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B cell TLRs and induction of immunoglobulin class-switch DNA recombination

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1. ABSTRACT

Toll-like receptors (TLRs) are a family of conserved pattern recognition receptors (PRRs). Engagement of B cell TLRs by microbe-associated molecular patterns (MAMPs) induces T-independent (TI) antibody responses and plays an important role in the early stages of T-dependent (TD) antibody responses before specific T cell help becomes available. The role of B cell TLRs in the antibody response is magnified by the synergy of B cell receptor (BCR) crosslinking and TLR engagement in inducing immunoglobulin (Ig) class switch DNA recombination (CSR), which crucially diversifies the antibody biological effector functions. Dual BCR/TLR engagement induces CSR to all Ig isotypes, as directed by cytokines, while TLR engagement alone induces marginal CSR. Integration of BCR and TLR signaling results in activation of the canonical and non-canonical NF-κB pathways, induction of activation-induced cytidine deaminase (AID) and germline transcription of IgH switch (S) regions. A critical role of B cell TLRs in CSR and the antibody response is emphasized by the emergence of several TLR ligands as integral components of vaccines that greatly boost humoral immunity in a B cell-intrinsic fashion.

2. INTRODUCTION

TLRs are a conserved and widely distributed family of PRRs that sense diverse types of microbe-associated molecular patterns (MAMPs). Other PRRs include NOD-like receptors (NLRs), CARD helicases, C-type lectins and scavenger receptors (1-4). The TLRs were originally discovered in mammals based on their homology to the *Drosophila* Toll receptor (5, 6), though the TLR family is more ancient and present in lower organisms such as sponges (7, 8). In mammals, TLRs are expressed mainly in macrophages, dendritic cells (DCs), epithelial cells, neutrophils and B lymphocytes (2, 9-12). They consist of an extracellular or intra-endosomal ligand-sensing domain composed of leucine-rich repeats (LRRs), a transmembrane helix and a cytoplasmic TIR domain that initiates signaling (10, 13, 14). TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on plasma membranes where they can bind MAMPs located on the surface of microorganisms, and TLR3, TLR7, TLR8, and TLR9 are expressed in endosomes, where they can sense internalized nucleic acid MAMPs (12, 15, 16) (Table 1).

TLRs function as a critical link between innate and adaptive immunity, as TLR engagement by MAMPs

Table 1. TLRs and their MAMP ligands

TLR	MAMP ligand(s)	MAMP-containing microorganisms	TIR adapters	Expressing cells
TLR1	Triacyl lipopeptides (e.g., Pam_3CSK_4)	Mycobacteria, bacteria,	MyD88/MAL	B cells, monocytes/Mϕ, mDC, neutrophils
TLR2	Diacyl- or triacyl- lipopeptides (e.g., Pam_2CSK_4, Pam_3CSK_4), lipotechoic acid, zymosan	Mycobacteria, bacteria, yeasts	MyD88/MAL	B cells, monocytes/Mϕ, mDC, neutrophils
TLR3	Double-strand RNA	Viruses	TRIF	B cells, monocytes/Mϕ, mDC
TLR4	LPS	Gram-negative bacteria	MyD88/MAL, TRIF/TRAM	B cells, monocytes/Mϕ, mDC, neutrophils
TLR5	Polymerized flagellin, flagella	Bacteria	MyD88	B cells, monocytes/Mϕ, mDC
TLR6	Diacyl lipopeptides (e.g., Pam_2CSK_4), lipotechoic acid, zymosan	Mycobacteria	MyD88/MAL	B cells, monocytes/Mϕ, mDC, neutrophils
TLR7	Single-strand RNA	Bacteria, viruses	MyD88	B cells, monocytes/Mϕ, pDC, neutrophils
TLR8	Single-strand RNA	Bacteria, viruses	MyD88	B cells, monocytes/Mϕ, mast cells, neutrophils
TLR9	Unmethylated CpG- containing DNA	Bacteria, viruses	MyD88	B cells, monocytes/Mϕ, pDC, neutrophils
TLR11	Profilin (from uropathogenic bacteria)	Toxoplasma	MyD88	B cells, monocytes/Mϕ

Abbreviations: Mϕ: macrophages; mDCs: myeloid DCs; pDCs: plasmacytoid DCs.

activates not only innate immunity (as extensively reviewed elsewhere (1, 9, 17, 18)) but also adaptive immunity (3, 19-23). Adaptive antibody responses are impaired, to various degrees, by mutations in genes encoding TLRs (24-32), TLR regulatory molecules, such as the TLR4 co-receptor CD14 (33, 34) or Unc93b1, which regulates endosomal TLR trafficking (35-37), TLR signaling adaptors, such as TIR-domain adaptors MyD88 (38-42) or TRIF (43), or downstream signal-relaying molecules, such as IRAK-4 (44, 45). For instance, antibody responses to capsular bacteria are compromised in patients deficient in MyD88 or IRAK4, resulting in recurring pyogenic infections (46, 47).

TLRs regulate and integrate functions of various immune cell types to mediate adaptive immunity. TLRs activate macrophages, leading to phagocytosis and "processing" of microbes and subsequently display on the cell surface of microbial peptide fragments in conjunction with major histocompatibility II (MHC II) molecules for presentation to T helper (T_H) cells (18, 48). TLR engagement in immature DCs results in maturation and activation of DCs, which, in turn, function as antigen-presenting cells and activate B and T cells (49, 50). Also, TLR-activated DCs, as well as epithelial cells, can secrete BAFF and APRIL, which, by engaging the BAFF-R, BCMA and/or TACI receptors on B cells, play important roles in B cell differentiation (51). Importantly, TLRs expressed in B cells can be directly engaged by MAMP ligands, as first suggested by findings showing B cells could be activated by LPS or polymerized flagellin well before the discovery of genes encoding TLRs (52-57). TLR engagement by MAMPs concominant with BCR crosslinking, as mediated by repetitive polysaccharidic or proteinic antigens that are typically "linked" naturally to MAMPs in bacteria, viruses or fungi, activates B cells for robust proliferation and differentiation, thereby leading to antibody production and playing a critical role in the antibody response (19, 23, 58, 59).

3. B CELL TLRS MEDIATE TI AND TD ANTIBODY RESPONSES

During the initial stages of the antibody response to infectious agents, naïve B cells are activated to secrete IgM antibodies, which are of low to moderate affinity and provide immediate, but limited protection against the invading pathogen. The multivalency of pentameric/hexameric IgM antibodies serves well in agglutinating bacteria at the onset of infections when pathogens are confined mainly in the bloodstream, but prevents these antibodies from crossing efficiently into extravascular spaces to clear pathogens systemically (60, 61). Maturation of the antibody response entails somatic hypermutation (SHM) and CSR. SHM inserts mainly point-mutations in V(D)J region DNA at a high rate, thereby providing a structural substrate for the positive selection of higher affinity Ig mutants; CSR replaces the Ig heavy chain (IgH) constant (C_H) region, e.g., Cμ, with a downstream Cγ, Cα or Cε region, resulting in IgG, IgA or IgE (62, 63). Class-switched IgG, IgA and IgE antibodies possess unique biological effector functions, including extravascular and systemic diffusion, longer half-life and ability to sensitize mast and NK cells (63-66). Protective antibodies produced in response to infection or vaccination are high affinity and class-switched (67, 68). Severe CSR deficiency, whether due to B cell intrinsic or extrinsic causes, results in hyper IgM (HIGM) syndrome, which is characterized by low or undetectable serum IgG, IgA and IgE levels and profound susceptibility to infections (66, 69).

In the antibody response, B lymphocytes undergo proliferation and differentiation into antibody-secreting plasma cells or memory B cells in a TI or TD fashion (70-73). TI responses are induced by antigens associated with bacteria or viruses that elicit weak or no T cell responses. These antigens fall into two categories, i.e., TI type I antigens, essentially MAMPs that can polyclonally activate B cells, and TI type II antigens, consisting of repetitive epitopes with low complexity, such as bacterial capsular polysaccharides, that can crosslink BCR (56, 74-76). Both TI type I and type II antigens have been used as immunogens in commercial vaccines (75, 77-79). TD antibody responses, which underlie the generation of neutralizing antibodies elicited by virtually all clinically relevant vaccines, are induced mainly by protein antigens and entail interaction of antigen-primed B cells with cognate $CD4^+$ T helper cells, leading to formation and development of germinal centers, the organized microanatomical structures in secondary lymphoid organs from which plasma cells and memory B cells expressing

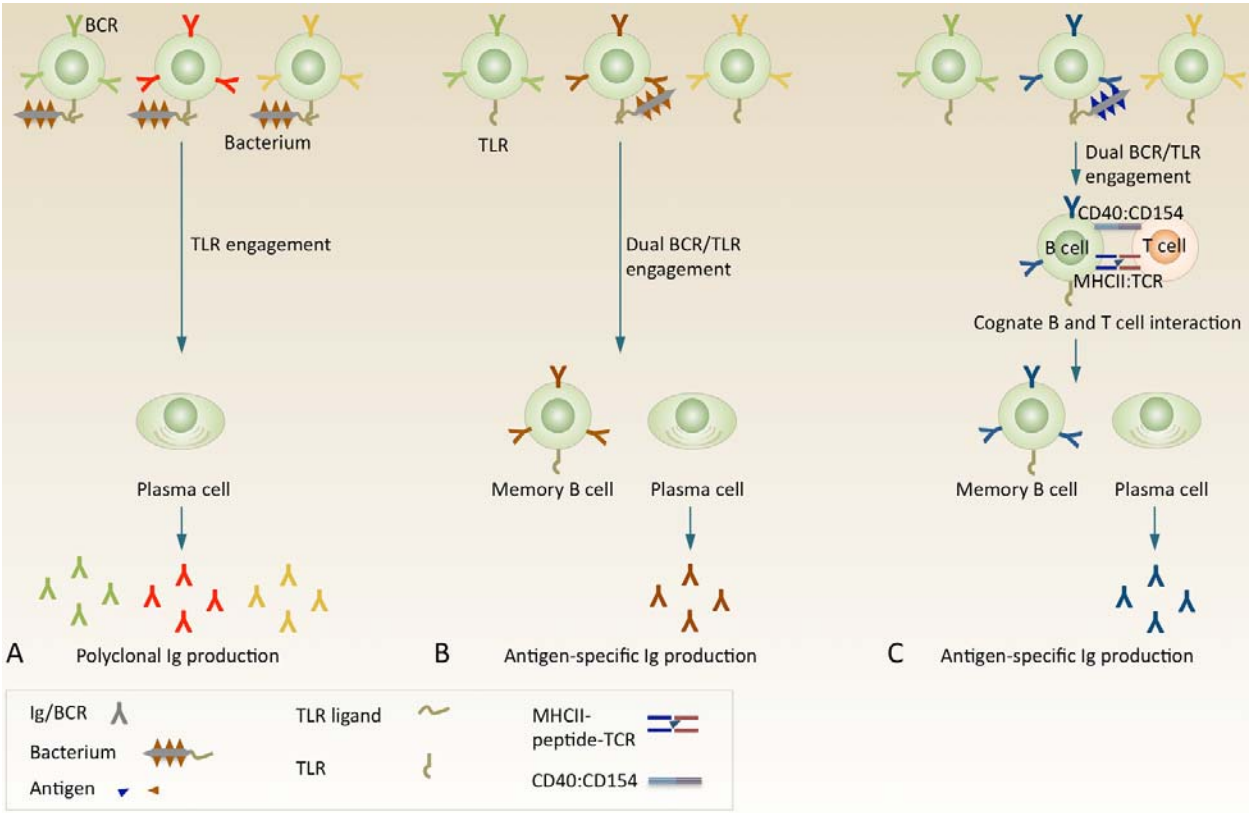

Figure 1. TLR engagement in B cells mediates TI and TD antibody responses. A microbe can directly induce B cell activation and antibody production by engaging germline-encoded TLRs as well as rearranged and clonally-distributed BCRs. (A) TLR activation of B cells alone may result in polyclonal activation and subsequent polyclonal antibody production. (B) Dual BCR and TLR engagement restricts the antibody response to antigen-specific B cells. (C) BCR/TLR-primed B cells enter the germinal center reaction to interact with cognate T cells, resulting in production of antigen specific antibodies.

high-affinity and class switched Igs emerge. B cells activated in a T cell-independent fashion may also give rise to plasma cells and memory B cells with similar, but not identical, features (80-83). As discussed below, TLRs can play important roles in polyclonal and antigen-specific TI antibody responses. They also play an important role in TD antibody responses in the early stages of infection before T cell help becomes available. TLRs do so by synergizing with BCR signaling to induce B cells to undergo CSR and to efficiently initiate/sustain the germinal center reaction (19, 23).

3.1. B cell TLRs in TI antibody responses

TLRs are highly expressed in peripheral B1 and marginal zone (MZ) B cells, which are also referred to as "innate-like" B cells, and in mature follicular B2 cells (11). TLR engagement by many microorganisms triggers production of natural IgM antibodies, which bind conserved microbial components, such as phosphorylcholine and polysaccharides (61, 84, 85). The role of TLRs in the generation of natural antibodies is further emphasized by findings that titers of natural antibodies present in germ-free mice increase significantly upon exposure to microbes or their components such as phosphorylcholine or LPS (84-89). These natural antibodies, which in general display a moderate affinity for microbial components, are generally sufficient to neutralize invading viruses and bacteria when their initial loads are relatively low (61, 84, 85, 88-93). This TLR-activated "frontline" antibody response is polyclonal in nature (Figure 1A), is mediated mainly by B1 and MZ B cells (84, 94-96) and, to a lesser extent, B2 cells (11, 89, 97). In addition to natural IgM antibodies, natural IgA antibodies are generated in the mucosa, as a result of chronic TLR stimulation from commensal bacteria, and play an important role in opposing invasion by many pathogens (98). TLR-mediated polyclonal activation of B cells is likely enhanced by BAFF and APRIL, both of which are intimately involved in CSR to IgA, and perhaps other cytokines (51, 99).

Most MAMPs on bacteria or viruses are naturally linked to repetitive polysaccharide or protein antigens, strongly suggesting that engagement of germline-encoded and non-clonally distributed TLRs occurs in concomitance with engagement of rearranged and clonally distributed BCRs, thereby recruiting antigen-specific B cells into the TI antibody response (Figure 1B). These B cells, unlike those polyclonally activated by TLRs in the absence of BCR crosslinking, would give rise to class-switched antibodies of at least moderate affinity, such as the specific IgA antibodies in isolated lymphoid follicles in the gut that

can control commensal and food-borne microbes (100). These antibodies may complement the high-affinity IgA antibodies produced in Peyer's patches in a TD fashion that neutralize microbial toxins and escaping pathogens (51, 99, 101). A critical role of dual BCR/TLR-mediated TI antibody responses is also emphasized by the production of polyoma virus-specific IgG antibodies in T cell-deficient mice immunized with viruses containing nucleic acid MAMPs, but not with empty virus-like particles (VLPs) (102). Importantly, some complex MAMPs such as LPS and polymerized flagellin (or intact flagella), contain repetitive epitopes that can, in addition to engaging TLRs, crosslink the BCR of a fraction of B cells (58, 103, 104), thereby eliciting anti-LPS or anti-flagellin class-switched antibodies. Consistent with a role of these dual BCR/TLR-engaging MAMPs in IgG responses, deficiency in MyD88 (a TIR domain-containing adaptor that critically transduces signaling from all TLRs with the exception of TLR3) or IRAK4 (a transducer of TLR signaling) results in decreased IgG to polysaccharidic antigens (46) and, conversely, deficiency in CD154 or CD40 does not affect CSR to IgG3, the IgG sub-class that predominates responses to polysaccharidic antigens (105-107).

3.2. B cell TLRs in TD antibody responses

Intact B cell-intrinsic TLR signaling is essential for effective TD antibody responses. LPS critically enhanced TD IgG1 responses to soluble protein antigens in a fashion dependent on expression of TLR4 and MyD88 in B cells (108). Likewise, the TLR9 ligand CpG substantially boosted IgG2b and IgG2c antibody responses to protein antigens administered in the same VLP, and mice deficient in B cell MyD88 were also defective in the antibody response to inactivated influenza virus (20). The TLR4 ligand monophosphoryl lipid A (MPL) together with the TLR7 ligand R837 administered in nanoparticles containing protein antigens induced high levels of antigen-specific antibody responses in a fashion dependent on B cell-intrinsic TLR signaling (21). In addition, immunization of mice with inactivated mouse-adapted H1N1 influenza virus in the presence of nanoparticles containing lipid A and R837, but not alum, conferred protection from subsequent challenge with a lethal dose of live virus (21). Lipid A and alum are the active ingredients of the AS04 adjuvant in the Cervarix® vaccine against human papillomavirus (109). Interestingly, haptenated proteins can elicit TD antibody responses specific to protein antigens, as dependent on TLR signaling, and to haptens, as independent to but amplified by TLR signaling, suggesting that a poorly understood immunostimulatory function of some haptens can mimick that of TLRs (110-112).

In spite of the critical role of TLRs in TD antibody responses, the underlying mechanisms are only starting to emerge. TD antibody responses are mainly effected by antigen-specific $Ig\delta^{hi}Ig\mu^{lo}$ follicular B2 cells. These B cells, recirculating between the periphery and secondary lymphoid organs, phagocytose antigen (113-116) and efficiently process internalized antigenic proteins for MHC II presentation of peptide fragments. TLRs upregulate B cell expression of MHC II, CD40 and CD80/86, thereby allowing B cells to efficiently engage T helper cells through MHC II:TCR, CD40:CD154 and CD80/86:CD28 interactions (93, 105-107, 117-120) (Figure 2). Activation of both B cells and T cells is boosted by TLR-stimulated DCs, which upregulate CD154, MHC II and CD80/CD86 and secrete cytokines, such as IL-4, IFN-γ, TGF-β, BAFF and APRIL (3, 49, 50). In addition, TLR activation likely plays an important role in priming antigen-specific B cells to initiate and sustain the germinal center reaction, which depends on T follicular helper (T_{FH}) cells (3, 14, 108, 121, 122). Dual BCR/TLR engaged-B cells express high levels of PNA-binding lectins, markers for germinal center cells, suggesting that, *in vivo*, TLR/BCR-engaged B cells undergo T cell-independent germinal center-like differentiation or are poised to receive T cell help to enter a germinal center reaction (119, 123) (Figure 2), thereby potentially contributing to SHM and positive selection to generate high-affinity Ig mutants. For instance, TLR4 signaling enhanced B lymphocyte trafficking into lymph nodes, cellular interactions of B lymphocytes within lymph node follicles, and access to the germinal center dark zones by antigen-specific B cells without prior antigen activation, thereby resulting in an overall increase in the generation of class-switched antigen-specific IgG1, IgG2a and IgG2b antibodies as well as plasma cells and memory B cells (123). Likewise, TLR-boosted germinal center reactions would lead to higher rate of SHM and positive selection of higher-affinity submutant clones for the generation of high-affinity neutralizing antibodies (124-126). Indeed, administration of antigen with TLR ligands in mice or humans generally results in production of antibodies with high-affinity for antigen and longer-lasting immune protection (127-138). Conversely, inactivation of TLR ligands present in a respiratory syncytial virus (RSV) vaccine results in production of low-affinity and non-protective antibodies (139).

In addition to actively participating in the T cell-dependent germinal center reaction to shape the overall antibody responses and maintaining the serological memory by polyclonally reactivating memory B cells (140), TLRs play an important role in the early stages of infection before T cell help becomes available, thus contributing to the prompt generation of protective IgM and isotype-switched IgG and IgA antibodies (19, 56-58, 108, 141-143). Follicular B2 cells recirculating between lymphoid organs and the periphery would constantly sample MAMPs and be induced by them to activate and proliferate, but only to a limited degree. MAMP-engaged antigen-specific B cells, i.e., those that undergo simultaneous TLR and BCR signaling, would be fully activated for robust proliferation. This is exemplified by the higher proliferation rates of B cells stimulated by MAMP ligands for TLR1/2, TLR4, TLR7 or TLR9, the five TLRs highly expressed in mature follicular B cells, together with agonistic anti–Igδ mAb conjugated to dextan (anti–δ mAb/dex), which crosslinks the BCR, than B cells stimulated by these MAMP ligands or anti–δ mAb/dex alone (23). Importantly, dual BCR/TLR engagement induced B cells to undergo efficient CSR, at a level comparable to that induced by CD40 engagement in T cell-

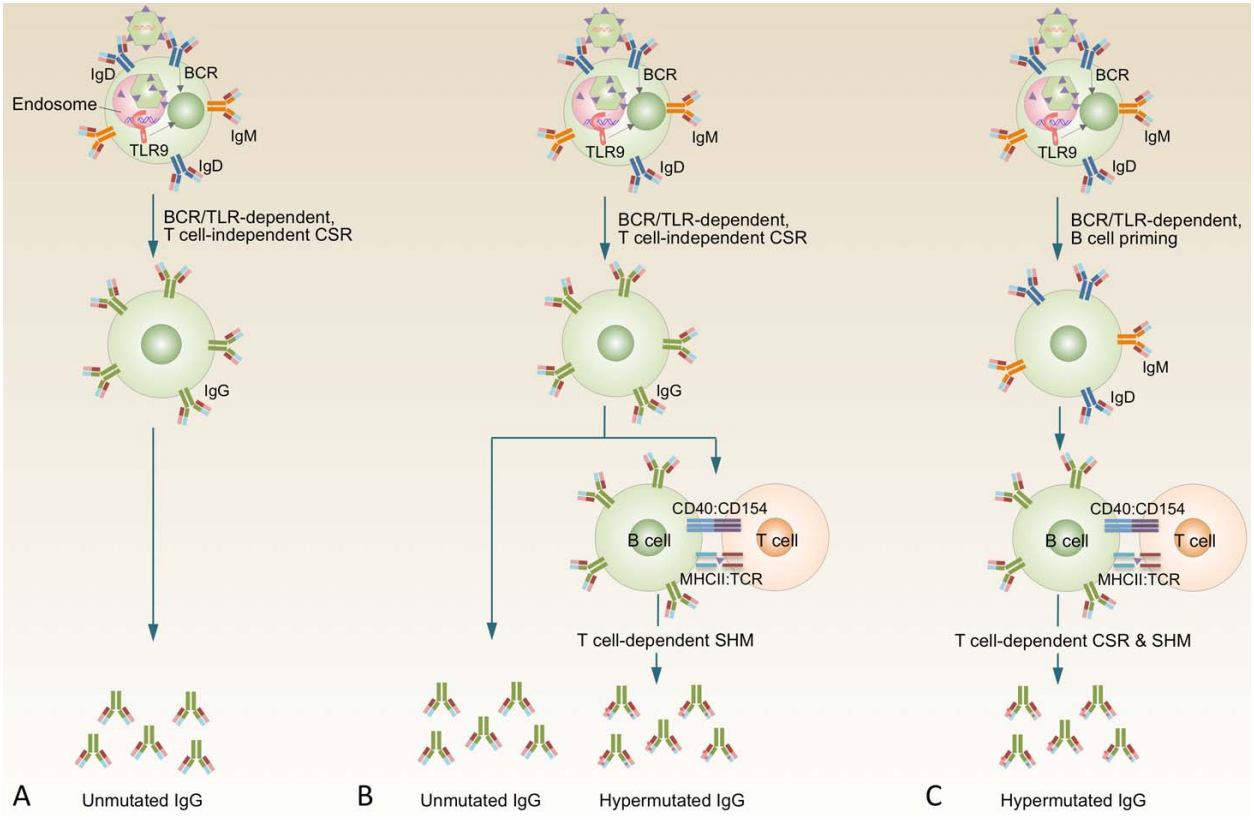

Figure 2. Dual BCR and TLR engagement in antigen-specific B cells induces T cell-independent CSR or primes B cells for T cell-dependent CSR and SHM. (A) Dual BCR and TLR engagement leads to T cell-independent B cell activation and CSR, but no SHM. (B) In addition to generating class switched but unmutated antibodies, dual BCR and TLR engagement upregulates MHC II, CD40, CD80/86 in class switched (e.g., IgG^+, as depicted) B cells, thereby priming these B cells to receive T cell help for induction of SHM in germinal centers. (C) Dual BCR and TLR engagement primes unswitched (IgM^+IgD^+) B cells to interact with cognate T cells in germinal centers, leading to T cell-dependent CSR and SHM.

dependent CSR, and at a much higher level than that induced by dual TLR/TACI or dual BCR/TACI engagement (23). Also, an important but less characterized aspect of dual BCR/TLR engagement is the boosting of specific antibody responses to tumor antigens, as suggested by significantly higher titers of anti-tumor antibodies elicited by vaccines that include TLR agonists (144-151).

4. CSR MECHANISMS

CSR and SHM, the two Ig diversification processes that critically underlie the maturation of the antibody response, require activation-induced cytidine deaminase (AID), a member of the AID/APOBEC deaminase family. AID deaminates deoxycytidines (dCs) in DNA, giving rise to dUs in the V(D)J regions (during SHM) and switch (S) regions (during CSR) (63, 152). dUs are not germane to DNA and must be dealt with by elements of the DNA repair machinery, mainly the base-excision repair (BER) and mismatch repair (MMR) pathways. Subsequent cleavage of S region DNA leads to generation of double-strand DNA breaks (DSBs), the obligatory intermediates for CSR (63, 153). S regions are located 5' of each of C_H region (except for Cδ) and contain recurring motifs in their "core" sequences, namely 5'-AGCT-3' repeats, which are specifically targeted by 14-3-3 proteins, a family of adaptors that recruit AID and protein kinase A (PKA) to S region DNA for CSR (154). After dC deamination, CSR proceeds through deletion of the intervening DNA and re-ligation of DSBs to form S-S junctions by non-homologous end joining (NHEJ) and alternative end joining (A-EJ) pathways (63, 155, 156). The deleted intervening DNA is looped out to form extrachromosomal DNA circles (157), which are transiently transcribed, giving rise to circle Iγ-Cμ, Iα-Cμ or Iε-Cμ transcripts, hallmarks of ongoing CSR to IgG, IgA or IgE (158). The recombined DNA sequences are transcribed to post-recombination Iμ-Cγ, Iμ-Cα or Iμ-Cε transcripts and "mature" V_HDJ_H-Cγ, V_HDJ_H-Cα or V_HDJ_H-Cε transcripts, which encode IgG, IgA or IgE (mature V_HDJ_H-Cμ transcripts encode IgM) (63).

The efficiency of CSR critically depends on the levels of AID; indeed, AID deletion ablates CSR and AID haploinsufficiency results in significantly decreased CSR (159, 160). AID gene (*AICDA* in the human and *Aicda* in the mouse) expression is under tight transcriptional regulation in a B cell-specific and B cell differentiation

stage-specific fashion, depending on the HoxC4 and NF-κB transcription factors (161, 162). As shown by us (161, 162) and others (163), HoxC4 binds to an evolutionarily conserved HoxC4/Oct-binding site in the *AICDA/Aicda* promoter, whereas NF-κB p52 subunit, an element of the non-canonical NF-κB pathway (164), and p65 subunit, an element of the canonical NF-κB pathway (165, 166), bind to the *AICDA/Aicda* promoter and upstream enhancers. Also, optimal *AICDA/Aicda* gene expression depends on a combination of several transcription factors, including E2A, STAT6, BATF and Smad (63, 167, 168). Furthermore, AID levels are regulated by hormones, e.g., potentiated by estrogen in a fashion dependent on HoxC4 upregulation, controlled by microRNA-mediated degradation of *Aicda* transcripts (169-171) and by proteasome-mediated degradation of AID in the nucleus (153, 172). Finally, AID activity in CSR is regulated by its phosphorylation (173), active nuclear export/cytoplasmic retention (174, 175) and binding to proteins such as 14-3-3 proteins and RNA exosome components (176).

CSR also requires germline IgH locus transcription, initiated by an I_H promoter (Iμ, Iγ, Iα or Iε) and proceeding through the upstream and downstream S and C_H regions engaged in the CSR process, to give rise to primary I_H-S-C_H transcripts, which are then spliced to generate "germline" I_H-C_H (Iμ-Cμ, Iγ-Cγ, Iα-Cα or Iε-Cε) transcripts (177, 178). Germline I_H-S-C_H transcription would increase the chromatin accessibility of recombining S regions to the CSR machinery, including 14-3-3 and AID. It would also provide AID substrates in S region DNA, that is, single-strand DNA transient transcription bubbles or "R-loops", the RNA-DNA hybrid secondary structures in which nascent RNA stably anneals with the transcribed DNA strand and displaces the non-transcribed strand as single-stranded DNA (63). In the presence of CD154 or LPS, cytokines IL-4, TGF-β and IFN-γ differentially activate transcription factors that induce germline transcription in selected S regions by specifically binding to the corresponding I_H promoters. IL-4 activates STAT6 and NF-κB to induce germline Iγ1-Sγ1-Cγ1 and Iε-Sε-Cε transcription for CSR to IgG1 and IgE, respectively; TGF-β activates Smad and Runx transcription factors to induce germline Iγ2b-Sγ2b-Cγ2b and Iα-Sα-Cα transcription for CSR to IgG2b and IgA, respectively; and IFN-γ activates germline Iγ2a-Sγ2a-Cγ2a transcription for CSR to IgG2a (63). IL-4 and TGF-β have also been shown to contribute to the induction of optimal AID gene expression through activation of transcription factor STAT6 and Smad, respectively (63).

5. TLRs AND BCR SYNERGIZE TO INDUCE T CELL-INDEPENDENT CSR

CSR is efficiently induced in a T cell-dependent fashion by CD154:CD40 engagement, which also induces high levels of AID, particularly in the presence of IL-4 (23, 154, 161). CSR can also be triggered in a T cell-independent fashion, mainly through TLR engagement by MAMPs (179-182). TACI, as engaged by BAFF and/or APRIL (soluble or multimerized), does not induce significant CSR, but synergizes with CD40 and TLRs to boost T cell-dependent and T cell-independent CSR, respectively (99, 183-186). B cells express high levels of at least eight TLRs, i.e., TLR1, TLR2 (TLR1/2 exists as a heterodimer), TLR4 (in the mouse) and TLR5 on the surface, and TLR3 (in the human), TLR7, TLR8 and TLR9 in endosomes (11). Since the discovery of genes encoding TLRs and identification of their expression in B cells, TLR induction of B cell differentiation, including CSR, has been a major subject of investigation (3, 12, 20, 21, 52, 53, 55-57, 108, 187-189). This led to the finding showing TLRs trigger CSR. CSR induced by TLRs, however, is inefficient with the only exception of that induced by LPS, which engages not only surface TLR4 but also the BCR (section 5.1). For instance, dsRNA engages TLR3, and CpG engages TLR9 to induce human B cells to undergo CSR to IgG, but only in the presence of IL-10 (179, 181). Likewise, CpG induces mouse B cells to undergo CSR to IgG3 and IgG2a (180) and, in the presence of IL-4, marginal CSR to IgG1 (182). Loxoribine or 8-mercaptoguanosine engage TLR7 and together with an agonistic anti–CD38 antibody induce low levels of CSR to IgG1 in the presence of IL-4 (190). Endosomal TLR3, TLR7 and TLR9 would sense their respective ligand upon fusion of TLR-containing endosomes, whose interior is topologically equivalent to the cell exterior, with autophagosomes (191-194). These "carry" TLR ligands released from disintegrating pathogens or internalized through TLR-, FcR- or BCR-mediated endocytosis (58, 113-116, 195), eventually triggering signaling pathways leading to limited activation of the CSR machinery.

5.1. TLRs and BCR synergize to induce CSR

As shown recently, MAMPs for surface TLR1/2 (Pam_3CSK_4) or TLR4 (lipid A), or endosomal TLR7 (R-848) or TLR9 (CpG) could all induce highly purified $Ig\delta^+$ B cells to undergo marginal CSR to IgG1, IgG2a, IgG3, IgA and IgE in the presence of the corresponding cytokines such as IL-4, IFN-γ or TGF-β (23). Anti–δ mAb/dex, which crosslinks Igδ to trigger BCR signaling and induces limited B cell proliferation but no CSR (23, 196) synergized with these TLR ligands to induce CSR to all IgG subclasses, IgA and IgE in a dose-dependent fashion, as shown by analysis of surface Ig expression, circle I_H-Cμ, post-recombination Iμ-C_H and mature VDJ-C_H transcripts, and secreted IgG, IgA and IgE. Dual BCR/TLR synergistic CSR induction was as potent as that induced by CD154 or LPS and occurred independently of anti–δ mAb/dex enhancement of TLR-induced B cell proliferation. By contrast, CD154-induced CSR, except that to IgA, was not significantly increased (or slightly decreased in CSR to IgG1) by BCR crosslinking, and GpC, a weak TLR9 agonist, and anti–δ mAb/dex induced only marginal CSR (23). In the absence of BCR crosslinking, CpG suppressed CSR to IgG1 and IgE induced by CD154 or LPS and IL-4 (197) through a poorly understood mechanism referred to as "tolerization" of heterologous TLR signaling (198, 199), thereby emphasizing the instrumental role of BCR in TLR-dependent CSR.

LPS – perhaps the most widely studied MAMP ligand – efficiently induces CSR to all Ig isotypes (IgG1,

IgG2a, IgG2b, IgG3, IgA and IgE) in the presence of appropriate cytokines (63). This bacterial component has a unique composition, containing a lipid A moiety, which engages TLR4, and a repetitive polysaccharidic moiety, which has been shown to elicit specific antibody responses, indicating that LPS not only triggers TLR4 signaling, but through its polysaccharidic moiety also engages BCRs of a considerable spectrum of the B cells. Indeed, LPS, but not lipid A, induced phosphorylation of CD79a Tyr182, one of the two ITAM Tyr residues that transduce BCR signaling. Phosphorylation of CD79a Tyr182 by LPS, however, was slower than that following induction by anti–δ mAb/dex or soluble/monomeric anti–Igμ antibody. Also, like anti–δ mAb/dex, which induced a continuous increase of free cytosolic Ca^{2+} ion levels, as a hallmark of persistent BCR signaling (200), LPS, but not lipid A or CD154, induced Ca^{2+} elevation, which was detectable within minutes of stimulation and further increased after a longer period of stimulation (23). Furthermore, B cells deficient in known BCR signaling elements, such as CD19, Btk, Blnk, Lyn or Vav, displayed greatly reduced responses to LPS (201-205). Finally, blocking of membrane-proximal BCR signaling with an anti–CD79a antibody inhibited LPS-induced CD79a phosphorylation and decreased CSR (23). Thus, LPS triggers TLR4 signaling and hallmark BCR signaling events, i.e., CD79a Tyr phosphorylation and Ca^{2+} mobilization, thereby inducing efficient CSR.

The critical role of BCR in synergizing with TLRs to express the full CSR potential is emphasized by a decrease in CSR in B cells knockout (KO) in p85α ($p85\alpha^{-/-}$ B cells), the predominant class IA regulatory subunit of the phosphoinositide-3-kinase (PI(3)K), which critically transduces BCR signals (23). CSR impairment in $p85\alpha^{-/-}$ B cells was due to impaired BCR signaling, but not TLR signaling, as $p85\alpha^{-/-}$ B cells stimulated with TLR ligand Pam_3CSK_4, lipid A, R-848 or CpG alone undergo limited CSR at levels comparable to their $p85\alpha^{+/+}$ counterparts (23). Interestingly, the surrogate BCR signaling activated by constitutively expressed EBV protein LMP2A in B cells with ablated surface BCR likely synergized with TLR signaling activated by constant stimulation by MAMPs in the gut to induce CSR in gut-associated lymphoid tissues in a fashion independent of B:T interactions, as these B cells cannot engage in cognate B:T cell interactions nor mount T cell-dependent immune responses in the spleen (206).

Overall, these findings have demonstrated that BCR signaling synergizes with activation of TLRs highly expressed in B cells to induce efficient CSR. This is a *bona fide* synergistic effect, rather than additive effect, as each signal alone induces either no (BCR) or marginal (TLR) CSR.

5.2. TLRs and BCR synergize to induce AID expression and germline I_H-S-C_H transcription

How do BCR and TLR signals synergize to induce CSR? CSR efficiency critically depends on the levels of AID expression and germline I_H-S-C_H transcription. AID is greatly induced by LPS or CD154, particularly in the presence of cytokines. It was also induced at lower levels by TLR ligands, such as lipid A, Pam_3CSK_4, R-848 and CpG (23, 126, 179, 181, 182). Consistent with the potentiation of TLR-dependent CSR by BCR crosslinking, AID expression induced by these TLR ligands was significantly increased by anti–δ mAb/dex, which alone does not induce AID, resulting in *Aicda* levels comparable to those induced by LPS; anti–δ mAb/dex, however, decreased CD154 plus IL-4-induced AID expression, consistent with its inhibition of CD40-dependent CSR to IgG1. BCR activation potentiates TLR-dependent CSR through upregulation of AID expression, as demonstrated by the ability of exogenous AID to largely substitute anti–δ mAb/dex in TLR9-dependent CSR. Anti–δ mAb/dex also enhanced, to variable degrees, germline I_H-S-C_H transcription induced by TLR ligands and appropriate cytokines. Consistent with these data, the defective CSR induced by dual BCR/TLR engagement (including that mediated by LPS) in $p85\alpha^{-/-}$ B cells is associated with decreases in both AID expression and germline I_H-S-C_H transcription compared to their $p85\alpha^{+/+}$ counterparts. Also, certain TLR ligands can induce germline I_H-S-C_H transcription in selected S regions without any additional cytokines; e.g., lipid A (and LPS) and Pam_3CSK_4 induce germline Iγ3-Cγ3 transcription, CpG induces germline Iγ2a-Cγ2a transcription (197) and BCR potentiates germline I_H-S-C_H transcription induced by TLR ligands alone (Casali and coll., unpublished). Finally, in addition to synergizing with TLRs to induce AID, BCR signaling would enhance expression of TLRs and/or facilitate endocytosis and trafficking of internalized TLR ligands to endosomes containing TLRs (16, 207, 208), likely leading to additional enhancement of TLR signaling in CSR.

6. INTEGRATION OF TLR AND BCR SIGNALING IN CSR

BCR and TLRs activate exclusive signaling pathways that do not crosstalk and confluent pathways, which can crosstalk, leading to convergence of signals at "nodes" (209, 210). Synergistic activation of the canonical and non-canonical NF-κB and other pathways by BCR and TLR signaling would account for the high levels of AID expression and CSR induced by dual BCR/TLR engagement in B cell differentiation and the antibody response.

6.1. TLR signaling

Upon binding of MAMPs, TLRs are thought to homo- and/or hetero-dimerize (or likely multimerize), leading to homotypic interactions of their intracellular TIR domains (211). Oligomerized/multimerized TIR domains in TLRs in turn recruit different TIR domain-containing signaling adaptor proteins (TIR adapters) via homotypic TIR:TIR interactions, thereby conferring signaling specificities to different TLRs. Among the four well studied TIR adapters in mammalian cells, i.e., MyD88, TRIF (also known as TICAM1), MAL (TIRAP) and TRAM (TICAM2), MyD88 and TRIF are main TLR signal transducers (Figure 3) – functions of another TIR adapter, SARM, are poorly understood (13). TLR4 signals through both MyD88, as bridged by MAL, and TRIF, as bridged by TRAM; TLR1/2, TLR2/6, TLR5, TLR7, TLR8 and TLR9 signal directly and only through MyD88 or MAL/MyD88;

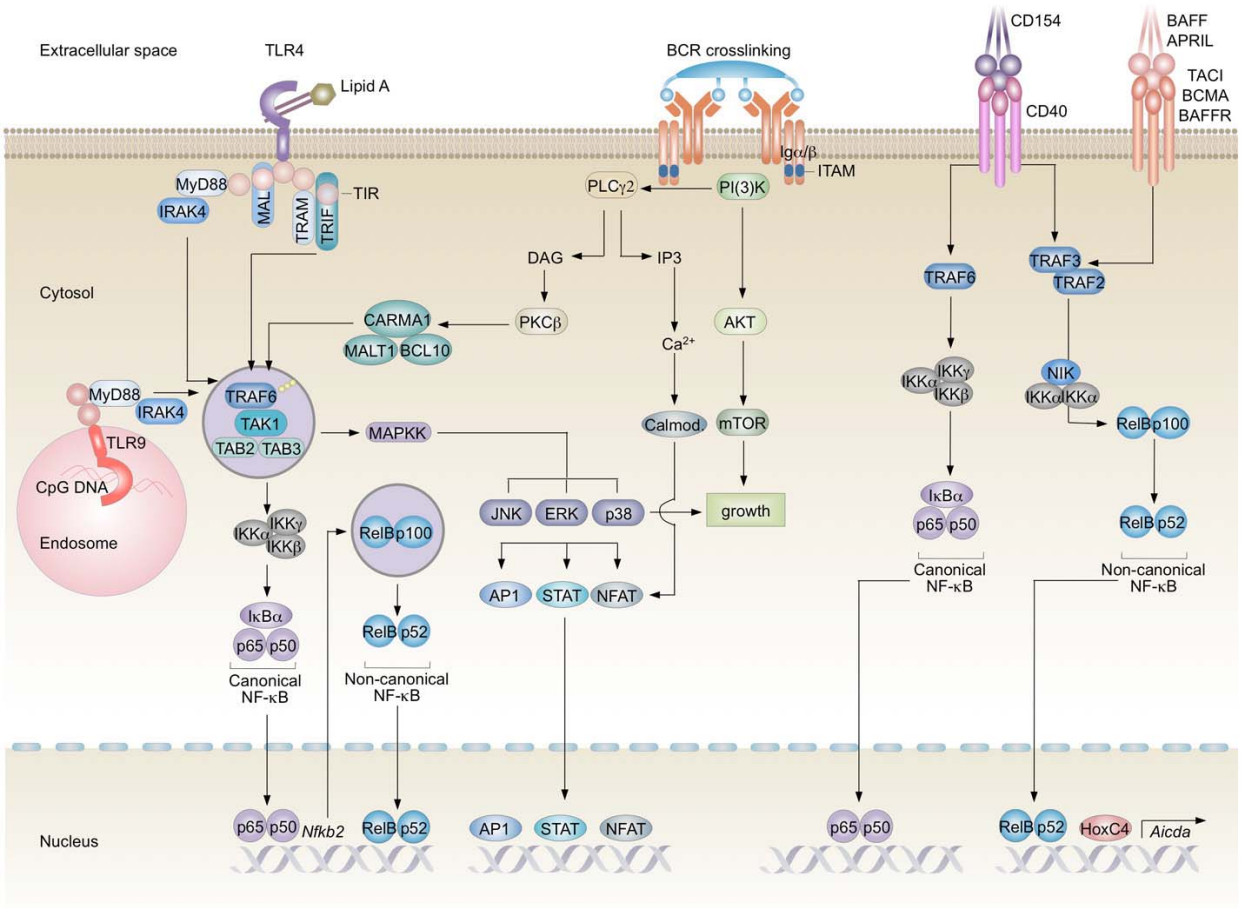

Figure 3. TLR and BCR signaling and integration in CSR and antibody responses. TLR ligands located on the surface of microorganisms are recognized by surface TLRs, whereas nucleic acid TLR ligands located inside microorganisms are recognized by endosomal TLRs. TLR signaling is initially relayed through homotypic TIR-TIR interactions with TIR-domain containing adapters such as MyD88 or TRIF, and eventually results in activation of several pathways, including the canonical NF-κB pathway. This induces, among other genes, expression of the non-canonical NF-κB precursor p100. Proximal BCR signaling activates the PI(3)K and PLCγ2 pathways, converging to nodes such as TAK1/TAB2/TAB3/TRAF6 for NF-κB and MAPK pathway activation. Dual BCR/TLR signaling integrates to induce p100 processing to p52, thereby activating the non-canonical NF-κB pathway, which is required for induction of AID. TNFR member CD40 induces both canonical and non-canonical NF-κB activation, thereby inducing CSR.

and TLR3 signals directly and only through TRIF (9, 10, 13, 212, 213).

Both MyD88 and TRIF recruit the IRAK family of serine/threonine kinases, that upon auto-phosphorylation, are activated to transmit the signal to TRAF6, which would play an important role in CpG-induced CSR in human B cells (179). Auto-polyubiquitination of TRAF6 in turn provides a "platform" for recruitment of the TAK1-TAB2-TAB3 complex and the IKKα-IKKβ-IKKγ complex, leading to phosphorylation of IKKα and IKKβ in the IKK complex by TAK1 (214, 215). Activated IKKα and IKKβ then phosphorylate IκB, leading to the ubiquitination and subsequent proteasome-mediated degradation of IκB (214), thereby releasing the canonical NF-κB p65:p50 and cRel:p50 heterodimers from inhibition by IκB. Following translocation into the nucleus and activation by phosphorylation, these canonical NF-κB heterodimers activate expression of target genes (Figure 3). Among signaling events in the TLR-dependent canonical NF-κB activation, TRAF6 ubiquitination, TAK1 phosphorylation and IKK phosphorylation can also be triggered by other signaling receptors, thereby serving as major nodes of signal integration (216, 217). In addition to activating the canonical NF-κB pathway, TAK1 (also known as MAP3K7) triggers MAPK cascades by phosphorylating MAP2K3/6 and MAP2K4, thereby activating the JNK, ERK and p38 pathways, respectively, leading to activation of gene expression by transcription factors that include AP-1, NF-AT and STATs (218).

Target genes of the canonical NF-κB pathway include those encoding proteins with specific functions, such as AID in B cells undergoing CSR. Alternatively,

they encode proteins with regulatory functions, such as transcription factors IRF-3, STAT1 and, likely, HoxC4 (188, 219). Importantly, activation of the IκBα-encoding *Nfkbia* gene will lead to re-synthesis of the IκBα inhibitor, thereby dampening the acute/transient canonical NF-κB activation, a feature of the NF-κB biology highly relevant to inflammation (220). Also, p100, the precursor of p52 in the non-canonical NF-κB pathway, is itself one of the target genes activated by canonical NF-κB (221), and processing of p100 to p52 is one major integrating node of TLR and BCR signaling (discussed in section 6.3).

6.2. BCR signaling

The BCR complex consists of an antigen-binding membrane-spanning Ig (mIg) molecule, flanked by the CD79a and CD79b signaling subunits and associated with CD19, CD21, CD22 and CD81 membrane proteins in lipid rafts (222, 223). Upon crosslinking of mIg by antigen (or experimentally, by anti–Ig antibodies), CD79a and CD79b are phosphorylated at ITAM Tyr residues, thereby triggering membrane-proximal BCR signaling. This occurs in a large complex termed the BCR signalosome, and the signal is mainly transduced by the (PI(3)K pathway, which activates a number of diverging pathways (Figure 3). PI(3)K generates phosphatidylinositol (3,4,5) triphosphate on the inner leaflet of the plasma membrane, where signaling molecules such as phospholipase Cγ2 (PLCγ2) and AKT are recruited upon binding phosphatidylinositol (3,4,5) triphosphate through their pleckstrin homology (PH) domain. Membrane-localized PLCγ2 cleaves phosphatidylinositol lipids to generate diacylglycerol (DAG), which activates protein kinase C-β (PKC-β) (224), and inositol triphosphate (IP3), which results in elevation of intracellular Ca^{2+} levels (200, 225, 226). PKC-β activates the CARMA1 signalosome complex composed of CARMA1, BCL10 and MALT1, resulting in TRAF6 ubiquitination and subsequent TAK1-mediated IKK phosphorylation for canonical NF-κB activation and/or TAK1-triggered MAPK cascades for JNK, ERK and/or p38 activation (227). Indeed, TAK1-deficient B cells show marked reduction in proliferation upon stimulation by BCR or TLRs (228). Other PI(3)K-dependent BCR signaling pathways include the Ca^{2+}–calmodulin–NF-AT and AKT–mTOR pathways (227, 229).

In experiments showing BCR activation of the TAK1-IKK-canonical NF-κB pathway, BCR signaling was triggered by soluble/monomeric anti–μ antibody, which, however, does not extensively crosslink BCRs in $Igδ^{hi}Igμ^{lo}$ mature B cells (200). BCR crosslinking by anti–δ mAb/dex did not induce immediate canonical NF-κB (p65) activation; rather, it increased the levels of the non-canonical NF-κB p52 subunit, which is processed from its precursor p100, within 24 – 48 h (the time frame of AID induction) in a fashion dependent on PI(3)K (23). B cell proliferation/differentiation induced by BCR crosslinking is in part dependent on p52, as suggested by the defective proliferation of $Nfkb2^{-/-}$ (p100/p52 KO) B cells upon stimulation with anti–δ mAb/dex that is reminiscent of the defective proliferation of these cells upon CD40 engagement (230); the latter induces both canonical and non-canonical NF-κB activation (164). Anti–δ mAb/dex also induces expression of p100, perhaps through a pathway independent of canonical NF-κB p65 and dependent on cRel, which is activated in B cells in a slower but more sustained fashion compared to p65 (231). As p100 expression, but not its processing to p52, can be induced by other signaling pathways, such as those triggered by TLR ligands (23) and soluble/monomeric anti–Igμ antibody (232), p100 induction and its processing to p52 make for a major node of signal integration, which, as discussed in the next section, is important for AID and CSR induction.

6.3. Integration of TLR and BCR signaling

CSR induction by dual BCR/TLR engagement provides another major proof-of-principle of the "two-signal" requirement for important pathways of immune cell activation, proliferation and/or differentiation (233, 234). BCR and TLR signals would integrate at two nodes, i.e., TRAF6 ubiquitination/TAK1 activation/IKK phosphorylation and p100 induction/p100 processing, leading to activation of both canonical and non-canonical NF-κB pathways and other transcription factors, such as JNK and p38 (Figure 3). Indeed, CpG- or lipid A-induced p65 phosphorylation, which is independent of PI(3)K, is accelerated and enhanced by anti–δ mAb/dex, and this is ablated in $p85α^{-/-}$ B cells deficient in PI(3)K signaling (23). Conversely, PI(3)K-dependent induction of p100 expression and processing by anti–δ mAb/dex are enhanced by CpG and lipid A. Likewise, LPS, which both engages TLR4 and crosslinks BCR, induces both canonical and non-canonical NF-κB activation in mature B cells. It also induces some non-canonical NF-κB activation in pre-B cells (235), in a fashion dependent on PI(3)K, further emphasizing the activation of the two NF-κB pathways by integrated BCR and TLR signaling.

Synergistic activation of canonical and non-canonical NF-κB by integrated BCR and TLR signaling underscores the importance of both NF-κB pathways in B cell differentiation, particularly CSR. The canonical NF-κB, which generally induces prompt gene activation in response to stimuli such as TLR ligands (165, 166) or TNF (236) in a fashion independent of new protein synthesis, would be critical for priming B cells to enter the cell cycle, thereby initiating CSR. By contrast, the non-canonical NF-κB pathway, which requires new protein synthesis, would provide slow but sustained signaling to support CSR and other B cell differentiation processes (164). Accordingly, CSR unfolds over 48 h after triggering by appropriate stimuli. Thus, canonical and non-canonical NF-κB activation would be critical for the initiation and sustained induction of high levels of AID, germline I_H-S-C_H transcription and likely 14-3-3 upregulation and histone post-translational modifications, all of which play a critical role in CSR and are induced by the same stimuli that trigger CSR. Like dual BCR/TLR signaling (including the signaling triggered by LPS), CD40 signaling activates the two (non-canonical and canonical) NF-κB pathways for AID induction, implying that despite their significant difference in the nature of triggering stimuli, T cell-

Table 2. TI and TD antibody responses

	T cell-independent		T cell-dependent
Specificity	Polyclonal	Antigen-specific	Antigen-specific
Signaling receptors	TLRs, TACI (and/or BCMA, BAFFR)	Dual BCR/TLR (or with TACI)	CD40/MHC II (or with dual TLR/BCR or TACI)
Molecular mechanisms	Not determined	Canonical and non-canonical NF-κB activation; MAPK pathway enhancement; AID expression	Canonical and non-canonical NF-κB activation; MAPK pathway enhancement; AID expression
Switched Ig isotypes (in mouse)	IgG3, IgA	IgG3, IgG2b, IgG2a, IgG1, IgE, IgA	IgG3, IgG2b, IgG2a, IgG1, IgE, IgA
SHM	Not determined	Not determined	Effective
Physiological functions	Control of commensal bacteria, maintenance of natural antibodies, maintenance of serological memory	Responses to TI antigens, containment of infection during early phase, priming and sustaining germinal center reactions	Eradication of infection, generation of immunological memory
Pathological consequences	Generation and/or amplification of autoantibodies by TLR-engaged autoreactive B cells, exhaustion of subsets of B cell clones	Generation of autoantibodies by TLR-stimulated autoreactive B cells, generation of class-switched pathogenic autoantibodies by dual TLR/BCR engagement	Generation of class-switched pathogenic autoantibodies, conversion of low-affinity autoantibodies to high-affiity autoantibodies by SHM/positive selection

independent and T cell-dependent CSR entail the same set of transcription factors that are critical for AID induction. CD40 employs different signal transducers, depending mainly on TRAF2/TRAF3 to activate both NF-κB pathways in CSR (237); TRAF2 and TRAF3 play no role in the BCR signaling pathway, thereby possibly accounting for the lack of synergy of BCR and CD40 in CSR (23, 238). Finally, TACI ligands can activate canonical and/or non-canonical NF-κB depending on their oligomerization states (99), thereby providing a mechanistic explanation for TACI-mediated enhancement of (BCR/TLR-induced) T cell-independent CSR and (CD40-induced) T cell-dependent CSR.

7. TLRS AND AUTOANTIBODIES

B cell TLR-dependent CSR can mediate the generation of protective antibodies in TI responses and in early stages of TD responses. Nevertheless, engagement of TLRs has also been associated with poorly controlled parasite responses, septic shock as well as autoimmunity (Table 2) (93, 239-246). In B cells, TLRs can be engaged by endogenous TLR ligands (247, 248), e.g., MAMP-like host molecules such as phospholipids (249) or nucleic acid molecules released from apoptotic bodies (246) under physiological conditions, e.g., in germinal centers in which B cells undergo high rates of apoptosis (250-253), or pathological conditions, e.g., during tissue injury (254, 255). Polyclonal activation of B cells induced by TLRs, perhaps augmented by BAFF (256, 257), would lead to emergence of polyreactive antibodies, which are generated particularly by B1 B cells – as shown by early work from our laboratory (84, 242, 258-263) – and possess low/moderate affinity for self-antigens, e.g., chromatin or nucleoli (246, 264). Accordingly, lupus-prone mice deficient in TLRs (265, 266) or TLR regulators, such as Unc93b1, display decreased autoantibody responses (245, 267).

Like antibodies at early stages of the antibody responses to microbial pathogens, autoantibodies produced initially are unmutated low/moderate affinity IgMs. Also like mature antibodies to pathogens, pathogenic autoantibodies are class switched and somatically mutated (263, 268-270), as exemplified by the highly pathogenic IgG2a anti-double strand DNA (anti-dsDNA) antibodies in lupus-prone MRL/$Fas^{lpr/lpr}$ mice that can readily pass into the extravascular spaces to mediate systemic tissue injury. Complex self-antigens that can simultaneously crosslink the BCR and engage TLRs (particular endosomal TLRs) in autoreactive B cells would lead to NF-κB activation and AID upregulation, and ultimately to the generation of class-switched autoantibodies. Such complex self-antigens would include chromatin, which by engaging endosomal TLR9 through its hypomethylated CpG-containing DNA component and crosslinking BCR through its repetitive histone component, elicits class-switched anti–chromatin IgG autoantibodies in systemic lupus erythematosus (SLE) (246, 264). Likewise, class-switched autoantibodies are elicited against repetitive cytoskeletal proteins, which are polymeric self-antigens that may crosslink the BCR of autoreactive B cells (271, 272). The important role of TLRs in mediating pathogenic autoantibody responses indicate that TLR signaling can be a potential target for therapeutics in systemic autoimmune diseases, including systemic lupus, or organ-specific autoimmune diseases, including rheumatoid arthritis (246, 267, 270, 273). Interestingly, perhaps in part due to its inhibition of endosomal TLR signaling (194, 274), chloroquine has been widely used in the treatment of SLE (275), and injection of G-rich DNA in MRL/$Fas^{lpr/lpr}$ mice reduced lupus symptoms likely by blocking engagement of TLR9 by endogenous hypomethylated CpG DNA (276).

8. CONCLUSIONS AND PERSPECTIVES

Overall, several recent findings have emphasized the important role of B cell TLRs in TI and TD antibody responses. They have also provided mechanistic insights into the newly discovered synergy between BCR and TLRs in inducing canonical and non-canonical NF-κB activation, AID expression and T cell-independent CSR, indicating that activation of the two NF-κB pathways would be a unifying mechanism underpinning T cell-independent and T cell-dependent CSR. A two-signal operational model may thus account for the selection of only antigen-binding B cells among the pool of B cells activated by TLR ligands, for further differentiation through CSR, thereby effectively mediating antibody responses to TI antigens or focusing the early adaptive response before T cell help becomes available. Indeed, new data have provided a proof of principle that the BCR/TLR-dependent and T cell-

independent CSR process can efficiently diversify antibody responses (20, 21). The dual BCR/TLR-dependent process of CSR and B cell differentiation would be evolutionarily conserved in jawed vertebrates, which express rearranged and clonally distributed BCRs and germline-encoded and non-clonally distributed TLRs (277). In organisms with a more primitive adaptive immune system, such as jawless vertebrates, dual BCR/TLR engagement would be substituted by cooperation of TLRs or perhaps other innate immune receptors such as NLRs with the VLRB antigen receptor, and play a major role in fighting invading microbial pathogens.

9. ACKNOWLEDGMENTS

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