al., 2004). Coordinated down-regulation of CXCR5 and up-regulation of CCR7 by T cells is required for the interactions at the T–B border that precede follicular migration (Cyster et al., 2000; Haynes et al., 2007). During this initial T–B cognate interaction, T cells provide signals that initiate Ig isotype switching (Toellner et al., 1996; Pape et al., 2003).

There is evidence to suggest that the nature of T cell help required to promote extrafollicular antibody responses may differ from that required to drive GC development. First, extrafollicular responses occur in response to pure polysaccharide antigens in the absence of T cell help, whereas T-independent GCs are only found in exceptional circumstances (de Vinuesa et al., 2000). Second, although SAP (SLAM-associated protein) expression in T cells is required for differentiation of Tfh cells and GC B cells (Qi et al., 2008; Cannons et al., 2010), extrafollicular antibody responses are less dependent on this adaptor molecule (Linterman et al., 2009a).

Although it is thought that Th1 and Th2 cells, which form in a Bcl-6–independent manner, can drive extrafollicular switched antibody responses, there is indirect evidence to suggest that Bcl-6 may play a role in these responses. Early antibody production is diminished in lymphocytic choriomeningitis virus–infected mice expressing low levels of Bcl-6 in T cells as a consequence of Bcl6 overexpression (Johnston et al., 2009). Also, in autoimmune lupus-prone MRL/Fas mice, T cells that share some but not all phenotypic markers of Tfh cells and are Bcl-6 dependent have been found in extrafollicular foci, and they appear to promote autoantibody production (Odegard et al., 2008; Poole et al., 2010). In contrast, the early antibody response to protein antigen was reported to be intact in irradiated Rag1−/− mice reconstituted with Bcl-6–deficient bone marrow (Fukuda et al., 1997). However, it is difficult to distinguish follicular from extrafollicular origin of antibody because most immunization strategies do not result in development of only one or the other pathway of B cell differentiation. Furthermore, isotype switching is comparable in both pathways, and up to 25% of the plasma cells found in splenic extrafollicular foci after standard TD immunization protocols are of GC origin (Sze et al., 2000).

In this study, we report the results of experiments from several different models in which we can distinguish antigen-specific plasmablasts and/or antibodies of either extrafollicular or follicular origin. We show that Bcl-6–expressing T cells are essential for extrafollicular production of IgG1 in responses to the model protein antigens hen egg lysozyme (HEL) and NP–KLH (4-hydroxy-3-nitrophenyl) acetyl keyhole limpet hemocyanin) and for the production of Salmonella enterica–specific IgG2c. Bcl-6–expressing helper T cells are initially situated at the interface between the B cell follicle and the T zone and, unlike Tfh cells located in the GC, do not express high levels of PD-1.

RESULTS AND DISCUSSION

Bcl-6–expressing PD-1hi CD3+ T cells are seen at the T–B border early in the course of an antibody response

We began by investigating whether Bcl-6 expression is confined to GC T cells during T–dependent immune responses by assessing the distribution and kinetics of Bcl-6–expressing cells during a T–dependent response to sheep RBCs (SRBCs). In unimmunized mice, there were virtually no Bcl-6+ T cells detected outside rare background GCs (Fig. 1 A). Between days 2 and 3, CD3+ Bcl-6+ PD-1hi cells were seen at the T–B border. By day 7, CD3+ cells expressing higher amounts of Bcl-6 and PD-1 appeared within newly formed GCs (Fig. 1, B and C). As has been reported before (Haynes et al., 2007; Linterman et al., 2009a), and most were Bcl-6+ CD3+ PD-1hi T cells were rarely seen at sites other than the GC. We conclude that high expression of PD-1 identifies GC Tfh cells. Together, these results suggest that the T cells that interact with B cells at the outer T zone have up-regulated Bcl-6, raising the possibility that expression of Bcl-6 influences B cell commitment to both the follicular and the extrafollicular pathways.

Bcl-6 expression in T cells boosts T-dependent extrafollicular antibody responses to a haptenated protein

To evaluate the influence of T cell–expressed Bcl-6 on the early antibody response to NP–KLH, we constructed mixed chimeras in which sublethally irradiated Rag1−/− mice were reconstituted with 80% Bcl6−/− CD45.2+20% Cd28−/− CD45.1 fetal liver. Cd28−/− fetal liver cells help maintain the chimeras’ health and provide a source of Bcl-6+ B cells and T cells that cannot differentiate into effector cells, including Tfh cells. A control group was reconstituted with 80% Bcl6+/+ CD45.2+20% Cd28−/− CD45.1 fetal liver.

Chimeric mice were immunized i.p. with NP–KLH in alum. 7 d after immunization, mice lacking functional Bcl-6–expressing T cells did not form GC (Fig. 2, A and B) or Tfh cells (Fig. 2, C and D). This concurs with previously published data showing that Bcl-6–expressing Tfh cells are required for the formation of GCs (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Mice lacking functional Bcl-6–expressing T cells had an ~50% reduction in switched Syndecan-1+ Bcl-6– T cells drive extrafollicular responses | Lee et al.
(CD138+) plasma cells in the spleen (Fig. S1, A and B). NP-specific IgG1 titers were reduced by >40-fold, and a more modest reduction in NP-specific IgM titers was also observed (Fig. 2 E). By day 7, few extrafollicular foci containing IgG1+ plasma cells were visible (Fig. 2 F) in the spleens of chimeras lacking Bcl-6 expression in T cells. Together, these results suggest that Bcl-6-expressing T cells are important for optimal extrafollicular antibody responses to protein antigens.

Bcl-6-expressing T cells are required for the development of antigen-specific extrafollicular plasmablasts (EFPBs)

Although the aforementioned results suggest a role for Bcl-6 in extrafollicular antibody responses, the origin of the antibody-producing cells cannot be precisely identified in this model. For example, an early wave of memory B cells that develops in parallel to the extrafollicular antibody response independently of GC, Bcl-6, ICOS (inducible T cell co-stimulator), and IL-21 has been now described by several groups (Toyama et al., 2002; Inamine et al., 2005; Chan et al., 2009; Linterman et al., 2010; Zotos et al., 2010). Another potential caveat of these experiments is that the absence of Bcl-6 expression in 80% of the B cells may influence the extrafollicular plasma cell response, although Bcl-6–deficient B cells would be expected to give rise to enhanced rather than decreased plasma cell responses as the result of unopposed Blimp-1 expression.

To control for these possibilities and accurately quantify the number of EFPBs produced, we took advantage of the SW\_HEL adoptive transfer system. CD45.1 SW\_HEL transgenic B cells bearing a rearranged HEL-specific VDJ\_H element targeted into the IgH chain locus combined with an HEL-specific κ L-chain transgene (Phan et al., 2003) were transferred i.v. into mixed Bcl6−/− CD45.2:CD28−/− CD45.2 or Bcl6+/+ CD45.2:CD28−/− CD45.2 chimeric mice in combination with HEL protein conjugated to SRBC. Thus, transferred SW\_HEL B cells have an intact Bcl6 gene, can undergo class switching, and develop into both GC B cell and EFPB populations in response to HEL (Phan et al., 2003; Paus et al., 2006). In this model, GC B cells and EFPBs can be identified by flow cytometry (Phan et al., 2003; Paus et al., 2006).

The development of HEL-binding, CD45.1 EFPBs and GC B cells was quantified 4.5 d after transfer. The gating strategies are shown in Fig. 3; GC B cells derived from transferred SW\_HEL cells are CD45.1 B220+ GL-7hi Fashi and HEL binding (Fig. 3 A). In the absence of Bcl-6–expressing T cells, the development of antigen-specific GC B cells was reduced to ~10% of the numbers found in the presence of Bcl-6–expressing T cells (Fig. 3 B), which is consistent with the previously described lack of functional Tfh cells in these chimeric mice (Yu et al., 2009).

The EFPB response derived from donor SW\_HEL B cells can be measured by enumerating CD45.1+ B220lo intracellular HEL-binding cells (Fig. 3 C; Phan et al., 2003; Paus et al., 2006). In the absence of Bcl-6–expressing T cells, the percentage of EFPBs was reduced to ~5% of that produced in the presence of Bcl-6–expressing T cells (Fig. 3 D). On day 5 of the SW\_HEL B cell response to HEL–SRBC, anti-HEL antibodies are produced exclusively by EFPBs (Paus et al., 2006). Quantification of serum anti-HEL IgM and IgG1 (the predominant

Figure 1. Bcl-6+ PD-1lo CD4+ cells are seen at the T–B border after SRBC immunization. (A–C) Immunofluorescence stains of spleen sections from C57BL/6 mice at 0 (A), 3 (B), and 7 d (C) after SRBC immunization. Boxed areas indicate the location of the zoomed-in images on the right, in the same order (from top to bottom). These data are representative of two independent experiments with four mice per experiment. F, follicle; T, T zone.
isotype found in SW_{HEL} B cell responses to HEL-SRBC; Phan et al., 2003) antibodies revealed negligible production of anti-HEL IgM in the mice lacking Bcl-6–expressing T cells (0.18% of the titer found in mice with Bcl-6–expressing T cells; Fig. 3 E) with no detectable anti-HEL IgG1 antibodies. These results demonstrate a requirement of Bcl-6–expressing T cells for the production of switched and unswitched EFPBs in response to a TD antigen.

**IL-21 acts early to promote follicular and extrafollicular antibody responses**

IL-21 is produced by Tfh cells and acts directly on B cells to maximize Bcl-6 expression and promote GC B cell growth and survival (Linterman et al., 2010; Zotos et al., 2010). IL-21 also promotes Blimp-1 expression and plasma cell formation (Ozaki et al., 2004). We compared side by side the effects of IL-21 in the course of a SW_{HEL} B cell–derived GC and EFPB response.

SW_{HEL} B cells sufficient or deficient for the IL-21R (IIl21r^{-/-}) were transferred to CD45.1 congenic C57BL/6 mice, which were immunized with HEL^{SRBC} (Fig. 4 A). HEL^{SRBC} is a mutant HEL protein that binds the SW_{HEL} BCR with lower affinity. In the absence of IL-21R signaling, the production of GC B cells was reduced to ~12% of the levels seen with IL-21R–sufficient donor cells at day 4.5 (Fig. 4 B), which is consistent with the described dependency of GC B cells on IL-21. The development of EFPBs was also impaired in the absence of IL-21R signaling, with only around 10% of the cell numbers seen with IL-21R–sufficient donor cells at day 4.5 (Fig. 4 C). In the absence of IL-21–mediated signaling, the extrafollicular response peaked 12 h later, on day 5. At this time point, there was a fourfold reduction in the number of EFPBs in the absence of IL-21R (Fig. 4 C). The GC and EFPB responses to SRBCs elicited by the host CD45.1 IIl21r^{-/-} B cells were comparable in both groups of adoptively transferred mice (Fig. S2, A and B).

The effects of IL-21 deficiency were apparent after 4 d in the course of both follicular and extrafollicular antibody responses (Fig. 4, A–C), suggesting that IL-21 produced by T cells acts at the stage of T–B interaction to enhance and accelerate B cell activation before their differentiation into either GC cells or extrafollicular plasma cells. IL-21 has recently been shown to promote GC B cell proliferation and survival, at least in part through maximizing Bcl-6 expression and survival (Linterman et al., 2010; Zotos et al., 2010). Our findings extend this effect of IL-21 to extrafollicular antibody responses and are consistent both with reports that IL-21 also promotes Blimp-1 expression and plasma cells accumulate when overexpressed (Ozaki et al., 2004) and with the paucity of extrafollicular plasma cells 7 d after NP-KLH immunization in IL-21R and IL-21 knockout mice (Zotos et al., 2010).

**Bcl-6–expressing T cells are required for the development of class-switched extrafollicular plasma cells in S. enterica infection**

We next sought to examine whether the requirement for Bcl-6 expression in T cells also occurs in the context of an extrafollicular antibody response to *S. enterica* infection, which has been shown to occur independently of IL-21 (Linterman et al., 2010). The immune response to infection with *S. enterica* induces a potent extrafollicular plasma cell response, resulting in the production of T-independent IgM antibodies and T–dependent class switching of B cells to produce IgG2a (IgG2c in C57BL/6 mice) and IgG2b antibodies (Cunningham et al., 2004; Linterman et al., 2009a). T dependency has been previously demonstrated by the absence of *S. enterica*–specific switched antibody responses in mice lacking CD28 (Cunningham et al., 2004; Linterman et al., 2009a). The GC response is considerably delayed, developing between days 20...
and 35 after infection (Cunningham et al., 2007). It has also been established that IFN-γ-producing Th1 cells are responsible for bacterial clearance, starting on week 2 after immunization (Hess et al., 1996). Thus, S. enterica infection is an ideal model in which to investigate whether Bcl-6–expressing T cells rather than conventional Th1 cells are required to drive the extrafollicular plasma cell response without the potential interference of GC B cell–derived antibodies or GC Tfh cells.

Bcl6+/+ CD45.2.Cd28−/− CD45.1 or Bcl6−/− CD45.2; Cd28−/− CD45.1 mixed fetal liver chimeric mice were infected i.p. with S. enterica. As shown previously (Cunningham et al., 2007), this immunization did not increase GC cells above background 7 d after infection (Fig. 5, A and B). At this same time point, anti–S. enterica IgM and IgG2c titers were measured by ELISA. Bcl-6 deficiency in T cells did not reduce the production of specific IgM (Fig. 5 C) but significantly reduced the titer of anti–S. enterica IgG2c antibodies (Fig. 5 D). This mirrored the requirement of CD28 for specific IgG2c but not IgM production in a cohort of C57BL/6 Cd28−/− mice (Fig. 5, E and F). Together, these results suggest that switched antibody responses to S. enterica require help from T cells in a Bcl-6–dependent manner.

Our data so far suggest that Bcl-6–expressing T cells rather than conventional Th1 cells are required for extrafollicular antibody responses. To confirm the presence of functional S. enterica–specific Th1 cells in mice lacking Bcl-6 expression in T cells, we investigated bacterial clearance, which has been shown to be critically dependent on the presence of functional Th1 cells (Hess et al., 1996). Although bacterial counts were increased by nearly 2 logs in nonchimeric Cd28−/− mice that lack Th1 cells (Fig. 5 H), lack of Bcl-6 expression in T cells did not alter bacterial load in the livers of chimeric mice 12 d after infection (Fig. 5 G).

Next, we investigated when and where Bcl-6+ T cells appear during the response to S. enterica. A population of CD3+ Bcl-6+ PD-1hi cells was detected at that outer T zone on day 4 after infection (Fig. 6, A and C), with total numbers increasing ~10-fold above background. This is consistent with our previous finding of a 20-fold increase in numbers of IgG2c switched plasma cells as early as day 4 after S. enterica infection (Cunningham et al., 2007). At this time point (day 4), CD3+ Bcl-6+ PD-1hi cells were absent (Fig. 6, A and C), but they became visible 35 d after immunization, when S. enterica–induced GCs appear, and they were located within the GC (Fig. 6 B). Together, these results highlight a necessary role for T cell–expressed Bcl-6 soon after infection to promote switching.

The expression of PD-1 and CXCR5 was evaluated on effector (CD4+ CD44hi) cells. On day 7, at which time S. enterica fails to induce GCs regardless of Bcl-6 expression, PD-1hi CXCR5hi GC Tfh cells (Fig. 6 D, red gate) that were seen at background levels in PBS-immunized chimeras
Bcl-6 is required for a T and B cell antigen–specific extrafollicular antibody response

A potential confounding factor in the aforementioned experiments is the increased proportion of activated B6l6+/− CD44hi CD4+ cells in mixed chimeras reconstituted with Bcl6+/− fetal liver (Fig. S3). To overcome this issue, we performed adoptive transfers of 10⁶ naive CD45.2 OT-II cells that express a transgenic TCR specific for OVA peptide 323–339 and were either deficient or sufficient in Bcl-6 expression (these cells expressed low amounts of PD-1 and CXCR5; Fig. S4 A) were equally represented in both sets of chimeras, irrespective of T cell expression of Bcl-6. Together, these data suggest that CD4+ CD44hi CXCR5+ Bcl-6+ PD-1lo cells may be important for the initiation of an extrafollicular antibody response.

mice (Fig. 1 B), OT-II T cells had up-regulated PD-1 on day 2, but the levels were approximately threefold lower than those of day 4 OT-II cells from immunized recipient mice analyzed on the same day (Fig. 7 B). CXCR5 expression did not increase significantly from day 2 to 4 (Fig. 7 B).

On day 5.5 after immunization, spleens were harvested, and distinct HEL-specific GC B cells and extrafollicular plasma cells were identified by flow cytometry on the basis of size and differential expression of B220, CXCR5, and HEL binding (Fig. 7 C). Recipients of B6l6+/− OT-II cells showed a parallel and comparable reduction in both GCs and EFPBs (Fig. 7, D–F). Staining with PD-1 and CXCR5 demonstrated the absence of PD-1hi CXCR5hi Tfh cells among OT-II B6l6+/− T cells on day 5.5 (Fig. 7, G and H). These results confirm that T cell–expressed Bcl-6 plays a role in B cells differentiating along the extrafollicular pathway. GL-7 has been recently described as a marker of GC Tfh cells (Yusuf et al., 2010). The appearance of GL−7th CD4+ cells among OT-II cells lacking Bcl-6 expression (these cells expressed low amounts of PD-1 and CXCR5; Fig. S5) suggests that GL−7th cells are a heterogeneous population comprised of activated CD4+ cells other than GC Tfh cells.

We observed significantly fewer switched EFPBs in the absence of T cell–expressed Bcl-6 across all experimental systems used in this study. A previous report described normal antibody titers in the first 2 wk after immunization of chimeric mice lacking Bcl-6 in T and B cells (Toyama et al., 2002). It is possible that the requirement for Bcl-6+ T cells may be overcome by signals from other cells; B cells differentiating along the extrafollicular route are also known to be boosted by IL-6 and/or APRIL (a proliferation-inducing ligand)–producing cells that colocalize with EFPBs (Mohr et al., 2009). It is also conceivable that the plasma cells and antibodies detected on day 14 in mice lacking Bcl-6+ T cells have arisen from an alternative route of differentiation. Indeed, several groups have reported a third B cell differentiation route into early memory B cells that is GC−, ICOS−, Bcl-6−, and...
IL-21\textsuperscript{−} independent (Toyama et al., 2002; Inamine et al., 2005; Chan et al., 2009; Linterman et al., 2010; Zotos et al., 2010).

Our data establish that Bcl-6 expression enables T cells to provide effective help to B cells for both follicular and extrafollicular antibody responses to protein antigens. PD-1 expression distinguishes outer T zone PD-1\textsuperscript{hi} Bcl-6\textsuperscript{+} pre-GC Tfh cells from the PD-1\textsuperscript{lo} Bcl-6\textsuperscript{+} Tfh cells that appear within the GC (GC Tfh cells). Bcl-6 has been shown to be critical for up-regulation of CXCR5 and down-regulation of CCR7 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), which are required for T and B cell localization to the T–B border (Cyster et al., 2000; Haynes et al., 2007). Thus, expression of Bcl-6 appears important to facilitate the initial T and B cell encounter at this location that results in the provision of T cell help required to initiate T-dependent B cell differentiation and Ig isotype switching. Indeed and contrary to common belief, there is evidence that production of >99% of switch transcripts occurs at this time and therefore precedes GC formation (Toellner et al., 1996; Pape et al., 2003). In agreement with this, we have shown here that in the absence of Bcl-6 expression by CD4\textsuperscript{+} T cells, the switched antibody responses to all antigens are completely abrogated, including the extrafollicular IgG2a response to S. enterica, against which only antigen–specific IgM, of probable T-independent origin, is maintained.

Our finding that T cell–expressed Bcl-6 acts at the time of T–B interaction does not exclude the possibility that Bcl-6 expression in T cells may also be required at the stage of plasmablast growth in extrafollicular foci. CXCR5\textsuperscript{lo} CXCR4\textsuperscript{hi} CD4\textsuperscript{+} T cells have been found in splenic extrafollicular foci of autoimmune MRL/Fas\textsuperscript{hi} mice (Odegard et al., 2008), and these are also Bcl-6 dependent (Poholek et al., 2010). This work suggests that Tfh are even more heterogeneous than we thought. Bcl-6\textsuperscript{+} T cells in the outer T zones appear competent to help B cells differentiate along either the follicular or extrafollicular pathway. This highlights the need for refining the nomenclature of B follicular helper T cells. As has been previously proposed (McHeyzer-Williams et al., 2009), pre-GC Tfh cells can be used to refer to T cells that prime B cells at the T–B border, which are shown here to be PD-1\textsuperscript{lo/int} Bcl-6\textsuperscript{+} pre-GC Tfh cells that appear within GCs, which are generally, albeit not universally, PD-1\textsuperscript{hi}.

**MATERIALS AND METHODS**

**Mice and immunizations.** C57BL/6, CD28\textsuperscript{−/−} mice and B66\textsuperscript{−/−} mice backcrossed for 14 generations into the C57BL/6 genetic background were maintained by the Australian National University Bioscience Services in specific pathogen–free conditions and had access to food and water ad libitum. SW\textsubscript{fem} CD45.1 mice, which carry a V\textsubscript{6}k10 light chain transgene and a knocked in VH10 Ig heavy chain in place of the endogenous IgH gene that encode a high-affinity antibody for HEL, were obtained from the laboratory of R. Brink (Garvan Institute, Sydney, New South Wales, Australia) and maintained by the Australian National University Bioscience Services. SW\textsubscript{fem} B21r\textsuperscript{+} CD45.2, SW\textsubscript{fem} B21r\textsuperscript{−/−} CD45.2, and recipient
Figure 6. Bcl-6+ T cells induced by S. enterica infection are PD-1lo and locate to the T–B border. (A and B) Immunofluorescence stains of spleen sections from C57BL/6 immunized 4 (A) or 35 d (B) previously with S. enterica. Boxed areas indicate the location of the zoomed-in images on the right, in the same order (from top to bottom). (C) Quantification of CD3+ Bcl-6- PD-1lo (left) and CD3+ Bcl-6+ PD-1hi (right) cells in the T–B border and GCs at the indicated times after immunizations. (D) Representative flow cytometric plots showing PD-1 versus CXCR5 expression on effector/memory CD4+ CD44hi cells from 80% Bcl6−/− CD45.2:20% Cd28−/− CD45.1 (Tc Bcl6−/−) chimeras or control 80% Bcl6+/+ CD45.2:20% Cd28−/− CD45.1 (Tc Bcl6+/+) fetal liver chimeric mice injected 7 d previously with S. enterica (green gate, non-Tfh effectors; blue gate, PD-1lo Tfh cells; and red gate, PD-1hi Tfh cells). These data are representative of two independent experiments with four mice per group in each cohort.
C57BL/6 CD45.1 mice were maintained in the animal facility at the Garvan Institute. For experiments involving SW<sub>HEL</sub> mice, 10<sup>5</sup> SW<sub>HEL</sub> B cells were transferred into recipients, which were simultaneously immunized i.v. with 2 × 10<sup>6</sup> SRBCs conjugated to HEL or HEL<sup>α</sup> protein (Paus et al., 2006). For experiments involving NP-KLH, immunizations with hapten (hydroxy-3-nitrophenyl) acetyl (NP) coupled to keyhole limpet hemocyanin (KLH) at a molar ratio of 16:1 were performed. 100 µg NP-KLH was precipitated in carbonate buffer (Na<sub>2</sub>CO<sub>3</sub> + NaHCO<sub>3</sub>, pH 9.6) overnight and subsequently precipitated in PBS/1% BSA. Serum was serially diluted onto the 96-well plates in PBS/1% BSA/0.05% Tween 20 buffer and incubated for 2 h at 37°C.

**Figure 7. Development of HEL-specific GC and EFPBs is impaired in the absence of OVA-specific T cells expressing Bcl-6.**

Naive OT-II B<sup>Bcl6<sup>+/-</sup></sup> or OT-II B<sup>Bcl6<sup>+/+</sup></sup> with or without SW<sub>HEL</sub> B cells were transferred i.v. 4 h apart into CD<sub>2<sup>8</sup></sub>-/- CD45.1 recipient mice, which were then immunized with HEL-OVA and/or OVA in alum i.p. (A and B) CXCR5 expression on naive (CD44<sup>-</sup>) and activated (CD44<sup>+</sup>) OT-II cells on either day 2 or 4 after adoptive cell transfer and immunization, analyzed on the same day (B). (C) Representative flow cytometric plots showing the gating strategy to identify donor-origin SW<sub>HEL</sub> GC B cells (CD45.2 B<sup>220</sup> HEL-binding<sup>CXCR5<sup>+</sup></sup> small forward scatter (FSC) and EFPBs (CD45.2 B<sup>220</sup> HEL-binding<sup>CXCR5<sup>+</sup></sup> large forward scatter) in the spleens of CD<sub>2<sup>8</sup></sub>-/- CD45.1 recipient mice 5.5 d after adoptive transfer. (D–F) Flow cytometric profiles (D) and quantification of SW<sub>HEL</sub> EFPBs (E) and GC B cells (F) in the same recipient mice 5.5 d after immunization with HEL-OVA. (G and H) Representative flow cytometric plot showing gating strategy (G) and quantification (H) of donor-origin Tfh cells identified as CXCR5<sup>hi</sup> PD-1<sup>-</sup> CD45.2 ( gated on CD<sub>4</sub><sup>+</sup> cells) after transfer of OT-II B<sup>Bcl6<sup>+/+</sup></sup> or OT-II B<sup>Bcl6<sup>-/-</sup></sup> T cells. These data are representative of two independent experiments with three to six mice per group.

**S. enterica inoculations and liver bacterial counts.** S. enterica serovar Dublin strain SL5631 (Segall and Lindberg, 1991) was grown in Luria-Bertani medium overnight. Mice were inoculated with 10<sup>6</sup> CFU's from a log-phase culture administered i.p. in PBS, with control mice receiving PBS only. Liver bacterial load was measured at day 12 after infection by homogenizing organs, plating serial dilutions in PBS onto Luria-Bertani agar, and incubating at 37°C overnight. For experiments with S. enterica serovar Typhimurium strain SL3261, mice were immunized i.p. with 5 × 10<sup>6</sup> bacteria in PBS.

**ELISA.** ELISAs were used to quantify serum titers of anti-HEL, anti-NP, and anti-S. enterica antibodies. To quantify anti-HEL and anti-S. enterica antibodies, 96-well plates were coated with either 1 µg/ml HEL protein or 250 µg/ml S. enterica lysate prepared as previously described (Valentine et al., 1998) in 0.05 M carbonate buffer (Na<sub>2</sub>CO<sub>3</sub> + NaHCO<sub>3</sub>, pH 9.6) overnight and subsequently blocked with PBS/1% BSA. Serum was serially diluted onto the 96-well plates in PBS/1% BSA/0.05% Tween 20 buffer and incubated for 2 h at 37°C.
After washing, antigen-specific antibodies were detected with anti-mouse IgM, anti-mouse IgG1, or anti-mouse IgG2a antibodies conjugated to alkaline phosphatase (Vector Laboratories). Bound antibody was detected with para-nitrophenylphosphate in glycine buffer. Plates were read at 405 nm with a microplate reader (Thermomax; Molecular Devices), and titers for serum samples were calculated as the log serum concentration required to achieve 50% maximum optical density. To detect anti-NP antibodies, 96-well plates were coated with 20 µg/ml NP14-BSA in PBS overnight. Serum samples were serially diluted on plates in block solution (PBS/1% FCS/0.6% skim milk powder/0.05% Tween 20) and incubated for at least 20 h at room temperature. Anti-NP was shown with goat anti-mouse IgG1 conjugated to horseradish peroxidase (HRP; SouthernBiotech) and visualized with ABTS substrate (2′-Azino-di-3-ethylbenzthiazoline sulphonate) diazonium salt; Sigma-Aldrich). Plates were read at 405 nm, with reference wavelength 490 nm (Molecular Devices).

**OT-II and SWHEL adoptive cell transfer experiments and HEL-OVA immunization.** For collaborative responses of SWHEL B cells and OT-II T cells, 10<sup>3</sup>-sorted CD45.2<sup>+</sup> SWHEL B cells and 20 µg HEL-OVA<sub>323-339</sub> peptide (Chan et al., 2009) were injected i.v. into CD28<sup>-</sup>CD45.1<sup>-</sup> mice. 4 h later, 10<sup>5</sup>-sorted naive CD44<sup>hi</sup> CD25<sup>-</sup> CD4<sup>+</sup> CD45.2<sup>+</sup> OT-II T cells were injected i.v. into the same recipient mice, and mice were immunized i.p. with 100 µg OVA (Sigma-Aldrich) in alum (Thermo Fisher Scientific) immediately after adoptive cell transfer.

**Flow cytometry.** To identify specific cell subsets, single cell suspension of splenocytes in ice-cold FACS buffer (2% FCS and 0.1% NaN<sub>3</sub> in PBS) were stained with the antibodies detailed below, all from BD unless specified, with thorough washing between stain layers. Flow cytometry data were acquired on a flow cytometer (LSRII; BD) running FACSdiva and analyzed with Flowjo version 8 (Tree Star). NP-binding cells were identified by staining with IgM Alexa Fluor 800 (conjugated in house; Invitrogen), NP-APC (conjugated in house; Smith et al., 2000), CD138-PE, PNA-FITC (Vector Laboratories), B220-PE Cy7, and CD45.1-Pacific blue (BioLegend). HEL-binding GC B cells were identified by first staining cells with 200 ng/ml HEL solution, followed by GL-7-FITC, CD95-PE, CD45.2-PerCP Cy5.5, anti-HEL HyHEL9–Alexa Fluor 647 (conjugated in house with an Alexa Fluor 647 antibody labeling kit; Invitrogen), B220-APC Cy7, and CD45.1-Pacific blue (BioLegend). HEL<sup>+</sup> EFPB cells were identified by treating spleens with collagenase and DNase; spleens were cut into 1-mm pieces and incubated at 37°C for 30 min with 1 ml of 1 mg/ml type II collagenase and 0.1% DNase in RPMI/10% FCS. After washing in FACS buffer as above, cells were stained with CD45.2-PerCP Cy5.5, B220-APC Cy7, and CD45.1-Pacific blue, permeabilized using the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions, and stained for intracellular HEL binding and IgG1 with HEL protein conjugated to Alexa Fluor 647 as above and anti-mouse IgG1-FITC, respectively. The presence of GC B cells in S. enterica-infected and uninfected mice was identified by staining cells with GL-7–FITC, CD95–PE, and B220–APC Cy7. T cell phenotypes were investigated using combinations of the following antibodies: CXCR5-biotin + streptavidin–PE Cy7, PD-1–PE, IL-21–APC (R&D Systems), CD4–APC Cy7, B220–FITC, CD44–Pacific blue (BioLegend), and CD25–PerCP Cy5.5.

**Immunohistochemistry.** NP-binding cells were visualized by freezing spleen samples in OCT (Tissue-Tek; Sakura). Spleen sections were prepared and stained as described previously (Toellner et al., 1996). Antibodies and conjugates used were B220-biotin (RA3-6B2), goat anti-mouse IgG1 (SouthernBiotech; HRP), and streptavidin alkaline phosphatase (SouthernBiotech). Antibodies were visualized with substrates AEC (Vector Laboratories) and FastBlue (Vector Laboratories).

**Immunofluorescence.** To visualize the responses to S. enterica and SRBC immunization, spleen samples were fixed for 20 min in ice-cold acetone (for S. enterica) or 4% paraformaldehyde for 2 h on ice, incubated in six changes of sucrose buffer overnight (for SRBCs), and embedded in Tissue-Tek OCT compound. Sections were blocked with streptavidin and biotin blocking kit (Vector Laboratories) before staining. Sections were stained for CD3 using anti-CD3 (BD) followed by anti–Armenian hamster Cy5 (Jackson ImmunoResearch Laboratories, Inc.). For Bcl-6, anti-mouse Bcl-6 (Santa Cruz Biotechnology, Inc.) was followed by donkey anti-rabbit FITC (Jackson ImmunoResearch Laboratories, Inc.) and then Alexa Fluor 488–conjugated goat anti-FITC (Invitrogen). PD-1 was stained with purified anti–PD-1 (BioLegend) followed by anti–rat Cy3 (Jackson ImmunoResearch Laboratories, Inc.). For AID, anti-mouse AID (Bioscience) was followed by biotinylated anti-rat IgG, and then streptavidin–HRP conjugates (Zymed) were followed by TSA(Tetramethylrhodamine) (PerkinElmer). Stained sections were mounted in Fluoromount-G (SouthernBiotech) and analyzed with a confocal laser-scanning microscope (DMIRXA2; Leica; SRBC experiments) or a laser-scanning confocal microscope (LSM510; Carl Zeiss) with a microscope (AxioVert 100M; Carl Zeiss) using a 40× objective. The latter images were subsequently analyzed on the LSM Image Browser (Carl Zeiss).

**Quantification of cells in tissue sections.** Quantification of Bcl-6<sup>+</sup> CD3<sup>+</sup> PD-1<sup }}>lo</sup> cells was performed as follows. 10 T zone areas were randomly selected. For each image, the number of CD3<sup>+</sup> Bcl-6<sup>+</sup> cells and their PD-1 status (bright/intermediate or negative) was quantified, and the total number of cells in each spleen was calculated as described previously (Cunningham et al., 2007). Cell numbers and densities were estimated using point counting, and counts were adjusted for the different sizes of the spleens seen throughout the study by multiplying the cells/cubic millimeter by the mass of the spleen.

**Statistical analysis.** Two-way analysis of variance was used to interrogate whether IL-21R signaling was a significant contributor to variance over a time course (Fig. 4, B and C). For other experiments, the nonparametric Mann-Whitney test (U test) was used.

**Online supplemental material.** Fig. S1 demonstrates that splenic switched plasma cells are reduced in mice lacking Bcl-6 expression in T cells in fetal liver chimeric mice immunized 7 d earlier with NP-KLH. Fig. S2 shows host-derived GC and EFPB responses 5 d after SRBC immunization. Fig. S3 shows that there is an increase in the proportion of effector cells among Bcl-6<sup }}>clo</sup> T cells from mixed chimeric mice. Fig. S4 shows that there is a decrease in the numbers of PD-1<sup }}>hi</sup> CD4<sup>+</sup> cells after S. enterica infection. Fig. S5 shows that PD-1<sup }>hi</sup> cells but not GL-7<sup }>hi</sup> cells fail to form in the absence of OT-II cell–expressed Bcl-6 after OVA immunization. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102065/DC1.

We thank Naresh Verma for help growing S. enterica, Jennifer Hogan for technical assistance, Michelle Townsend for tissue sectioning, and Matthew Cook for critical reading of the manuscript. This work was funded by a National Health and Medical Research Council (NHMRC) program grant to C.G. Vinuesa and R. Brink and an NHMRC project grant to C.G. Vinuesa. C.G. Vinuesa is supported by a Viertel Senior Medical Research Fellowship. A.F. Cunningham is the recipient of a UK Medical Research Council New Investigator award. The authors have no conflicting financial interests.

Submitted: 29 September 2010
Accepted: 1 June 2011

**REFERENCES**

Cannons, J.L., H. Qi, K.T. Lu, M. Dutta, J. Gomez-Rodriguez, J. Cheng, E.K. Wakeland, R.N. Germain, and P.L. Schwartzberg. 2010. Optimal germinal center responses require a multistage T cell: B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity*. 32:253–265. doi:10.1016/j.immuni.2010.03.010

Chan, T.D., D. Gatto, K. Wood, T. Camidge, A. Basten, and R. Brink. 2009. Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the differentiation or extrafollicular migration of early plasmablasts. *J. Immunol.* 183:3139–3149. doi:10.4049/jimmunol.0901690
Ye, B.H., G. Cattoretti, Q. Shen, J. Zhang, N. Hawe, R. de Ward, C. Leung, M. Nouri-Shirazi, A. Orazi, R.S. Chaganti, et al. 1997. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nat. Genet. 16:161–170. doi:10.1038/ng0697-161

Yu, D., S. Rao, L.M. Tsai, S.K. Lee, Y. He, E.L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, et al. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity. 31:457–468. doi:10.1016/j.immuni.2009.07.002

Yusuf, I., R. Kagayama, L. Monticelli, R.J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). J. Immunol. 185:190–202. doi:10.4049/jimmunol.0903505

Zotos, D., J.M. Coquet, Y. Zhang, A. Light, K. D’Costa, A. Kallies, L.M. Corcoran, D.I. Godfrey, K.M. Toellner, M.J. Smyth, et al. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. J. Exp. Med. 207:365–378. doi:10.1084/jem.20091777
Author/s: 
Lee, SK; Rigby, RJ; Zotos, D; Tsai, LM; Kawamoto, S; Marshall, JL; Ramiscal, RR; Chan, TD; Gatto, D; Brink, R; Yu, D; Fagarasan, S; Tarlinton, DM; Cunningham, AF; Vinuesa, CG

Title: 
B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells

Date: 
2011-07-04

Citation: 
Lee, S. K., Rigby, R. J., Zotos, D., Tsai, L. M., Kawamoto, S., Marshall, J. L., Ramiscal, R. R., Chan, T. D., Gatto, D., Brink, R., Yu, D., Fagarasan, S., Tarlinton, D. M., Cunningham, A. F. & Vinuesa, C. G. (2011). B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. JOURNAL OF EXPERIMENTAL MEDICINE, 208 (7), pp.1377-1388. https://doi.org/10.1084/jem.20102065.

Persistent Link: 
http://hdl.handle.net/11343/265013

File Description: 
Published version

License: 
CC BY-NC-SA