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Toll-like Receptors and B-cell Receptors Synergize to Induce Immunoglobulin Class Switch DNA Recombination: Relevance to Microbial Antibody Responses

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Abstract

Differentiation of naïve B cells, including immunoglobulin (Ig) class switch DNA recombination (CSR), is critical for the immune response and depends on the extensive integration of signals from the B cell receptor (BCR), tumor necrosis factor (TNF) receptor family members, Toll-like receptors (TLRs) and cytokine receptors. TLRs and BCR synergize to induce CSR in T cell-dependent and T cell-independent antibody responses to microbial pathogens. BCR triggering together with simultaneous endosomal TLR engagement leads to enhanced B cell differentiation and antibody responses. The requirement of both BCR and TLR engagement would ensure appropriate antigen-specific activation in an infection. Co-stimulation of TLRs and BCR likely plays a significant role in anti-microbial antibody responses to contain pathogen loads until the T cell-dependent antibody responses peak. Furthermore, the temporal sequence of different signals is also critical for optimal B cell responses, as exemplified by the activation of B cells by initial TLR engagement, leading to the upregulation of co-stimulatory CD80 and MHC-II receptors, which, in turn, result in more efficient interactions with T cells, thereby enhancing the germinal center (GC) reaction and antibody affinity maturation. Overall, BCR and TLR stimulation and the integration with signals from the pathogen or immune cells and their products, determine the ensuing B cell antibody response.

Keywords

activation-induced cytidine deaminase (AID); adaptive immunity; adjuvant; antibody; autophagy; B cell receptor (BCR); class-switch DNA recombination (CSR); CD40; CpG; cytokine; innate immunity; LPS; NF-κB; somatic hypermutation (SHM); Toll-like receptor (TLR); vaccine

I. B CELL DIFFERENTIATION VIA INNATE AND ADPAPTIVE IMMUNE RECEPTORS

A. The Innate Immune Response

The protection from a nearly unlimited number of pathogens encountered during the lifetime of an individual is achieved through the integration of innate and adaptive immune responses, which are intrinsically linked by crosstalk through cells and molecules.[1–6] The immune system detects and eliminates invading pathogenic microorganisms, as well as

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infected and neoplastic self-cells, by distinguishing them or associated molecules from "normal" self-cells and self-molecules. While lower organisms have only an innate immune system, higher organisms, beginning with jawed vertebrates, in addition possess a more sophisticated system: the adaptive, or acquired, immune system.[7–10] Innate immunity provides an early, though non-specific, initial response to pathogens.[7,11–14] In contrast, adaptive immunity requires a lag period for activation, is specific, diverse and highly efficient in targeting of pathogens[8,9,15,16] (Table 1).

The innate immune system is a universal and ancient form of host defense against infection.[7,10,17] In mammals, it is composed of diverse cell types, including macrophages, dendritic cells (DCs), neutrophils, basophils, mast cells, eosinophils and natural killer (NK) cells, and molecules and molecular systems, such as anti-microbial peptides, acute-phase proteins (collectins, ficolins, and pentraxins) and complement.[8,9,13] Innate immune cells constantly sample the environment for the presence of pathogens through their germline-encoded pattern recognition receptors (PRRs).[3,7,9,13,18] PRRs primarily bind to "non-self" molecules that have conserved features known as microbe-associated molecular patterns (MAMPs); an alternative term is pathogen-associated molecular patterns (PAMPs). MAMPs are unique chemical structures found in the microorganism but generally absent in the higher hosts. For example, LPS contains sugars and lipids composed of chemical bond arrangements not found in higher eukaryotes, thereby constituting a unique MAMP. Another MAMP is unmethylated, CG-containing DNA (CpG) from bacteria and viruses.[19] LPS and CpG are recognized by an important subset of PRRs known as TLRs.[3,20–24] Other PRR classes include NOD-like receptors (NLRs), CARD helicases, C-type lectins, and scavenger receptors.[3,10,25] (Table 2). Some of these receptors may be functional in B cells, however their effects on B cell antibody responses are not known, and they will not be considered further here. After sensing pathogens via their PRRs, macrophages and mast cells are activated to phagocytose the pathogen, display peptide fragments on major histocompatibility II (MHC II) receptors, and secrete pro-inflammatory cytokines and lipid mediators. Likewise, stimulation of PRRs on immature DCs leads to their maturation and activation, which in turn activates adaptive immunity.[7,10,13,26]

B. The Adaptive Immune Response

Evolutionarily, adaptive immunity emerged after innate immunity by the need to respond to specific and changing antigens through the use of antigen-specific receptors on the surfaces of B and T lymphocytes.[1,4,15,27,28] Even though only jawed vertebrates possess a true adaptive immune system, recent evidence indicates that jawless vertebrates, such as lamprey and hagfish, contain lymphocyte-like immune cells expressing variable leucine-rich receptors (VLRs), which are triggered to rearrange upon infection with pathogen, and display a specificity for antigen similar to antibodies of the jawed vertebrate adaptive immune system.[29–36] The adaptive immune response enables higher vertebrate animals to adapt their response to a pathogen to be more specific and effective, and to mount a faster and stronger response during subsequent encounters with the same pathogen (which is referred to as immunological memory).

The mechanisms of generating antigen-specific receptors in the adaptive system involve extensive variability and rearrangement of receptor gene segments. During their development, B and T lymphocytes rearrange those portions of their genomes, which code for BCRs and T cell receptors (TCRs), respectively. Lymphocytes display on the extracellular surface of their plasma membranes multiple (hundreds to thousands) identical copies of their BCR or TCR with unique antigen specificity among the pool of total lymphocytes.[37,38] The rearrangement and clonal distribution of antigen receptors is different from the previously described PRRs, which do not rearrange and usually have the same sequence in all cells that express them in an organism (Table 1). When antigen receptors

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bind an antigen on a bacterium, virus, or parasite, they cluster together, thereby bringing into proximity membrane-associated and intracellular signaling molecules, contributing to lymphocyte differentiation. Such an antigen receptor crosslinking process only occurs in those B and T lymphocytes that express receptors with an above-threshold affinity for antigen to ensure that only antigen-specific lymphocytes are amplified and differentiate into effector and memory cells, which have the same specificity for the antigen that initiated and drove the response.[39,40] Antibodies (and BCRs) are further diversified by CSR and SHM.

C. B Cell Differentiation

B lymphocytes play major roles in the immune response by producing antibodies against antigen, as well as by functioning as professional antigen presenting cells (APCs), and by producing cytokines.[41–44] B cells produce high-affinity antibodies which can directly inactivate pathogens,[45–47] can opsonize pathogens to assist innate immune cells in eliminating pathogens, and, finally, can activate complement.[8,9,48] Conversely, B cells are directly and indirectly informed about the presence and nature of pathogens by innate immune elements such as TLRs,[10,13,49] and are indirectly informed by complement factor C3d.[50–52] Dysregulated and mis-targeted B cell antibody responses could result in autoimmunity, whereas impaired antibody responses during an actual infection could result in an immune deficiency.

In mammals, B cells begin their development in the bone marrow from lymphoid progenitor cells, which in turn derive from pluripotent hematopoietic stem cells. B cells derive their name from the *Bursa* of Fabricius in chickens, which is the equivalent organ of bone marrow in mammals. Antibody diversification during B cell development occurs by sequential Ig gene recombination where noncontiguous Ig variable (V), diversity, and joining (J) gene segments are recombined into functional V(D)J genes.[37,38] The development of mature B cells and the generation of a diversified antigen receptor repertoire are crucial processes, but they are beyond the scope of this review. Here, we will focus on events that occur after B cells have completed their development in the bone marrow and have matured in peripheral organs such as the spleen and lymph nodes, where they are ready to respond to infection.

Upon encountering antigens, naïve mature B cells (IgM^{lo} IgD^{hi}) exit the resting state, increase metabolic activity and enter the cell cycle to initiate proliferation and concomitant differentiation, eventually leading to fully differentiated effector cells which produce high affinity antibodies against pathogenic targets.[53–56] Full differentiation of B cells typically results in non-cycling short- or long-lived plasma cells (also referred to as plasmacytes, to include cells at different stages of differentiation), which are specialized to produce large amounts of antibodies, and memory B cells which can be quickly differentiated into plasma cells upon reinfection.

Prolonged and continuous engagement of multiple receptor types is required for B lymphocytes to be activated, to proliferate, to differentiate and to maintain their survival.[57,58] B cells begin to proliferate within 24 hours after induction from stimuli, such as BCR crosslinking by antigens, and engagement of other surface receptors, including CD40 and TLRs (Fig. 1); after the initial cell division B cells divide continuously every 6–8 hours.[55,59,60] DCs and T cells require prolonged stimulation before differentiation,[61,62] whereas innate immune cells, such as macrophages and neutrophils, respond faster upon sensing pathogen and are attracted to pathogens via chemotaxis by following concentration gradients of pathogenic components,[63,64] reflecting the differential roles of different types of immune cells in innate and adaptive immunity.

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The initial differentiation of naive B cells in secondary lymphoid organs upon antigen recognition can result in their quick differentiation into short-lived plasmacytes, which secrete a burst of mostly IgM antibodies, usually of low-affinity, to limit the spread of pathogens and blunt the infection. Most activated B cells enter the GC reaction to undergo CSR to generate antibody isotypes with different biological effector functions, and SHM to generate antibody mutants as substrates for antigen-mediated positive selection of higher affinity antibodies. Both CSR and SHM are initiated by the enzyme activation-induced cytidine deaminase (AID),[65,66] which is preferentially expressed in activated B cells, especially those in GCs.

Activated DCs in the secondary lymphoid organs can process and present peptides to T follicular helper cells (T_{FH})[67] in GCs[68–71] of spleen, lymph nodes, Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) in the gut.[72] Internalization and processing of pathogenic proteins lead to the display of antigen-derived peptides on the MHC II receptors of antigen-specific B cells, which then make cognate interactions with T_{FH} cells (that are specific for the same antigen) via MHC II-antigenic peptide-TCR interactions. In addition, B cells and T cells further contact each other through the membrane protein receptor pairs CD28:CD80/CD86, and inducible costimulator (ICOS): ICOS ligand (ICOSL), which are located on the surfaces of T cells and B cells, respectively. Activation of T_{FH} by DCs or cognate B cells leads to upregulation of the membrane protein receptor CD154 (CD40L) on T cells. The engagement of CD40, a member of the TNF family of transmembrane receptors located on the surface of B cells,[73,74] by CD154 results in vigorous B cell proliferation and differentiation, as will be detailed in section II.C. In addition to membrane protein receptor interactions, several types of interleukin (IL) molecules greatly influence the nature of the differentiation program for T and B cells.

The clonal expansion of a few parental B lymphocytes that are activated, and their concomitant differentiation to plasmacytes, which secrete thousands of antibodies per second,[75–78] leads to the secretion of millions of affinity-matured and class-switched antibody molecules targeting specific antigens. Following antigen clearance, the expanded B cell pool contracts, likely due to engagement of inhibitory Fc receptors (FcRs) on B cells by excess antibodies,[79–84] and by competition for homing in the bone marrow stromal environment,[85,86] thereby ensuring that optimal titers of high-affinity antibodies are produced.

D. CSR in Humoral Immune Responses

The prominence of antibodies in immunity is illustrated by the fact that all current clinical vaccines function at least in part by eliciting protective antibodies,[87,88] which are usually class-switched and of high affinity, similar to those generated during natural infections. Naïve B cells display IgM and IgD on their surface, and CSR substitutes the original IgM isotype with IgG, IgA and IgE by replacing the constant region of heavy chain (C_H) of antibodies[65,89–97] (Fig. 2).

The first antibodies to be produced are of IgM class, and are likely encoded in the germline, as the B cells that produce them have not yet completed the GC reaction and thus have not undergone SHM. The intrinsic *affinity* of the initial IgM for antigen is relatively low, however the overall *avidity* of IgM for antigen is relatively high, as its pentameric structure containing a total of ten identical Fab arms results in a gain in local entropy when antigen binding has been initiated in any one of the ten arms, enabling these early IgMs to efficiently coat bacterial and viral antigens during the early stages of infection.[98 99] The large size of the initially produced pentameric IgM restricts its distribution mainly to the blood and prevents it from crossing efficiently into extravascular spaces and binding pathogens systemically.

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In contrast to IgM, all other antibody isotypes diffuse efficiently to extravascular sites, and, therefore, their generation is critical during an infection. Class-switched antibodies possess diverse biological effector functions, including direct pathogen or toxin/virulence factor neutralization, opsonization, complement activation, sensitization of mast and NK cells, extravascular diffusion, improved transport properties and longer half-lives.[9,92,96,97,100,101] A deficiency in CSR, whether due to B cell intrinsic or extrinsic causes, would lead to an impaired antibody response during an infection, as occurs in humans with the Hyper IgM (HIGM) syndrome.[97,100–102] These individuals are highly susceptible to infection and require injections of IgG fractions from pooled sera of many healthy donors to provide passive antibody-mediated protection against potential pathogens.

B cells express both the exquisite receptor for adaptive immunity, i.e. BCR, and receptors for innate immunity, such as TLRs. In addition to being stimulated by T-dependent signals, B cells can be directly stimulated by engagement of their TLRs by the respective ligands. Innate and adaptive immune signals can therefore be integrated in the same B cell.[17,54]

II. THE ROLES OF BCR, CD40, TLRS AND CYTOKINE RECEPTORS IN CSR

Signaling pathways from BCR, CD40, TLRs, and cytokine receptors play dominant roles in B cell antibody responses. While T cell help is critical for optimal B cell differentiation, including CSR and SHM, recent evidence indicates that T-independent signals from BCR and TLRs, also significantly contribute to the antibody responses against microbial pathogens. Mutations that interfere with BCR signaling, such as those in genes encoding several tyrosine kinases, phosphoinositide 3 kinase (PI3K), and phospholipase Cγ2 (PLCγ2) and their several signaling adapters, while they result in varying deficiencies in B cell development, compromise antigen recognition and subsequent signaling, resulting in severe impairment of antibody production and CSR.[16,103–105] Humans[106–110] and mice[111–114] carrying mutations in TLR genes or in genes encoding TIR-domain adapters (such as MyD88[115–118], TRIF[119]) and TLR regulatory molecules (such as CD14,[120,121] which is a co-receptor for TLR4, and Unc93b1,[113,122,123] which is involved in correct endosomal TLR trafficking) display varying impairments of innate and adaptive immune responses, as reviewed recently.[124–127] However, the extent to which the defect directly impairs B cell responses remains unclear.[20,49,128,129] While one signal alone can directly activate B cells, the response is limited in terms of the antibody isotypes produced, and combining multiple signals together broadens the scope of the antibody response by leading to increased B cell differentiation. It is likely that B cells are optimally differentiated by more than one signal,[17,54] with BCR and TLR signaling contributing to the generation of optimal antibody responses.

A. BCR Signals

One of the primary B cell activating signals is provided by BCR crosslinking, where several BCR units composed of the membrane Ab, Igα, Igβ, CD19, CD21, CD81 and associated adapters (Fig. 1) are brought together into close proximity to initiate downstream signaling.[104,130,131] BCR crosslinking leads to phosphorylation of immunotyrosine-based activation motif (ITAM) receptors, which in turn activate several enzymes, in particular PI3K and PLCγ2. These two enzymes generate second messenger molecules, such as phosphorylated phosphotidylinositols (PtdIns) by PI3K, and inositol triphosphate (IP3) and diacylglycerol (DAG) by PLCγ2, leading to the release of free intracellular calcium ions (Ca^{2+}). Subsequent nuclear factor bound to κB (NF-κB) activation results in an increase in the levels of transcription factors required for entering the cell cycle and differentiation pathways. However, in the absence of other stimulating signals, B cell proliferation is followed by activation-induced cell death (AICD),[56,132–135] a process probably involved in the elimination of B cells activated by self-antigens in the absence of an infection. Thus, BCR

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signaling *per se* is insufficient for full B cell differentiation, including CSR, but it synergizes with strong signals from different receptor families, such as CD40 or TLRs, to induce B cell terminal differentiation, including CSR.

B. Cytokine Receptor Signals

Cytokines, such as IL-4, IFN-γ and TGF-β, influence B cell differentiation by combining with other signals to direct the B cell response, such as CSR to specific isotypes.[67,136–138] Cytokine receptor signaling in B cells acts in part by inducing IgH germline transcription (I_H-C_H) of heavy chain (C_H) regions participating in CSR. [91,92,96,136,138] Each C_H gene cluster consists of three distinct and sequential components: the I_H promoters for germline transcript initiation, the switch (S) regions where DNA breaks for CSR occur, and finally, the C_H genes themselves (Fig. 2B).

In the presence of CD40 or LPS engagement, IL-4 induces the activation of the transcription factors NF-κB and STAT-6, whose DNA binding sites include those located in Iγ1 and Iε promoters. After NF-κB and STAT-6 bind to these promoters, germline Iγ1-Cγ1 and Iε-Cε transcription for IgG1 and IgE, respectively, is initiated. Likewise, Iγ2b and Iα promoters contain binding sites for the transcription factors Smud and Runx, which are induced upon stimulation with TGF-β to mediate CSR to these two classes.[91,92,96,139] As cytokines are mainly, although not exclusively, secreted by T_H1 and T_H2 cells, and can be classified into T_H1 (IFN-γ and TGF-β) or T_H2 (IL-4) classes[138], the antibody isotypes directed by cytokines can also be classified, in the mouse, into T_H1 (IgG2a, IgG2b, IgG3) and T_H2 (IgG1, IgE) isotypes, with IgM and IgA not falling exactly into either category.

Germline I_H-C_H transcription plays a critical role in CSR, likely by either opening up DNA, and/or by routing RNA polymerase II to deliver AID to S regions. S regions contain an unusual abundance of the '5-WRCY-3' hotspot,[140,141] and particularly the '5-AGCT-3' iteration. These sequences could play important roles in recruiting CSR factors, including AID, and could also serve as the preferred substrate for AID[142]. In S regions, AID deaminates cytosine (dC) to uracil (dU).[96,142] dU can be excised by apurinic/apyramidinic endonuclease (APE). Excision of nearby dUs on opposite DNA strands results in dsDNA breaks, which are then thought to be re-ligated mostly by components of the nonhomologous end-joining (NHEJ) machinery.[96,143,144] The entire process replaces one C_H gene with a downstream C_H gene, e.g. it replaces the Cμ gene with the Cγ1, in the case of CSR to IgG1[95,96,141,145] (Fig. 2B).

C. TNFR Superfamily Signals

The TNF receptor (TNFR) superfamily consists of members such as CD40, B cell activating factor of the TNF family receptor (BAFFR), B cell maturation antigen (BCMA), and transmembrane activator and calcium signaling modulating and cyclophilin ligand [CAML] interactor (TACI).[146] Each member is a homotrimeric type II membrane protein consisting of an extracellular ligand-binding domain, a membrane-spanning domain, and an intracellular signaling domain. There is a difference in the ligands that these members bind, with CD40 engaging CD154 on the surface of T cells, and BAFFR, BCMA and TACI binding one or both of the soluble proteins BAFF and a proliferation-inducing ligand (APRIL).

BAFF and APRIL are produced mainly by innate immune cells as well as by epithelial cells. BAFFR binds BAFF, BCMA binds APRIL, and TACI binds both BAFF and APRIL.[147,148] BAFF and APRIL function to promote the maturation of T1 immature transitional B cells to mature B cells to maintain appropriate levels of mature B cells in a negative feedback fashion, to help with the establishment of plasma cells to their niches in the bone marrow,

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[147–150] and, finally, to induce or enhance B cell differentiation, including CSR.[151–155] One report suggested the direct induction of CSR by engagement of TACI or BAFFR,[152] however, in our experiments, BAFF did not induce CSR, although BAFF enhanced LPS- or CD40-driven CSR, in mouse B cells stimulated *in vitro*. Thus, BAFF may enhance CSR when B cells are activated by an additional stimulus, as evidence suggests in the case of B cell activation by viral components[151,153,155] or epithelial cell-secreted BAFF and APRIL[156–159] as will be described in section D1 below. Finally, BAFF can be produced by IgD-armed basophils upon binding bacteria, thus establishing a link between initial IgD production and sustained, local B cell differentiation, including CSR.[160]

In B cells, the main stimulatory surface receptor belonging to the TNFR superfamily that induces robust B cell differentiation is CD40. CD40 is found on the surfaces of other APCs, and is generally required for their activation. This receptor has been found to be essential in mediating a broad variety of immune responses, including T-dependent CSR, SHM, GC reaction, and plasma and memory B cell formation.[74,161–163] CD40 signals are transmitted by interaction of CD40 cytoplasmic tail with TNF receptor-associated factor (TRAF) adapter proteins, including TRAF2, TRAF3, TRAF5, TRAF6, and TTRAP,[164,165] which ultimately results in the activation of transcription factors such as NF-κB and activator protein 1 (AP-1).[163] The most important TRAFs that transduce CD40 signals are TRAF2 and TRAF6. These subsequently interact with germinal center kinase (GCK), which then activates the c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) pathways.[165] TRAFs mediate NF-κB induction by activating the inhibitor of NF-κB kinase (IKK) complex to phosphorylate the inhibitor of κB (IκB) proteins, thereby releasing NF-κB for nuclear translocation and binding to its target DNA[166] (Fig. 1). NF-κB then orchestrates a transcriptional program, which includes the induction of molecules necessary for B cell differentiation, including induction of AID for initiation of CSR and SHM.[164] As the T-dependent B cell response *in vivo* is MHC-II restricted, *in vitro* studies of B cell differentiation by CD40 should perhaps also involve simultaneous MHC-II engagement.[167]

D. TLR Signals

1. TLRs in Immune Cells—The involvement of TLRs in mammalian immunity was first discovered based on their homology to the *Drosophila* Toll receptor, where Toll plays a dual role in the dorso-ventral segmentation during development and in the production of antifungal peptides during an infection.[54,168,169] Most mammalian species contain ten to fifteen different TLRs, each of which senses one or a limited number of MAMPs (Table 3), and, in a few cases, host-derived molecules.[21,170–172] TLRs are expressed in innate immune cells, B cells and in some non-immune cell types, including epithelial cells. In particular, epithelial cells sense bacteria via TLRs, and then secrete BAFF or APRIL, which activates B cells residing in the periphery and promotes their CSR, thereby containing the bacteria.[156–159] Non-immune functions of TLRs, as in development, cancer or tissue injury[173] are outside the scope of this review.

TLRs consist of an extracellular or endosomal sensing domain that contains leucine-rich repeats (LRRs), a transmembrane region, and a cytoplasmic Toll/interleukin I/resistance protein (TIR) domain.[23,174,175] LRRs fold into a sickle-shaped β-coil domain and confer specificity in sensing a particular class of ligands, mainly through their concave, leucine-rich surface.[22] The intracellular and variable TIR domain confers signaling specificities to different TLRs, enabling each of them to interact with a limited number of TIR-domain signaling adaptor proteins via homotypic TIR-TIR interactions.[21,22,24,174,176,177] Comparative sequence analysis of the human TLRs have revealed that the members of the TLR family can be divided into five subfamilies, as represented by TLR2, TLR3, TLR4, TLR5, and TLR9. The TLR2 family consists of TLR1, TLR2, TLR6, and TLR10; the TLR9

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subfamily consists of TLR7, TLR8, and TLR9; the other three subfamilies, TLR3, TLR4 and TLR5, contain only the one indicated member.[178]

Upon engagement by their ligands, TLRs are thought to dimerize and signal as homodimers (or possibly homomultimers), with the exception of TLR1/TLR2 and TLR2/TLR6, which signal as heterodimers. Signaling pathways emanating from different TLRs are complex and often redundant (Fig. 1). It is generally accepted that there are two main transmission pathways in the propagation of TLR signals, mediated by two TIR domain-containing adaptors: the myeloid differentiation factor 88 (MyD88) pathway and the TIR domain-containing adaptor inducing interferon-β (TRIF) pathway, respectively. The other three known TIR domain-containing adapters are MyD88-adaptor-like (MAL, also known as Toll/interleukin 1 receptor domain containing adaptor protein, TIRAP), TRIF-related adaptor molecule (TRAM), and the recently discovered sterile and armadillo motifs (SARM). TLR4 signals through both the MyD88 pathway, as bridged by TIRAP, and the TRIF pathway, as bridged by TRAM; TLR9 signals directly and only through the MyD88 pathway. With the exception of SARM, the other four adaptors utilize IL-1 receptor associated kinases (IRAKs) to transmit signals to TRAFs, which ultimately leads to the nuclear translocation and target gene promoter binding of transcription factors such as NF-κB and AP-1, thereby initiating gene transcription of IRF-3 and STAT-1, and to induction of AID expression to mediate CSR and SHM in activated B cells.[176,177] SARM has been suggested to negatively modulate signaling,[174] possibly to restore the pre-activation non-signaling state or to dampen the response.

In monocytes, in which TLRs are highly expressed, TLR engagement induces their differentiation into macrophages or DCs, and further TLR stimulation can modulate immune functions of these cells, including enhancement of phagocytosis of pathogens.[179,180] In DCs, TLRs appear to be expressed in a cell subset-dependent manner: myeloid DCs in peripheral tissues express most TLRs except TLR9, while plasmacytoid DCs in blood express mostly TLR1, TLR6, TLR7, and TLR9.[26,181,182] Binding of the appropriate ligand induces DC maturation, including enhanced antigen presentation and cytokine secretion, and upregulation of the stimulatory receptor CD86, all of which greatly influence the adaptive immune response.[181] The presence and potential function of TLRs in T lymphocytes, which generally sense antigen presented by DCs and B cells, is currently unclear, though there is some evidence indicating that TLR2 engagement may remove the T_{reg} suppressive functions during ongoing infection.[183,184]

2. TLR Signaling in B Cell Antibody Responses—B cells express high levels of most TLRs and respond to stimulation by TLR ligands associated with pathogens, likely those present in pathogens phagocytosed by B cells, as well as released, soluble TLR ligands. The details of the TLR signal transduction pathways in cells such as macrophages and DCs[21,22,185] described in the previous section are likely relevant to B cells as well (Fig. 1). TLR engagement by MAMPs such as LPS or CpG induces potent B cell differentiation,[19] similar in magnitude to that induced by CD40, and much stronger than that induced by BCR crosslinking alone. Even though direct B cell differentiation and antibody production in response to TLR ligands, such as LPS or flagellin, was reported since the 1960s,[17,54,186–189] only recently has the significance and scope of these data received due attention.[10,17,20,49,54,177] While TLRs are clearly functional in B cells, relatively little is known about how TLRs influence B cell differentiation, including CSR and SHM.[176,177,190]

The role of TLRs in B cell antibody responses has been somewhat controversial, at least in part due to different outcomes from different experimental settings. In one study, TLR signaling in B cells was found to be indispensable for optimal B cell antibody responses against native proteins, using LPS or flagellin as TLR ligands in immunizations employing

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wild-type, TLR4- or MyD88-deficient B cells adoptively transferred into B cell-deficient mice.[49] However, in other studies, antibody responses to haptenated proteins mixed with different adjuvants, many of which contain TLR agonists, was not significantly impaired in mice doubly deficient in MyD88 and TRIF.[128,175,191] One explanation for these data is that antibody responses to haptenated proteins may be different from those against natural and unhaptenated proteins, perhaps due to the alternation of the protein structures by the haptenation process.[175,191] Alternatively, it has been proposed that the repetitive arrays of haptens cross-link BCR with high efficiency such that TLR signaling may not be essential for the B cell responses, whereas soluble protein antigens that cannot efficiently crosslink the BCR may require TLR stimulation to elicit a robust response.[192] The fact that B cells highly express most TLRs and are efficiently activated by engagement of their ligands points to a role for TLRs in optimal B cell differentiation, as will be detailed in the subsequent sections.

TLR stimulation of B cells in combination with cytokines has been used to drive CSR to specific isotypes. In fact, well before the discovery of CD154 as one of the main T_H cell receptors, LPS was used to activate mouse B cells,[187] and was subsequently found to do so by engaging TLR4.[111,193] LPS stimulation of B cells also led to the historical discovery of NF-κB.[194–196] Since LPS stimulation of B cells induces NF-κB and AID, and results in CSR to IgG3, the specificity of CSR could be partially influenced by TLR ligands such as LPS, but is greatly influenced by cytokines, as was mentioned earlier.

III. TLR AND BCR SIGNALING SYNERGIZE TO INDUCE CSR

A. Surface TLRs in the Induction of CSR

The surface TLR1, TLR2, TLR4, TLR5, and TLR6 bind MAMPs that are expressed on the surface of microorganisms, while the endosomal TLR3, TLR7, TLR8, and TLR9 bind nucleic acid MAMPs located inside microorganisms. TLR4, which is expressed on the plasma membrane, binds LPS present on the surface of bacteria, and is internalized together with LPS,[197,198] which could be a general feature of TLR recognition and downstream trafficking and signaling events.[199–202] TLR agonists are potent drivers of B cell differentiation, including NF-κB and AID expression, which coupled with cytokine-directed germline I_H-C_H transcription, induces CSR to specific isotypes. Experimentally, LPS stimulation of mouse B cells induces CSR to IgG3; LPS in combination with IL-4 induces CSR to IgG1 and IgE; LPS in combination with IFN-γ induces CSR to IgG2a; finally, LPS in combination with TGF-β induces CSR to IgG2b and IgA.[91,92,96] Likewise, we have recently found that stimulation of TLR1/6 by Pam_3CSK_4 results in CSR to the isotypes specified by cytokines.[203] Therefore, TLR signaling results in context-dependent modulation of both the magnitude and the fine structure of the B cell antibody responses.

TLR ligands may stimulate not only their cognate TLRs, but they may also happen to bind and crosslink the BCR in a small fraction of B cells, thereby activating an entirely different stimulation pathway.[204–206] In particular, TLR ligands are thought to behave in two different ways: a T-independent type I (TI-I) and a T-independent type II (TI-II) way.[8,9,54,189,204,207] At saturating doses, TI-I antigens, including LPS, will bind all B cells via their TLR4 and, therefore, virtually all B cells will be (polyclonally) activated (mitogenic stimulation). At much lower LPS concentrations, those fewer B cells whose BCR also happens to bind the O-saccharide component of LPS with high affinity, are thought to receive enough signals from both TLR4 (engaged by the lipid A component of LPS) and BCR, hence the name TI-II. Even though TLR4 engagement by LPS does not require BCR crosslinking to induce B cell differentiation, BCR crosslinking can further enhance TLR4-induced B cell differentiation[198,208] and CSR;[203] the same also holds for TLR1/6-induced CSR.[203] Furthermore, while *in vitro* stimulation of B cells with high (mitogenic) doses of

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LPS, in the presence of cytokines can induce CSR to specific isotypes, the contribution of BCR crosslinking by LPS under these *in vitro* conditions may skew the response to a TI-II response (TLR + BCR), which engages the BCR and thus results in the amplification of antigen-specific B cells, rather than a genuine TI-I (TLR alone) response.

B. Endosomal TLRs in the Induction of CSR

The endosomal location of TLR3, TLR7, TLR8, and TLR9 may be optimal in allowing their sensing of internalized nucleic acid MAMPs from viruses and bacteria bacteria, while likely avoiding sensing host nucleic acids.[202,209] While the dynamics of distribution and maturation of endosomal TLRs is still a matter of active research,[210] several reports indicate that each endosomal TLR may not be located in exclusive endosomes, but rather they may be located to common endosomes hosting several different TLRs, where each TLR senses its respective ligand as it is gradually exposed in a disintegrating microorganism.[211,212]

Stimulation of the endosomal TLR9 by unmethylated CpG DNA has been thought to suppress class switching to IgG1 and IgE isotypes but promote class switching to IgG2a in B cells activated by LPS or CD154 and IL-4, likely by regulating germline transcription.[213] However, there has been a great variability in the data of different research groups, particularly in the magnitude and the identity of the TLR9-enhanced or -suppressed isotypes,[213–218] and the controversial involvement of T box expressed in T cells (T-bet),[213,219–221] a transcription factor important for T_H1 cell polarization. CpG stimulation was reported to suppress CSR to IgG1 and IgE induced by LPS or CD40 and the cytokine IL-4, and at the same time promote CSR to the T_H1 isotype IgG2a under these T_H2 conditions,[213] suggesting that CpG stimulation could somehow divert the CSR machinery to other target S regions, which is a role usually played by cytokines.

CpG can also modulate B cell differentiation to plasmablasts/plasmacytes,[86,222] thereby resulting in complex changes in the titer of isotype-switched antibodies in culture supernatants, and therefore complicating the monitoring of CSR. Furthermore, IFN-γ and TGF-β, two cytokines critical for CSR to IgG2a and IgG2b, respectively, are known to decrease B cell proliferation and survival, whereas CpG is a potent proliferation and survival stimulus, which adds additional complexity to the overall antibody response.[203]

C. BCR signaling synergizes with TLR signaling

TLR-induced CSR depends on the correct cellular trafficking and localization of the engaged TLRs, as well as whether other signals, particularly those from the BCR, are also activated.[6,199–202,223,224] TLR ligands such as LPS or flagellin, can elicit antibody responses directed against them, complicating the delineation of the respective roles of BCR and TLRs in anti-microbial antibody responses. B cells phagocytose antigen in a FcR- or BCR-dependent way,[225–228] efficiently process internalized antigenic proteins, as well as sense various MAMPs present in the phagocytosed pathogen. BCR and TLR9 signaling synergize in the induction of NF-κB in B cells,[199,223,229] and BCR signaling recruits TLR9-containing endosomes to autophagosomes,[199,223] possibly as a way of efficiently coordinating the sensing of nucleic acid TLR ligands present when a B cell internalizes pathogens.

Signaling cross-talk between ITAM receptors and TLRs could modulate responses in immune cells.[6,224] For example, DCs responded to ssRNA by producing IFN-γ only if the viruses had been previously opsonized with antibodies, which crosslink Fc receptors on the DC membrane;[230] crosslinking of activating FcRs in DCs and other cells can serve the same function as the crosslinking of BCR on B cells, as both are ITAM-bearing receptors. In

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another study, vesicular stomatitis virus (VSV) induced IFN-α in DCs upon its internalization to autophagosomes and simultaneous engagement of TLR7 by viral RNA.[231]

In our studies, we have found that CpG stimulation alone results in robust B cell proliferation, but not in significant CSR, as indicated by the low frequency of B cells expressing surface IgG, IgE, or IgA measured by flow cytometry.[203] However, in the presence of BCR crosslinking (and appropriate cytokines), CpG stimulation could lead to robust CSR to all respective isotypes, as well as to plasma cell formation and antibody secretion.[203] Furthermore, contrary to previous reports,[213,217,232] our data indicate that TLR9 stimulation in the absence of BCR crosslinking suppresses LPS- (or CD154-) and cytokine-induced CSR not only to T_H1 but also to T_H2 isotypes, and suppresses plasma cell formation.[203] However, in the presence of BCR crosslinking, LPS and CpG, as well as LPS and Pam_3CKS_4, synergized in inducing CSR in a dose-dependent way.[203] Similar synergistic and antagonistic effects of have been reported for cytokine production by B cells[43] and PBMCs[233] stimulated with specific pairwise combinations of TLR ligands.

CpG and other surface and endosomal TLR ligands, such as Pam_3CSK_4 and R848, might not display a T_H1 vs T_H2 bias in inducing B cells to undergo CSR to specific Ig isotypes (like cytokines do), but like LPS, these may act as inducers of NF-κB and AID, with the latter initiating CSR to isotypes specified by cytokine-induced germline transcription. The mechanism by which CpG antagonizes LPS- and CD40-induced CSR in the absence of BCR crosslinking could be due to sequestration of signaling adapters, or due to signal paralysis. Another possibility could be that in the absence of strong BCR crosslinking, CpG may enter the cell via BCR-mediated uptake, setting off weak BCR signaling (low ITAM signaling), which has been proposed to negatively regulate TLR and cytokine signaling in macrophages.[6,224] Conversely, during dual stimulation with BCR crosslinking and CpG (mimicking the internalization of microorganisms by BCRs that have high-affinity for their external epitopes), the strong ITAM signaling synergizes with TLR9 signaling, and results in differentiation, as hallmarked by CSR (Fig. 3).

Considering CpG as a general inducer of B cells to undergo CSR to isotypes directed by the different cytokines, without itself displaying an intrinsic T_H1 vs T_H2 isotype bias, does not contradict the reported T_H1 vs T_H2 polarizing effect of CpG in other innate immune cell types, and its use as a predominantly T_H1 vaccine adjuvant.[234–238] It is likely that cells such as DCs and macrophages sense the pathogen type present in an infection by engagement of various PRRs (including TLRs) by corresponding pathogenic MAMPs, and then produce T_H1 or T_H2 cytokines that initiate the induction of T_H1 or T_H2 cells, which then amplify the polarization and induce B cell differentiation and CSR to specific T_H1 or T_H2 isotypes.[13,26,239–241] Whether B cells, after directly sensing the type of pathogen via engagement of their TLRs by the corresponding pathogenic MAMPs, can undergo CSR to the most appropriate isotypes, as is the case for LPS-induced CSR to IgG3, remains to be determined.

While the role of TLRs in autoimmunity is beyond the scope of this review, the reader is referred to several recent reviews on the subject.[172,242–249] Briefly, it appears that under conditions where TLRs sense host ligands that resemble MAMPs, such as phospholipids that resemble LPS and could engage TLR4, or self RNA or DNA that could engage TLR3, TLR7/8 and TLR9, B cells are activated to produce autoantibodies.[250–253] This could be particularly important in the generation of anti-DNA class-switched autoantibodies, which are the main cause of systemic lupus erythrematosus (SLE).

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IV. TLR- AND BCR-INDUCED B CELL ANTIBODY RESPONSES AGAINST MICROBIAL PATHOGENS

A. TLR Signaling in T-independent B Cell Differentiation

1. TLR Signaling During the Initial Phase of B Cell Differentiation—TLRs were likely one of the first immune receptors that recognized conserved molecular patterns of pathogens.[4] A recent report described the rearrangement of lamprey antigen receptors, where an APOBEC-related enzyme was found to deaminate the gene segments encoding these receptors, leading to their rearrangement and thus diversification to a limited number of configurations.[32] Another report on lamprey immunity revealed the presence of B- and T-like cells, which sense pathogens via membrane-expressed VLRs, with the B-like cells progressing through blast formation and secreting soluble antigen-specific VLRs.[36] Thus, it is possible that TLRs, VLRs or related primordial PRRs were the original antigen receptors, and during evolution were replaced by the newer and more and versatile BCRs, which are based on the Ig domain fold. TLRs continued to provide information regarding the presence and type of pathogen to innate immune cells and B cells.

TLR-mediated differentiation of B cells may be a critical component of T-independent, but early and effective B cell clonal amplification and differentiation leading to the production of class switched, potentially hypermutated, protective antibodies during early infection when pathogen loads are relatively low and T cell help is not yet fully available. LPS stimulation of mouse B cells *in vitro* results in IgM and IgG3 antibody production. The first antibodies to be produced *in vivo* against an antigen encountered for the first time are IgM, which can contain the pathogen in the first hours to days of infection. Studies in mice selectively deficient in secreted IgM,[254] suggest that the initial T-independent production of IgM serves not only to contain pathogens, but by forming complexes with pathogens, serves as an autocrine factor for continued local B cell proliferation via IgM-mediated multimerization of soluble antigen that can now efficiently crosslink the BCR. In the absence of timely T cell help, other cell types, such as follicular dendritic cells (FDCs), may provide a level of stimulation sufficient for B cell activation and early IgM secretion.[255]

2. TLR Signaling During B Cell Differentiation in the Absence of T Cell Help—Even though optimal CSR requires T cell help, some CSR does occur without T cell help following challenge with viral, bacterial and fungal pathogens (Table 4). While the titers of class switched antibodies in the absence of T cell help are usually much lower than the optimal titers produced in the presence of normal T cell help, in several cases the titers are enough to confer some protection against infection. Class-switched antibodies can be generated against invading pathogens without T cell help because the enzyme AID can be induced in part by the conserved homeodomain transcription factor HoxC4[145,256,257] in a T-independent fashion.[17,54,176,177,189,192,258–260]

CSR that occurs in the absence of T_H cells[261] is particularly applicable to antiviral antibody responses.[258,262] Immunization of T cell-deficient mice with polyoma virus resulted in the production of virus-specific class switched IgG, although in lower titers compared to wild type mice, whereas immunization with either a viral antigen or virus-like particles (VLPs) did not result in IgG production, possibly due to the presence of dual TLR and BCR engagement in the case of whole virus and lack of TLR engagement in the case of antigen alone or VLP immunization.[263] In other studies, natural IgA antibodies were produced in response to TLR ligands of commensal gut bacteria,[264] natural IgE antibodies were generated to activate innate immune mast cells without T cell help,[265] and IgG2a antibodies were produced outside GC follicles three days after immunization.[266] Considering the low level CSR that occurs in the absence of T cell help, it is possible that this can have

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functional significance in helping to contain the low pathogen loads during the early stages of infection when T cell help has not yet peaked[198,267] (Fig. 4).

B. TLR Signaling may Play Roles During the T-Dependent Phase of B cell Differentiation

Initial IgM production by B cells is likely due to their activation by various TLRs and the BCR, since antigen-specific T helper cell numbers do not peak until several days after their initial activation. IgM production, in addition to providing some early and direct protection by targeting pathogenic epitopes, also serves in the activation of complement, which then results in microbe coating with C3d, which binds the CD21/CD35 receptors on the B cell surface and enhances B cell activation. [267] The antibody-pathogen complexes are drained in secondary lymphoid organs, where they are destroyed by professional phagocytes such as macrophages, and in the process stimulate the T-dependent GC reaction.[99]

Direct TLR stimulation of B cells during the GC reaction, may help to sustain and shape the processes of antibody affinity maturation, by acting as a reporter of pathogen load and infection conditions.[10,20,49,175,191] For example, initial TLR-mediated activation of B cells leads to the upregulation of co-stimulatory CD80/CD86 and MHC-II receptors, thereby priming B cells for more abundant and efficient interactions with T cells and DCs.[268] T cell help alone may not be sufficient for optimal antibody responses, with direct TLR stimulation of B cells contributing to optimal antibody titers and CSR[49], though this issue remains unclear[128,129,175,191]. During the GC reaction, TLR signals in B cells are likely integrated with signals from the BCR, T cells and DCs, eventually giving rise to plasma and memory B cells, whose Ig genes encode antigen-specific, high-affinity and class-switched antibodies.[199,223,269]

Immunization with CpG and hepatitis antigen resulted in higher antibody affinities than immunization with antigen alone.[270] When TLR ligands in a respiratory syncytial virus (RSV) vaccine were inactive, the resulting antibodies were low-affinity due to impaired SHM and were not protective.[271] These reports suggest that TLRs play a role in SHM, since their engagement contributes to the generation of high-affinity antibodies, though the molecular and cellular mechanisms responsible have not yet been elucidated. The immune system, including B cells, need to continually recognize and bind pathogens in order to ultimately clear them. In particular, nucleic acid TLR ligands may be important signals for SHM, since they would signal the presence of replicating, and potentially hypermutating, microorganisms.[272]

C. TLR and BCR Signaling in Vaccine Research

1. Vaccine Adjuvants—Well before the discovery of the TLRs, vaccinologists empirically used adjuvants such as Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) and Ribbi's adjuvant, which, in addition to containing oils for forming depots upon injection, also contain mycobacterial components (which include various TLR ligands) in order to elicit robust immune responses to soluble protein antigens.[7,10,175,273,274] Since the discovery of the crucial adjuvanting properties of TLR and other PRR ligands, there has justifiably been an explosion in the application of defined adjuvants for use in vaccine development.[274–297]

All current clinical vaccines elicit protective antibodies,[87,88] frequently also eliciting the mobilization of other immune cells, including NK cells, cytotoxic T cells, T_H cells, DCs, macrophages, etc. [274] As mentioned previously, several studies have reported that CpG or antigen-CpG fusion immunization in humans and mice result in higher antibody titers, and/or antibody affinities for antigen and/or longer-lasting protection,[19,234,235,269,270,298–303] possibly due to enhanced CSR and SHM and expansion of high-affinity memory B cells.

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The reader may refer to a recent review for an extensive list of examples where prior CpG administration has resulted in some protection before challenge with pathogens.[304] A comprehensive list of current clinical and experimental vaccines that target T-independent bacterial antigens has also been recently reviewed.[305]

2. New Generation Modular Vaccines—Based on the promise of defined TLR agonists for use in vaccines, a new generation of vaccines is currently being developed. These vaccines employ particles made of biodegradable materials, lipid vesicles, or self-assembling protein subunits, that in addition to containing protein antigen, are infused with internal or external TLR ligands[192,306–312] (Fig. 5). This approach allows for modular, systematic and controlled testing of the best combinations of protein antigens from the pathogens and natural or synthetic TLR agonists, taking into account the dual TLR and BCR nature of B cell differentiation that was described in the previous section. Generally, a rigid array of a minimum of 15–20 repeating antigens induces optimal BCR signaling.[192]

While modular vaccine design is still under development, as a general approach, the particle and the TLR ligand combinations used should mimic the pathogen as closely as possible, e.g. it may not be beneficial to use flagellin to develop modular viral vaccines since it does not naturally occur in viruses. The TLR ligands can be LPS, Pam_3CSK_4, flagellin etc, which should be incorporated on the outside of the vaccine particle, and CpG, ssRNA, dsRNA or agonists such as imiquimod or R848, which should be best incorporated inside the particle. Future studies may, however, report beneficial, synergistic combinations of TLR ligands that are not normally found in the same pathogen, which may apply especially in the cases of co-infection by two or more pathogens.

Taking this approach one step further, recent studies have explored the feasibility of developing broad-spectrum vaccines, where immunity is provided against more than one pathogenic strain, or against several related or even unrelated pathogens.[313,314] In this case, antigens from several different pathogens are incorporated in the same particle or separately in mixtures of different particles, with TLR ligands typical of each pathogen also incorporated in the particles. In principle, this approach should allow for mobilization of B cells that produce antibodies specific for each pathogen. Issues remaining to be resolved relate to the safety and efficacy of CpG and other TLR agonists in eliciting broad-spectrum immunity to emerging and re-emerging pathogens.[313,315]

V. CONCLUSIONS

The synergy of BCR and TLR signaling critically regulates B cell antibody responses, and in particular CSR. BCR crosslinking facilitates the encounter of endosomes containing TLR9, and likely other TLRs, to autophagosomes as a way of surveilling the presence of nucleic acid TLR ligands during an infection. BCR stimulation provides information on the affinity of antigen for antibody, and the spacing and topology of antigenic arrays on the pathogen, whereas TLR stimulation may instruct B cells on the nature of the pathogen, e.g., the presence of a $Gram^{+ve}$ or $Gram^{-ve}$ bacterium or of an RNA or DNA virus. It would be particularly useful for B cells to sense multiple TLR signals from a pathogen in a combinatorial fashion, thereby leading to a more appropriate and specific response.

The dual presence of BCR and TLRs on B cells allows them to respond to a greater variety and combination of signals than previously thought, including traditionally innate signals, thereby allowing for fine-tuned responses that best deal with the pathogenic threat. The interplay and synergy between BCR, TLRs, and T-dependent signals is also proving to be both challenging and rewarding. The ultimate task remains to predict B cell behavior during an infection *in vivo* based on studies of responses to defined combinations of stimuli under

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different conditions *in vitro* and *in vivo*. Knowledge of fundamental B cell signals and typical responses would be beneficial for the design of therapies for immune deficiency or autoimmunity, and for the development of effective vaccines.

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Owing to space limitations, we could cite only a fraction of the papers relevant to the topics discussed in this review article. We apologize to the other authors of publications that were not cited. We would like to thank members of the Casali laboratory for invaluable discussions and critical reading of this manuscript. This work was supported by NIH grants AI 045011, AI 079705 and AI 060573 to P.C.

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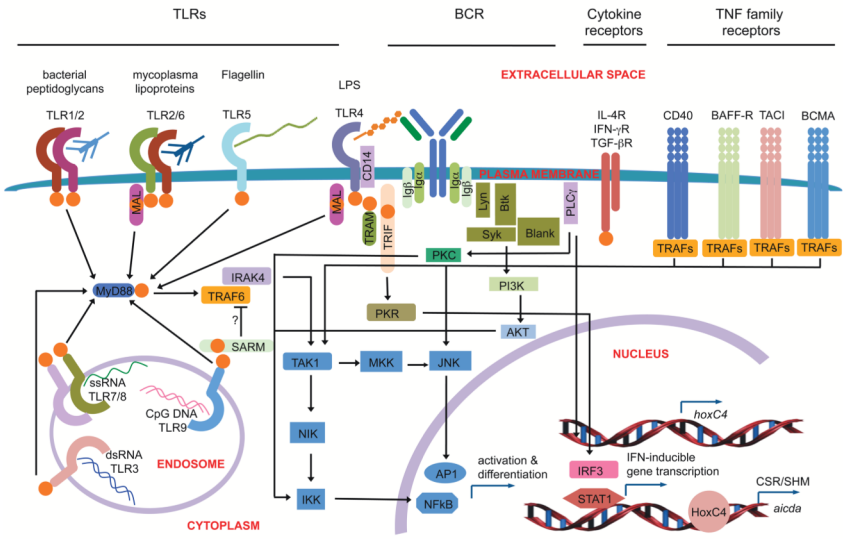

FIGURE 1.

Four main signals for B cell differentiation and antibody responses. During an infection, naïve mature B cells receive several types of stimuli that are then summed up before determining the appropriate response. DCs and T_H cells interact with B cells via surface receptors, such as CD40, and by cytokine receptors, such as IL receptors. A pathogen would directly induce signals by crosslinking the BCR (as shown by the proximity of the O-sccharide of LPS) and by activating innate receptors such as the TLRs (as shown by the interaction of TLR4 with the lipid A component of LPS). TLR ligands are sensed by the extracellular, or endosomal, sickle shaped domains, and signals are initially relayed to the nucleus via homotypic TIR-TIR interactions (orange spheres) with TIR adapters. These signals are integrated and initiate a response by inducing NF-**κ**B and AP-1, inflammatory gene transcription, IFN-inducible gene transcription, and induction of AID activity, leading to CSR and SHM

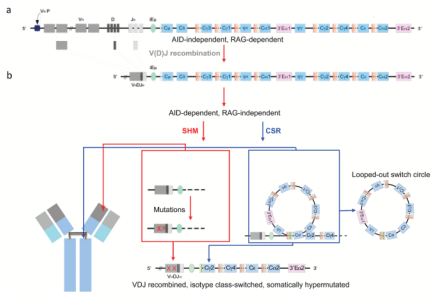

FIGURE 2.

Overview of CSR and SHM. **a**. Human B cells diversify the variable portion of their antigen receptors during development in the bone marrow through V(D)J recombination, which is mediated by recombination activating gene 1 (RAG1) and RAG2 recombinases. **b**. During an infection, naïve mature B cells in the spleen and lymph nodes undergo rearrangement of the constant portion of the IgH (i.e., CSR) to endow it with new biological effector functions, as well point mutations in the variable regions (i.e., SHM) to further increase its affinity, both of which depend on AID. Each C_H region is indicated in blue color, beginning with C**μ** on the left. The intronic IgH enhancer (iE**μ**, light green color), which is essential for optimal IgH gene expression, is located upstream of C**μ**. The different orange colored thin segments just upstream of each C_H region are the I_H promoters followed by the S regions. I_H promoters are activated in response to particular cytokines, and serve to drive germline transcription through S and C_H regions, possibly opening up local chromatin structure for AID activity, or delivering AID to the S regions by RNA pol II or other trans-acting factors.

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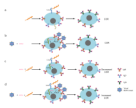

FIGURE 3.

The interplay of surface TLR4 and endosomal TLR9 in CSR. **a**. LPS induces CSR to isotypes, as directed by cytokines. **b**. CpG by itself does not induce significant CSR; however, in the presence of BCR crosslinking (indicated by a virus which is bound by BCR; experimentally it can be mimicked by reagents that crosslink the BCR polyclonally), it behaves similar to LPS in inducing CSR to the isotypes specified by cytokines. **c**. CpG suppresses LPS-induced CSR; however CpG synergizes with LPS in a dose-dependent way in the presence of BCR crosslinking (**d**).

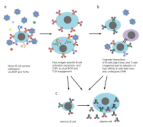

FIGURE 4.

Model on the role of endosomal TLRs in B cell antibody responses. **a**. B cells in the secondary lymphoid organs can be directly activated by viruses which crosslink the BCR. This then leads to their phagocytosis, double membrane autophagosome formation, and trafficking of the autophagosomes to TLR-containing endosomes. If any present viral TLR ligands are sensed by TLR3, TLR7/8, or TLR9, an appropriate response is then mounted by the B cells, which includes their differentiation (and in some cells, T-independent CSR), the upregulation of receptors that interact with T_H cells and DCs, thus enhancing the GC reaction (**b**). The result of T-independent (**a**) and T-dependent (**b**) B cell differentiation is the formation of antigen-specific plasma cells, and memory B cells, which can be quickly differentiated to plasma cells upon subsequent re-infection (**c**).

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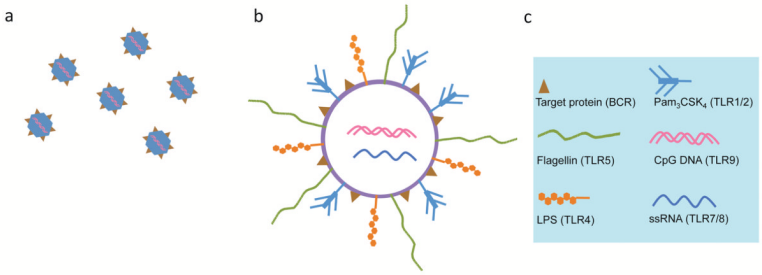

FIGURE 5.

The design features of modular vaccines. **a**. Virus-mimicking biodegradable particles with favorable pharmacokinetics, which by themselves are not immunogenic, are used as scaffolds for the incorporation of protein antigens from one or several pathogens. Initial screens determine the most immunogenically suitable protein antigens (brown triangles) to use. In addition, combinations of one or more natural or synthetic TLR ligands are included, usually mimicking their occurrence in the actual pathogen(s). **b**. Particles mimicking bacteria are coated with bacterial protein antigens as well as TLR ligands characteristic of each bacterium. These composite vaccines (**a**, **b**) activate both innate immune cells as well as B lymphocytes, which will differentiate to plasma cells that secrete antigen-specific antibodies, as well as to memory B cells which can be activated upon potential future re-infection. **c**. Legend indicates the protein antigens and the various TLR ligands used in modular vaccines.

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TABLE 1

Features of innate and adaptive immunity

Feature	Innate Immunity	Adaptive Immunity
Specificity	Low	Very high
Receptor clonal distribution	No	Yes
Receptor diversity	Encoded in germline	Recombined gene segments
Response timeline	No lag	Lag time for induction
Efficiency of response	Moderately efficient	Highly efficient
Memory	No	Yes

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TABLE 2

Pattern recognition receptors

PRR	Ligand(s)	Immune Cells
TLRs	Several surface and intracellular MAMPs	Neutrophils; monocytes/Mφ; DCs; B cells
NLRs	MDP; anthrax toxin; RNA; flagellin	Monocytes/Mφ; DCs
CARD helicases	dsRNA	Neutrophils; monocytes/Mφ; DCs; B cells
C-type lectins	Glycosylated microbial ligands	Monocytes/Mφ; DCs
Scavenger receptors	Lipid-containing microbial ligands	Monocytes/Mφ; DCs

Mφ: macrophages

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TABLE 3

TLRs and their ligands

TLR	Ligand(s)	Microorganism	TIR Adapters	Immune Cells
TLR1	Triacyl lipopeptides, e.g. Pam_3CSK_4	Bacteria, mycobacteria	MyD88; TIRAP	Neutrophils; monocytes/Mφ; mDC; B cells
TLR2	Diacyl lipopeptides, e.g. Pam_2CSK_4; Lipotechoic acid; Zymosan	Bacteria, mycobacteria, yeasts	MyD88; TIRAP	Neutrophils; monocytes/Mφ; mDCs; B cells
TLR3	dsRNA	Viruses	TRIF	mDCs; Mφ; B cells
TLR4	LPS	Gram-negative acteria	MyD88; TIRAP; TRAM; TRIF	Neutrophils; monocytes/Mφ; mDCs; B cells
TLR5	Flagellin	Bacteria	MyD88	Monocytes/Mφ; mDCs; B cells
TLR6	Diacyl lipopeptides	Mycoplasma	MyD88; TIRAP	Neutrophils; monocytes/Mφ; mDCs; B cells
TLR7	ssRNA	Bacteria and viruses	MyD88	Neutrophils; monocytes/Mφ; pDCs; B cells
TLR8	ssRNA	Bacteria and viruses	MyD88	Neutrophils; monocytes/Mφ; mast cells; B cells
TLR9	Unmethylated CpG containing DNA	Bacteria and viruses	MyD88	Neutrophils; monocytes/Mφ; pDCs; B cells
TLR11	Profilin	Toxoplasma	MyD88	Monocytes/Mφ; B cells

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

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TABLE 4

T cell-independent anti-microbial class switched antibody responses

Pathogen/Antigen	Mouse Model	Ig produced	Refs.
Polyoma virus	TCRβ$^{-/-}$ x δ$^{-/-}$	IgM, IgG	316
	B cell transfer in SCID	IgM, IgG	317
	CR2$^{-/-}$ B cell transfer in SCID	IgM, IgG	318
VSV	TCRα$^{-/-}$; TCRβ$^{-/-}$	IgM, IgG2a	319
Rotavirus	TCRαβ$^{-/-}$ x γδ$^{-/-}$	IgM, IgG, IgA	320
	TCRαβ$^{-/-}$	IgA	321
LCMV, VSV, Pichinde virus	CD154$^{-/-}$	IgM, IgG2a	322
Influenza virus	CD4$^{-/-}$	IgM, IgG1, IgG2b, IgG3	323
	CD40$^{-/-;}$ MHCII$^{-/-}$	IgM, IgG	324
Ehrlichia muris	CD4$^{-/-}$; CD8$^{-/-}$; MHCII$^{-/-}$	IgM, IgG1, IgG2c, IgG2b, IgG3	325
Ehrlichia chafeensis	CD4$^{-/-}$	IgG1, IgG2a, IgG2b, IgG3	326
Borrelia burgdorferi	TCRβ$^{-/-}$; TCRβ$^{-/-}$ x δ$^{-/-}$	IgM, IgG3	327
Bacillus anthracis	TCRα$^{-/-}$	IgG	328
Yersinia enterocolitica	nude	IgG	329
Citrobacter rodetium	CD4$^{-/-}$	IgM, IgG1, IgG2c, IgG2b, IgG3, IgA	330
Porphyromonas gingivalis	TCRα$^{-/-}$	IgG, IgA	331
Melanin (*C. neoformans*)	nude	IgM, IgG1, IgG2b, IgG3	332
Cryptosporidium parvum	nude	IgM, IgA	333
Protoscolex (*E. granulosus)*	CD4$^{-/-}$	IgG1, IgG2a, IgG2b, IgG3	334
Echinococcus multilocularis	nude; TCRβ$^{-/-}$; MHCI$^{-/-}$; MHCII$^{-/-}$	IgM, IgG1, IgG2a, IgG2b, IgG3	335