Peripheral B lymphocyte tolerance

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Abstract. This lecture discusses two interrelated topics, B cell tolerance in the peripheral immune system and BAFF. Using the 3-83 antibody transgenic mouse bred to mice carrying cognate antigen in the liver, we previously found that clonal elimination drastically reduced the precursor frequency of autoreactive cells. The consensus model to explain this tolerance is the 2-signal hypothesis, which proposes that in the absence of T cell help BCR stimulation is a negative signal for B cells. However, this model fails to explain how these same B cells can respond to T-independent type II (TI-2) antigens, raising the question of how they distinguish TI-2 antigens from multimeric self determinants. We propose that B cells use NK-like missing self recognition to provide the needed specificity, as foreign antigens are unlikely to carry self markers. The model has implications for the evolution of the immune system, B lymphocyte signaling, tissue specificity of autoimmunity, and microbial subversion of the immune system. Overexpression of the critical B cell survival cytokine BAFF/BLyS has been associated with autoimmunity. We have discovered a novel splice isoform that regulates BAFF activity and may play a role in limiting B cell activity. The novel form, called Δ BAFF, is able to heteromultimerize with normal BAFF and can suppress receptor binding and proteolytic release from the cell surface. Preliminary studies from transgenic mice overexpressing wild type or Δ BAFF are consistent with a possible regulatory role for Δ BAFF, raising the possibility that the relative expression levels of BAFF and Δ BAFF regulates tolerance. (Keio J Med 53 (3): 151–158, September 2004)

Key words: antibody, BAFF, TNF13B, inhibitory signaling, tolerance, B lymphocyte

Introduction

A new theory of self/non-self discrimination in mature B cells

A coherent theory to explain mature B cell responsiveness and tolerance does not exist. A number of lines of evidence indicate that mature B cells are tolerizable. The consensus model to explain this tolerance is the 2-signal hypothesis,¹ which proposes that in the absence of T cell help BCR stimulation is a negative signal for B cells, leading to death or functional inactivation. However, these same B cells can respond to T-independent type II (TI-2) antigens, which have the properties of high multivalency and an absence of T cell determinants. (TI-2 antigens by definition lack intrinsic mitogenicity and therefore likely do not activate toll-like

receptors.) Indeed, responses to TI-2 antigens can occur normally in T cell deficient mice. These considerations raise the question of how B cells can distinguish TI-2 antigens from multimeric self determinants.

A competing alternative to the 2-signal model, initially suggested by Lederberg, is that tolerance sensitivity of B cells is dependent on the age of the individual; cells from immature individuals are tolerance sensitive, whereas those of adults are activatable only. Work of several laboratories has indeed indicated that recently formed splenic B cells or neonatal B cells may be especially susceptible to tolerance induction.² Unlike newly formed bone marrow B cells,^{3–5} these cells do not appear to undergo efficient receptor editing, but are tolerized through an apoptotic process.⁶ In addition, the ability of B cells to respond to TI-2 antigens is acquired relatively slowly after birth.⁷ While transi-

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Fig. 1 TI-2 Ags (**A**) may be distinguished from self (**B**) by a second marker on self cells. Receptors for the putative second signal could be expressed by soluble factors in the plasma, by putative non-T-cell leukocyte “helpers,” or, as indicated here, by the B-cell itself.

tional B cells are more easily tolerized in some experimental situations, mature B cells are clearly tolerizable *in vivo*, at least in the case of some experimental ligands such as anti-IgD, lysozyme or MHC class I.^{8–13} Given that these tolerizable cells are able to respond to TI-2 antigens, despite the high multivalency of TI-2 antigens and their ability to evoke antibody responses even in the total absence of T cells,^{14–15} it is not clear how TI-2 antigens avoid inducing tolerance and promote activation. In an attempt to resolve this paradox, we have recently proposed the hypothesis that B cells use NK-like missing self recognition to provide the self/non-self discrimination needed to distinguish foreign TI-2 antigens from multivalent self.¹⁶

“Missing self” recognition is believed to be an important aspect of natural killer (NK) cell signaling.¹⁷ In NK cells, several receptors are expressed that can stimulate activation of killer function.¹⁸ These activatory receptors are counteracted by the signaling of more numerous inhibitory receptors. Both activating and inhibitory receptors often recognize MHC class I molecules or related structures. Because of the balance of signals mediated by these receptors, NK cells can be activated under physiological conditions by target cells that lose the expression of a subset of self MHC molecules. This is the basis for such phenomena as hybrid resistance, which is the ability of an F1 hybrid of two inbred strains to reject parental bone marrow grafts through NK-mediated killing. NK recognition is thought

to be important for killing of infected or defective host cells, which may lose MHC class I expression as part of a microbial strategy or tumor selection to hide from the CD8 cytotoxic T cells of the adaptive immune system.

As illustrated in Fig. 1, we propose that unlike foreign antigens, self antigens, even those that are displayed in a multimeric array, are associated with one or more widely expressed “self markers” that are capable of stimulating a B cell’s inhibitory receptors. Putative self markers would include particular carbohydrate moieties, such as Sia2-6Galβ1-4GlcNAc, the widely expressed host ligand for the B cell-restricted inhibitory receptor CD22. Another established class of self markers is the MHC class I antigens; B cells carry at least one cell surface inhibitory receptor with apparent reactivity to MHC class I molecules, PirB, but its functional significance is unknown. Interestingly, both PirB and CD22 genes have strong homology to NK inhibitory receptors (KIRs) and their genes are located in the genome adjacent to the major KIR homology cluster in both human (chr 19q13) and mouse (chr 7), within the leukocyte receptor cluster (LRC).¹⁹ Upon activation, PirB and CD22 proteins are phosphorylated at immunoreceptor tyrosine inhibitory motifs (ITIMs; consensus [L/I/V/S]xYxx[L/V]) and recruit the tyrosine phosphatase SHP-1,^{20–23} a mode of action essentially identical to the authentic NK inhibitory receptors.²⁴ Other B cell receptors with inhibitory function also map to the LRC region, including LAIR and CD66a.¹⁹ Additional in-

hibitory receptors on B cells that contain ITIM motifs and recruit SHP-1, SHP-2 or SHIP-1 phosphatase activities include PD-1, FcγRIIb, CD72, CD31 and additional members of the FcR superfamily.^{25–30} Therefore, B cells express a diversity of inhibitory receptors similar to those of NK cells that may participate in “missing self” recognition.

In contrast to their expression of a multiplicity of NK-like inhibitory receptors, B cells do not appear to express any NK-like activatory receptor except the BCR itself. Activatory receptors on NK cells are similar to BCR and TCR in their modes of intracellular signaling. Like the antigen receptors, activatory NK receptors do not have intrinsic signaling function; they are transported to the plasma membrane with signal transducer proteins.¹⁸ The transducers themselves are small dimeric transmembrane proteins with conserved cytoplasmic immunoreceptor associated tyrosine activatory motifs (ITAMS); consensus: [D/E]xxYxx[L/I]xxxxxxYxx[L/I].³¹ These ITAM motifs are sites of tyrosine phosphorylation and recruitment of ZAP70 and/or syk protein tyrosine kinases. Signal transducers for T cells, NK cells, and myeloid cells include CD3ζ, γ, δ, ε, DAP12 and FcεRIγ. B cells lack these transducers, but express the related heterodimer Igα/β. Interestingly, like the NK cell ITAM-containing transducer DAP12, Igα is encoded near the KIR locus at 19q13.2. Igα and Igβ are required both for B cell antigen receptor transport to the plasma membrane and coupling of antigen recognition to the signaling machinery of the cell through cytoplasmic ITAM motifs.³¹

A common feature of activatory receptors is the presence in the transmembrane region of polar or charged amino acid residues (usually K or R) that interact with a residue of opposite charge (D or E) in a chain of one of the associated transducers.^{18–31} A consequence of this design is that expression of functional activatory receptors is restricted by transducer expression and specificity of association. Because Igα and Igβ do not appear to directly associate with activatory receptors other than sIg (nor to dimerize with other transducers), the lack of expression in B cells of transducers besides Igα/β insures that B cells cannot use non-immunoglobulin activatory receptors, even those that are expressed inside the cells, such as PirA.³² In contrast, NK and myeloid cell types express multiple transducers that convey signals from a broader array of receptors. For example, NK cells express CD3ζ and FcεRIγ in addition to DAP12. Some activatory receptors, such as NKp46, interact with all of these transducers, while others associate solely with particular homodimers of DAP12 (human KIR2DS) or FcεRIγ (mouse NKR-PI)¹⁸; NKp46 can also signal through CD3ζ-FcεRIγ heterodimers. In any case, the suppression by B cells of alternative signal transducers along

Fig. 2 Hypothetical evolutionary progression differentiating lymphocyte function from its primordial NK activity, to NK and adaptive lymphocyte subsets. Shapes in gray represent inhibitory receptors, those in white, activatory receptors. The model suggests that with the invention of V(D)J recombination at a midpoint in evolution, a subset of lymphocytes discontinued expression of most or all non-rearranging activatory receptors, increasingly focussing on the rearranging activatory receptors BCR or TCR, while retaining multiple inhibitory receptors.

with the specificity of Igα/β for association with sIg appears to insure that a single type of activatory receptor, the BCR, is functional in B cells.

We therefore propose that in the evolution of the adaptive immune system, B and T lymphocytes suppressed expression of innate activatory receptors after the invention of V(D)J recombination, but retained the recombining activatory receptor, along with a significant collection of inhibitory receptors (Fig. 2). One vestige of the proposed NK origin of lymphocytes is the focus of NK and CD8 T cells on similar MHC class I antigens. A second vestige is the continued expression on a subset of T cells of the NK activatory receptor NK1.1, which in part defines the NKT cell.³³ (Upon activation, CD8 cells express a different type of NK receptor associated with the PI3K-recruiting adapter DAP10).³⁴ This hypothesis may also help explain odd NK-like features sometimes seen in lymphocytes that promote monoallelic distribution of receptors, such as parental imprinting of the PirB gene.³⁵

The notion that B cells express inhibitory receptors that recognize widely expressed self ligands has numerous implications for immune tolerance and autoimmunity. It is well known that tissues protect themselves from the toxic effects of antibody-mediated complement activation by expressing various regulators of the complement system, such as CD46, CD55 and

CD35; such inhibition works at the effector phase of antibody responses.³⁶ We suggest that the role of self marker expression by tissues is in part to suppress the initiation phase of such antibody responses through engagement of inhibitory receptors on B cells.

However, tissues may differ in their expression levels of self markers because there are likely to be multiple self markers and cognate receptors involved in the tolerance process. This heterogeneity, coupled with allelic variation in self markers such as MHC class I molecules, may lead to a situation in which in certain individuals particular tissues are more vulnerable than others to a breakdown in tolerance. This target tissue component of immune tolerance may explain how even general defects in lymphocytes can lead to tissue specific autoantibody disease.³⁷ A second hypothetical contributor to autoantibody disease could be aberrant expression of non-BCR activatory receptors on B cells. For example, aberrant expression of Fc ϵ RI γ and Fc γ RIII in mature B cells is predicted to result in B cell hyperreactivity. Interestingly, these receptors are expressed by early progenitors of T and B cells, but are normally turned off with maturation.³⁸

Pathogenic microbes have evolved means to suppress antibody responses. One way they can do so is to produce ligands for inhibitory receptors. The human cytomegalovirus is known to produce a pseudo MHC class I ligand UL18 that engages not only NK inhibitory receptors, but also the B cell inhibitory receptor ILT2, the human homologue of PirB.^{39–40} B cells and phagocytes recognize sialic acid moieties on self cells using a number of inhibitory receptors of the so called Siglec family.³⁹ B cells may express as many as 5 different Siglecs, including CD22.³⁹ Because the ability to produce sialic acids is a relatively recent evolutionary invention, lacking in most non-deuterostome organisms, it is significant that several pathogenic bacteria have independently evolved or captured the enzymatic machinery to do so from their hosts.³⁹ This bacterial adaptation should suppress antibody responses early in infection, prior to the development of robust T cell help. Another potential way that microbes could perturb the B cell system is to introduce activatory signaling that is independent of the BCR. This is known to occur in the case of two viruses, Epstein-Barr virus of humans and Bovine leukemia virus, which encode ITAM-containing proteins that can promote cell activation, growth and survival of infected cells.⁴¹ Such dysregulation probably promotes B cell neoplasia.

The regulation of B cell survival by BAFF

In the preceding section, we have proposed that B cell recognition of self by inhibitory receptors, a cell intrinsic mechanism, facilitates the self/non-self dis-

Table 1 Genes Implicated in B Cell Tolerance or Autoimmunity

B cell intrinsic	References
Fas	(59)
Cr2 (CD21/CD35)	(60, 61)
CD40	(62, 63)
Fc γ RII	(64)
Lyn	(65–67)
SHP-1	(68, 69)
CD22	(70, 71)
Bcl-2	(72)
Bim	(73, 74)
TLR9	(75)
TACI	(76)
B cell extrinsic	
FasL	(77–79)
Complement C1q, C4	(80, 81)
CD40L	(63, 82–84)
IL-4	(62, 85, 86)
BAFF	(50, 87, 88)
Tlr4 (LPS)	(89)

crimination. The outcome of BCR signaling leading to B cell anergy, activation, survival or death will reflect thresholds of positive and negative signals, along with extrinsic signals that shift these thresholds. Table 1 lists genes expressed in B cells or non-B cells whose overexpression or deficiency is implicated in promoting autoimmunity and breakdown in B cell self-tolerance. In transgenic models where autoreactive B cells have been studied, the fate of autoreactive cells is dependent on the presence of competing, non-autoreactive B cells, indicating that B cells compete for a limiting survival factor.^{42–43} As we discuss below, a key B cell survival factor whose overexpression is implicated in humoral autoimmunity is BAFF.

Most B cells require at least two signals to survive in the peripheral immune system: intrinsic expression of BCR (i.e., activatory receptor) on the cell surface⁴⁴ and extrinsic availability of the TNF family cytokine BAFF.⁴⁵ Furthermore, BCR and BAFF signaling are also important in the TI-2 antibody response.^{46,47} BAFF overexpression *in vivo* promotes autoimmune lupus-like disease and potentiates antibody responses.^{47–50} On the other hand, mice deficient in BAFF or the BAFF receptor (BAFF-R) lack long-lived follicular B cells and are hyporesponsive to immunization and germinal center maturation.^{48,51–53} BAFF also interacts with two other receptors, TACI and BCMA, which are also able to bind to APRIL, BAFF's closest homologue. Importantly, treatment of lupus-prone mice or a mouse model of collagen-induced arthritis with soluble Fc fusion proteins of TACI, which binds to BAFF, can reduce disease incidence and severity.⁵¹ On a normal, non-autoimmune background, short-term treatment

Fig. 3 BAFF transgenic (Tg) mice, but not Δ BAFF transgenics, have preferential augmentation of serum IgA levels. Independent male founder mice transgenic for BAFF, Δ BAFF, or non-Tg controls were tested for serum levels of IgM, IgA, IgG1, and IgG2a/b.

with BCMA-Fc fusion protein reduced B cell numbers by two-fold.⁵⁴ Because overexpression or underexpression of BAFF has pathogenic consequences, it is likely that natural antagonists of BAFF action exist.

We recently identified a novel splice isoform of BAFF that exists in both mouse and human.⁵⁵ This form, which is called Δ BAFF because it lacks a 57-bp exon, appears to be abundant in quiescent macrophages, while the full-length form predominates in activated macrophages, in which total BAFF levels are greatly induced. Furthermore, Δ BAFF can assemble disulfide-linked complexes both with itself and with wild type BAFF. Interestingly, unlike full length BAFF, Δ BAFF fails to be efficiently cleaved from the cell membrane. These features, along with the ability of Δ BAFF coexpression to limit BAFF bioactivity in a mouse model system, suggest that Δ BAFF may play a role in limiting antibody, and autoantibody, responses. Δ BAFF may modulate BAFF activity by heterotrimerizing with newly made BAFF to affect receptor specificity or potency, or by preventing efficient release of BAFF from the plasma membrane. Alternatively, or in addition, Δ BAFF may heterotrimerize with other TNF family molecules, such as APRIL, which may suppress B cell response or survival through TACI or BCMA signaling. We have begun to probe these possibilities using a transgenic overexpression strategy.

Method

Transgenic constructs were generated by introducing BAFF or Δ BAFF cDNA sequences downstream of a 2.9 kb human CD68 promoter.⁵⁶ The human CD68 promoter construct has been used in transgenic mice to generate myeloid specific expression.⁵⁶ Vector sequences were removed and isolated insert DNA was microinjected into [(DBA/2XC57Bl6/J)F1 X (C3H/

HeJXC57Bl6/J)F1] zygotes. Young adult mice were analyzed for plasma immunoglobulins by solid phase ELISA assay. Flow cytometry analysis was used to quantify the numbers of B lymphocyte subsets in the lymphoid organs of young adult offspring of founder mice bred to C57Bl6/J. A detailed description of the transgene constructs and transgenic mouse characterization will be presented elsewhere (Gavin, *et al.* in preparation).

Results

Disparate in vivo biological effects of BAFF and Δ BAFF

We have compared the *in vivo* functions of BAFF and Δ BAFF in a series of transgenic mice expressing BAFF or Δ BAFF under the control of the human CD68 promoter, which drives expression in myeloid and dendritic cells, where BAFF is normally expressed.⁵⁶ As shown in Fig. 3, several independent founder lines carrying BAFF transgenes have roughly 100-fold increased levels of IgA, but only ~10 fold higher levels of IgM and marginal increases in IgG. In contrast, Δ BAFF transgenics lack elevated (or reduced) IgA levels relative to wild type. While preliminary, this finding suggests that BAFF may have an especially significant, selective adjuvant effect on humoral mucosal immune responses. In addition, Δ BAFF transgenics manifested a reduced level of plasma IgG2a/b and reductions in overall B cell numbers, particularly in the spleen where marginal zone B cell numbers were much lower (Table 2).

Discussion

While our analyses of BAFF and Δ BAFF transgenic mice are still quite preliminary, our results are

Table 2 Quantitation of Splenic Marginal Zone B Cell Numbers

MZ cells*	Littermate controls	Delta BAFF Tg	BAFF Tg
Mean (Std Dev)	3.8 (1.2) $\times 10^6$	2.4 (0.7) $\times 10^6$	13.2 (4.6) $\times 10^6$
n =	15	13	11
P value**	N/A	>0.001	>0.0000001

* Phenotype defined by flow cytometry staining (B220+ CD21hi CD23lo). ** P value from two tailed, two sample equal variance Student's T Test comparing transgenic to control.

consistent with other studies indicating that BAFF overexpression can increase B cell numbers, and preferentially augment IgA and, to a lesser extent, IgM serum levels.⁵⁷ Of greater novelty were the results using the Δ BAFF expressing transgene. We had guessed that mice expressing this construct should suppress the effects of wild type BAFF, resulting in a phenotype resembling BAFF-deficient mice.⁴⁸ Our results seem to confirm the prediction that Δ BAFF expression partly neutralizes BAFF activity, because the Δ BAFF overexpressing mice had the predicted reductions in B cell numbers. However, little reduction in serum IgM or IgA levels was seen relative to wild type mice. In contrast, IgG2a/b levels were reduced in Δ BAFF transgenic mice. These results may suggest a role for Δ BAFF in regulating isotype switch, antibody forming cell survival, or the expression of other cytokines that regulate these processes. Gorelik *et al* demonstrated that a major source of BAFF expression is radio resistant stromal cells.⁵⁸ The CD68 expression of these is unknown and might explain why our Δ BAFF transgenic mice do not more resemble a BAFF-deficient phenotype. So far, the most dramatic phenotype in Δ BAFF transgenic mice has been the reduced numbers of marginal zone phenotype cells, perhaps reflecting the myeloid cell expression pattern of the transgenic promoter.

However, the phenotype of Δ BAFF transgenics might be based not only on the ability of Δ BAFF to act as a dominant negative of BAFF in CD68 positive cells; Δ BAFF subunits might associate with other TNF family proteins, such as APRIL, or form homotrimers with unique biological functions. Δ BAFF homomultimers can form and be transported to the plasma membrane of transfected 293 cells or S17 stromal cells.⁵⁵

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