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Tyani D. Chan
Sandra Gardam
Dominique Gatto
Vivian M. Turner
John Silke
Robert Brink

In vivo control of B-cell survival and antigen-specific B-cell responses

Authors' addresses
Tyani D. Chan[1], *Sandra Gardam*[1*], *Dominique Gatto*[1,2], *Vivian M. Turner*[1], *John Silke*[3], *Robert Brink*[1,2]
[1]Garvan Institute of Medical Research, Darlinghurst, NSW, Australia.
[2]St. Vincent's Clinical School, University of New South Wales, Darlinghurst NSW, Australia.
[3]Department of Biochemistry, La Trobe University, Vic., Australia.

Correspondence to:
Robert Brink
Garvan Institute of Medical Research
384 Victoria St
Darlinghurst, NSW 2010, Australia
Tel.: +61 2 9295 8454
Fax: +61 2 9295 8404
e-mail: r.brink@garvan.org.au

*Present address: Department of Biomedical Molecular Research, Ghent University, Technologiepark 927, 9052 Gent-Zwijnaarde, Belgium.

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Summary: Targeted modification of the mouse genome provides the capability to manipulate complex physiological processes in a precise and controlled manner. Investigation of B-lymphocyte biology has benefited not only from the targeted modification of genes controlling B-cell survival and responsiveness, but also from the manipulation of antigen specificity made possible by targeting endogenous immunoglobulin loci. In this review, we discuss recent results obtained from our laboratory using gene-targeted mouse models to investigate the *in vivo* regulation of B-cell survival and responsiveness. The control of BAFF-dependent survival signals by the TRAF2- and TRAF3-signaling proteins is discussed as is the potential involvement of these molecules in B-lineage malignancies. We also outline the development and use of the SW_{HEL} model for analyzing antigen-specific B-cell responses *in vivo*. This includes insights into the control of early decision-making during T-dependent B-cell differentiation, the affinity maturation and plasma cell differentiation of germinal center B cells, and the identification of EBI2 as a key regulator of B-cell migration and differentiation.

Keywords: *B cells, BAFF, TRAFs, germinal center, plasma cells, EBI2*

Introduction

One of the practical advantages of studying B-lymphocyte biology is that the relative ease with these cells can be extracted from lymphoid tissues and subsequently cultured *in vitro*. The ability to study B cells under these controlled conditions has revealed much about their potential to proliferate and differentiate into antibody-secreting plasma cells in response to a wide range of T-cell-derived and T-cell-independent stimuli. Although this approach has been valuable for defining some of the key response capabilities of B cells, predicting *in vivo* B-cell behavior by extrapolating from *in vitro* observations is often difficult. For example, although mature B cells survive with a half-life of 6–8 weeks *in vivo* (1, 2), they die relatively rapidly when placed under non-mitogenic culture conditions, even in the presence of their obligate survival factor BAFF [B-cell activating factor belonging to the tumor necrosis factor (TNF) family] (3). Moreover, the differentiation of activated B cells into germinal center (GC) B cells,

a hallmark of *in vivo* T-dependent humoral immune responses, is not achievable through the addition of any known combination of soluble factors *in vitro* (4). Thus, the *in vivo* context of B cells, in particular the various microenvironments they can occupy within lymphoid tissues, must provide a combination of extracellular inputs to B cells that are difficult if not impossible to reproduce *in vitro*. This is almost certainly also true for the many other immune cell types that influence B-cell behavior *in vivo*. Clearly, therefore, rigorous *in vivo* experimentation is critical in order to obtain the best possible understanding of B-cell behavior under physiological conditions.

The identification of genetic defects associated with primary immunodeficiencies and autoimmune diseases in man is beginning to yield important clues as to how the human immune system operates *in vivo* (5). Although these data often confirm predictions derived from mouse experimentation, this is not always the case (6). Nevertheless, the manipulability and genetic homogeneity of inbred mice means that they remain the primary research tool for elucidating how immunocytes such as B cells function *in vivo*. Classically, the identification of the key molecules that control the *in vivo* behavior of B cells and other immune cell types has been achieved by administering mice with antibodies and soluble receptor proteins that either block or activate molecular function or deplete specific cell types. Although this approach has provided many valuable insights, it is typically limited to targeting extracellular molecules (cell surface or secreted) and prone to eliciting 'side effects' through constant region interactions with Fc-receptor expressing cells and occasionally other unpredictable *in vivo* phenomena (7). The ability to manipulate the mouse genome, either through the introduction of transgenic gene constructs or the specific targeting of endogenous loci, has provided a versatile and highly controlled approach to identifying how B cells survive and respond *in vivo*. For example, global and conditional gene inactivation as well as the transgenic overexpression of various gene products has revealed many of the intracellular and extracellular proteins that are fundamental to controlling B-cell physiology. In addition, the genetic manipulation of B-cell antigen receptor (BCR) specificity has provided the opportunity to track antigen-specific B cells *in vivo* and to explore the complex relationship between the nature of the antigens recognized by B cells and their ultimate physiological fate.

Over the last 10 years, our laboratory has developed a number of genetically modified mouse lines designed to explore the genetic and antigenic factors that control the survival and responsiveness of primary B cells *in vivo*. This has included the production of (i) mice specifically lacking the expression of key molecules thought to regulate B-cell survival and migration, and (ii) mice in which B cells express a defined BCR directed against a model antigen, hen-egg lysozyme (HEL).

This review provides a summary of the major findings that have emerged from our investigations and, in conjunction with related work from other groups, the impact of these findings on our knowledge of *in vivo* B-cell behavior. We also discuss the future directions of research in this area as well as some of the challenges that lie ahead.

Molecular control of B-cell survival

The various stages of B lymphopoiesis in the adult bone marrow are tightly linked to the rearrangement and successful expression of the immunoglobulin (Ig) heavy- and light-chain genes. In particular, the combination of Ig heavy and light chains to form a functional BCR is a fundamental requirement for the continued survival of B-lineage cells both at the conclusion of Ig gene rearrangements in the bone marrow and after their maturation and migration into the peripheral B-cell pool (8). The nature of the survival signals 'constitutively' delivered to B cells remain controversial but are likely to include inputs from both the classical NF-κB and phosphoinositide 3-kinase (PI3K) pathways (9, 10). However, BCR signals alone are not sufficient to sustain B-cell survival and must be complemented by signals delivered by the TNF superfamily protein BAFF through its B-cell membrane receptor BAFF-R.

BAFF, BAFF-R, and B-cell survival

BAFF (also known as BLyS or TNFSF13B) was identified over a decade ago to be a potent survival factor for B cells that is essential for preventing spontaneous apoptosis of naive mature B cells *in vivo* (11–14). Although BAFF binds to three separate members of the TNF receptor (TNFR) superfamily (TACI, BAFF-R, and BCMA), gene knockout experiments combined with a naturally occurring B-cell-deficient mutant strain (A/WySnJ) have identified BAFF-R (TNFRSF13C) as the receptor required for the transduction of BAFF-dependent survival signals to B cells (14–16). More detailed descriptions of the biology of BAFF, its closely related TNF superfamily homolog APRIL, and their receptors can be found in this issue (17) and elsewhere (18).

BAFF is expressed constitutively within the body in both soluble and membrane-bound forms by a range of hemopoietic and non-hemopoietic cell types (19). Because the signals delivered by BAFF are essential for B-cell survival, the levels of BAFF expressed *in vivo* effectively controls the size of the mature B-cell pool. Thus, mice that overexpress BAFF have a

greatly expanded numbers of mature peripheral B cells whereas these cells are virtually absent in mice that lack BAFF (12–14). BAFF appears to both facilitate the entry of immature B cells into the mature B-cell pool and subsequently maintain their survival (20, 21).

The nature of the survival signals delivered to B cells through the interaction of BAFF with BAFF-R has been investigated in some detail (18, 22). A complication in assessing the specific survival signals contributed by BAFF-R activation is the synergy between the signals delivered by the BCR and BAFF-R and the often overlapping nature of these signals (e.g. canonical NF-κB activation, PI3K activation). However, the activation of the non-canonical NF-κB pathway in naïve B cells, characterized by the proteolytic processing of the p100 NF-κB2 protein to its active p52 form, is completely dependent on both BAFF and BAFF-R (23, 24) and is known to play a critical role in maintaining B-cell survival (23, 25–27). A significant component of our work has focussed on the link between BAFF-R and the activation of NF-κB2 processing, in particular the role of members of the TNFR-associated factor (TRAF) family of intracellular signaling proteins.

TRAF signal transduction proteins

Members of the TNFR superfamily typically can be divided into those that do and do not contain death domain structures in their cytoplasmic-signaling domains. Although death domain TNFRs (e.g. TNFR1, CD95) typically recruit cytoplasmic death domain proteins (e.g. FADD, TRADD) to trigger intracellular signaling, non-death domain TNFRs (e.g. BAFF-R, CD40) activate intracellular signaling pathways by directly binding members of the TRAF family (28). There are six mammalian TRAF molecules, each of which has a carboxy-terminal TRAF domain (mediates receptor recruitment), an adjacent coiled-coil domain (mediates TRAF homo- and hetero-trimerization), and, besides TRAF1, an amino-terminal RING finger domain. In the case of TRAF6, and possibly also TRAF2 and TRAF3, these RING domains can act as lysine 63 (K63) E3 ubiquitin ligases that directly conjugate K63-linked polyubiquitin chains to specific target proteins, thereby directly or indirectly facilitating the activation of downstream signaling enzymes (29–33). This can result in the activation of multiple downstream pathways by TNFRs (28).

Nearly, all non-death domain members of the TNFR superfamily recruit multiple TRAFs to their cytoplasmic domains. BAFF-R, however, has only a single TRAF-binding site that is specific for TRAF3 (34) (*Fig. 1A*). The link between the recruitment of TRAF3 to BAFF-R and p100 NF-κB2-processing centers around the critical role of the serine–threonine kinase NIK. Active NIK is required to phosphorylate and activate another serine–threonine kinase, IKK1, which in turn phosphorylates p100 and targets it for proteasomal processing to p52 (35). Cell line studies identified TRAF3 as a suppressor of the alternative NF-κB pathway as it binds strongly to NIK and targets it for degradative proteolysis in the proteasome (36) (*Fig. 1B*). In this case, BAFF treatment was shown to cause extensive proteolysis of TRAF3, with the result that NIK levels gradually increased thereby promoting NF-κB2 processing (36). To investigate the relevance of this mechanism to the survival of primary B cells and the potential involvement of other TRAF molecules, we generated mice in which B cells lacked the expression of either TRAF2 or TRAF3.

Conditional inactivation of TRAF2 and TRAF3 in B cells

By the year 2000, conventional gene knockout mice had been produced for all six of the TRAF family members. Of these, *Traf2*$^{-/-}$, *Traf3*$^{-/-}$, and *Traf6*$^{-/-}$ mice all exhibited pre- or peri-natal lethality (37–39). Clearly, therefore, mice in which the *Traf2* and *Traf3* genes could be conditionally inactivated were required in order to meaningfully examine the roles of TRAF2 and TRAF3 in *in vivo* B-cell development and survival. Although not physically linked in either the human or mouse genomes, the *Traf2* and *Traf3* genes have arisen from a relatively recent gene duplication event and have a similar overall intron/exon structure (40). In particular, the length of the second coding exon of each gene is not divisible by three, making these exons suitable targets for 'floxing' with loxP sites in order to achieve Cre-dependent gene inactivation. This was performed for both genes using the C57BL/6 ES cell line Bruce4 (41) and with subsequent removal of the neomycin resistance cassette (42, 43).

To achieve B-cell-specific inactivation of either TRAF2 or TRAF3 expression, homozygous *Traf2*$^{flox/flox}$ and *Traf3*$^{flox/flox}$ mice were bred to carry a *Cd19*-cre transgene that directs B-cell-specific expression of Cre recombinase (43, 44). As will be discussed further below, the phenotypes of these mice (designated *Traf2ΔB* and *Traf3ΔB*, respectively) were virtually indistinguishable at the cellular level, with B cells deficient in either protein exhibiting greatly extended survival and accumulating in large numbers *in vivo* (43). Interestingly, *Traf2*$^{flox/flox}$ mice were initially crossed with the non-B-cell-specific, interferon-α/β-inducible *Mx1*-cre transgene, and yet exhibited the same B-cell phenotype as *Traf2ΔB* mice due to the preferential *in vivo* survival of the relatively small numbers

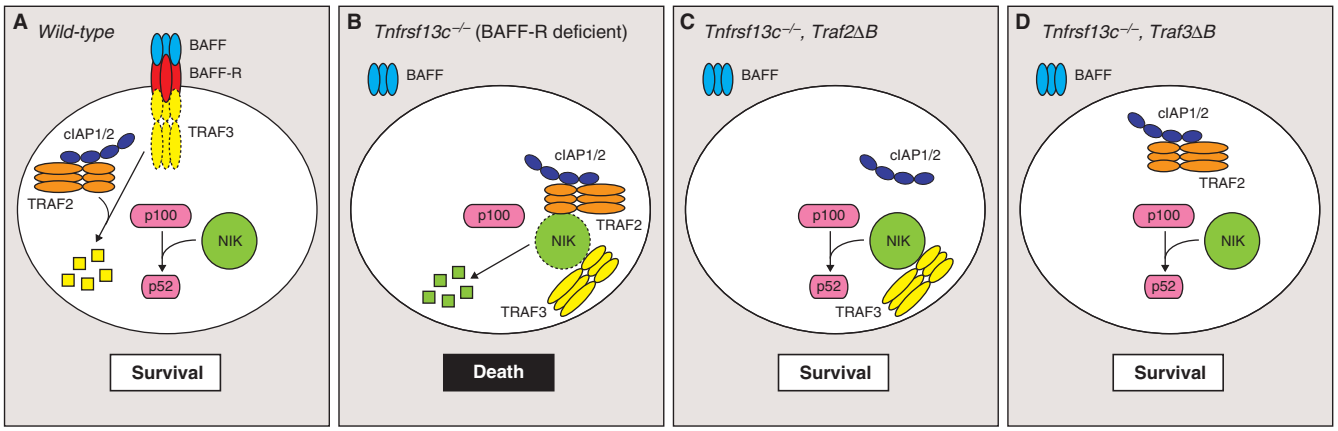

***Fig. 1.* Model for the control of B-cell survival by BAFF-R and the TRAF2 and TRAF3 signaling proteins.** (A) Under normal circumstances the binding of BAFF to BAFF-R recruits TRAF3, which is subsequently degraded in a TRAF2-dependent fashion. This liberates the serine/threonine kinase NIK, which in turn facilitates the processing of NF-κB2 p100 to p52 and promotes B-cell survival. (B) In the absence of BAFF-R (TNFRSF13C), TRAF3 remains bound to NIK that facilitates the binding to NIK of TRAF2 and associated cIAP proteins. NIK is then targeted for degradation, thus inactivating p100 processing resulting in B-cell death. (C and D) Since TRAF2 and TRAF3 are both required for NIK degradation, the absence of either protein alone results in constitutive NIK stabilization, activation of p100 processing, and BAFF-R-independent B-cell survival.

of TRAF2-deficient B cells produced due to 'leaky' expression of the *Mx1*-cre transgene (40).

TRAF2 and TRAF3 cooperate to regulate B-cell survival

As mentioned above, the major overt phenotype of mice that specifically lacked the expression of either TRAF2 or TRAF3 in their B cells was the accumulation of mature B cells in peripheral lymphoid tissues. This was particularly apparent for the marginal zone B-cell subset of the spleen and the B cells within peripheral lymph nodes, the numbers of these cells typically being approximately five-fold higher compared with wildtype controls (42, 43). This was found to be due to the longer lifespan of TRAF2- and TRAF3-deficient B cells which, unlike wildtype B cells, could survive for many days when cultured *in vitro* in unsupplemented medium (42, 43). The striking similarity between B-cell phenotypes of *Traf2ΔB* and *Traf3ΔB* mice was also reflected at the molecular level. In particular, both the processing of NF-κB2 p100 to p52 and the amount of nuclear p52 were greatly increased in TRAF2- and TRAF3-deficient B cells (42, 43), indicating enhanced activation of the alternative NF-κB pathway in each case. *In vivo* accumulation, extended survival, and heightened activation of the alternative NF-κB pathway in TRAF3-deficient B cells have been independently confirmed by another group (45), and we showed in addition that B cells lacking the expression of both TRAF2 and TRAF3 had a similar but not enhanced phenotype (43). Together, these data indicated that TRAF2 and TRAF3 play non-redundant, critical functions in negatively regulating NF-κB2 activation and B-cell survival.

Based on the *in vitro* data described above (36), the effect of deleting TRAF3 from B cells could have been predicted. Thus, recruitment and degradation of TRAF3 upon binding of BAFF to BAFF-R and subsequent liberation of NIK from being itself degraded should facilitate NF-κB2 processing and thus sustain B-cell survival (*Fig. 1A*). However, as TRAF2 does not appear to be directly recruited to BAFF-R (34), it was puzzling at first that the deletion of either TRAF2 or TRAF3 in B cells should result in such a precise phenocopy. An explanation was suggested by the fact that TRAF2 binds directly to NIK (46) and so may cooperate with TRAF3 to target NIK for degradation. Although the low levels of NIK protein in primary B cells have made this difficult to test directly in this cell type, TRAF2-deficient fibroblasts have both elevated NIK and NF-κB2 processing (47). Further detailed *in vitro* cell line studies (31, 48) support a model whereby binding of TRAF2 to NIK is facilitated by TRAF3 and results in the K48-ubiquitylation of NIK by the TRAF2-associated cIAP1 and/or cIAP2 proteins (*Fig. 1B*). This is consistent with data showing the importance of the cIAP-binding site of TRAF2 for the induction of NIK degradation in fibroblasts (49). Our data have also indicated that TRAF2 is required for the BAFF-R-mediated degradation of TRAF3 in primary B cells (43) (*Fig. 1A*), raising the possibility that cIAP1/2 may be involved in this process as well. However, the role of the cIAP molecules in BAFF-R signaling and B-cell survival remain to be defined. Mice lacking the expression of either cIAP1 or cIAP2 alone do not have any apparent B-cell phenotype (50, 51) indicating that, if these molecules do play significant roles in regulating B-cell survival, they do so in a redundant manner.

B cells deficient in TRAF2 or TRAF3 do not require BAFF-R

As mentioned above, the major overt phenotype of mice that specifically lack the expression of either TRAF2 or TRAF3 in their B cells is the *in vivo* accumulation and extended survival of these B cells. This is consistent with the constitutive and cooperative negative regulation of NIK, NF-κB2 processing, and survival by these molecules (*Fig. 1B*). All these events are outcomes of BAFF-R signaling, which triggers these responses by directing the proteolytic degradation of TRAF3 (*Fig. 1A*). The data obtained to this point, however, did not indicate the extent of the importance of the pathways negatively regulated by TRAF2 and TRAF3 to B-cell survival. Are these pathways simply one component of the survival signals delivered via BAFF-R or are they the sole means by which BAFF-R sustains B-cell survival?

To address this question, we produced mice that lacked BAFF expression (*Tnfsf13b*$^{-/-}$ mice) but that in addition did not express TRAF2 in the B-cell lineage (*Traf2Δ B*). Strikingly, B cells that lacked TRAF2 developed and survived *in vivo* despite the absence of BAFF (43). More recently, we have bred BAFF-R-deficient mice (*Tnfrsf13c*$^{-/-}$ mice) with *Traf2Δ B* and *Traf3Δ B* mice and demonstrated that the absence of either TRAF2 or TRAF3 from B cells also allows them to develop without the requirement for BAFF-R signaling (S. Gardam, V.M. Turner, R. Brink, manuscript in preparation) (*Fig. 1C, D*). In other words, the absence of either TRAF2 or TRAF3 from B cells is sufficient to activate all the pathways normally triggered by BAFF-R to sustain B-cell survival. What pathways this may include in addition to the alternative NF-κB pathway remains to be determined. It seems clear, however, that the TRAF2-dependent degradation of TRAF3 that is induced by BAFF-R is the key to B-cell survival.

Are TRAF2 and TRAF3 B-lineage tumor suppressors?

In view of the extended, BAFF-independent survival of B cells in *Traf2Δ B* and *Traf3Δ B* mice, it is perhaps not surprising that inactivating mutations in *TRAF3* and to some extent *TRAF2* have been frequently observed in human B-lineage tumors. These include a wide range of malignancies including B-cell chronic lymphoid leukemia, non-Hodgkin's lymphoma, marginal zone lymphoma, Waldenstrom's macroglobulinemia, and multiple myeloma (MM) (52–56). In the case of MM, recent evidence indicates that the inactivation of TRAF3 and other related proteins drives activation of the alternative and/or the classical NF-κB pathways and that this is likely to be the mechanism by which these mutations contribute to oncogenesis (57). Our future studies with *Traf2Δ B* and *Traf3Δ B* mice will include analysis of the development of B-lineage tumors and identification of the mechanisms by which inactivation of TRAF3 or TRAF2 contributes to tumor formation in both B cells and plasma cells.

A model for analyzing antigen-specific B-cell responses

Both the B- and T-cell repertoires are characterized by an incredible diversity of clonal, cell surface, antigen receptors. The advantage of this to the immune system is clear – the greater the number of unique antigen combining sites in the repertoire, the greater the chance of recognition of and responsiveness to an invading foreign antigen. One of the costs of lymphocyte antigen receptor diversity, however, is the resulting scarcity of antigen-specific lymphocyte clones within the primary repertoire. As a consequence, the delivery of an effective adaptive immune response typically requires a period of proliferative expansion of antigen-reactive lymphocytes before they are present at sufficient frequencies to mount a significant effector response. For immunologists, the scarcity of antigen-specific clones has presented a longstanding problem in identifying and characterizing the lymphocytes participating in either positive or negative responses to foreign and self-antigens. The ability to manipulate lymphocyte antigen specificity genetically has made a major contribution to overcoming this difficulty and provided new insights into the consequences of antigen encounter under different scenarios.

SW_{HEL} mice

The onset of mouse transgenesis in the 1980s transformed immunological research as it allowed the generation of mice in which most lymphocytes expressed a defined antigen specificity determined by the variable region genes of their antigen receptor transgenes (58). By greatly increasing the frequency of antigen-specific lymphocytes, such mice provided the first real opportunity to observe antigen-specific responses *in vivo*. In B-cell biology, probably the most successful model has involved 'MD-4' Ig-transgenic mice. These mice, which were first described in 1988, produce B cells that are specific for the well-characterized model antigen HEL (59). In conjunction with mice expressing HEL as a transgenic 'neo-self' antigen, MD-4 mice have provided many of the key findings on how self-reactive B cells generated in the primary repertoire are controlled (21, 60). However, because these original anti-HEL Ig-transgenic mice cannot undergo Ig class switch recombination (CSR) or somatic hypermutation (SHM), they have been of limited use in examining the B-cell fate during active immune responses to foreign antigen. To facilitate such

studies while retaining the advantages of the anti-HEL system, we have used a gene-targeting approach to develop the 'SW_{HEL}' strain of mice, in which anti-HEL B cells are produced that are capable of both CSR and SHM (61).

MD-4 and SW_{HEL} mice both produce B cells that have the anti-HEL specificity of the HyHEL10 mAb (62). These two lines of mice carry an identical κ light-chain transgene. However, although MD-4 mice carry the HyHEL10 heavy-chain variable region (V_H10) in a separate V_H10-μδ transgene, in SW_{HEL} mice V_H10 is targeted to the head of the endogenous C57BL/6 *Igh* locus (61), that is the position where recombined VDJ exons are found under normal physiological conditions. Initial analysis of B cells from SW_{HEL} mice confirmed that they can indeed switch to all downstream heavy-chain isotypes (61) and, when challenged with antigen, enter GCs and undergo SHM of their Ig variable region genes (63).

'Low-affinity' HEL and challenge of SW_{HEL} B cells *in vivo*

One of the potential drawbacks of both MD-4 and SW_{HEL} mice is that the HyHEL10 specificity carried by the anti-HEL B cells has an affinity for HEL (2×10^{10} M^{-1}) (62) that is 3–4 orders of magnitude higher than that exhibited by most naive B cells for either foreign or self-antigen. To provide a system that more accurately reproduces the antigen-binding affinities one would expect to find in the primary B-cell repertoire, we generated a panel of recombinant HEL proteins carrying one or more point mutations that altered the amino acid side chains involved in interactions with HyHEL10 (62). By choosing substitutions that were present in lysozyme molecules from other species (e.g. echidna, water buffalo), mutant HEL proteins were produced that bound HyHEL10 with decreased affinity but which folded normally and showed unchanged binding to other anti-HEL mAbs such as HyHEL5 and HyHEL9 (64). Mutation of one, two, or three HyHEL10 contact residues produced the HEL^{1X}, HEL^{2X}, and HEL^{3X} proteins, respectively. These proteins, which are expressed in yeast and purified from culture supernatant, bind HyHEL10 with progressively decreasing affinity (2.5×10^8, 8×10^7, and 1.5×10^6 M^{-1}, respectively) and are required at progressively increasing concentrations in order to trigger optimal intracellular tyrosine phosphorylation within SW_{HEL} B cells (64).

Although the combination of SW_{HEL} B cells and the various HEL mutants in theory provides a good system for analyzing *in vivo* B-cell responses, the relatively high frequency of anti-HEL B cells in SW_{HEL} mice (10–20% of spleen) is once again 3–5 orders of magnitude higher than would be expected for most antigen-specific lymphocytes in the primary repertoire (65). To study responses from B cells present at physiological precursor frequencies, spleen or lymph node cells containing small numbers ($1–3 \times 10^4$) of anti-HEL SW_{HEL} B cells are transferred into unirradiated CD45 congenic recipient mice for antigen challenge. As only approximately 10% of donor lymphocytes successfully migrate to the spleen (63), this approach results in an anti-HEL SW_{HEL} B-cell precursor frequency of approximately 0.001%. Although the pursuit of a physiological precursor frequency may appear to negate the objective of visualizing the early stages of *in vivo* antigen-specific B-cell responses, this approach allows anti-HEL SW_{HEL} B cells to be detectable by flow cytometry during even the earliest stages of the response through the use of antibodies directed against donor and recipient CD45 allotypes in combination with antigen (HEL) binding and autofluoresence exclusion gates (58, 66). Early localization of responding B cells is also detectable using immunofluorescence histology (63, 66). To drive *in vivo* responses to the various HEL proteins, direct conjugates with sheep red blood cells (SRBCs) are made and co-injected intravenously with donor SW_{HEL} B cells into recipient mice. This provides a strong source of endogenous anti-SRBC T cell help to the SW_{HEL} B cells and drives their T-dependent proliferation, GC B-cell differentiation, plasma cell differentiation, and antibody production (58, 63, 64, 66, 67).

Tracking affinity maturation in the GC reaction

Using the adoptive transfer system described above, SW_{HEL} B cells transferred into CD45 congenic host mice and challenged with SRBC-conjugated HEL proteins form GCs after 4–5 days. These GCs can persist for 4–6 weeks, during which time responding SW_{HEL} B cells undergo extensive Ig gene SHM, which can be monitored by single cell sorting of SW_{HEL} GC B cells and sequence analysis of the PCR-amplified V_H10 exon (64).

SW_{HEL} GC B cells from mice challenged with wildtype HEL coupled to SRBCs (HEL^{WT}-SRBC) show no selection for any specific somatic mutation (67). This lack of affinity maturation indicates that it is not possible to generate further somatic mutations in V_H10 that significantly increase the already very high affinity of the SW_{HEL} BCR for HEL^{WT} (2×10^{10} M^{-1}) and is consistent with the proposition that there is a 'ceiling' for affinity maturation when antigen affinity reaches approximately 10^{10} M^{-1} (68). In contrast, challenge of SW_{HEL} B cells with either HEL^{3X}-SRBC or HEL^{2X}-SRBC results in strong selection for the Y53D amino acid substitution in V_H10. This substitution increases the affinity of the SW_{HEL} BCR for the HEL^{3X} and HEL^{2X} proteins by 85- and 6-fold, respectively (58, 67).

Because SW_{HEL} GC B cells undergo affinity maturation in response to HEL^{3X}-SRBC or HEL^{2X}-SRBC, in theory they should exhibit an increased capacity to bind HEL^{3X} as the response progresses. As IgG1 is the major class-switched BCR expressed by SW_{HEL} GC B cells in these responses, we used flow cytometry to measure both the levels of BCR on $IgG1^+$ GC B cells and the amount of different HEL proteins bound to these antigen receptors (staining with HEL protein first and detecting bound HEL with HyHEL5-Alexa647). Examples of the binding obtained at the time of GC formation (day 5) and after 5 and 10 days of affinity maturation (days 10 and 15) in response to HEL^{3X}-SRBC are shown in Fig. 2. Staining of $IgG1^+$ GC B cells with 50 ng/ml of HEL^{3X} shows a progressive increase in the binding of the antigen over the course of the response. This is independent of BCR density and thus reflects increased affinity for the HEL^{3X} immunogen (Fig. 2*A*). On the other hand, the affinity maturation of the response to HEL^{3X} results in a reduced BCR affinity for HEL^{WT} (Fig. 2*B*), consistent with the reduced affinity of HEL^{WT} for Y53D-mutated HyHEL10. The ability to track affinity maturation of GC B cells using this system is a unique and powerful tool that should help provide insights into the molecular mechanisms that underlie this still largely mysterious process. Indeed, this system was recently employed in a collaborative study that identified a key role for the Rho–Rac GTP-exchange factor DOCK8 in the selection and survival of GC B cells (69).

Regulation of *in vivo* T-dependent B-cell differentiation

The adoptive transfer system described above provides the ability to study the earliest phases of B-cell responses to antigen. Thus, despite the fact that these responses derive from naive B cells present at physiologically low precursor frequencies, we have been able to gain fresh insights into the early migration, proliferation, and differentiation of B cells engaged in a T-dependent humoral immune response.

Early migration and proliferation

Within secondary lymphoid tissues such as the spleen and lymph nodes, B and T cells occupy distinct but adjacent areas zones by virtue of their expression of the chemokine receptors CXCR5 and CCR7. Naïve B cells exhibit a $CXCR5^{hi}CCR7^{lo}$ phenotype, which promotes their chemotaxis toward the chemokine CXCL13 produced by follicular dendritic cells (FDCs) and other stromal cells within the B-cell follicle (70). Naïve T cells, on the other hand, have a $CXCR5^{lo}CCR7^{hi}$ phenotype that directs their migration to the CCL19 and CCL21 chemokines produced by stromal cells present within the T-cell zone (71). Previous studies using HEL-stimulated MD-4 cells demonstrated that an early event triggered by the binding of antigen to the BCR is the upregulation of CCR7 and the subsequent migration of activated B cells to the border

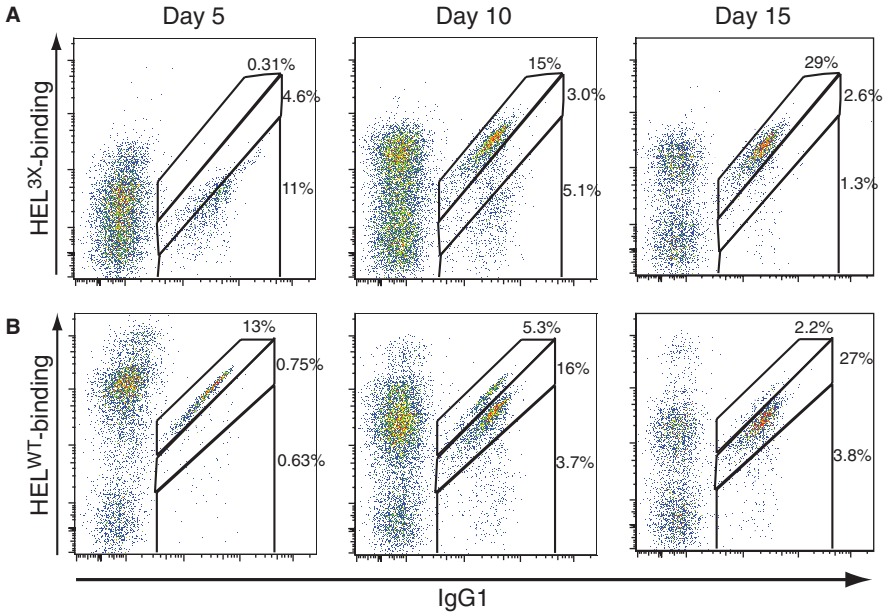

***Fig. 2.* Tracking the affinity maturation of SW_{HEL} B cells in response to HEL^{3X}-SRBC.** SW_{HEL} B cells were challenged with HEL^{3X}-SRBC in adoptive transfer as described in the text. Recipient spleens were harvested 5, 10, and 15 days after challenge and stained with a range of fluorochromes including anti-IgG1-Pacific Blue and 50 ng/ml of either HEL^{3X} (A) or HEL^{WT} (B) followed by HyHEL5-Alexa647. Plots represent data from donor-derived B cells ($CD45.1^-$, $CD45.2^+$, $B220^+$). Affinity maturation of the response is indicated by the accumulation of IgG1-switched B cells with increased HEL^{3X}-binding capacity (A). Note that this corresponds with reduced affinity for HEL^{WT} (B).

between the B-cell follicle and the T-cell zone (72). As activated $CD4^+$ T-helper cells also move to this border region by virtue of their upregulation of CXCR5 (73), the early movement of antigen-stimulated lymphocytes favors the formation of cognate T:B cell interactions and thus propagation of the response.

As would be predicted, SW_{HEL} B cells stimulated with either HEL^{WT}-SRBC, HEL^{2X}-SRBC, or HEL^{3X}-SRBC rapidly upregulate CCR7 and localize to the T:B cell border region in the spleen (63, 66). Between days 1.0 and 1.5 of the response, responding B cells remain at this site, presumably interacting with anti-SRBC T helper cells but without yet commencing cell division (66). By day 2.0, however, significant changes begin to occur. First, anti-HEL B cells move from the T:B border region and into the follicle proper. This corresponds with (and is probably due to) the return of CCR7 expression to the low levels seen on naive B cells. Second, extensive cell division commences at this time, with some responding B cells dividing up to three times in 12 h (66). Proliferative expansion of responding B cells then continues within the follicle for another 24 h in the absence of any evidence of differentiation into GC B cells or plasma cells. This follicular expansion of undifferentiated B-cell blasts has also been observed in responses to Arsonate-conjugated KLH (74), suggesting that this is a general feature of T-dependent B-cell responses.

By day 3.5 of the response of SW_{HEL} B cells to HEL^{2X}-SRBC, differentiation into both GC B cells and extrafollicular plasma cells is evident (66). Histologically, a subset of responding B cells begins migrating into central areas of the follicle that are devoid of naïve IgD^+ B cells and represent early GCs. Other responding anti-HEL cells migrate toward the poles of the follicles and into extrafollicular regions of the tissue where the early plasmablasts congregate (75). Phenotypically, early GC B cells are also recognizable at day 3.5 by virtue of their downregulation of CD38 and surface BCR levels, while the plasmablasts downregulate surface B220 and, if donor SW_{HEL} B cells also carry the Blimp-GFP reporter gene (76), are identifiable by GFP expression (66). All responding cells continue to proliferate strongly within the GC and the extrafollicular zones and expand in numbers until day 5.0 of the response. At this point, the extrafollicular plasmablasts cease proliferating and undergo an abrupt wave of synchronized apoptosis such that virtually none remain 24 h later (66). This is typical of extrafollicular responses, which serve to provide a rapid source of unmutated antibodies to initiate the neutralization of foreign antigen (75). The GC response, on the other hand, persists and acts to generate the high-affinity antibody specificities required for long-term immunity.

Ig class switching

A feature of T-dependent B-cell responses is the switching of Ig heavy-chain isotypes by the process of CSR. The class of Ig resulting from this process is thought to be heavily influenced by the cytokines produced by the T-helper cells driving the B-cell response. Thus, interferon-γ-producing Th1 cells drive responses primarily comprised of IgG2a/c antibodies, whereas interleukin-4 producing Th2 cells support mostly IgG1 and IgE responses (77). Classically, the T-helper cells elicited by SRBC immunization are thought to be Th2-like, as IgG1 antibodies and $IgG1^+$ GC B cells are the major products of these responses (78). Although this is generally also true of the responses of SW_{HEL} B cells to HEL-SRBC conjugates, it is apparent that anti-HEL antibodies of all the IgG subclasses (i.e. IgG1, IgG2b, IgG2c, IgG3) are produced in these responses (66) and that significant numbers of anti-HEL extrafollicular plasmablasts switched to each of the IgG subclasses can be detected histologically (authors' unpublished data).

To study the genesis of class switched cells in more detail, surface expression of all the IgG subclasses was analyzed throughout the earliest stages of the response of SW_{HEL} B cells to HEL^{2X}-SRBC. Responding B cells expressing either IgG1, IgG2b, IgG2c, or IgG3 were readily detectable as early as day 3.0 of the response (66, authors' unpublished data). At this point, responding cells showed no evidence of commitment to either the GC B-cell or plasma cell lineage, indicating that significant CSR occurs before these differentiation decisions have been made. This is consistent with previous observations of CSR during the very early stages of T-dependent B-cell responses (79, 80) and indicates that the commonly held assumption that CSR occurs primarily in the GC is erroneous. Both the GC and plasmablast populations carried similar ratios of cells switched to each of the IgG subclasses at the peak of the response on day 5.0 (66, authors' unpublished data). Clearly, therefore, it is possible to elicit responses involving switching to a range of Ig classes rather than a single dominant class.

BCR signal strength controls the early antibody response

The generation of a range of HEL proteins with different affinities for the SW_{HEL} BCR provided the opportunity to examine the impact of initial antigen affinity on B-cell responses. Challenge of SW_{HEL} B cells with high (HEL^{WT}-SRBC), intermediate (HEL^{2X}-SRBC), or low (HEL^{3X}-SRBC) affinity antigen resulted in equivalent GC B-cell responses in each case. However, although high- and intermediate-affinity antigen generated robust extrafollicular plasmablast and early antibody

responses, these were greatly reduced when low-affinity antigen was used (64). Although similar observations were subsequently made in a different experimental system (81), previous investigations of responses to the hapten nitrophenyl (NP) conjugated to chicken γ globulin carrier (NP-CGG) failed to show any obvious impact of antigen affinity on the extrafollicular plasmablast response. Thus, in this case, B cells with affinities ranging as low as 10^5 M^{-1} efficiently entered both the extrafollicular plasmablast and GC compartments (82–84).

One way to reconcile these two sets of apparently conflicting results was to propose that it is not antigen affinity *per se* that is the critical variable determining the contribution of B cells to the extrafollicular plasmablast response but the overall strength of the interaction between the BCR and antigen. In this case, haptenated antigens such as NP-CGG could drive low-affinity B cells into the extrafollicular plasma cell response because of their high epitope (hapten) density (e.g. typically 16 NP groups per CGG monomer) (83). To test this proposition, SW_{HEL} B cells were challenged antigens carrying intermediate (HEL^{2X}-SRBC) or low (HEL^{3X}-SRBC) affinity HEL proteins but with the density of these proteins on the SRBC surface either decreased or increased, respectively. In each case, the size of the extrafollicular plasmablast response varied directly according to the change in epitope density. GC B-cell responses, on the other hand, were not significantly altered by these changes in epitope density (64). Evidence from several systems therefore points to the conclusion that antigen-reactive primary B cells contribute to the early plasma cell response according to the strength of their interaction with the immunizing antigen. In this way, specificities with the greatest potential to neutralize antigen are enlisted into the early antibody response, even if their affinity is relatively low.

We have recently employed the SW_{HEL} system to investigate the mechanism underlying the preferential contribution to the early plasmablast response of B cells that interact strongly with antigen. Comparison of the early stages of responses to intermediate (HEL^{2X}-SRBC) and low (HEL^{3X}-SRBC) affinity antigens (conjugated at equivalent densities) revealed no apparent differences in proliferation or even initial commitment to the plasma cell lineage. Indeed on day 4.5, similar numbers of extrafollicular plasma cells could be detected by both flow cytometry and immunohistology (66). After this time point, however, the plasmablasts produced in response to HEL^{3X}-SRBC abruptly disappeared due to both a decreased rate of proliferation and an increased rate of apoptosis in comparison with the HEL^{2X}-SRBC response (66). It would appear, therefore, that daughter cells of clones that bind relatively weakly to the antigen are not impaired in their ability to undergo differentiation along the plasma cell lineage but form a smaller extrafollicular plasmablast response due to their relatively poor ability to proliferatively expand at this point of the response. The reason for this remains unclear but may be related to their ability to maintain productive interactions with extrafollicular T-helper cells that have been observed in this as well as other systems (66, 85).

Antigen affinity drives plasma cell differentiation of GC B cells

The importance of antigen affinity/interaction strength to the first wave of antibody production from unmutated extrafollicular plasmablasts led us to examine whether the second wave of plasma cell production, that is from somatically mutated GC B cells, might be similarly controlled. Evidence that this may be the case comes from previous investigation of post-GC plasma cells from the bone marrow that indicated that they are typically of higher affinity than memory B cells generated from GC precursors (86, 87). To directly test this possibility, we took advantage of our ability to identify low- and high-affinity GC B cells in SW_{HEL} B-cell responses to HEL^{3X}-SRBC (*Fig. 2B*) and, when SW_{HEL} donor B cells carrying the Blimp-GFP reporter gene were used (76), to track committed plasma cells through their expression of GFP. Two pieces of evidence obtained in this system indicated that successful differentiation of plasma cells from GC B-cell precursors requires prior acquisition of high affinity for the immunizing antigen. First of all, only approximately 25% of GC B cells had acquired the high-affinity Y53D mutation by day 10 of the response, whereas the frequency of this mutation in the plasma cell population at same point was >85% (67). Second, the frequency of GFP^+ plasma cells within the high-affinity cells present on day 10 was approximately 10-fold greater than within the low-affinity cells present at this point. It remains to be determined to what extent the mechanism of action of antigen affinity in regulating this late phase of plasma cell differentiation reprises the role of antigen affinity/interaction strength during the extrafollicular plasmablast response. Nevertheless, the interaction between antigen and BCR clearly plays a major role in shaping both early- and late-stage antibody production *in vivo*.

EBI2 controls early B-cell migration and differentiation

As discussed earlier, the differential expression of CCR7 on responding B cells plays a major role in guiding them to the appropriate microenvironments within secondary lymphoid

tissues during the early stages course of T-dependent responses. The differentiation of responding B cells into GC B cells and plasmablasts is accompanied by changes in the expression of two other chemokine receptors, namely CXCR4 and CXCR5. In particular, although both GC B cells and plasmablasts acquire a $CXCR4^{hi}$ phenotype, GC B cells retain high expression of CXCR5 while this receptor is rapidly downregulated during plasmablast differentiation (66). The $CXCR4^{hi}CXCR5^{lo}$ phenotype of plasmablasts is consistent with their movement away from the follicle where CXCL13 is produced and toward the extrafollicular regions where stromal cells express the CXCR4 ligand CXCL12. On the other hand, GC B-cell expression of CXCR5 retains them within the follicle whereas CXCR4 expression, which is subsequently modulated over the course of the GC response, appears to regulate movement within the GC (88).

Although the balanced actions of CCR7, CXCR4, and CXCR5 can explain much about the directed movement of B cells during T-dependent responses, it is more difficult to explain phenomena such as the localization of responding B cells into GCs (88) suggesting that perhaps other chemotactic receptors may also be involved in regulating B-cell migration. CCR7 was initially named Epstein–Barr virus induced 1 (EBI1) by virtue of the fact that it's mRNA was a greatly upregulated in Epstein–Barr virus-infected B cells (89). It is usually forgotten that this same study identified an mRNA encoding a second 7-transmembrane receptor, EBI2, which was similarly upregulated by Epstein–Barr virus infection (89). Although EBI1 was subsequently identified as a chemokine receptor (CCR7), its ligands identified (CCL19, CCL21), and its central role in leukocyte migration established (90), EBI2 remained for over 15 years an orphan receptor with no known function. EBI2 (also known as GPR183) is not a member of the chemokine receptor family (91, 92) but, like many chemokine receptors, signals through the pertussis-sensitive $G\alpha_i$ protein (92). The pattern of expression of EBI2 suggests that it may play a role in B-cell biology. Thus, *Ebi2* is expressed primarily in naïve B cells (89, 92), is acutely upregulated following BCR stimulation (93), and is downregulated in GC B cells via the action of the transcriptional repressor Bcl-6 (94).

To investigate the potential involvement of EBI2 in B-cell responses, we produced gene-targeted mice in which the *Ebi2*-coding exon was specifically and completely removed ($Ebi2^{-/-}$ mice) (95). Although EBI2-deficient mice were overtly healthy and showed no obvious defect in the organization of lymphocytes within secondary lymphoid tissues, immunization of these mice with SRBCs elicited a greatly reduced extrafollicular plasmablast response, despite the presence of an intact GC response (95). To further investigate the nature of this response defect, SW_{HEL} mice were crossed onto the $Ebi2^{-/-}$ background and EBI2-deficient SW_{HEL} B cells challenged in adoptive transfer with HEL^{2X}-SRBC as described previously. An advantage of this approach is that, as the B cells are transferred into normal wildtype recipients, any defect in the response can be unequivocally ascribed to the EBI2-deficient SW_{HEL} B cells. These cells did indeed produce a defective response, as the extrafollicular plasmablast response was once again greatly compromised. Detailed immunohistological examination of the early stages of the response identified that the responding EBI2-deficient SW_{HEL} B cells migrated aberrantly within the spleen. In particular, anti-HEL B cells were seen to congregate primarily around the central follicle area, with very few migrating to the poles of the follicle and into the extrafollicular areas where the early plasmablast response forms (95). Strikingly, prevention of the downregulation of *Ebi2* normally associated with GC differentiation had almost the opposite effect to EBI2-deficiency. Thus, enforced expression of EBI2 via transduction of SW_{HEL} B cells with an *Ebi2*-expressing retrovirus abrogated the GC response and resulted in most responding cells entering the extrafollicular plasmablast response (95). Similar results were obtained in a separate study (96), thus establishing EBI2 as an important regulator of T-dependent B-cell differentiation.

To address the question of how EBI2 achieved this regulation, more detailed analysis was made of the migratory properties of naïve EBI2-deficient B cells. Mixed chimeras reconstituted with wildytpe and $Ebi2^{-/-}$ bone marrow revealed that EBI2-deficient B cells tended to congregate around the central areas of the follicle, with EBI2-sufficient B cells occupying the peripheral areas (96). Transfer of $Ebi2^{-/-}$ B cells into wildtype mice resulted in a similar scenario, with the transferred EBI2-deficient B cells colocalizing with FDCs in the central follicle area. Significantly, in secondary follicles where a GC was present, EBI2-deficient B cells were largely excluded from the GC but still avoided the peripheral areas of the follicle and formed a 'ring' around the GC (*Fig.* 3).

These two recent studies indicate that EBI2 does indeed act as a chemotactic receptor on B cells. In particular, EBI2 expression appears to draw B cells toward the peripheral areas of the follicle, especially to extrafollicular regions such as the splenic bridging channels. Is it possible, therefore, that the regulation of B-cell migration by EBI2 explains its key role in regulating early T-dependent B-cell differentiation? The data obtained thus far are certainly consistent with this proposition. First, the failure to downregulate EBI2 expression greatly inhibits GC B-cell differentiation. As the loss of EBI2 facilitates

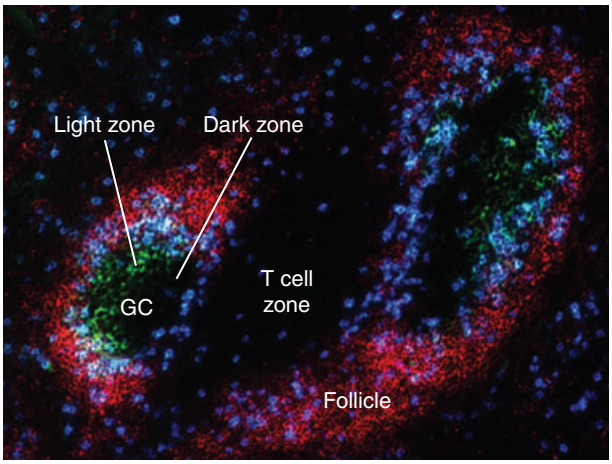

***Fig. 3.* EBI2-deficient B cells cluster around the germinal center (GC) rim in secondary follicles.** CD45.1 congenic C57BL/6 mice were immunized with SRBCs and injected intravenously with 10^7 B cells purified from C57BL/6 *Ebi2*$^{-/-}$ mice (CD45.2$^+$) 7 days later. Recipient spleens were harvested after a further 16 hours, sectioned, and then stained for IgD (red), CD21/35 (green), and CD45.2 (blue). Two distinct B-cell follicles can be seen in red, each of which contains an IgD$^-$ GC. Note the CD21/35-expressing FDCs in the GC light zone and the T-cell zone-proximal dark zone devoid of FDCs. Blue EBI2-deficient B cells can be seen forming a 'ring' around each of the GCs. Very few of these cells either access the GC proper or distribute throughout the peripheral regions of the follicle.

migration to the central follicle where GCs form, it is possible that responding B cells simply cannot enter the GC microenvironment and receive the appropriate differentiation signals (e.g. from FDCs, Tfh cells) without downregulating *Ebi2*. However, as naïve EBI2-deficient B cells are 'attracted' to GCs but do not enter (*Fig.* 3), separate activation-induced changes are required in addition to EBI2 downregulation in order for B cells to efficiently enter the GC. Second, the absence of EBI2 inhibits migration to the extrafollicular regions where the early plasmablast response occurs. As EBI2-deficiency also downregulates this component of the response, it is possible that differentiation along the plasma cell lineage may be reinforced by extrafollicular versus follicular migration. Whatever the precise mechanisms may be, these data on EBI2 function during B-cell migration and differentiation point to a potentially important link between lymphocyte migration and differentiation decisions (97).

Concluding remarks

The studies carried out in our laboratory over the last 10 years using genetically modified mouse models have revealed important information on the mechanisms that regulate B-cell survival and responsiveness *in vivo*. Future challenges will be to extend our knowledge of these basic mechanisms and to use this information to better understand the basis of B-cell-dependent disease. In relation to B-cell survival, an immediate challenge is to identify the role of the cIAP proteins in this process and to establish how mutation of the genes encoding TRAF2, TRAF3, and potentially the cIAP proteins contributes to B-lineage cancers such as MM. Future studies using the SW_{HEL} system should continue to be informative as much remains to be discovered in relation to antigen-specific B-cell responses. Projects examining the regulation of B-cell memory, B-cell responses to HEL-expressing infectious agents, IgA and IgE antibody production, and positive and negative selection in the GC are all in progress and promise to provide insights into mucosal immunity, allergic responses, autoimmunity, and vaccinology. As ever, the ultimate challenge will be to eventually translate these findings into improved patient outcomes. The ability to investigate physiological processes using the controlled *in vivo* system provided by genetically modified inbred mice will continue, either directly or indirectly, to form a major component of our efforts to understand and treat human disease.

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