Protective Extrafollicular B Cell Responses are Driven by Intrinsic and Extrinsic TLR-Mediated Signals

By

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Abstract

Affinity for antigen is not only crucial for antibodies' protective capacity but is also considered the main inducer of plasma cells that secrete them. This has been demonstrated through use of model antigens where reductions in epitope valency or disruptive conformational alteration led to reductions in formation of antibody-secreting cells (ASCs). Surprisingly, the presence of abundant antigen does not always lead to high-affinity plasma cell generation such as during Salmonella and Ehrlichia infections. During these challenges, extrafollicular B cell responses (EFRs), foci of local, rapidly-formed, and short-lived ASCs known as plasmablasts, dominate the antibody response but fail to generate antigen-specific antibodies. This coincides with the kinetics of affinity maturation towards hapten antigen, where early antibody responses are of lower affinity than ones generated by highly matured germinal center (GC) reactions.

Contradictory to this, antibody affinity towards vesicular stomatitis virus (VSV) antigen in mice does not increase over the course of infection, indicating a lack of overall affinity maturation within the anti-VSV repertoire. Additionally, mice either infected or inoculated intravenously (i.v.) with influenza generate high-affinity and neutralizing antibodies against hemagglutinin (HA) before formation of GCs, indicating that under certain physiological circumstances, EFRs likely generate early, protective antibodies. Given annual influenza outbreaks and the current COVID-19 pandemic, it is of the utmost interest to generate protective antibodies as quickly as possible, as vaccines generally elicit protective serum antibody levels only after multiple weeks or multiple boosts. Using influenza infection and immunization in mice as a model, we sought to characterize EFRs, determine the signals responsible for their generation, and ascertain the protective capacity of EFR-derived antibodies.

Firstly, this work demonstrates EFRs generated after influenza infection produce fully protective antibodies, independent of GCs, while subcutaneous immunization with influenza

virion in alum produced no EFRs. This indicated that infection-induced signals were required for EFRs generation and sustainment. Single knockout of a plethora of inflammatory and anti-viral signaling pathways had no impact on early EFRs formation. Only did complete loss of Toll-like receptor (TLR) signaling result in changes to EFR dynamics in both a B cell intrinsic and extrinsic manner, with complete deletion of TLR adaptors MyD88 and TRIF leading to both defective early EFR formation and loss of immune protection. Therefore, TLR-derived signaling is required for optimal generation of EFRs against influenza.

Intrinsically, B cells required the presence of TLR components, from either MyD88/TRIF or unc93b and TLR2/4, for antigen-stimulated survival and expansion *in vitro*. This correlated with the inability of TLR-null B cells to upregulate IRF4, the master transcription factor of B cell differentiation. Additionally, TLR-null B cells had dysfunctional nuclear localization of c-Rel, an NF-kB factor that regulates IRF4 expression, and could not sustain upregulated c-Rel expression post-activation. Thus, B cells require TLR-mediated activation of c-Rel for survival and expansion after antigen-mediated stimulation, even in the absence of explicit TLR ligands, and likely need additional inflammatory stimuli to overcome abrogation of this signaling circuit.

While subcutaneous immunization with alum-adjuvanted influenza virus particles produced no EFRs, boosting with a lipopolysaccharide (LPS), a TLR4 agonist that activates both TLR signaling adapters MyD88 and TRIF, led to robust, antigen-specific EFRs. Boosting with both influenza virus and LPS longer-term suppressed antigen-specific GC responses while enhancing antigen-specific EFRs, generating higher concentrations of antigen-specific serum antibodies which led to greater protection against lethal influenza infection than serum from mice boosted with virion alone.

Thus, EFRs are an un-tapped source for kinetically advantageous, protective antibodies whose effectors form from a crucible of antigen and innate, inflammatory signals. This work not only elucidates crucial inputs of EFR generation, but also further clarifies the nature of B cell responses, which should be applied to other physiological and disease contexts.

Chapter 1

Introduction: B Cell Responses to Influenza

Jonathan H. Lam

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B cell–derived antibodies (Abs) generated in response to influenza virus are critical for preventing death from acute respiratory tract infections, promoting a more rapid and full recovery, and providing continued protection from future infection-induced illness and/or death. This is despite the fact that the yearly re-emerging seasonal strains of influenza virus undergo rapid point mutations, “antigenic drift,” which reduces the effectiveness of strain-specific Abs generated from 1 year to the next (1). Nonetheless, pre-existing, non-neutralizing humoral immunity, even if unable to prevent reinfection, can prevent serious disease and/or reduce mortality. This is well illustrated by the fact that individuals most at risk from dying following influenza infection are the very young and old or those with immuno-deficiencies, patients in whom an effective immune system has either not developed or is compromised. In addition to the mostly strain-specific Abs generated following an infection, influenza-specific B cell responses can include rare responses against highly conserved antigenic epitopes. Responses to such epitopes can provide cross-protection against multiple influenza strains (i.e., induce “heterosubtypic” influenza-specific immunity). The specificity of these cross-protective Abs and their protective capacities has been a recent focus of anti-influenza vaccine development efforts and are reviewed in detail elsewhere (2). Advances in our understanding of B cell responses to infections have revealed their complexity. In addition to the generation of Abs, B cells also generate cytokines with which they can regulate the immune response, and they can act as APCs to CD4 and CD8 T cells (3). Their elaboration of cytokines and interaction with T cells is likely to induce responses that are distinct from those provided by other APCs, shaping T cell immunity in yet to be determined ways. Moreover, the presence of virus-induced innate responses can also affect B cell functions, and this may differ depending on the tissue in which B cells receive such signals, thus indicating further complexities based on B cell tissue location and interaction with innate immune cells. Further complexities are the extensive heterogeneity among B cells with regard to their developmental origins, initial BCR affinity for influenza antigen (Ag), and their differentiation state, all of which shape B cell response outcomes.

Here, multiple aspects of B cell immunity to influenza virus infection are discussed, focusing on experimental mouse models and citing human studies when possible. B cell responses to innate signals and B cell–derived cytokines involved in antiviral responses are described as well as how B cells transform these signals into functional changes. Additionally, innate-like B cell responses, early extrafollicular plasmablast responses (EFRs), and later germinal center (GC) responses are detailed, with discussion of how EFRs and GC responses are of equal importance in the development of strong and durable humoral immunity to influenza virus infection.

B cells shape the early immune response to influenza infection

Mice never exposed to influenza virus and even those kept under germ-free conditions nonetheless have circulating “natural” IgM Abs, a fraction of which can bind to a variety of different influenza virus strains (4–6). This IgM Ab is generated in the bone marrow and spleen of mice by differentiated, neonatally derived subsets of innate-like B cells called B-1 cells (6, 7) and can reduce viral loads and mortality rates from infection (8, 9). In addition to this steady-state function, innate-like B-1 cells also respond to influenza virus infection with rapid migration from the pleural cavity to the draining mediastinal lymph nodes (medLN) (5, 10). Accumulation in the medLN, which occurs as early as 2 d after influenza infection, depends on type I IFN production in the respiratory tract, which was shown to cause integrin CD11b activation on the surface of pleural cavity B-1 cells, facilitating their retention in the medLN (10). Once there, some B-1 cells begin to produce IgM, including IgM that can bind multiple strains of influenza virus (4, 5). The mechanisms leading to induction of Ab secretion by these cells remains unknown.

Secreted IgM is a potent facilitator of complement activation (11) and can bind the complement receptors (CR1/2) as well as the Fc receptor for IgM (FcmR) expressed on dendritic cells (DCs), macrophages, and B cells (12, 13). Lack of IgM in mice led to delayed

influenza clearance (8, 9, 14, 15). IgM-mediated protection from influenza infection is mediated at least in part through activation of complement (15). In addition, lack of secreted IgM and/or lack of FcmR expression on B cells caused decreases in influenza-specific IgG, likely further contributing to reduced viral clearance (9). Thus, early production of IgM in response to influenza optimizes the overall Ab response and is a potential catalyst in priming both innate and adaptive immunity.

Immediate early responses by bone marrow–derived conventional follicular B cells (also termed B-2 cells), which make up the vast majority of B cells in lymphoid and non-lymphoid tissues, are less well characterized. Some potential functions may be inferred from other infection models. In addition to populating secondary lymphoid tissues, naive B cells are dispersed throughout the conductive airways and lung parenchyma at homeostasis (16) and are thus able to respond rapidly to respiratory infections. In support of this, lung-associated B cells were observed transporting Ag to the spleen, but not the lymph nodes, within 2 h after intranasal immunization of mice with virus-like particles (17). Following intratracheal administration of anthrax spores, B cells were found to deliver Ag within just 6 h to the lung-draining lymph nodes in a BCR-independent manner (18). These observations indicate early functional roles of B cells in Ag delivery from the periphery to secondary lymphoid tissues. The nature of the Ag and/or the inflammatory responses induced to distinct pathogens may affect subsequent localization of these B cells and therefore the initiation of the adaptive response.

B cell responses during influenza infection are present in both medLN and spleen. However, splenic responses appear to be minimal and of shorter duration compared with those in the medLN, indicating that the medLN are the main sites of B cell response activation after intranasal influenza infection, at least in experimental settings. In humans, where severe infections usually proceed from the upper to the lower respiratory tract, the involvement of upper respiratory tract draining cervical lymph nodes likely play a greater role compared with

experimental infections of mice, in which the virus is usually applied deep into the respiratory tract.

The medLN significantly increase in size and cellularity within 1–2 d post-infection (19). Influenza Ag-carrying CD11b+ DCs migrate into these lymph nodes, thereby promoting the activation of CD4 and CD8 T cells. Interestingly, lymph node expansion following lymphocytic choriomeningitis virus (LCMV) infection was shown to depend on the provision of lymphotoxin a1b2 by B cells (20). Furthermore, 2 d after immunization with the Ag keyhole limpet hemocyanin in CFA, B cells facilitated a 6-fold increase in lymph node cellularity by secreting vascular endothelial growth factor A (VEGF-A) (21), which promotes both blood and lymph vasculature remodeling and growth (22). Without B cells or B cell–derived VEGF-A, DC trafficking and lymph node cellularity were reduced to homeostatic levels (21). Thus, B cells actively regulate immune responses by shaping secondary lymphoid tissue remodeling post-infection.

Within the lymph nodes, B cells become subjugated to cues for activation provided by innate signals. Global gene expression analysis on medLN B cells of day 2 influenza-infected mice revealed a type I IFN signature (23). Type I IFN was shown to cause rapid global B cell activation, likely prior to first Ag encounter, including immediate upregulation of TLR3 and 7 and increases in expression of CD69 and CD86 (23). This renders B cells more responsive to innate signals and increases their ability to interact with T cells by trapping them in the lymph node as well as increasing expression of costimulatory molecules. Indeed, these innate signaling–derived activation signals were shown to be important for B cell response induction, as B cell–specific deletion of the type I IFN receptor diminished the influenza-specific Ab response as early as 3 days post-infection (23). Thus, adaptive B cell response activation is integrated in, and dependent on, the network of infection-induced innate signals. Further exploration of how innate activation of B cells affects the quality and quantity of anti-influenza responses will be

important for the development of adjuvants that support stronger and longer-lasting humoral immunity.

B cell activation leads to rapid induction of EFRs and slower development of GC responses in draining lymph nodes after influenza infection

As discussed above, respiratory tract draining lymph nodes are the main site of influenza-induced B cell activation. A critical outcome of B cell activation is the induction of Ab-secreting cells (ASCs) producing class-switched, protective Abs (24, 25). Strong influenza-specific Ab responses are produced by EF B cells well before GC-derived plasma cells are generated (Fig. 1). EF responses are situated in the lymph node medulla, where rapid and strong B cell proliferation is followed by differentiation of these cells into short-lived plasmablasts that secrete IgM, IgA, and IgG (26).

As explored below, the explicit mechanisms that drive B cells into either the EF or the GC fate have not yet been completely resolved. Of importance, the kinetics of EFRs correlate with the peak and resolution of primary influenza infection (27), indicating that this rapid, strong, and early humoral response contributes to virus clearance. In contrast, GC responses do not usually form until the infection has nearly resolved, indicating GCs are geared toward generating and maintaining immunological memory by producing long-lived Ab-secreting plasma cells and memory B (B_{mem}) cells. The latter importantly enhances the precursor frequency of influenza-specific B cells, some of which can respond to the next influenza virus challenge either by initiating rapid EFRs or by forming new GCs (Figure 1).

Apart from the above-discussed migration of Ag-carrying B cells into secondary lymphoid tissues, most B cells likely come across Ag in the lymph node follicles through stochastic movement that bring them near the follicle's capsular plane, where they encounter Ag tethered on subcapsular macrophages (28). B cells can also find cognate Ag elsewhere in the lymph node (reviewed in Ref. 29), although whether B cell response quality differs depending on

the type and site of Ag encounter is unknown. The Ag-specific activation of B cells and CD4 T cells induce genetic reprogramming that strongly promotes their interaction, specifically their chemokine-mediated migration toward each other. This is facilitated initially by the upregulation of CCR7 and downregulation of CXCR5 in B cells and the inverse in CD4s, which leads to their congregation at the paracortical ridge (30), otherwise known as the T:B border. Movement of B cells is also affected by the orphan receptor Ebi2, which is expressed differentially at the many stages of B cell activation and differentiation in the lymph node (31). Furthermore, Ag stimulation induces secretion of CCL3 and CCL4 by B cells, which will further attract activated CCR5-expressing CD4 T cells and thus enhance T–B interaction and humoral immunity (32).

EFRs. Ag-activated B cells can form EF foci independent of CD4 T cell "help," and such T cell–independent B cell responses can confer some protection against influenza virus infection (33, 34). However, most protective B cell responses to influenza, including those generated during EFRs, are T cell–dependent. The local expansion and rapid differentiation of B cells into plasmablasts, seen in EFRs, requires B cell genetic programs to polarize toward an ASC fate. The ASC state is characterized by expression of transcription factors Blimp1, XBP1, and high expression of IRF4, which is upregulated upon initial B cell activation together with IRF8. However, IRF4 and IRF8 become mutually antagonistic after reaching certain expression thresholds, influencing polarization toward an effector (IRF4) or a GC (IRF8) fate (35).

The signals that preferentially induce IRF4 over IRF8 and thereby an ASC fate are the subject of ongoing investigation. Elegant previous work has begun to shed light on some potential mechanisms. Of particular significance, a strong correlation was observed between BCR affinity for Ag and the ASC fate (36–38). Reducing a high-affinity interaction of B cells to the model Ag hen-egg lysozyme, by alterations made to the cognate Ag, showed that only high-affinity BCR–Ag interaction could promote EFRs (36). This is supported also by *in vivo* studies showing that GC-derived, B_{mem} cells will rapidly form EFRs during a recall response preferentially over generating new GCs (39). At the molecular level, and consistent with those

observations, IRF4 is known to be upregulated by increasing affinities of the BCR for Ag. Furthermore, IRF4 is induced in a dose-dependent manner by the engagement of B cell–expressed coreceptor CD40 with CD40L on T cells (40). These observations strongly suggest that BCR signal strength, as well as T cell help, contribute toward differentiation of activated B cells into plasmablasts. Whether strong BCR signaling alone can overcome the need for CD40 engagement in the formation of T cell–independent EFRs or whether IRF4 induction might be induced by innate signals in addition to BCR signaling remains to be determined.

A model of rapid EFR induction that depends on the presence of high-affinity BCR is consistent with data demonstrating that these responses can provide Ab rapidly and of sufficient quality to contribute to immunity after influenza infection (27). However, it also suggests that rapid EFRs are formed only when B cells of “sufficient” Ag affinity are present in a given host prior to an infection. The high morbidity and mortality rates observed in newborns and the very young after influenza infection might be in part explained by the fact that these individuals lack such a robust and broad naive B cell repertoire (41) and that they do not yet carry influenza-specific B_{mem} cells that could provide clones specific for influenza (see also below).

Once an activated B cell differentiates into a plasmblast, their populations begin to expand rapidly in the lymph node medulla and secrete predominantly class-switched Ab, peaking between 7 and 14 d after influenza infection (27, 42). In humans, influenza-specific plasmablast numbers in the peripheral blood peak around 7 d after symptom onset during infection (43) and after immunization (44). However, post-immunization plasmablasts appear at a much-reduced frequency compared with infection (45) and have even higher levels of somatic hypermutation than circulating B_{mem} cells (46), indicating they were recruited from memory pools and thus previously generated in GC rather than EF.

Once EFRs are initiated, its maintenance does not appear to require further T cell help (47). Interestingly, it was shown that an increased presence of neutrophils in the draining lymph nodes led to increases in plasmablast numbers after s.c. CFA immunization (48). The

mechanisms of that interaction were inferred to be dependent on the cytokine BAFF secreted by neutrophils. However, other studies more explicitly showed that the survival factor A proliferation-inducing ligand (APRIL), also secreted by neutrophils, was vital in maintaining ASC populations within niches of the lower respiratory tract submucosa (49) as well the splenic marginal zone (50). Plasmablasts from the EF response thus appear to be maintained or at least significantly supported by Ag-independent means, including through the secretion of survival factors by effectors of the innate compartment.

GC responses. GC-derived expansion of B cell clones is minimal during initial influenza infection and virus clearance (i.e., the first 7 to 10 d after virus exposure). Instead, GC-associated B cell numbers peak well into the contraction phase of the immune response (51) (Fig. 1). To initiate GC formation, B cells activated by Ag and cognate CD4 T cell help return to the B cell zone (follicle) from the T:B border, along with their cognate CD4 T cells, in which follicular DCs display non-processed Ag (52). Ag-binding B cell clones then undergo massive proliferation as well as somatic hypermutation, the latter supported by the expression of the enzyme aicda (AID), forming the dark zone of the GC with cells known as centroblasts. This process leads to B cell subclones with genetic alterations in the Ag-binding site of their BCR. Centroblasts then migrate to the light zone of the GC for validation of Ag specificity. Once there, these cells, known as centrocytes, engage with CD4 follicular helper T cells to determine B cell fate (53). Positive selection will result in either a return to the dark zone for further rounds of proliferation and mutations or in an exit from the GC as either B_{mem} cells or terminally differentiated plasma cells. It is not known what signals instruct the differentiation of GC clones to either a B_{mem} or plasma cell fate, but lower-affinity clones were shown to preferentially accumulate within the B_{mem} pool (54–56), whereas high-affinity clones are shunted preferentially toward a plasma cell fate (25–27) in much the same manner as seen during EFRs. Therefore, it appears that both plasmablasts and post–GC plasma cells may use the similar differentiation pathways to reach an ASC state. However, although EF-derived ASCs appear to be short-lived

and confined to the site of their induction, the post–GC plasma cells migrate to the bone marrow in a CXCR4-dependent manner. There, they can secrete Ab and survive long term, supported by local, stromal cell–derived survival signals (57).

Extensive Ig sequencing studies of circulating B_{mem} cells in humans have shown that strong Ab repertoire diversity is generated in response to repeated infection with, or exposure to, seasonal influenza viruses. The data suggest similar strong, ongoing GC responses to occur in humans following influenza infection as those studied in experimental mouse models (58). Given that both the influenza virus and the B cells are relying on mutations to out-compete each other, the GC response is likely the main B cell response engaged in the “arms race” between the virus and the humoral response, as somatic hypermutation during EFRs is limited (59,60). Indeed, sequencing studies have been able to trace the evolution of B cell responses that occur in response to repeated exposures to seasonal influenza virus strains (61–63), clearly revealing a GC-derived B_{mem} cell repertoire that is shaped by repeated exposures to mutated viruses.

Humoral immunity to influenza integrates the B-1 response and B-2–derived EF and GC responses

Thus, humoral immunity to influenza can be categorized into immediate early, early, and late responses, each facilitated by a distinct set of B cells. Control of an acute influenza infection by B cells involves both innate-like B-1 cells as well as rapidly responding conventional B cell–derived EFRs, both having the capacity to control early tissue virus load. This control is critical as an early containment of this rapidly dividing virus can reduce the risk of overshooting cytokine and CD8 T cell responses, which can cause extensive and excessive accumulation of leukocytes in the lung parenchyma, lung pathology, and eventual organ failure.

GC B cell responses, in contrast, must fulfill distinct roles in combating influenza infection. The kinetics of these responses precludes important roles during primary infection. Yet the induction and then maintenance of GC responses for months after the clearance of the

virus suggests that their main function is the strengthening of the initial line (i.e., B-1 and EF) of humoral defense during recall responses, either through generation of Ab-producing plasma cells or through provision of a large repertoire of B_{mem} cells. The production of affinity-matured, neutralizing Abs by plasma cells per se seems of limited value against an infection that can rapidly mutate targeted epitopes. Therefore, the generation of a broad repertoire of B_{mem} cells seems very important as these B cells are generated to a variety of influenza Ag and can respond, depending on their initial affinity to Ag, by either rapidly generating Abs or by inducing strong GC responses. It may also explain why such B_{mem} cells are not of as high affinity for the inducing Ag as the effector plasma cells; the next round of infections might bring mutated Ag that bind differently to these BCR. Recent studies with human B_{mem} cells support the notion that this compartment has a broad repertoire and includes cross-reactive BCR-bearing cells that are continuously shaped by repeat exposure to seasonal influenza virus strains (58, 64). The broadening of this repertoire would ensure that there are B cell pools available to bind viral Ag, even if altered by mutations, as seen during the seasonal encounters with influenza by humans.

Although EF and GC responses to influenza are generated by conventional B cells, early sequencing studies showed a remarkable lack of overlap in the V-gene usage of hemagglutinin (HA)-specific B cells early and later after immunization with influenza virus (27, 65). Recent elegant studies by the Yewdell group (51) confirmed these early studies using influenza virus infection of mice. The data are consistent with the above-discussed findings that only high-affinity Ag–BCR interactions drive EF responses (36), whereas lower-affinity interactions initiate GC responses. Given the strength of the EF response post-infection, this suggests that humoral responses to influenza virus infection, and likely other infections, do not begin with the elaboration of low-affinity Abs that, over time, affinity mature into higher-affinity Ab responses. Rather, high-affinity Ab responses produced by the EF response are replaced later by high-affinity Ab responses generated from the GC response.

If our analysis is correct, this would be a significant departure from the textbook idea of Ab responses that “mature” over time but very consistent with conclusions drawn from previous studies with VSV infection of mice that failed to demonstrate overall changes in serum Ab affinity over the course of that virus infection (66). For an acute infection, like infections with influenza virus, the immediate early activation of B cells into ASC from pre-existing high-affinity clones may make the difference between life and death, although during more protracted infections the influence of the EF response may be more modest.

Respiratory tract B cell activation during influenza infection

In addition, and similar to other mucosal sites, strong and sustained respiratory tract tissue injury, including injury induced by influenza infection, can result in the formation of bronchus-associated lymphoid tissue (BALT) in the submucosa of the upper respiratory tract and within the parenchyma of the lower respiratory tract airways (67–69). Although the importance of BALT formation in immune defense is still not resolved, such inducible BALT was shown to contain GCs and to generate influenza-specific Ab responses after influenza infection in mice that lacked all secondary lymphoid organs, including all draining lymph nodes (67), indicating their potential to catalyze adaptive T and B cell responses. Interestingly, following local ablation of BALT with diphtheria toxin, significant reductions in influenza-specific serum Ab titers were noted after intranasal influenza inoculation (70). However, another study showed that after influenza infection, BALT formed only if mice had received repeated intranasal administration of LPS as neonates (71). Differences in virus strain and mouse models may be the cause of these discrepancies. Together, the data indicate that tertiary lymphoid tissues in the respiratory tract may contribute to immunity during strong and/or chronic immune activation.

The GC generates ASCs and B_{mem} cells capable of recall responses and long-term influenza-specific Ab production

Following the resolution of influenza infection, ASC populations are found long term in bone marrow as well as locally in the respiratory tract (72). The differentiation pathways of the local ASCs have not been resolved, although those residing in the bone marrow likely arise from GC responses. Both influenza infection–induced bone marrow and lung ASCs were shown to require TACI (a receptor for APRIL) for long-term survival, whereas lymph node and spleen ASCs were unaffected by the loss of that receptor (73). Given that lymph node ASCs are derived mainly from EF responses, as they disappear with the resolution of the EF response and before robust GC responses develop (42), this could suggest that cell-fate decisions between plasmablasts and plasma cells seem to be the result of broadly overlapping genetic programs. Differences might still exist between individual ASC populations based on tissue location and/or whether their differentiation pathways involved EF or GC responses.

GC-derived plasma cell development leads to sustained influenza-specific serum Ab titers. Ag-specific Ab has been detected decades post-infection and sometimes after immunization in humans (74). Carbon dating on plasma cells in the gastrointestinal tract confirmed this subset's aging capacity (75). Similarly, survivors of the 1918 influenza pandemic showed sero-reactivity to the 1918 virion 90 years later (74). This indicates that influenza infection can generate essentially life-long humoral protection against the same (homosubtypic) virus strain. Whether boosting by subsequent seasonal influenza exposures contributes toward maintenance of such Ab responses is not known and should be considered.

Few data are available revealing the lifespan and aging dynamics of B_{mem} cells. It has been well demonstrated, however, that these cells respond acutely to secondary challenges, partaking in both EF and GC responses in local lymph nodes (39, 76), as well as migrating directly to sites of insult (77, 78). More than 5 months after influenza challenge, class-switched, CD38+ B_{mem} cells were found in spleen, medLN, and lungs of BALB/c mice (77). Furthermore,

reactivation of lung B_{mem} populations was shown to contribute to enhanced immunity during virus challenge (77, 79) and the loss of B_{mem} cells, as seen in HIV-infected patients, reduced the patient's ability to respond to influenza vaccinations (80). As outlined above, repeated exposure to influenza virus continuously alters the virus-specific class-switched B_{mem} compartment of humans, indicating that B_{mem} cells respond to and help control influenza infections, likely including many that cause subclinical infections.

B cells as regulators of T cell–dependent antiviral immunity

Several influenza virus infection studies in mice demonstrated B cell synergy with either CD4 or CD8 T cells in optimizing protection against a primary viral challenge. Although lack of CD8 T cells delayed clearance of influenza A/PR8 (H1N1) significantly in mice (81), so did a lack of B cells, albeit not as strongly (14). The targeting and elimination of DCs by cytotoxic CD8 T cells may also affect B cells directly by reducing priming of robust CD4 T cell responses that support development of virus-specific Abs. Consistent with that, the lack of CD8 T cells actually led to increased Ab responses (82). Absence of both CD8 T cells and B cells led to uncontrolled viral dissemination and death (34), indicating CD4 T cells alone cannot protect against this infection. The same held true for CD8 T cells, as lack of both CD4 T cells and B cells resulted in significant mortality after low-dose H1N1 infection (83).

This raises the question of how B cells cooperate with T cells to prevent influenza virus–induced immunopathology and death. A lack of Ag-specific Ab in a Listeria–LCMV challenge mouse model led to uncontrolled CD4 T cell activity, cytokine storm, and death, which was abrogated upon transfer of Ag-specific B cells (84). In that case, Ag-specific Ab was shown to reduce Ag load and thereby prevent continued, overly strong activation of effector CD4 T cells. Consistent with that data, influenza-infected mice lacking B cells and chronically depleted of CD8 T cells, but with an intact CD4 T cell compartment, rapidly succumbed to infection, although replenishment with virus-specific B cells rescued these mice (34). The data indicate

that generation of protective Abs provides a bridge between CD4-driven, innate, and Ag-directed responses.

Inclusive with this dynamic of suppressive feedback, B cells also promote CD4 T cell activity. Absence of B cells in influenza-infected mice was associated with reduced systemic CD4 T cell proliferation and reduced polarization of Th1 cells in the lungs, which resulted in decreased viral clearance (14). This might be due at least in part through the ability of B cells to produce numerous cytokines, as B cell–derived IFN-g, IL-6, and TNF-a were shown to support Th1 responses by CD4 T cells (85–87). In addition, production of IL-6 by B cells was shown to promote the development and/or maintenance of CD4 follicular helper T cells and thereby the regulation of antiviral Ab responses (88).

Triggering of cytokine production by B cells might be induced through their expression of a wide array of pattern-recognition receptors, including TLRs, retinoic acid inducible gene I–like receptors, and NOD-like receptors (89–91). Specifically, the activation of TLR7 on murine B cells has been shown to induce IFN-a production during influenza, although not to EBV infection (92, 93). B cells also express inflammasome complexes (94) and thus can promote Caspase-1–mediated processing of the inflammatory cytokines IL-1, IL-18, and IL-33 into their active forms (95). IL-1b is particularly interesting as IL-1 knockout mice showed increased morbidity and mortality following influenza infection, which correlated with decreases in amounts of serum and airway influenza-binding IgM (96).

Cytokine production by B cells may also modulate potential overshooting cytokine responses, the so-called “cytokine storm”, observed following infection with particularly pathogenic influenza viruses. IL-10 can suppress such overshooting responses (97), and IL-10 production by regulatory B cells (“Bregs”) and plasma cells has been shown to be effective in modulating a number of inflammatory conditions (reviewed in Ref. 98). Given the close interaction of B cells with recently activated CD4 T cells in the bridging channels of activated lymph nodes, further analysis of immunomodulation of antiviral T cell responses by cytokine-

producing B cells may provide important mechanistic insight into the regulation of effective antiviral immunity to influenza.

Effective heterosubtypic immunity to influenza

Effective immunity to influenza virus requires the presence of T and B cells that respond to more than one sub-strain of influenza, so called heterosubtypic immunity. Studies conducted following the 2009 pandemic showed that humans harbored influenza-specific memory CD4 T cells that were cross-reactive between seasonal and 2009 pandemic H1N1 strains (99), as well as cross-reactive B_{mem} cells. CD8 T cell immunity to influenza has long been known to target highly conserved internal proteins of influenza, particularly the nuclear protein, which can provide some heterosubtypic immune recognition (100).

The contribution of each adaptive immune compartment to heterosubtypic immunity was evaluated in mice, demonstrating a most potent role for T cell–dependent Ab responses. Genetic ablation of CD4 T cells in mice following a sublethal dose of a H3N2 virus infection resulted in significantly enhanced mortality to subsequent challenge with a lethal dose of H1N1 (33). The same studies with B cell–deficient mice resulted in even greater mortality, although depletion of CD8 T cells had no effect (33). This was despite existing cross-reactivity of H3N2-primed CD8 T cells. As expected, CD8 T cell–deficient mice generated protective levels of neutralizing serum Ab against the heterosubtypic virus, although influenza-specific Ab titers in CD4 T cell–deficient mice barely rose above that of their naive controls (33).

Recent studies have identified the highly conserved “stalk” domain of the trimeric HA molecule as a target of broadly cross-reactive and indeed cross-protective Abs (reviewed elsewhere Ref. 2). A current challenge for exploiting this information for the better design of vaccines is the finding that these epitopes are neither immunodominant nor do they induce neutralizing Abs (i.e., Abs that directly prevent the binding of the virus to target cells). Their effectiveness instead relies on Fc-mediated effector functions by innate immune cells, including

the enhancement of Ab-directed cell cytotoxicity (ADCC) facilitated by the binding of specific Ig isotypes to activated Fc receptors (101, 102). Indeed, broadly neutralizing mAbs with disrupted Fc receptor binding did not protect against lethal influenza infection in mice, whereas identical clones with full Fc receptor binding potential completely prevented infection-induced mortality (103, 104). Unfortunately, immunization of mice with Ag holding ADCC-associated epitopes led to increased alveolar damage and mortality after boost with intact virus (105), demonstrating the double-edged sword of Fc-mediated ADCC during influenza infection.

Given the limitations of these naturally induced broadly protective Abs, it appears likely that the observed naturally developing T cell–dependent, B cell–mediated heterosubtypic immunity is provided not only by such cross-reactive protective Abs but also by the ongoing reshaping of the overall influenza-specific B_{mem} compartment by seasonal influenza virus exposures. Such exposures constantly boost and shape an increasingly broad repertoire of B_{mem} cells, generating a range of Ab specificities that are both neutralizing and non-neutralizing. Together, they provide strong protection, if not always from virus-induced disease, from serious illness and death. When failure of protection is observed (i.e., in the very young as well as the elderly), the lack of a functional broad and robust repertoire of responding
B cells, either because they are underdeveloped or no longer functional, is likely the main driver of this failure of immune protection.

It seems counterintuitive then that the presence of pre-existing, humoral memory to influenza virus has been shown to prevent the induction of strong neutralizing responses to a related but distinct influenza strain under certain conditions (106, 107), a phenomenon known as “original antigenic sin.” In those situations, the presence of a broad repertoire of pre-existing, influenza-specific B_{mem} cells might lead to the activation of non-protective B cells over naive, but potentially neutralizing B cells. Alternatively, the rapid capture of Ag may prevent strong immune response activation. Although animal models show clear evidence for such a phenomenon, the

extent to which such scenario may contribute to the lack of protection in humans remains unclear.

It cannot be understated how involved B cells are in every facet of the adaptive immune response, from early priming events that set the stage for proper T and B cell activation, to the formation of memory after pathogen clearance to prevent repeated assaults on the host. While each of these facets needs to be given proper interrogation, it is the generation of protective and neutralizing antibodies that have the highest impact on human and animal health, with EFRs producing the earliest, Ag-specific antibody response. The studies conducted in this dissertation aimed to elucidate the timing and dynamics of EFRs after influenza infection and immunization and to determine the signals required for robust EFRs. Most importantly, these studies also aimed to test the protective capacity of EFR-derived antibodies and interrogate under what circumstances they may be generated in the context of immunization.

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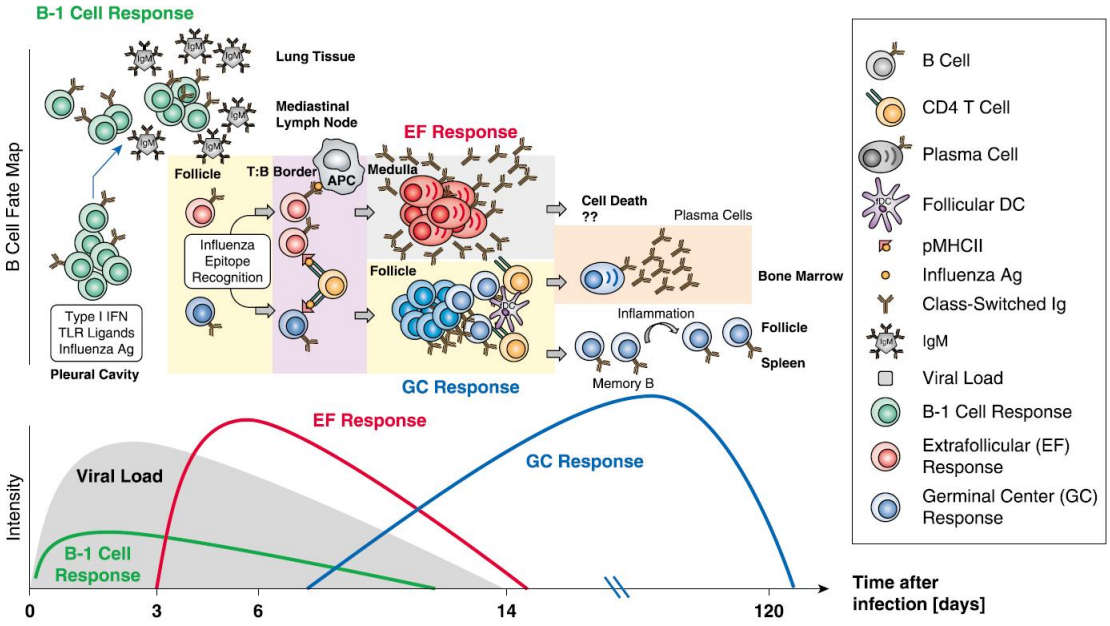

FIGURE 1.1. The heterogenous nature of B cell responses to influenza virus infection. Top, B-1 cells, activated by various innate signals, including type I IFN, respond to initial influenza infection with migration to the draining medLN, where these cells differentiate to IgM-producing cells. Conventional B cells, activated by influenza Ag, will migrate to the T–B border, where they receive “T cell help”. Thus activated, B cells will differentiate along one of two pathways: EF, which induce strong and rapid clonal expansion and differentiation to Ab-secreting plasmablasts, and GCs in the B cell follicles. EF-derived plasmablasts are thought to live for only 3–5 d, whereas the outcome of GC responses is the development of long-lived Ab-secreting plasma cells in the bone marrow and circulating non-secreting B_{mem} cells. Inflammatory signals may activate these B_{mem} cells to rapidly migrate to spleen and lymph nodes and to differentiate to ASCs or undergo new diversification in GC responses. Bottom, B-1 and EF responses are the only B cell responses that are sufficiently fast to influence viral clearance after a primary challenge.

Chapter 2

Extrafollicular B cell responses to influenza infection generate protective antibodies and require Toll-like receptor signaling for optimal generation

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Abstract

Influenza is a globally endemic virus that can cause severe respiratory tract tissue damage, either directly through virus-induced tissue damage or following the initiation of inflammatory and cytotoxic immune responses. Therefore, rapid influenza virus clearance is crucial for preventing high levels of morbidity and mortality following infection. Influenza-specific antibodies are produced during primary infection in the lung-draining lymph nodes (dLN) due to the rapid and transient expansion of plasmablasts in extrafollicular B cell responses (EFRs), However, EFR were not formed in the dLN after subcutaneous immunization, indicating that infection-induced signals are required for EFRs. Here, we show that deletion of numerous individual innate and inflammatory signaling pathways implicated in the anti-influenza response did not alter early EFRs. Yet, a combined lack of both Toll-like receptor (TLR) adaptors, MyD88 plus TRIF, disrupted early EFR formation and caused a significant reduction in EFR-derived antibody-mediated immune protection. In contrast, ablation of all TLR signaling through deletion of TLR2, 4, and Unc93b while leading to significantly higher viral loads did, not alter early EFR formation after influenza infection, nor affect the serum passive protective capacity. When restricting the TLR defects to only B cells, via generation of mixed bone marrow irradiation chimeras with B cells from either the MyD88 plus TRIF, or TLR2/4/Unc93 knock outs, showed both significant reductions in EFRs. The data identify B cell- intrinsic and extrinsic TLR signaling as regulators of EFRs. Furthermore, the data suggest that the aggregate of the inflammatory signals generated in response to a viral infection provide critical support of the EFRs.

Introduction

Acute respiratory tract infections induce neutralizing antibody responses that are critical for long lasting protection. Germinal center (GC) responses are considered the most effective in generating protective antibodies, as antigen (Ag)-specific GC B cells undergo extensive somatic hypermutation resulting in long-lived antibody-secreting plasma cells (ASCs) that generate high-affinity, strongly neutralizing antibodies. However, after primary influenza virus infection, GCs appear relatively late, usually after viral contraction, and thus are unlikely to contribute towards virus clearance (1). Instead, early antibodies are produced from plasmablasts of the extrafollicular B cell responses (EFRs) that develop in the respiratory tract-draining mediastinal lymph nodes (medLN) shortly after infection and before GC formation (2).

Differentiation of B cells to ACSs following immunization with model Ag was shown to require high-affinity interactions between Ag and the B cell receptor (BCR) (3,4), as those signals trigger the induction of the transcriptional regulators of plasma cell differentiation, IRF4 and Blimp-1 (5). However, EFRs have been demonstrated to be low affinity and lack extensive somatic hypermutation (6,7), suggesting their emergence prior to GC development may use other signals in addition to high-avidity interactions. EFRs are also associated with chronic inflammatory diseases including autoimmunity (8), suggesting sustained inflammatory stimuli support persistence of local EFRs. Recently, it was shown that human patients suffering from severe COVID-19 disease had elevated levels of circulating, EFR-derived plasmablasts compared to patients with milder infections (9), establishing a correlation of EFR magnitude with disease severity. I therefore explored the potential role of inflammation in EFRs after primary influenza infection to assess what signals were either required or influenced EFR development and functionality.

Materials & Methods

Mice. Male and female 8- to 12-wk-old C57BL/6 (WT; CD45.2), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), B cell–deficient (μMT) mice as well asTNFAR1/2 KO, IFN-gamma KO, IL-12 KO, CD19-Cre IFNAR KO, IL-1R KO, TLR3 KO, TLR4 KO, TLR7 KO were commercially obtained (The Jackson Laboratories). Breeding pairs of MyD88/TRIF DKO and TLR2/4/unc93b TKO mouse strains were gifts from Dr. Barton (UC Berkeley). Breeding pairs of S100A9 KO mice were a kind gift of Dr. Rafatellu (UC San Diego). Mice were bred and held under SPF housing conditions with ad libitum access to food and water. All experiments conducted were approved by the UC Davis Institutional Animal Care and Use Committee.

Mixed bone marrow (BM) chimeras were generated by adoptively transferring 4 x 10^6 total mixed BM cells from sIgM-deficient (CD45.2, 75%) and either C57BL/6 (WT; CD45.2), MyD88/TRIF double knockout (CD45.2), or TLR4/TLR2/Unc93b triple knockout (CD45.2) BM (25% each) into 5-6 week-old B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice, lethally irradiated by exposure to a gamma irradiation source 24 h prior to transfer. Chimeras were rested for at least 6 weeks before infection and analysis.

Infections, and immunizations. Mice were anesthetized with isoflurane and infected intranasally with a sublethal dose (10 PFU/ml) of influenza A/Puerto Rico/8/34 (A/PR8) in 40 μl volumes in PBS. Virus was grown in hen eggs and each virus batch was titrated for its effect on mice prior to use. Specifically, sublethal infection doses were chosen that incurred no more than 20% weight loss. For immunizations, mice were inoculated subcutaneously with 1x10^7 PFU A/PR8 in a 50:50 alum to PBS mixture.

Adoptive serum transfer for passive protection. Indicated strains of mice were infected with 10 PFU A/PR8. Blood from terminally anesthetized mice at 10 dpi was collected via cardiac puncture and spun down for serum separation. Serum from each strain was pooled and naïve C57BL/6 mice were subsequently injected i.v. with a mixture of 50μl pooled serum and 150μl 1x

PBS. These mice were then inoculated i.n. with 100 PFU A/PR8 one day later and measured for weight loss.

Flow cytometry. Single-cell suspensions from mediastinal lymph nodes (medLN) were made and labeled for phenotyping. Briefly, after Fc receptor block with anti-CD16/32 (5 mg/ml for 20 min on ice), cells were stained with the following antibody-fluorophore conjugates at temperatures and times according to manufacturer/provider: HA-PE and HA-APC oligomers (kindly provided by Dr. Frances Lund, UAB), BV786 anti-CD19 (1D3) (BD Bioscience), APC-eFluor780 anti-CD45R (RA3-6B2), PE-Dazzle 594 anti-CD38 (90) (both Thermo Fisher), BV711 anti-CD24 (M1/69), BV605 anti-CD138 (281-2) (both Biolegend), eFluor450 anti-GL-7 (GL7), PE or PE/Cy7 anti-IRF4 (3E4), PerCP-eFluor710 anti-IRF8 (V3GYWCH), eFluor450 anti-Ki67 (SolA15) (all Thermo Fisher), FITC anti-IgM (331, in-house), and BV650 anti-IgD (11-26c.2a) (Biolegend). For a non-B cell "dump", the following antibodies on AlexaFluor 700 were used: anti-CD90.2, anti-CD4, anti-CD8a, anti-Gr-1, anti-CD11b, anti-NK1.1, anti-F4/80 (all Thermo Fisher). The Foxp3 Staining Buffer Set (Thermo Fisher) was used for fixation and permeablization of cells for staining of transcription factors according to manufacturer's protocol. For cytoplasmic only staining, Cytofix/cytoperm buffer set (BD Biosciences) was used according to manufacturer's protocol. B cells from 7 dpi medLN were sorted by flow cytometry for ELISPOT using pooled antibodies for dump channel, anti-CD19, anti-CD45R, anti-CD24, and anti-CD38. Purity of sorted cells was assessed immediately afterwards (>96%).

ELISA. Influenza binding serum IgM, IgG, IgG1, IgG2a, IgG2c, and IgG3 concentrations and avidity were measured. Briefly, ELISA plates were coated with 400 HAU of purified A/PR8 virus overnight. Following a 1h RT incubation with blocking buffer, two-fold serially diluted samples in PBS were incubated for 2h at RT. Binding was revealed with goat anti-mouse IgM and IgG, IgG1, IgG2a, IgG2c, and IgG3 biotin (SouthernBiotech) and with SA-HRP (Vector

Laboratories) incubated each for 1h. The avidity index was determined by incubating sample replicates in the presence or absence of a 5 M urea wash following serum Ab incubation.

ELISPOT. A/PR8-specific Ig-secreting cells were measured. Briefly, ELISPOT plates were coated with 500 HAU of purified A/PR8 overnight, then blocked for non-specific binding for 1 hour. Serial dilutions of FACS-sorted EF PBs and pooled non-EF B cells were incubated overnight at 37℃. Ab-secreting cells (ASC) were revealed with goat anti-mouse IgM, IgG-biotin (Southern Biotech) followed by SA-HRP (Vector Laboratories) and 3-amino-9-ethylcarbazole (Sigma-Aldrich).

Viral-load qRT-PCR. Infected mice were euthanized, and lung tissue was extracted and homogenized using Gentle Macs (Miltenyi) in 1 ml PBS. Tissue was pelleted and supernatant was aliquoted and frozen. Viral RNA was purified from aliquots using the QIAamp viral RNA mini-kit (Qiagen). Presence of influenza was detected through amplification of influenza M gene using rtPCR. Primers used were AM-151 (5'-CATGCAATGGCTAAAGACAAGACC-3') and AM-397 (5'-AAGTGCACCAGCAGAATAACTGAG-3') and primer/probe AM-245 (6FAM-5'-CTGCAGCGTAGAGCTTTGTCCAAAATG-3'-TAMRA). Reverse transcription and amplication were done using TaqPath Multiplex Master Mix (Thermo Fisher). Samples were quantified to a standard of A/PR8 virus stock.

Statistical analysis. All data were analyzed using Graphpad Prism software. Quantified data are represented as mean with 95% confidence interval error bars. P-values indicated in figure legends.

Results

EFRs produce Ag-specific antibodies and are generated after intranasal influenza infection but not subcutaneous immunization

Intranasal infection of C57BL/6 mice resulted in the appearance of pre-GC/GC-like (GC) B cells (CD45Rhi/CD19hi/CD38lo/CD24hi) at 7 days post infection (dpi) that were also interferon regulatory factor 8 (IRF8) high, a transcription factor associated with GC polarization (10) (**Fig. 2.1a, top**). These cells also co-stained for the GC marker GL-7 (not shown). Early-formed plasmablasts from EFRs (EF PBs) were identified as CD45Rlo/CD19+/CD38lo/CD24+ as well as IRF4-high, associated with an ASC fate (5,10), with many also expressing CD138 (**Fig. 2.1a, bottom**), a canonical marker of ASCs. EF PBs and GC B cells had both lost surface expression of IgD and most cells had lost IgM by 7 dpi (**Fig. 2.1b**), indicating high levels of class-switching. While B cell frequencies in the medLN remained relatively constant throughout the timecourse **(Fig. 2.1c)**, drastic changes in both the EF and GC compartments took place. Relatively few GC B cells could be found until after 9 dpi (**Fig. 2.1d**), while EF PBs were seen as early as 5 dpi and peaked on 9 dpi, before contracting by 14 dpi (**Fig. 2.1e**). Only EF PBs, purified by flow cytometry, secreted pathogen-specific antibodies at 7 dpi, detected as influenza-bound total Ig and IgG2c by ELISPOT on cells (**Fig. 2.1g**), demonstrating that EF PBs contain the only functional, influenza-specific ASCs in the medLN at this timepoint. Use of two distinct fluorophore-labeled, recombinant hemagglutinin (HA) of A/PR8 identified similar kinetics of HA-specific (HA) B cells to the total EF B cell responses (**Fig. 2.1h-k**). HA B cells comprised as much as 15% of the entire EFR compartment at the early time points. The independence of EFR formation from GCs during influenza infection, which has been alluded to previously (11), was confirmed with the presence of EF B cells in infected Mb-1-Cre Bcl6 f/f mice that are unable to form GCs **(Fig. 2.2)**. Thus, EFRs are responsible for earliest Ag-specific antibody response to influenza infection and are independent of GCs.

A different quality of B cell response was seen after subcutaneous immunization with influenza virions in alum adjuvant. Compared to infection, immunizations yielded smaller GCs and little to no EFRs (**Fig. 2.3a**) in draining LN at 3, 7 and 10 days post-immunization (**Fig. 2.3b**). GCs were only 3x smaller but EFRs were barely detectable, at least 40x smaller after immunization (**Fig. 2.3c**). Consequently, fewer HA-specific B cells were detected over the course of immunization compared to infection (**Fig. 2.3d,e**). The HA-specific B cells that were present expressed no CD138 and only a few expressed Ki67 (**Fig. 2.3d,e**), a marker of cell cycling. Increasing the virus Ag-dose for immunization increased GC B cell numbers but had no effect on the size of EFRs (**Fig. 2.4**). We conclude that infection-induced signals are required for strong EFRs to influenza.

Ablation of total TLR signaling drastically alters EFR kinetics to influenza

To identify the influenza infection-induced signals that support EFRs, we first considered inflammatory cytokines that were previously identified as contributing towards B cell differentiation and ASC maintenance, as well as S100A9, a damage-associated molecular pattern protein produced by stressed and dying cells and released during influenza infection (12). Among the cytokines tested, IL-1, Type I interferons (IFN), IL-6, and TNFa are induced early after influenza infection (13-15) and support ASCs (16-18). IL-12 and the effector cytokine it supports, IFNg, which is produced by T cells, NK cells, and ILC1s, are known to support ASC maintenance (19,20). However, mice deficient in each of these soluble cytokines or their receptors showed EFRs similar to their wild type (WT) controls at 7 dpi (**Fig. 2.5a**). B cells are also importantly affected through innate signals received via Toll-like receptors (TLRs). Influenza pathogen-associated molecular patterns (PAMPs) activate endosomal TLR3 (21), TLR7 (22), while TLR4 has a role in infection-mediated pathology (23). However, infection of mice lacking individual TLR (TLR3, TLR4, and TLR7) had no significant effects on the number

of total EF PBs and CD138+ EF PBs compared to their WT controls (**Fig. 2.5b**). Thus, individual cytokines or innate signaling receptors appeared either not necessary or redundant in the regulation of EFRs.

The potential for redundancy of inflammatory signaling contributing to the regulation of EFRs was addressed with mice double- deficient for both TLR adaptors, TRIF (24) and MyD88 (DKO), which also transduce IL-1 and IL-18 signaling (25). Indeed, the DKO mice showed significantly reduced EFRs at 7 dpi (**Fig. 2.6a**) and DKO serum provided substantially reduced passive protection against lethal infection (**Fig. 2.6b**). Infection of another TLR-null model, through deletion of genes for TLR2 (26), TLR4 (27) and a missense mutation of Unc93b (28) (TKO), surprisingly showed EFRs similar to WT controls (**Fig. 2.6a**) and fully protective serum against lethal infection (**Fig. 2.7a**), despite slight but significant reductions in CD138+ EF PBs at 7 dpi (**Fig. 2.6a**). This indicated divergent roles for MyD88/TRIF and upstream TLR signaling in regulation of EFRs.

To tease out the source of these regulatory differences, mixed bone marrow irradiation chimeras, in which only B cells lacked either MyD88 and TRIF (DKO BMC) or all TLRs (TKO BMC), were infected with influenza and analyzed at 7 dpi (**Fig. 2.6c**). Both DKO and TKO BMCs showed reduced EF and GC responses compared to WT chimera controls (**Fig. 2.6d**), demonstrating similar B cell intrinsic roles for both MyD88/TRIF and upstream TLR signaling in B cell responses overall, but distinct B cell extrinsic mechanisms from each that lead to kinetic and functional differences, specifically in EFRs. Indeed, global TLR knockout mice had increased viral loads compared to WT controls at the same timepoint (**Fig. 2.7b**), but all chimeras had similar viral loads at the same timepoint (**Fig. 27c**). The lack of viral control in the global knockouts correlated with the appearance of significantly greater, delayed EFRs in the knockouts as well (**Fig. 2.7d**). Therefore, lack of TLR signaling leads to intrinsic defects in EFRs but can be overcome by extrinsic factors related to delayed virus clearance.

Finally, the anti-influenza serum antibody composition, concentration, and overall avidity was addressed. Surprisingly, both DKO and TKO mice had higher average levels of total, influenza-specific serum IgG at 10 dpi compared to WT **(Fig. 2.8a, middle top)**. This was attributed to significant increases in IgG1 **(Fig. 2.8a, lower left)**. However, the overall avidity of DKO and TKO IgG1 was significantly lower than WT **(Fig. 2.8b, lower left)**, as was IgG3 **(Fig 2.8b, lower right)**, indicating that lack of TLRs led to changes in the anti-influenza IgG profile as well as reductions in IgG1 avidity.

Discussion

These data provide much needed insight into the generation, timing, and functionality of EFRs after acute respiratory tract infection. The data show that influenza strongly induces EFRs that contain Ag-specific plasmablasts, while s.c. immunization leads to minimal expansion and/or maintenance of these effectors. Thus, influenza infection provides signals for initiation and maintenance of EFRs that immunization does not supply.

The question then is what are these signals? Are they discrete pathways that specifically promote B cell expansion and differentiation in an extrinsic or intrinsic manner? Or is it more holistic, where the upregulation of any of several pathways can lead to a proper milieu for the development of extrafollicular niches? It appears that the answer is both. While knockout of many individual pathways that are associated with inflammation and/or antibody responses, including individual TLRs, failed to significantly change early EFR generation, the ablation of all TLR signaling severely delayed EFRs, wherein the magnitude of DKO and TKO EFRs were similar to WT by 10 dpi, but reduced at 7 dpi. This indicates that there is a scale of inflammatory signals even in the presumed presence of Ag surplus. When inflammation is low, as following immunizations, EFRs are minimal. As the inflammation is ramped up as seen in infection, so is

the magnitude and speed of EFR generation. It is likely that, in TLR-null mice, the lack of signals is compensated by a medley of alternative inflammatory pathways, leading to a latent development of EFRs rather than a complete abrogation. Indeed, inflammatory STAT pathways such as IL-4 (29), IL-21, and IFNγ are all present after influenza with IL-21 and IFNγ especially important in developing protective antibodies in the absence of GC development (11). Thus, these inflammatory pathways may take over during the absence of TLR signaling in B cells to promote EFR initiation and expansion. However, unlike the model for Ag affinity, there is a ceiling to the benefits of these early inflammatory stimuli, as other infection models have demonstrated that too much inflammation caused by high, persistent pathogen burden results in a suppression of Ag-specific antibody responses (6,7). Therefore, while TLRs mediate the optimal development of EFRs, the “goldilocks” level of inflammation can be achieved eventually, perhaps through a collective of alternative pathways. Modulating the level or type of inflammation thus may lead to enhancements in protective antibody generation that would either reduce tissue damage during infection or improve the time lag between vaccinations and levels of protective titers.

As there were several knockouts tested for EFR dysfunction, the data are limited by having only a single timepoint of comparison. There might be differences in kinetics of the EFR that we missed, as seen in the global TLR-null mice. Furthermore, while the overall magnitude of EFRs was compared, differences in serum antibody levels and repertoire of anti-influenza antibody of these single knockouts were not measured in these studies. It would be of interest to know if specific inflammatory signatures in the medLN lead to changes not only in Ig isotype, but the clonality and affinity of the anti-influenza repertoire as well. Indeed, DKO mice, which had significantly fewer passively protective antibodies, had actually higher average anti-influenza serum IgG concentrations, especially IgG1. However, TKO mice showed a similar profile in anti-influenza serum IgG profile and avidity as the DKO mice but passive protection with TKO serum

from 10 dpi animals was fully protective against lethal infection. Such inconsistencies between TLR-null models may be due to alterations in the anti-influenza antibody repertoire, where EFR-derived TKO antibodies have largely different target epitopes than EFR-derived DKO antibodies, which would result in differences of neutralization and protection. This would indicate that inflammatory stimuli, be it B cell intrinsic or extrinsic, influence more than the isotype and may dictate the profile of targeted epitopes through discrete or stochastic selection of the Ag-specific repertoire. As different signaling pathways lead to modulations in expression of the same target genes, i.e. genes potentially associated with B cell proliferation and differentiation, further work that explores the contribution of specific inflammatory signals on gene expression programs of EFR-derived, high-affinity ASCs would be highly informative.

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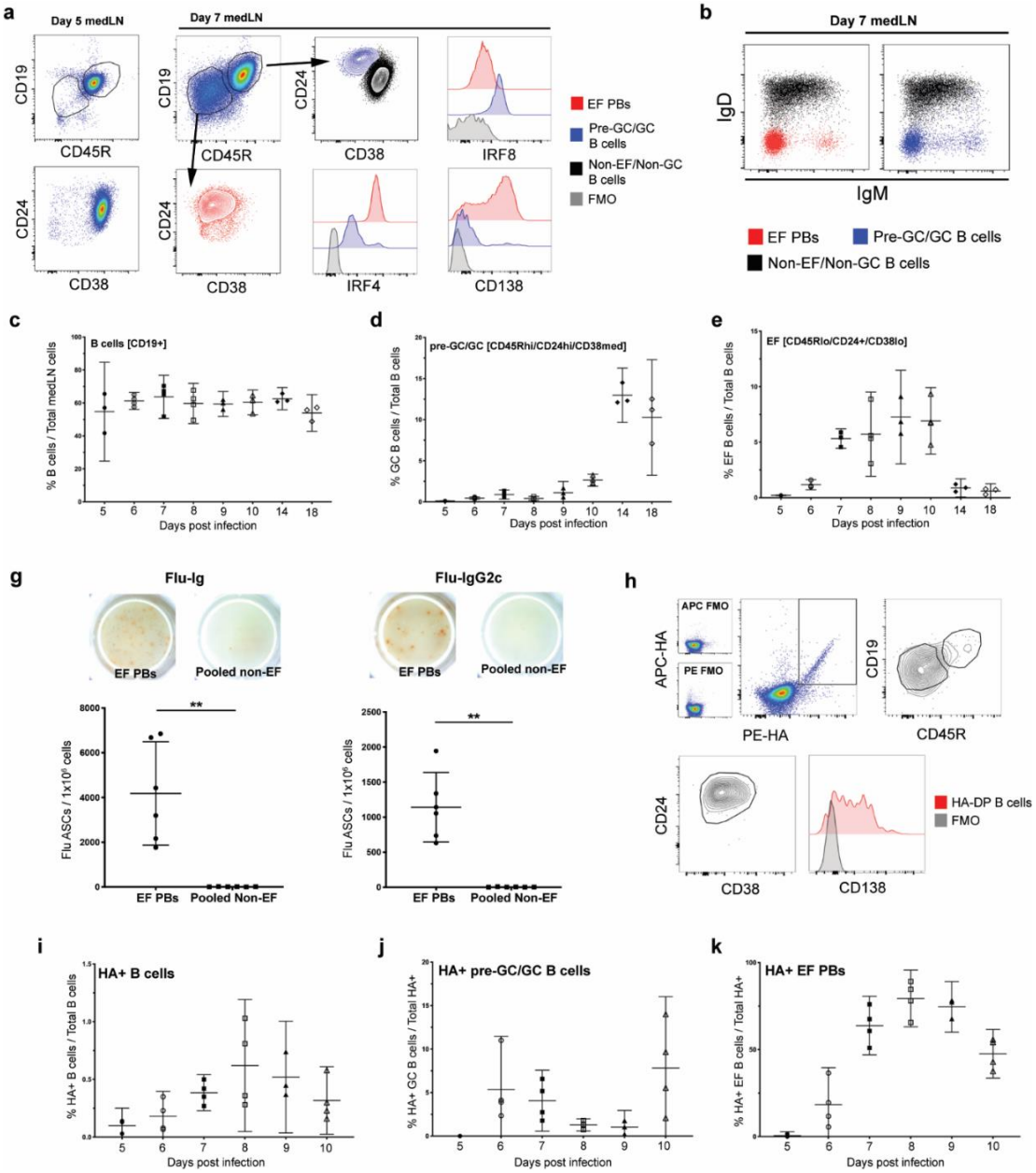
a
Day 5 medLN
Day 7 medLN
CD19
CD45R
CD24
CD38
IRF8
IRF4
CD138
EF PBs
Pre-GC/GC B cells
Non-EF/Non-GC B cells
FMO
b
Day 7 medLN
IgD
IgM
EF PBs
Pre-GC/GC B cells
Non-EF/Non-GC B cells
c
B cells [CD19+]
% B cells / Total medLN cells
Days post infection
d
pre-GC/GC [CD45Rhi/CD24hi/CD38med]
% GC B cells / Total B cells
Days post infection
e
EF [CD45RIo/CD24+/CD38Io]
% EF B cells / Total B cells
Days post infection
g
Flu-Ig
Flu-IgG2c
EF PBs
Pooled non-EF
Flu ASCs / 1x10^6 cells
**
h
APC FMO
PE FMO
APC-HA
PE-HA
CD19
CD45R
CD24
CD38
CD138
HA-DP B cells
FMO
i
HA+ B cells
% HA+ B cells / Total B cells
Days post infection
j
HA+ pre-GC/GC B cells
% HA+ GC B cells / Total HA+
Days post infection
k
HA+ EF PBs
% HA+ EF B cells / Total HA+
Days post infection

Figure 2.1. Primary influenza infection induces strong early EFRs prior to GC formation. Shown are flow cytometric analyses of mediastinal lymph nodes (medLN) from C57BL/6 mice infected with influenza A/PR8 intra-nasally (i.n.) at seven days post-infection (dpi). (**a)** Identification of extrafollicular plasmablasts (EF PBs) and pre-GC/GC B cells by flow cytometry. (**b)** IgM and IgD expression on EF PBs, pre-GC/GC B cells, and non-EF/non-GC B cells. **(c-f)** C57BL/6 mice were infected and medLN were collected on the days specified, measuring B cell frequencies of total cells **(c)**, pre-GC/GC frequency of B cells **(d)**, EF frequency of B cells **(e)**. (**g**) Influenza-specific ELISPOTS of sorted EF PBs and pooled non-EF cells for total Ig (left) and IgG2c (right). (**h**) Flow plots of HA-specific B cells using double HA-tetramer staining. (**i-k**) Time course of HA-specific B cell subsets during influenza infection as in (c-e), measuring frequency of HA-specific clones (**i**), HA-specific pre-GC/GC clones (**j**), and HA-specific EF PBs (**k**). Graphs are representative of two experiments (n>/=3). Error bars represent 95% confidence interval (CI), p-values determined by unpaired Student's t-test with Welch's correction. **: $p<0.01$

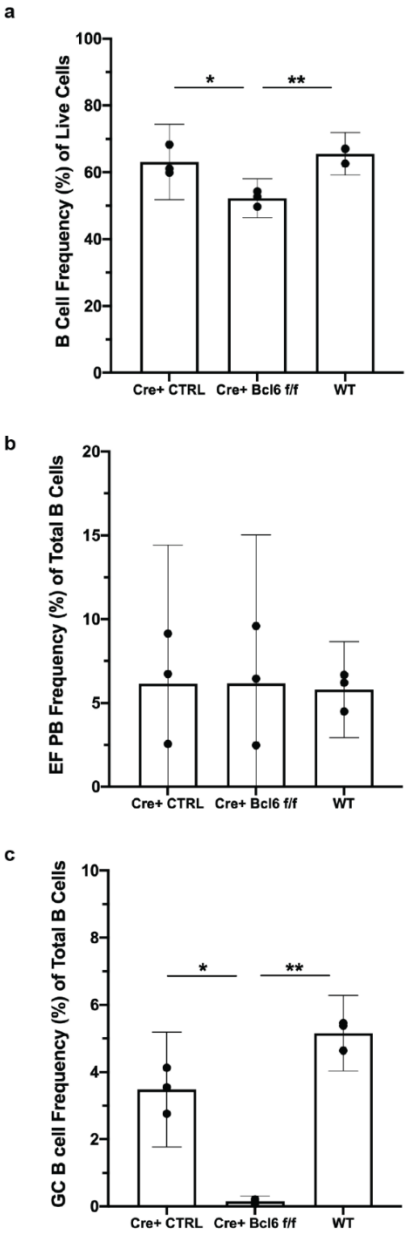

Figure 2.2. EFRs are not generated through GC reactions. WT, Mb-1 Cre+ control, and Mb-1 Cre+ Bcl6 f/f mice were infected with 10 PFU A/PR8 and medLNs were analyzed at 10 dpi. **(a)** B cell frequency of total cells. **(b)** EF PB frequency of total B cells. **(c)** GC B cell frequency of total B cells. Error bars represent 95% CI, p-values determined by unpaired Student's t-test with Welch's correction. *: $p<0.05$, **: $p<0.01$.

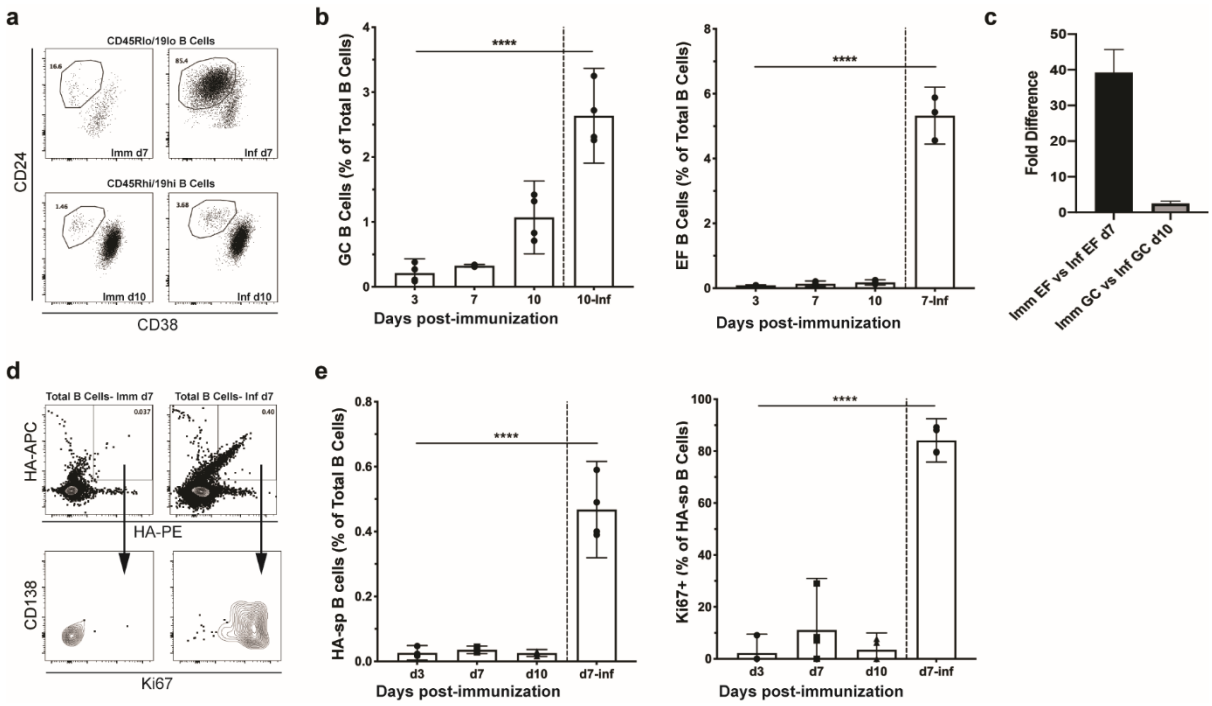

Figure 2.3. Subcutaneous immunization with influenza and alum does not elicit EFRs. (**a-e**) C57BL/6 mice were immunized s.c. with 1x 10^7 PFU influenza A/PR8 in alum and inguinal LNs were analyzed on days indicated. **(a)** Flow plots comparing immunization to infection EF and GC formation. **(b)** Kinetics of EF and pre-GC/GC B cells compared to infection. (**c**) Fold-difference of EF and GC responses compared to infection. **(d)** Flow plots comparing HA-specific B cell populations during immunization and infection. **(e)** Kinetics of total (left) and proliferating (right) HA-specific B cells compared to infection. Graphs are representative of two experiments (n=4). Error bars represent 95% CI, p-values determined by one-way ANOVA (b, e). ****: $p<0.0001$.

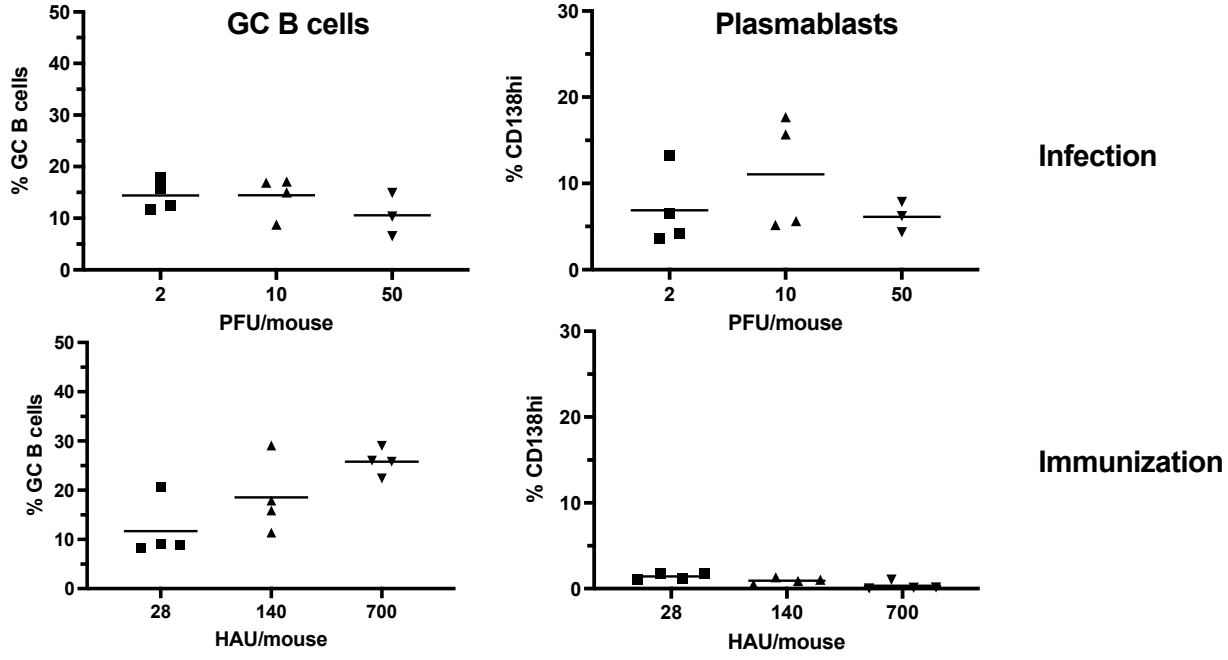

Figure 2.4. Antigen-dose increases germinal centers but not plasmablast responses. Scatter plots indicate frequencies of B220+ CD38+ CD24hi germinal center B cells (left) and B220lo CD138hi plasmablasts in draining lymph nodes of BALB/c mice (n=4 per group) either infected intranasally with indicated PFU influenza A/PR8 (top) or immunized s.c. with indicated HAU sucrose-gradient purified influenza A/PR8 virion emulsified in Complete Freund's adjuvant. Analysis was done on day 7 after infection/immunization. All data are frequencies of cells after gating for live, lymphocytes.

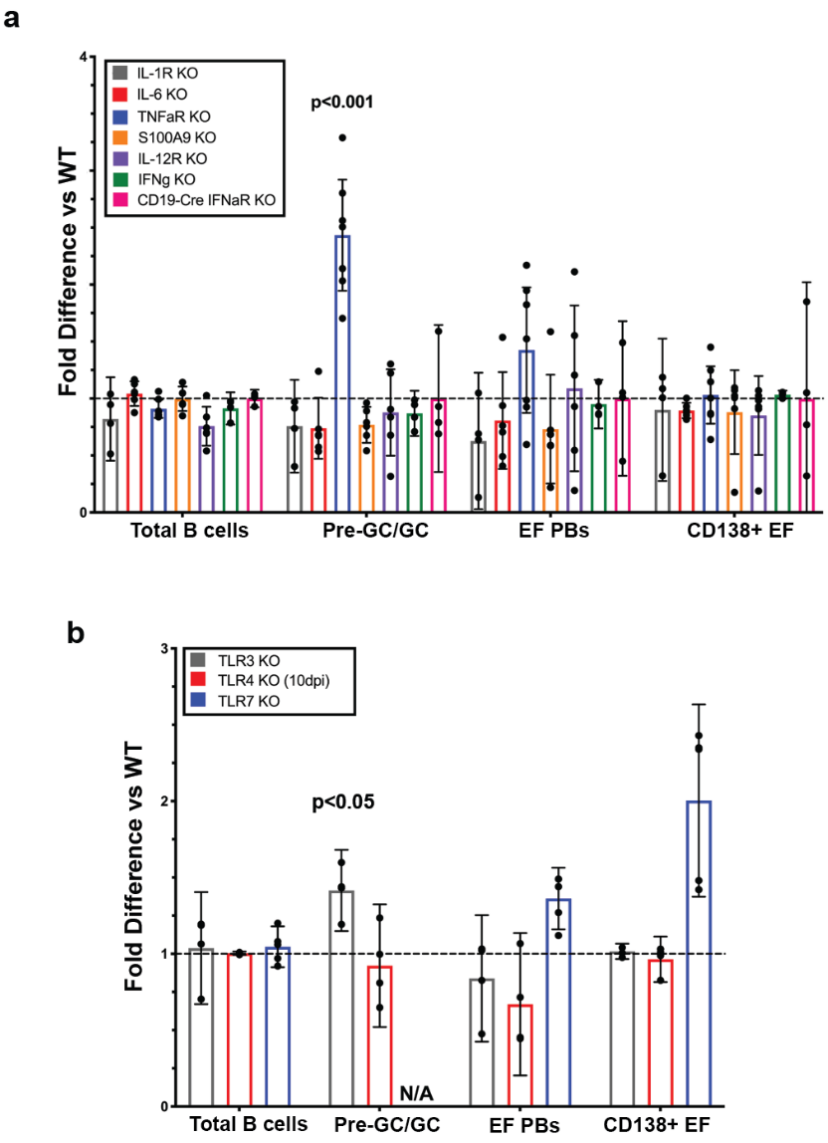

Figure 2.5. Lack of single cytokine or TLR pathway does not affect EFRs to influenza.

Knockout and WT mice were infected with 10 PFU A/PR8 and medLNs were collected at 7 days post-infection (dpi). Shown are fold-differences in major B cell subsets in C57BL/6 WT and indicated gene-targeted mice (**a**), as well as **(b)** single TLR-knockouts. Error bars represent 95% CI, Statistical significance determined by unpaired Student's t-test with Welch's correction. P-values indicated on charts.

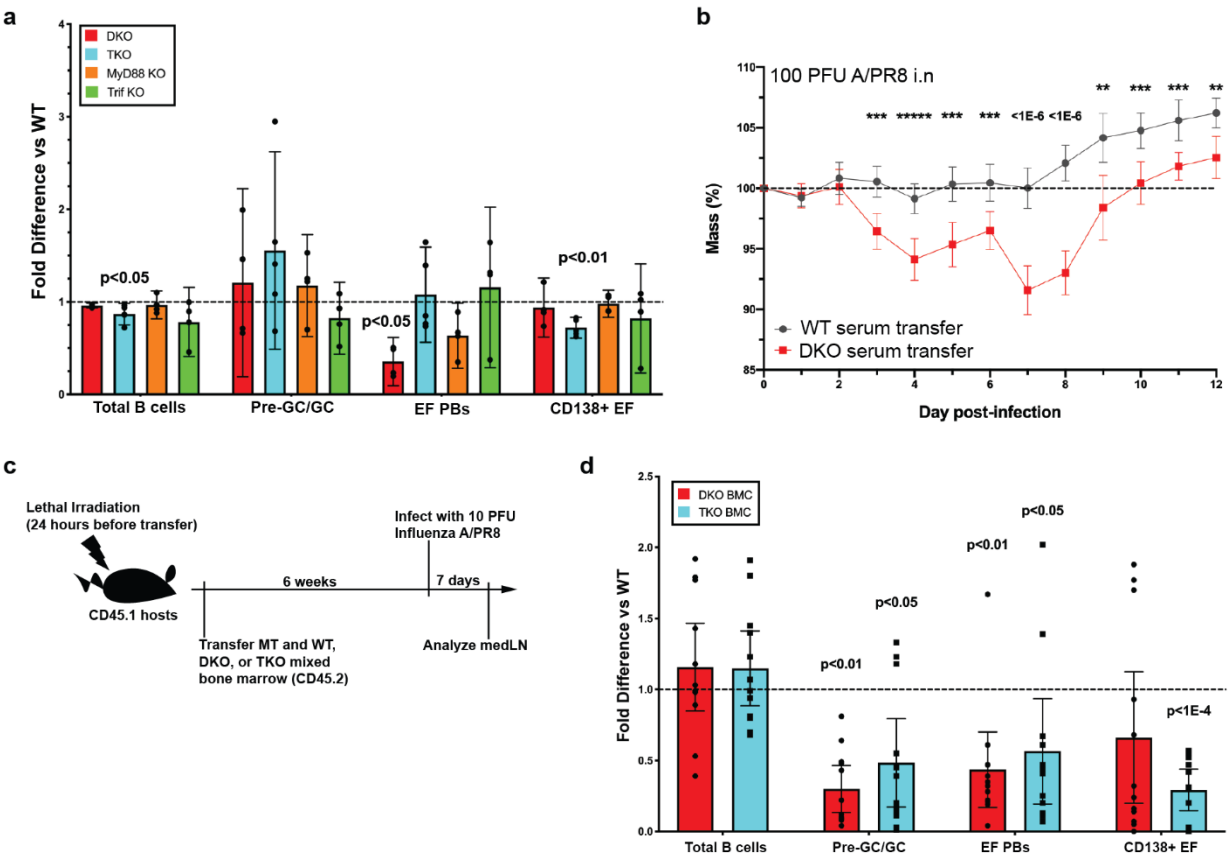

Figure 2.6. Optimal EFR kinetics and protective antibodies require MyD88 and TRIF. Knockout and WT mice were infected with 10 PFU A/PR8 and medLNs were collected at 7 days post-infection (dpi). **(a)** Fold-difference of B cell subsets in TLR-deficient versus WT mice at 7 dpi. **(b)** Serum from influenza-infected MyD88/TRIF-deficient (DKO) mice at 10 dpi was transferred to C57BL/6 mice prior to infection with a lethal dose (100 PFU) of influenza A/PR8 the next day. Shown is % change in weight over the course of infection. **(c)**. Mixed bone-marrow chimeras (BMC) established with irradiated CD45.1 C57BL/6 host mice reconstituted with µMT donor BM and BM from either DKO or TKO, then infected with 10 PFU A/PR8 6 weeks later. **(d)** Quantification of DKO and TKO BMC compared to WT BMC controls of B cell subsets at 7 dpi. Graphs are representative of two or more experiments (n=10 **(b)**), all else n>/=3). Error bars represent 95% CI. Statistical significance determined by unpaired Student's t-test with Welch's correction. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001 or indicated in subfigures.

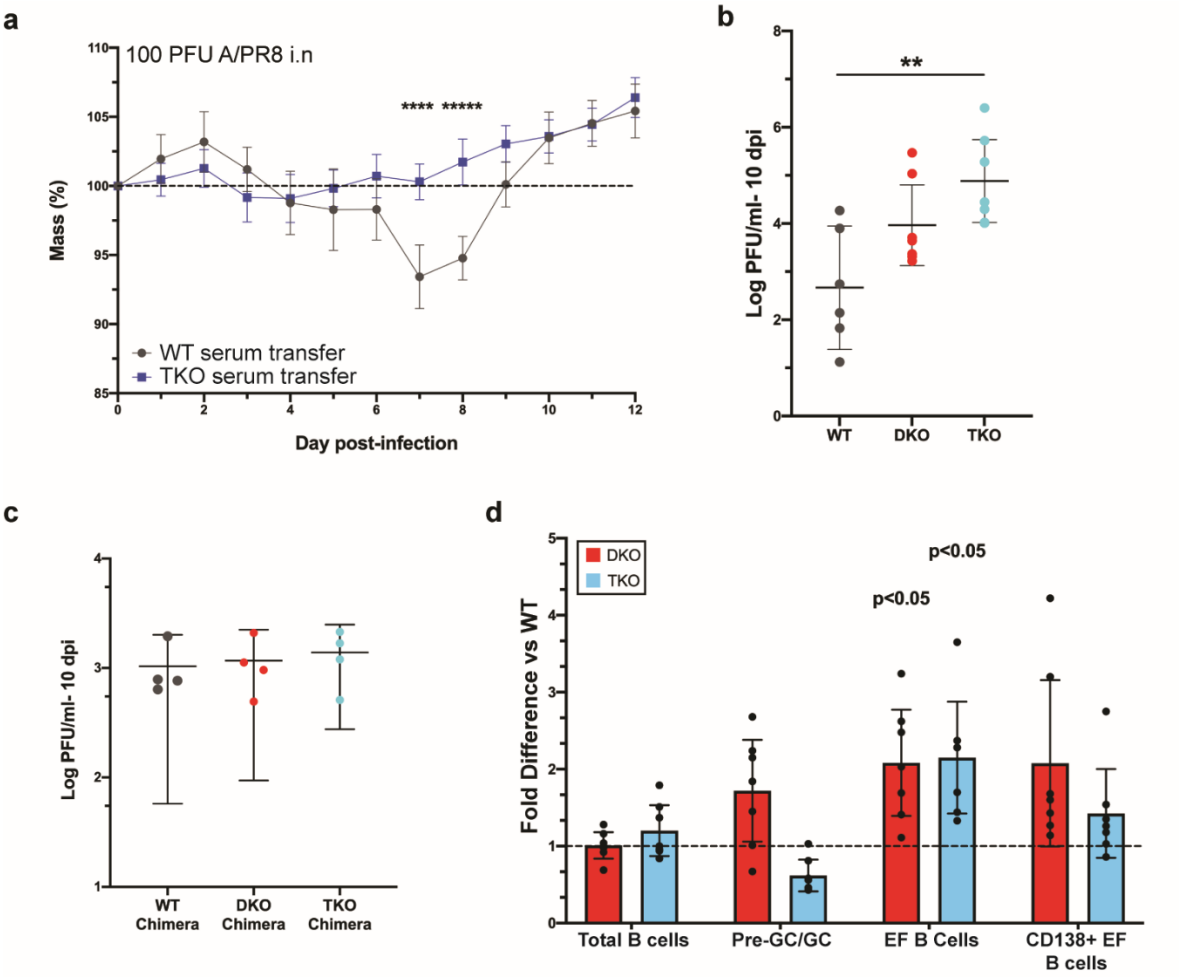

Figure 2.7. **Passive protection with TKO serum and late-stage viral loads and EF responses.** (**a**) Shown are the weight loss curves of C57BL/6 mice injected with serum from age-matched, influenza A/PR8-infected WT and TKO mice taken at 10 dpi and then challenged with a lethal dose (100 PFU) of influenza A/PR8 the next day. **(b)** Viral loads as analyzed by viral qRt-PCR on lung homogenates at 10 dpi for global knockouts (left) and chimeras (right). **(c)** Fold-differences of B cell subsets in DKO, TKO and WT mice at 7 dpi. Graphs are representative of two experiments (n=10 **(a)**, n>4 **(b,c)**). Error bars represent 95% CI. Statistical significance determined by one-way ANOVA (b) and unpaired Student's t-test with Welch's correction. *: p<0.05 (or shown) **: p<0.01 ***: p<0.001, ****: p<0.0001. *****" p>1E-5.

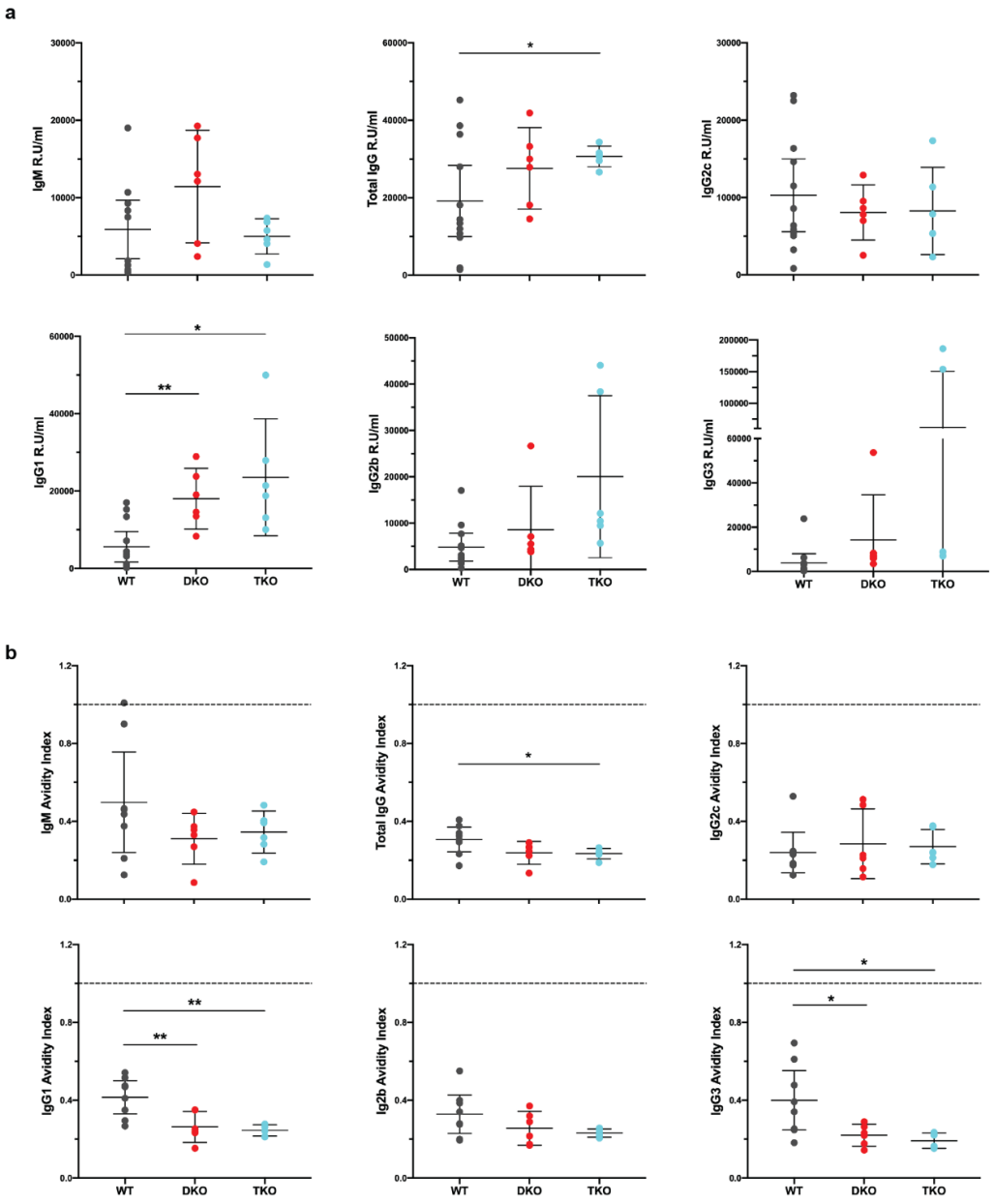

Figure 2.8. Differences between WT, global DKO, and global TKO anti-influenza serum antibodies at 10 dpi. Mice were infected as described previously, and serum was analyzed by ELISA. **(a)** Relative concentrations of different anti-influenza serum antibody isotypes. **(b)** Relative avidity of different anti-influenza serum antibody isotypes. Error bars represent 95% CI. Statistical significance determined by one-way ANOVA and unpaired Student's T-test with Welch's correction. *: $p<0.05$ **: $p<0.01$.

Chapter 3

Toll-like receptor signaling is an intrinsic requirement for antigen-mediated B cell survival and proliferation

Jonathan Lam

Chapter adapted from: Lam, Jonathan and Baumgarth, Nicole. Toll-like receptor signaling directs B cells towards protective extrafollicular responses during infection and immunization. In preparation.

Abstract

B cells express similar innate receptors to myeloid-derived professional antigen-presenting cells (APCs) along with bespoke antigen receptors. While the contribution of major innate receptors in guiding the function of professional APCs is well characterized, the dynamics between innate and adaptive signaling within the B cell, and how that may affect B cell fate and subsequent antibody responses, are less clear. Data presented here demonstrate that TLR signaling enhances antigen-mediated B cell activation *in vitro* primarily through increased proliferation along with concomitant upregulation of IRF4, a transcription factor required for differentiation of B cells into a plasma cell. Most profoundly, a complete lack of TLR signaling, either through MyD88/TRIF (DKO) or TLR2/4/Unc93b (TKO) ablation, led to severe defects in anti-IgM-mediated activation even in the absence of explicit TLR ligation. Specifically, DKO and TKO B cells showed reduced viability and completely failed to proliferate after B cell receptor (BCR) stimulation in an anti-IgM dose-dependent manner, both in the presence or absence of BAFF and CD40L. While BCR signaling in TLR-null B cells appeared normal when measuring calcium flux and activation of various downstream signaling pathways, they were resistant to downregulation of surface IgD. Furthermore, TLR-null B cells failed to upregulate IRF4 following IgM-BCR stimulation, which correlated with their dysfunctional activation and a failure to induce NF-kB factor c-Rel nuclear localization, which controls IRF4 expression. Thus, the TLR axis directly supports priming of B cells, and it modulates the outcome of IgM-BCR-mediated stimulation by co-opting the TLR:IRF4 circuit to enhance proliferation and differentiation after exposure to cognate antigen.

Introduction

Many studies have aimed to dissect the integration of B cell receptor (BCR) signaling with canonically distinct pathways such as toll-like receptors (TLRs). Lipopolysaccharide (LPS)-induced activation of B cells via the TLR and IL-1R adaptor, MyD88, was shown to require the presence of a BCR. Furthermore TLR-stimulation was shown to directly phosphorylate Syk, a major downstream effector of BCR signaling (1). The BCR was also necessary for TLR9-mediated B cell activation (2), indicating that the BCR may act as a platform for integrating innate signaling pathways. Furthermore, many types of diffuse-large B cell lymphomas (DLBCLs) carry an activating mutation in MyD88, but interestingly not the germinal center-like B cell (GCB) subgroup (3). The mutation is associated with upregulated gene signatures for survival and proliferation, specifically NF-kB, Myc, and interferon regulatory factor 4 (IRF4) (3), whose high expression is associated with differentiation into antibody-secreting cells (ASCs) upon BCR or CD40 stimulation in an antigen dose-dependent manner (4). TLRs have also been shown to synergize with BCR stimulation in the context of autoimmunity, where self-antigen complexes are recognized by both the BCR and either TLR7 (5) or TLR9 (6) upon endosomal uptake. This presumably activates MyD88, leading to similar gene expression profiles seen in the MyD88-mutant DLBCLs, resulting in survival and proliferation of, in this case, antigen-specific clones. The role of the other TLR signaling adaptor, TRIF, which is downstream of both TLR3 and TLR4 (7), in enhancement of or integration with the BCR signalsome, is unknown and therefore an interesting subject for exploration.

Influenza infection activates multiple TLRs (8,9,10), allowing for BCR-TLR synergism as seen in both cancerous and autoimmune BCR signaling. This precipitates the possibility that extrafollicular B cell responses (EFRs) are the result of a highly regulated crosstalk between activated adaptive and innate signaling. Lacking input from either presumably would lead to an abrogation of EF foci formation. Indeed, EFRs are known to be highly proliferative, but short-

lived (11). This may be due to lack of antigen (BCR activation) and/or innate stimuli (TLR activation) as infections resolve. Further investigation is required into how each affects EFR generation, maintenance, and contraction. Here I assessed the role of TLRs in B cell activation in the context of anti-IgM mediated stimulation and analyzed subsequent downstream signaling events and gene expression.

Materials & Methods

Mice. Male and female 8- to 12-wk-old C57BL/6 (WT; CD45.2), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), and B cell–deficient (μMT) mice were commercially obtained (The Jackson Laboratories). Breeding pairs of MyD88/TRIF DKO and TLR2/4/unc93b TKO mouse strains were gifts from Dr. Barton (UC Berkeley). Mice were bred and held under SPF housing conditions with ad libitum access to food and water. All experiments conducted were approved by the UC Davis Institutional Animal Care and Use Committee.

Mixed bone marrow (BM) chimeras were generated by adoptively transferring 4 x 10^6 total mixed BM cells from sIgM-deficient (CD45.2, 75%) and either C57BL/6 (WT; CD45.2), MyD88/TRIF double knockout (CD45.2), or TLR4/TLR2/Unc93b triple knockout (CD45.2) BM (25% each) into 5-6 week-old B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice, lethally irradiated by exposure to a gamma irradiation source 24 h prior to transfer. Chimeras were rested for at least 6 weeks before infection and analysis.

Infections. Mice were anesthetized with isoflurane and infected intranasally with a sublethal dose (10 PFU/ml) of influenza A/Puerto Rico/8/34 (A/PR8) in 40 μl volumes in PBS. Virus was grown in hen eggs and each virus batch was titrated for its effect on mice prior to use. Specifically, sublethal infection doses were chosen that incurred no more than 20% weight loss.

Magnetic B cell enrichment. Splenic B cells were treated with Fc Block (anti-mouse CD16/32, clone 2.4.G2) and were then enriched using a mixture of biotinylated Abs (anti-

CD90.2 (30-H12), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-Gr-1 (RB6-8C5), anti-CD11b (M1/70), anti-NK1.1 (PK136), anti-F4/80 (BM8), anti-CD5 (53-7.3), anti-CD9 (MZ3), anti-CD138 (281-2) and anti-biotin MicroBeads (Miltenyi Biotec). Nylon-filtered, stained splenocytes were separated using autoMACS (Miltenyi Biotec). Purities of enriched mouse B cells were >98% as determined by subsequent FACS analysis.

B cell cultures. Magnetically enriched B cells were cultured at 5 x 10^6 cells/ml at 37℃. Cells were incubated with anti-IgM $(Fab)_2$ and/or LPS in culture media at the indicated concentrations for 30 minutes, one, two, and three hours. Three-hour anti-IgM pulsed B cells were washed twice with PBS, and then cultured in culture media containing 200 ng/mlCD40L (Peprotech) and 5 ng/ml BAFF (R&D Systems) in 96-well round-bottom plates for 48 hours at 5% CO_2.

Flow cytometry and phosphor-flow. Flow cytometric analysis was done using Fixable Aqua, PE anti-c-Rel (1RELAH5) (both Thermo Fisher), BV786 anti-CD19, eFluor450 anti-Ki67, PE/Cy7 anti-IRF4, PerCP-eFluor710 anti-IRF8 and APC anti-IL-21R (4A9) (all eBioscience). The Foxp3 Staining Buffer Set (Thermo Fisher) was used for fixation and permeabilization of cells for staining of transcription factors according to manufacturer's protocol. For cytoplasmic only staining, Cytofix/cytoperm buffer set (BD Biosciences) was used according to manufacturer's protocol. For phospho-flow, APC anti-p-Syk (moch1ct), PerCP-eFluor710 anti-p-p38 (4NIT4KK), PE/Cy7 anti-p-mTOR (MRRBY), and PE anti-p-p65 (B33B4WP) were stained according to manufacturer's protocol (Thermo Fisher).

Nuclear fraction ELISA. c-Rel nuclear localization was measured. Briefly nuclear, and cytoplasmic protein fractions were extracted from cultured, purified B cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher) according to manufacturer's protocol. ELISA plates were coated at 4 μg/ml dilution of polyclonal anti-c-Rel (Thermo Fisher) overnight,

then blocked for non-specific binding for 1 hour. Bound c-Rel was detected using 4 µg/ml monoclonal anti-c-Rel (1RELAH5). Binding was revealed by SA-HRP (Vector Laboratories).

Calcium flux assay. To measure changes in cellular calcium concentrations, B cells were stained with 2µM cell-permeant Fluor-3 and 4µM FuraRed (both Thermo Fisher) according to manufacturer's protocol and stimulated with 10 µg/ml anti-IgM(fab)$_2$ fragments prior to analysis by flow cytometry. The ratio of the calcium-excitable (Fluor3) and calcium-quenched (FuraRed) dyes were calculated to determine free-intracellular concentrations.

Statistical analysis. All data were analyzed using Graphpad Prism software. Quantified data are represented as mean with 95% confidence interval error bars. P-values indicated in figure legends. A p-value of < 0.05 was considered statistically significant.

Results

TLR stimulation enhances BCR-mediated activation in B cells

It has previously been demonstrated that co-activation of the IgM-BCR and TLR4 enhances the activation of NF-kB and induces activation-induced deaminase (AID) (12). The latter is necessary for the formation of class-switched antibody secreting cells (ASCs), which are present in EFRs after influenza infection (**Fig. 2.1**). To further dissect the intrinsic effect of TLR activation on B cell physiology, negatively-enriched, naïve, follicular B cells were cultured with graded doses of anti-IgM(Fab)$_2$ (pulsed for 3h) and the TLR4 agonist LPS, which activates both MyD88 and TRIF. Anti-IgM with LPS co-treatment showed a modest additive effect on viability compared to LPS stimulation alone (**Fig 3.1a**) and a more profound effect on proliferation. LPS plus anti-IgM greatly increased expression of Ki67 compared to either stimulus alone at equivalent doses (**Fig. 3.1b**). Co-stimulation of anti-IgM with LPS also showed strong induction of IRF4 and IRF8, critical transcriptional regulators of the B cell fate (**Fig. 3.1c**), while anti-IgM enhanced and LPS inhibited induction of IL21R expression, a cytokine receptor required for the

generation of ASCs (13) (**Fig. 3.1d**). Taken together, TLR stimulation alone greatly promoted B cell survival. It also enhanced BCR-induced entry into the cell cycle and potentiated B cell differentiation.

B cells require the presence of a functional TLR signaling axis for BCR-mediated proliferation

As stated previously, a growing body of research has demonstrated integration of canonical TLR components with the BCR (1) and with TNF superfamily receptors (14), suggesting TLR signaling-deficient B cells may be altered in their response to signals induced via the BCR, or via co-stimulation through CD40 or BAFFR. Indeed, stimulation of naïve, follicular MyD88/TRIF double knockout (DKO) and TLR2/4/Unc93b-ablated (TKO) B cells pulsed with graded doses anti-IgM$(Fab)_2$ for three hours, followed by incubation with CD40L and BAFF for 48 hours (**Fig. 3.2a**), showed reduced viability in an anti-IgM dose-dependent manner (**Fig. 3.2b, c**). and a near inability to enter the cell cycle as measured by Ki67 staining (**Fig. 3.2d**) compared to wild type (WT)-derived cells. MyD88 and TRIF single KO B cells showed similarly reduced survival (**Fig. 3.3a**) and proliferation (**Fig. 3.3b**) under the same culture conditions with frequencies approximately half those of WT B cells. The data indicated that MyD88 and TRIF support BCR-mediated activation signals in a non-redundant, additive manner. Overall similar results were obtained with BCR-stimulation alone (**Fig 3.3c, d**). Consistent with these data, non-EF/GC B cells isolated from draining lymph nodes of influenza infected DKO and TKO B cell chimeras showed significantly reduced expression of Ki67 compared to controls (**Fig. 3.2f**) at 5 days post infection (dpi), a timepoint at which IRF4 levels are at their highest within this B cell population (not shown). The few HA-specific clones present in DKO and TKO chimeras at this timepoint also showed reduced Ki67 expression (not shown). Thus, lack of TLRs or their adaptors in B cells significantly reduces B cell survival and cell cycle entry early after influenza infection.

Lack of TLRs does not negatively affect immediate activation events downstream of the BCR

To determine which BCR-induced signaling pathways might be affected by the intrinsic lack of TLRs or adaptor molecules, early signaling events after BCR stimulation were assessed. No significant defects were observed in induction of intracellular calcium following BCR stimulation in either DKO or TKO B cells (**Fig. 3.4a**). Phosphorylation of effector proteins downstream of BCR activation did not show significant differences prior to stimulation compared to WT controls (**Fig. 3.4b**) and activation of mitogenic pathway MAPK p38 (15) and the pro-inflammatory NFkB1 (16) were roughly similar after 30-minute BCR stimulation (**Fig. 3.4c upper left, lower right**). Surprisingly, there were dose-dependent increases both in pSyk (**Fig. 3.4c, upper right**), a major activation node for several BCR-mediated signaling pathways (17), and the pro-growth regulator mTOR (18) in both TLR-signaling deficient B cells compared to WT controls (**Fig. 3.4c, lower left**). Together, these data indicate that early BCR-mediated signal transduction was either comparable or even increased in B cells lacking TLR-signaling compared to WT.

Following IgM-BCR stimulation, activated B cells downregulate surface IgD, whose level of expression is associated with anergy (19). After observing higher IgD expression in TLR-null HA-specific B cells, IgD regulation was measured *in vitro*. IgM-BCR signaling-induced loss of surface IgD was strongly attenuated in DKO and TKO B cells relative to WT, which lost IgD expression in an anti-IgM dose-dependent manner (**Fig. 3.5a**). Along with the enhanced induction of cell death and inability to proliferate to antigen-mediated activation, despite apparently normal early downstream BCR signal transduction, the TLR-signaling deficient B cells resembled anergic B cells.

TLRs are required for optimal activation of the NF-kB c-Rel:IRF4 circuit in B cells after BCR stimulation

B cell activation initiates concomitant increases in IRF4 and IRF8 expression, followed by increased ratios of IRF4 to IRF8 for cells fates towards plasma cell differentiation, or the opposite for GC commitment (20). Non-differentiated B cells from DKO and TKO chimeras showed significantly lower expression of IRF4 and overall lower expression of IRF8 than WT at 5 dpi with influenza (**Fig. 3.5b**). HA-specific B cells showed a similar trend (not shown). *In vitro*, BCR stimulation caused an increase in IRF4 but not IRF8 expression in an anti-IgM dose-dependent manner in B cells from WT mice. Strikingly, B cells from both DKO and TKO mice failed to upregulate IRF4 in response to anti-IgM stimulation in the presence of CD40L and BAFF (**Fig. 3.5d, e**), while IRF8 expression remained comparable to WT (**Fig. 3.5d, f**). *Ex vivo* baseline levels of IRF4 in all strains were similar overall (**Fig. 3.5d**).

NF-kB c-Rel is known to promote IRF4 expression upon nuclear re-localization and is downstream of both BCR and TLR4 (21). Reductions in cytoplasmic c-Rel expression, which infers translocation to the nucleus, were seen in WT B cells by flow cytometry as early as 30 minutes after BCR stimulation in an anti-IgM dose-dependent manner, while these changes were significantly attenuated in TLR-signaling deficient B cells (**Fig. 3.5g**), independent of whether or not respective c-Rel baseline levels were altered (**Fig. 3.6a**). Consistent with these data, WT but neither DKO nor TKO B cells showed significant accumulation of c-Rel in the nucleus 1h after anti-IgM and LPS stimulation, as assessed by ELISA on isolated nuclear-fractions (**Fig. 3.6b**). Differences in anti-IgM-induced nuclear c-Rel had normalized by 2h continuous stimulation in TLR-null B cells compared to the WT controls (**Fig. 3.6c**) but were concomitant with significant increases in total c-Rel expression (**Fig. 3.6d**). However, this normalization of c-Rel was short-lived, as sustained c-Rel expression, which is associated with initialization of the B cell differentiation program (22), remained drastically lower in B cells

lacking TLR-signaling than in WT B cells 48h after anti-IgM pulse (**Fig. 3.5h**). Thus, even in the absence of an explicit TLR agonist, B cells require the presence of TLRs for full activation of c-Rel circuitry and long-term maintenance of c-Rel expression in response to antigen-mediated stimulus.

Discussion

These data address the ability of B cells that lack TLRs to become activated upon BCR stimulation and exposure to B cell help factors. Both DKO and TKO B cells failed to proliferate in response to anti-IgM and CD40L/BAFF treatment, along with a failure to downregulate IgD and to sustain high expression of IRF4. This indicates that the TLR machinery play a crucial role in signal transduction through the IgM-BCR complex.

While TLR stimulation leads to the activation of multiple gene programs, a defect in NF-kB c-Rel nuclear localization and upregulation after BCR stimulation was specifically observed in DKO and TKO B cells. As IRF4 induction is dependent on c-Rel nuclear translocation after both TLR4 and BCR activation (21), it follows that there is also a defect in IRF4 induction. We did observe an eventual normalization of BCR-mediated c-Rel localization in TLR-null B cells after two hours. However, as c-Rel has multiple c-terminal phosphorylation sites (23), perhaps TLR components are required for an optimal phosphorylation signature in addition to release from IkBs. Indeed, it was observed that despite functional dimerization, nuclear localization, and DNA binding of c-Rel with a truncated c-terminus, its regulatory activity was abnormal (24). Therefore, ablation of any functional TLR axis may dictate the nuclear activity of c-Rel, while maintaining localization potential. Further work is needed to determine how TLRs affect phosphorylation of the c-terminal trans-activation domain of c-Rel and how specific gene regulation is altered in their absence.

Additionally, while total c-Rel levels did increase after 48 hours in TLR-null B cells, they were still significantly below levels observed in respective WT controls at every concentration of anti-IgM treatment measured. Therefore, the level of IRF4 expression is concomitant to the level of c-Rel expression and it appears that B cells need to reach a certain threshold of c-Rel in order to optimally induce IRF4. Indeed, c-Rel dominates B cells' NF-kB program after antigen-mediated activation (22), potentiating an activated clone for several rounds of proliferation and enabling access to genes associated with terminal differentiation into plasma cells.

Thus, a model can be articulated where the BCR co-opts TLR components specifically for the purposes of improving survival and optimizing proliferation after antigen recognition. This initial blasting provides greater probability that daughter clones will find and interact with T helper cells to both continue clonal expansion and further potentiate differentiation into an ASC. Once a clone is fully activated, it may then travel to an ASC niche, where it presumably is maintained and expanded through inflammatory inputs, an important B cell-extrinsic aspect addressed previously. Therefore, the initial activation and expansion of antigen-specific B cells may relay mostly on B cell-intrinsic TLR mechanisms, while their maintenance and expansion as effectors may rely more on extrinsic, indirect signals. More work is necessary to determine at which junctions of activation B cells rely most on intrinsic versus extrinsic inflammatory cues and how this may affect fate determination, specifically the bifurcation into either GC or EFR. Elucidation of these mechanisms may enable the development of more effective vaccine platforms that induce EFRs and thus provide faster protective antibody production, thus faster induction of immune protection and/or more rapid reductions in pathogen spread within a host and thereby the levels of inflammation, morbidity and mortality.

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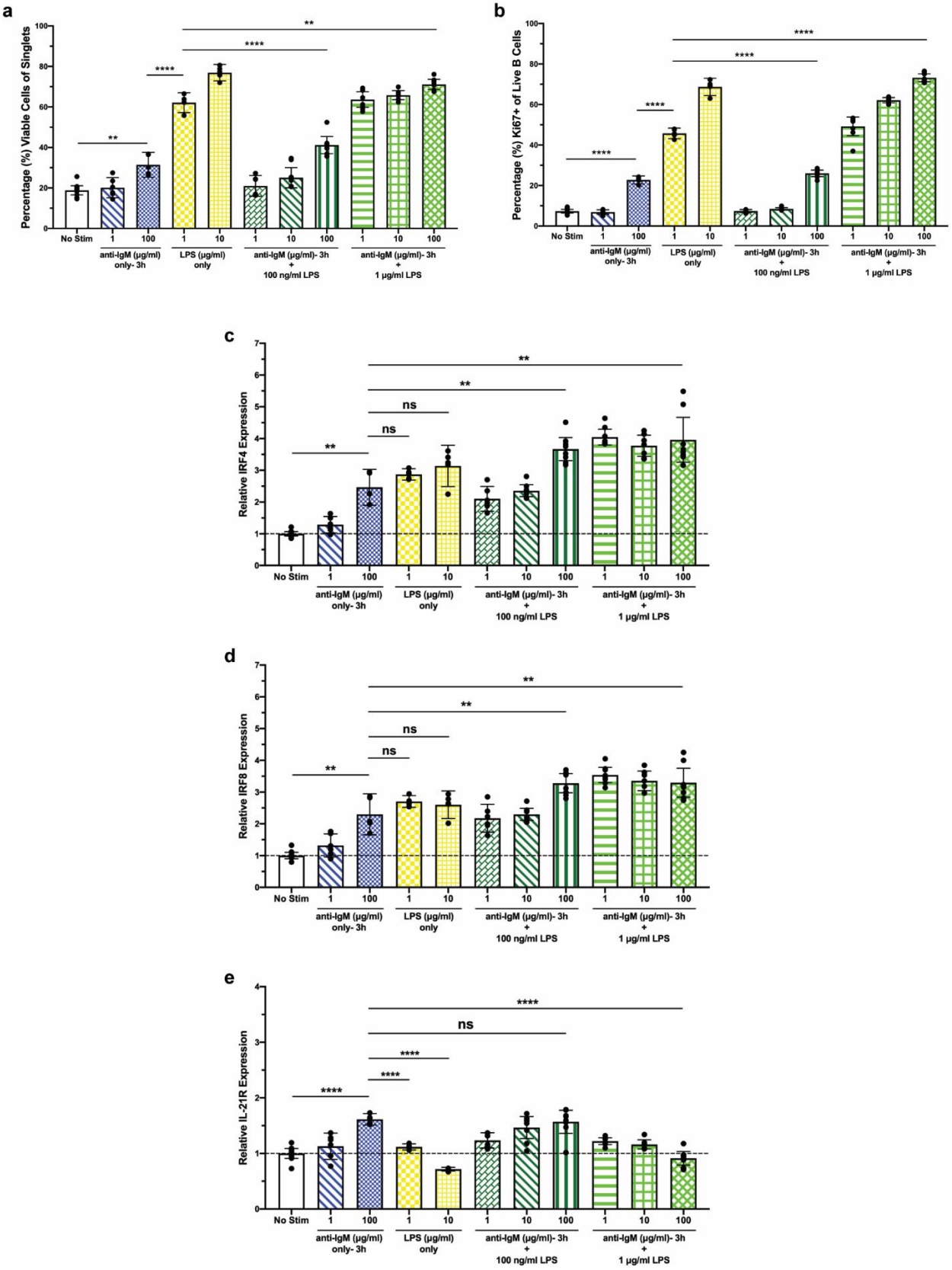
a
Percentage (%) Viable Cells of Singlets
b
Percentage (%) Ki67+ of Live B Cells
c
Relative IRF4 Expression
d
Relative IRF8 Expression
e
Relative IL-21R Expression
No Stim
anti-IgM (µg/ml) only- 3h
LPS (µg/ml) only
anti-IgM (µg/ml)- 3h + 100 ng/ml LPS
anti-IgM (µg/ml)- 3h + 1 µg/ml LPS
ns

Figure 3.1. BCR and TLR4 stimulation have additive effects on cell viability, proliferation, and activation phenotype. Pooled and enriched splenic and lymph node WT B cells were cultured with indicated graded concentrations anti-IgM 3h pulsed or graded, sustained concentrations of LPS and assessed for **(a**) cell viability, **(b**) proliferation, (**c**) IRF4 (**d**) IRF8, and (**e**) IL-21R expression. Graphs are representative of two experiments (n=4). Error bars represent 95% CI. Statistical significance determined by one-way ANOVA (all $p<0.01$) and unpaired Student's t-test with Welch's correction. *: $p<0.05$ **: $p<0.01$ ***: $p<0.001$, ****: $p<0.0001$.

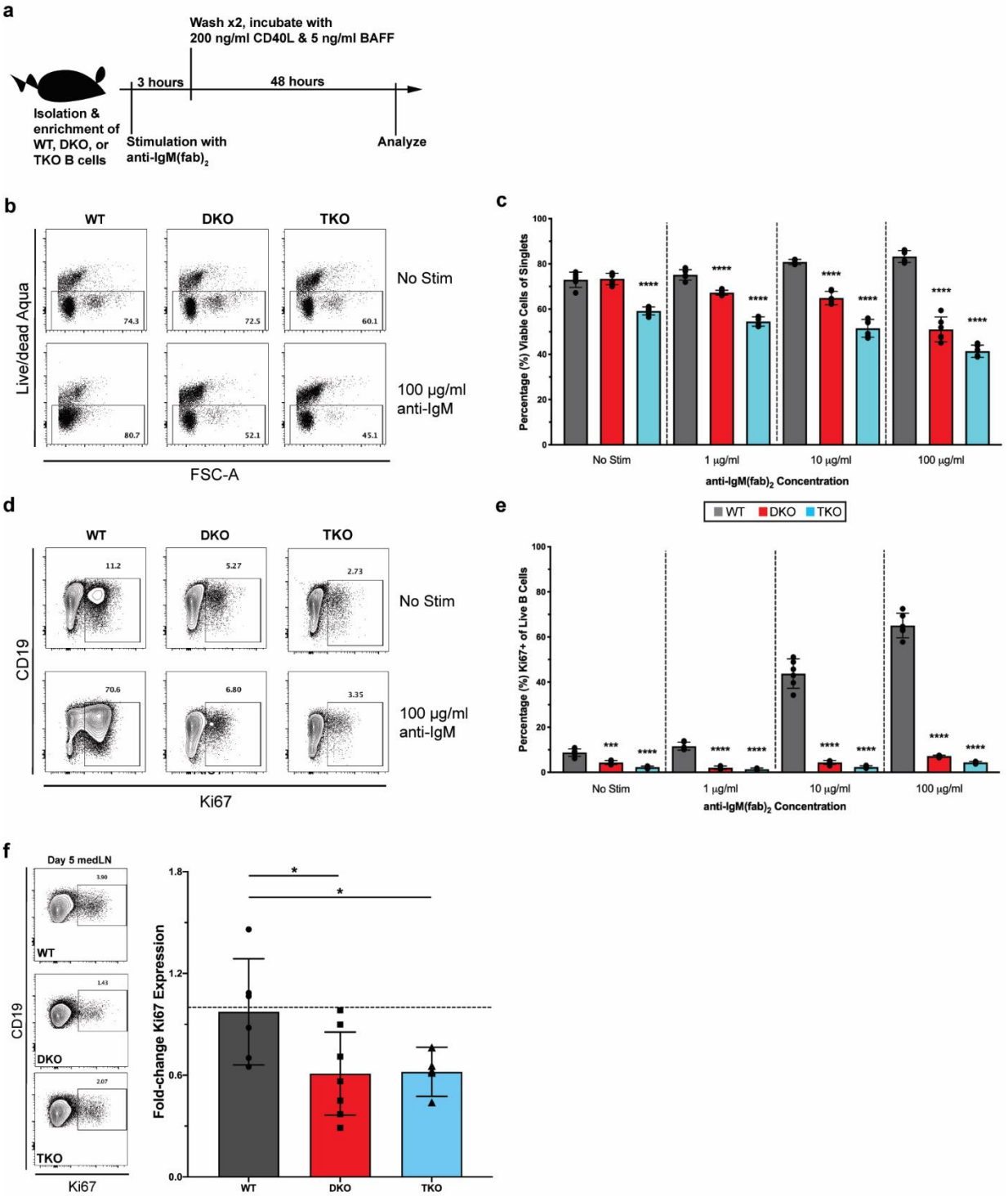
a
Wash x2, incubate with
200 ng/ml CD40L & 5 ng/ml BAFF
3 hours
48 hours
Isolation & enrichment of WT, DKO, or TKO B cells
Stimulation with anti-IgM(fab)2
Analyze
b
WT
DKO
TKO
Live/dead Aqua
No Stim
100 µg/ml anti-IgM
74.3
72.5
60.1
80.7
52.1
45.1
FSC-A
c
Percentage (%) Viable Cells of Singlets
No Stim
1 µg/ml
10 µg/ml
100 µg/ml
anti-IgM(fab)2 Concentration
d
WT
DKO
TKO
CD19
No Stim
100 µg/ml anti-IgM
11.2
5.27
2.73
70.6
6.80
3.35
Ki67
e
WT DKO TKO
Percentage (%) Ki67+ of Live B Cells
No Stim
1 µg/ml
10 µg/ml
100 µg/ml
anti-IgM(fab)2 Concentration
f
Day 5 medLN
WT
DKO
TKO
CD19
Ki67
Fold-change Ki67 Expression
WT
DKO
TKO

Figure 3.2. BCR-mediated survival and proliferation are defective in the absence of TLR signaling. (a) Experimental layout. Pooled splenic and LN B cells from WT, DKO, or TKO B cells negatively enriched (>98% purity) were pulsed with graded levels of anti-IgM for 3 hours, then stimulated with CD40L and BAFF for 48 hours. Representative flow plots (**b**) and quantification (**c**) of cell viability. Representative flow plots (**d**) and quantification **(e**) of cell proliferation. **(i)** Ki67+ non-EF/GC B cells in chimeras from 5 dpi. Graphs are representative of two experiments (n>/=4). Error bars represent 95% CI, p-values were determined by unpaired Student's t-test with Welch's correction. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$. Inter-strain differences in (f) $p<0.01$ by one-way ANOVA.

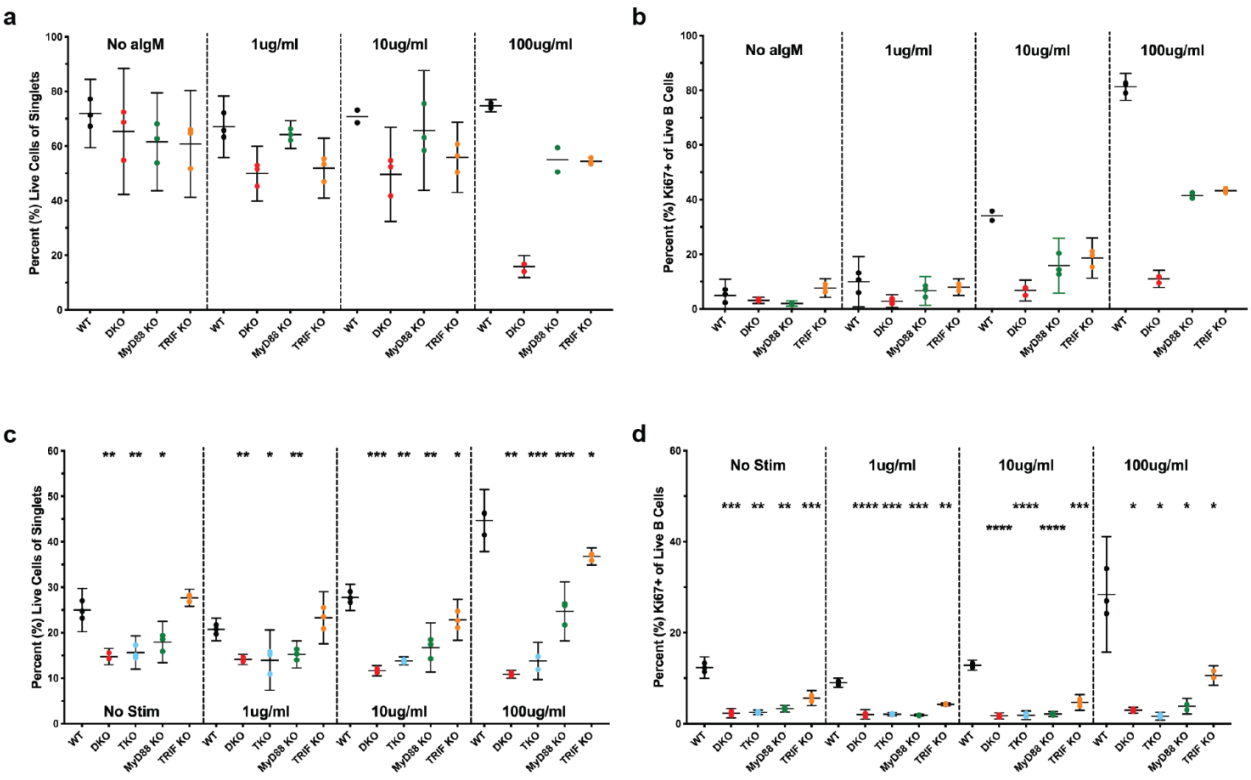

Figure 3.3. MyD88 and TRIF non-redundantly regulate B cell viability and proliferation in response to BCR stimulus. Pooled splenic/LN WT, DKO, MyD88 KO, and TRIF KO B cells were negatively enriched (>98%) and pulsed with graded anti-IgM for 3 hours, then stimulated with CD40L and BAFF for 48 hours and assessed for **(a)** viability and **(b)** proliferation. **(c,d)** Cells as in **(a)** were pulsed with graded doses anti-IgM for 3 hours followed by incubation in complete medium only for 48h, then assessed for (**c**) cell viability and (**d**) proliferation. Error bars represent 95% CI, p-values (c,d) were determined by unpaired Student's t-test with Welch's correction comparing KO to respective WT control. Intra-strain differences in (c,d) all p<0.01 by one-way ANOVA. *: p<0.05 **: p<0.01 ***: p<0.001, ****: p<0.0001.

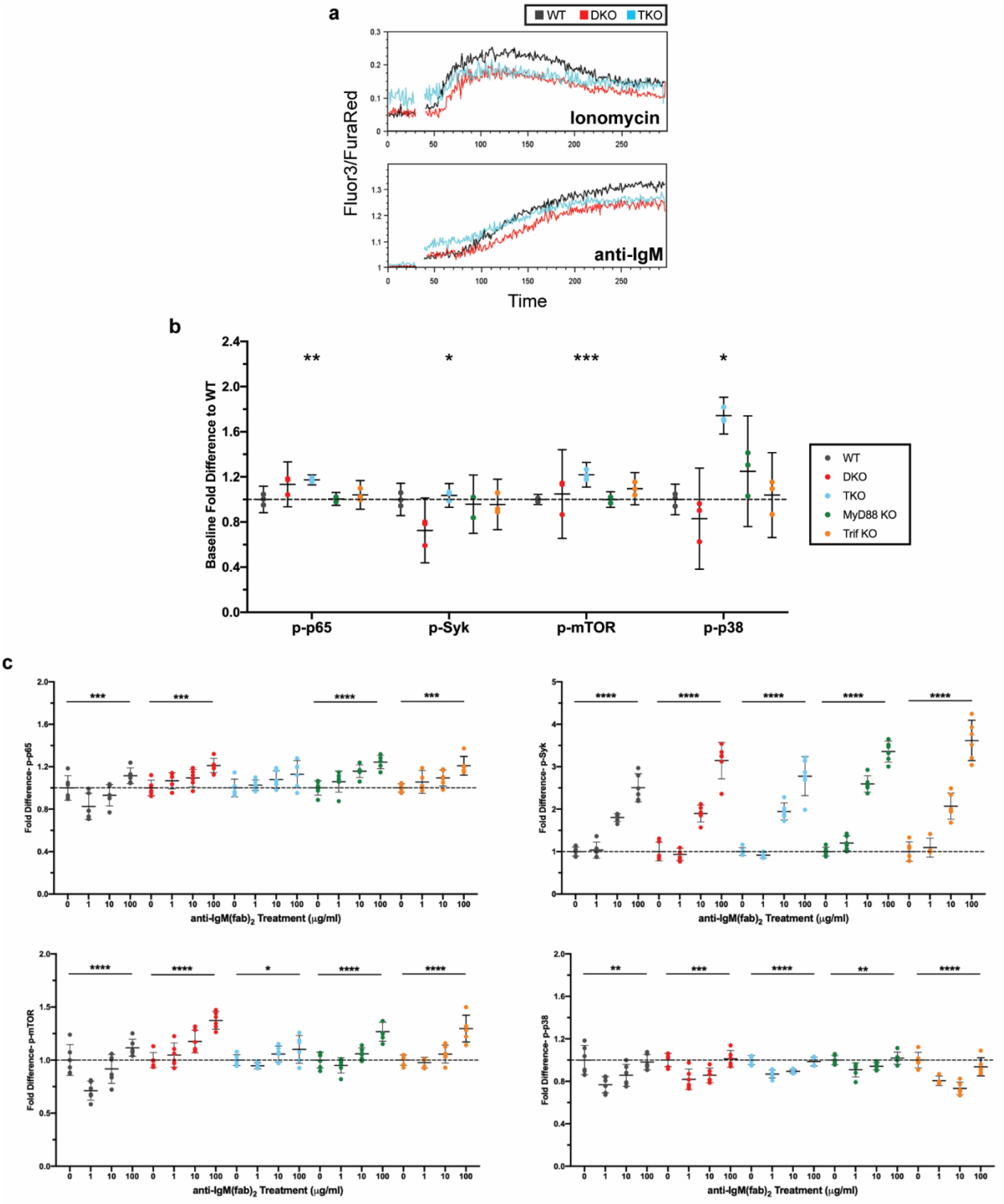
a
WT
DKO
TKO
Fluor3/FuraRed
Ionomycin
anti-IgM
Time
b
Baseline Fold Difference to WT
**
*
***
*
WT
DKO
TKO
MyD88 KO
Trif KO
p-p65
p-Syk
p-mTOR
p-p38
c
Fold Difference- p-p65
Fold Difference- p-Syk
Fold Difference- p-mTOR
Fold Difference- p-p38
anti-IgM(fab)2 Treatment (μg/ml)

Figure 3.4. TLR-signaling deficient B cells have nominal immediate activation and functional downstream effector protein activation. (**a**) Calcium flux of BCR-independent (iono) and BCR-mediated (anti-IgM) activation in purified WT, DKO, and TKO B cells. (**b,c**) Pooled splenic/LN WT, DKO, TKO, MyD88 KO, and TRIF KO B cells were negatively enriched (>98%) and stimulated with graded anti-IgM for 30 minutes, then assessed for phosphorylated, intracellular proteins. (**b**) Baseline fold-difference in phosphorylation between WT and knockout B cells. (**c**) Fold-changes in protein phosphorylation of target proteins relative to non-stimulated conditions for each strain. Graphs are representative of two experiments (n=6) except for (b) (n=3). Error bars represent 95% CI, p-values determined by one-way ANOVA. *: $p<0.05$ **: $p<0.01$ ***: $p<0.001$, ****: $p<0.0001$.

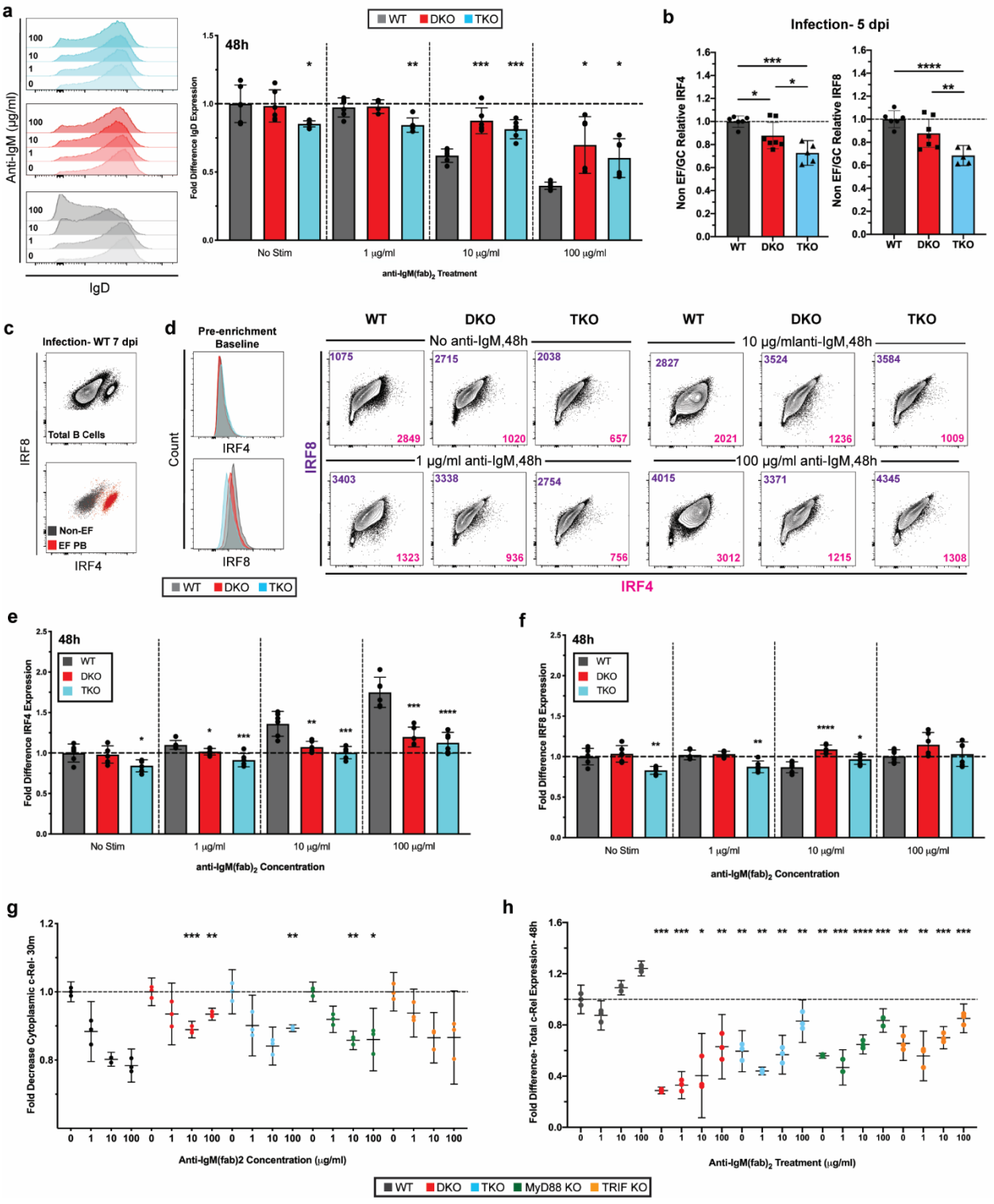
a
Anti-IgM (µg/ml)
IgD
WT DKO TKO
48h
Fold Difference IgD Expression
No Stim
1 µg/ml
10 µg/ml
100 µg/ml
anti-IgM(fab)2 Treatment
b
Infection- 5 dpi
Non EF/GC Relative IRF4
Non EF/GC Relative IRF8
c
Infection- WT 7 dpi
Total B Cells
Non-EF
EF PB
IRF8
IRF4
d
Pre-enrichment
Baseline
Count
IRF4
IRF8
WT DKO TKO
No anti-IgM,48h
10 µg/mlanti-IgM,48h
1 µg/ml anti-IgM,48h
100 µg/ml anti-IgM,48h
1075
2849
2715
1020
2038
657
2827
2021
3524
1236
3584
1009
3403
1323
3338
936
2754
756
4015
3012
3371
1215
4345
1308
e
48h
Fold Difference IRF4 Expression
anti-IgM(fab)2 Concentration
f
48h
Fold Difference IRF8 Expression
anti-IgM(fab)2 Concentration
g
Fold Decrease Cytoplasmic c-Rel- 30m
Anti-IgM(fab)2 Concentration (µg/ml)
h
Fold Difference- Total c-Rel Expression- 48h
Anti-IgM(fab)2 Treatment (µg/ml)
WT DKO TKO MyD88 KO TRIF KO

Figure 3.5. Lack of functional TLR signaling leads to altered BCR complex dynamics and failure to upregulate IRF4. (**a**) Histogram overlays of IgD expression (left) for WT, DKO, and TKO B cells stimulated as in Fig. 5a; Summary of fold-difference in IgD expression (right). (**b**) Fold-difference in IRF4 (left) and IRF8 (right) non-EF/GC B cells from 5 dpi. (**c**) Representative flow plots showing IRF4 and IRF8 expression in infected mice (top), highlighting clustering of EF PBs (bottom). (**d**) Pre-enrichment baseline of IRF4 and IRF8 in B cells of each strain (left) and representative IRF4 versus IRF8 flow plots from cells stimulated with indicated anti-IgM concentrations (right). Colored numbers in plots correspond to each like-colored axis. Fold-change compared to non-stimulated WT B cells in IRF4 **(e)** and IRF8 expression (**f**) after treatment outlined in Fig. 5a. (**g**) Fold-change in cytoplasmic c-Rel measured by flow cytometry after 30-minute anti-IgM relative intra-strain non-stimulated treatment. (**h)** Fold-differences in total c-Rel expression after a 3h anti-IgM pulse and 48h culture in complete media only. Error bars represent 95% CI. Statistical significances determined by one-way ANOVA and unpaired Student's t-test with Welch's correction. Stars in (g,h) are Student's t-test comparison to respective WT control. *: $p<0.05$ **: $p<0.01$ ***: $p<0.001$, ****: $p<0.0001$.

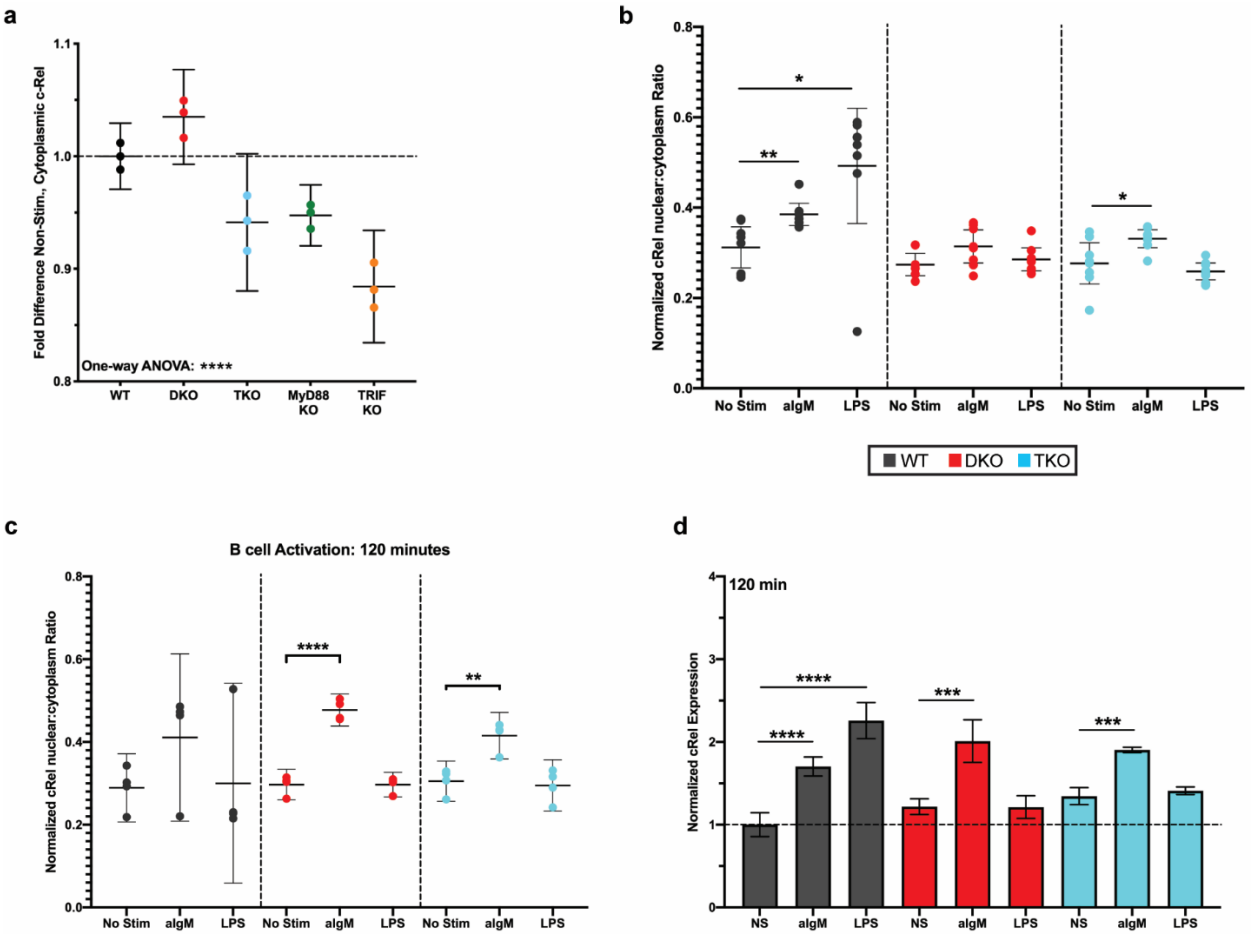

Figure 3.6. Immediate activation of c-Rel after BCR stimulation is defective in TLR-signaling deficient B cells. **(a)** (**b-c**) Shown are the ratios of nuclear vs cytoplasmic c-Rel in WT, DKO, and TKO B cells as determined by ELISA on nuclear versus cytoplasmic protein extracts at 60 minutes **(b)** and 120 minutes **(c)** after anti-IgM or LPS stimulation. (**d**) Total c-Rel expression after 120-minute anti-IgM or LPS stimulation. Error bars represent 95% CI. Statistical significance determined by one-way ANOVA (a, intra-strain comparisons in b-d) and unpaired Student's t-test with Welch's correction (b-d). *: $p<0.05$ **: $p<0.01$ ***: $p<0.001$, ****: $p<0.0001$.

Chapter 4

Toll-like receptor-mediated inflammation generates highly protective extrafollicular-derived antibodies following influenza Immunization

Jonathan Lam

Chapter adapted from: Lam, Jonathan and Baumgarth, Nicole. Toll-like receptor signaling directs B cells towards protective extrafollicular responses during infection and immunization. In preparation.

Abstract

Subcutaneous immunization with alum-adjuvanted influenza virus particles produced no extrafollicular B cell responses (EFRs), while lack of Toll-like receptor (TLR) signaling adaptors MyD88 and TRIF compromised EFR generation and protective capacity. This indicates that explicit activation of MyD88/TRIF is integral to EFR generation and may be absent during immunization. Here, mice primed subcutaneously with influenza and primed/boosted with lipopolysaccharide (LPS), a TLR4 agonist that activates both TLR signaling adapters MyD88 and TRIF, generated robust, antigen-specific EFRs compared to antigen prime alone. Additionally, repeated application of antigen as well as LPS led to the preferential expansion and the differentiation of hemagglutinin (HA) -specific B cells into plasmablasts instead of germinal center (GC) B cells, while repeated provision of antigen alone heavily favored HA-specific B cell entry into the GC compartment. This biasing of the B cell fate towards EFRs led to increased influenza-specific serum IgG in LPS-boosted mice along with improved passive protection from lethal homologous challenge. TLR-mediated inflammation thus sets the stage for the preferential entry of antigen-specific clones into the EFR over GCs, leading to the rapid and robust generation of protective antibodies.

Introduction

Immune responses are stochastic reactions to inputs from the local tissue environment, where locally- or systemically-sourced molecular signals culminate in a network of said inputs known as the inflammatory milieu. This network “speaks” to effector and support cells, ideally leading to a highly regulated response proportional to the insult.

As demonstrated earlier, antigen-specific ASCs can be formed outside the GC given specific cues provided by the inflammatory milieu. What those initiating cues are is important to understanding how local, microanatomical niches form that support these ASCs generated from extrafollicular B cell responses (EFRs). Such responses are known to be transient in nature (1), suggesting that as both antigen and innate stimuli wane as infections resolve, so will EFRs.

EFRs have been observed in models of Salmonella and Ehrlichia infection where mice experience high local pathogen burden and inflammation (2, 3). This suggests that 1) antigen affinity and density are not the only factors determining early ASC differentiation and maintenance either locally at the infection site or in secondary lymphoid organs; and 2) EFRs provide locally responding, short-lived effectors, analogous to effector T cell responses. Indeed, secondary, heterologous challenge of mice with influenza virus elicited rapid propagation of ASCs in the lungs by memory B cells (4), which responded locally to distinct epitopes that were not covered by the previously generated plasma cell repertoire. As memory B cells are known to respond to inflammation-induced chemokines CXCL9 and CXCL10 (5), it follows that the inflammatory nature of the secondary infection likely contributed to propagation of subsequent EFRs. Furthermore, the RNA of sheep red blood cells (SRBCs), which are commonly used as adjuvant-carriers for model antigens, stimulate RNA-sensing pattern-recognition receptors MAVS and toll-like receptor (TLR) 3 (6). For example, SRBCs are used in the hen-egg lysozyme model, where high affinity interactions between B cell receptor (BCR) and antigen have been

associated with robust EFRs (7), suggesting again that innate, inflammatory signaling contributes towards the priming of antigen-specific EFRs.

As subcutaneous (s.c.) immunization with influenza virion yielded no EFRs (**Fig. 2.3**), I speculate that the threshold for inflammation required for EFR induction had not been reached within the draining lymph node (LN). Given that lack of MyD88 and TRIF led to poor EFRs and antibody-mediated immune protection after infection (**Fig. 2.6**), and that stimulation of the MyD88/TRIF-activating receptor TLR4 led to enhanced B cell activation following immunization (**Fig. 3.1**), I therefore interrogated how local, TLR-mediated inflammation affected B cell fate decisions following s.c. immunization.

Materials & Methods

Mice. Female 8- to 12-wk-old C57BL/6 (WT; CD45.2) were commercially obtained (The Jackson Laboratories). Mice were held under SPF housing conditions with ad libitum access to food and water. All experiments conducted were approved by the UC Davis Institutional Animal Care and Use Committee.

Infections and immunizations. Mice were anesthetized with isoflurane and infected intranasally with a lethal dose (100 PFU/ml) of influenza A/Puerto Rico/8/34 (A/PR8) in 40 µl volumes in PBS. Virus was grown in hen eggs and each virus batch was titrated for its effect on mice prior to use. For immunizations, mice were inoculated subcutaneously with $1x10^7$ PFU A/PR8 in a 50:50 alum to PBS mixture. For some experiments immunizations were supplemented with 3µg LPS, or mice were in addition boosted repeatedly with $1x10^6$ PFU A/PR8 and 3µg LPS in PBS or PBS alone as indicated.

Adoptive serum transfer for passive protection. Indicated strains of mice were infected with 10 PFU A/PR8. Blood from terminally anesthetized mice at 10 days post infection (dpi) was

collected via cardiac puncture and spun down for serum separation. Serum from each strain was pooled and naïve C57BL/6 mice were subsequently injected i.v. with a mixture of 50µl pooled serum and 150µl 1x PBS. These mice were inoculated i.n. with a lethal dose of 100 PFU A/PR8 one day later. Mice were also weighed before and following influenza infection to monitor the course of infection. They were humanely euthanized via exposure to $C0_2$ when they reached weightloss of > 20%.

Flow cytometry. Single-cell suspensions from the draining inguinal lymph node were made and labeled for phenotyping as previously outlined. Briefly, after Fc receptor block with anti-CD16/32 (5 mg/ml for 20 min on ice), cells were stained with the following antibody-fluorophore conjugates at temperatures and times according to manufacturer/provider: HA-PE and HA-APC oligomers (kindly provided by Dr. Frances Lund, UAB), BV786 anti-CD19 (1D3) (BD Bioscience), APC-eFluor780 anti-CD45R (RA3-6B2), PE-Dazzle 594 anti-CD38 (90) (both Thermo Fisher), BV711 anti-CD24 (M1/69), BV605 anti-CD138 (281-2) (both Biolegend), eFluor450 anti-GL-7 (GL7), PE or PE/Cy7 anti-IRF4 (3E4), PerCP-eFluor710 anti-IRF8 (V3GYWCH), eFluor450 anti-Ki67 (SolA15) (all Thermo Fisher), FITC anti-IgM (331, in-house), and BV650 anti-IgD (11-26c.2a) (Biolegend). For a non-B cell “dump”, the following antibodies on AlexaFluor 700 were used: anti-CD90.2, anti-CD4, anti-CD8a, anti-Gr-1, anti-CD11b, anti-NK1.1, anti-F4/80 (all Thermo Fisher). The Foxp3 Staining Buffer Set (Thermo Fisher) was used for fixation and permeabilization of cells for staining of transcription factors according to manufacturer’s protocol.

Statistical analysis. All data were analyzed using Graphpad Prism software. Quantified data are represented as mean with 95% confidence interval error bars. P-values indicated in figure legends. A p-value of < 0.05 was considered statistically significant.

Results

Sustained presence of TLR-mediated inflammation enhances antigen-specific EFRs during immunization

While robust EFRs were generated against influenza after infection (**Fig. 2.1**), subcutaneous (s.c.) immunization of mice with influenza virions in alum did not generate EFRs (**Fig. 2.2**). As TLR4 stimulation enhanced critical aspects of B cell priming and antigen-mediated expansion (**Fig. 3.1**), the total system effect (B cell intrinsic plus extrinsic) of TLR signaling on B cell fate (EFR versus GC) was assessed. Groups of C57BL/6 mice were inoculated s.c. with influenza in alum plus LPS, along with repeated LPS boosting thereafter (Ag+LPS; **Fig. 4.1a**). Indeed, LPS-treated mice showed greatly increased total B cells and extrafollicular plasmablasts (EF PBs) compared to mice receiving influenza in alum (Ag Only) by both frequency and total number (**Fig. 4.1b-c**). While GC numbers were increased several-fold in in LPS-treated mice (**Fig. 4.1c, bottom row**), there was no difference in GC B cell frequencies (**Fig. 4c, top row**), indicating a preferential expansion of EF PBs in the presence of LPS. More importantly, twice as many influenza hemagglutinin (HA) binding B cells were found in LNs of Ag+LPS mice compared to mice receiving antigen alone, with several-fold increases in the EF PB but not the GC compartment **(Fig. 4.1d**). The data indicated that TLR activation not only increased expansion of antigen-specific B cells but preferentially shunted them towards an EFR fate. HA B cells from Ag+LPS mice had strongly increased expression of ASC markers CD138 and IRF4, which was not seen in the other two groups of animals (**Fig. 4.1e,f**). Antigen-specific B cells from mice immunized with LPS+antigen also showed improved survival after immunization (**Fig. 4.2a**), which was also seen in HA-specific B cells from draining lymph nodes of mice at 7 dpi with influenza (**Fig 4.2b**). Furthermore, HA B cells from LPS+antigen immunized mice were strongly positive for Ki67, indicating vigorous clonal expansion of HA B cells in the presence of LPS (**Fig. 4.1e,f**). Thus, TLR4 stimulation during immunization led to significantly

increased expansion of antigen-specific ASCs compared to antigen administration alone, without noticeable suppressing the antigen-specific GC response.

Repeated antigen exposure requires sustained, TLR-mediated inflammation to preferentially expand protective, antigen-specific EFRs

It has been reported recently that increasing antigen valency (8) or availability (9) favors EFRs, where differentiation into ASCs is driven by an overall increase in avidity from increasing the stoichiometric ratio between epitope and total BCR. Additionally, there are contradictory observations of EFRs in different models of sustained antigen availability in the presence of inflammation. For example, EFRs are either absent (2,3) or delayed (10,11,12) during infections of high pathogen burden but have been seen in autoimmune diseases, where EFR-derived, self-antigen specific antibody complexes drive pathology (13). We therefore sought to determine how inflammation influences B cell fate dynamics and EFR-derived antibody functionality through a model of repeated antigen exposure.

Groups of mice were primed with both influenza and LPS s.c., then boosted with additional antigen twice, either alone (Ag Boosted) or with LPS (Ag+LPS Boosted), along with a control group that received no antigen (LPS Boosted) (Fig. **4.3a**). Again, groups receiving LPS boosts had significantly greater frequencies of B cells in the draining lymph node (**Fig. 4.3b**), while overall GC B cell frequencies were similar between both Ag Boosted and Ag+LPS Boosted groups (**Fig. 4.3c**). As expected from my previous studies, groups boosted with LPS had significantly higher EFR frequencies than the Antigen Boosted group (**Fig. 4.3d**).

HA B cell frequencies were similar between Ag boosted and Ag+LPS boosted mice (**Fig. 4.3e**), as was the level of cell cycle entry as measured with staining for Ki67 (**Fig. 4.3f**). However, mice boosted with Ag alone had significantly higher GC HA B cells than the other groups (**Fig. 4.3g**), with a ratio of GC versus EF PBs (**Fig. 4.3h**) about 2 to 1. This indicated that repeat antigen exposure does not favor polarization of antigen-specific B cells towards EFRs.

Again, Ag+LPS boosted mice favored entry into the EFR versus GC at a ratio of about 2 to 1 (Fig. **4.3g,h**), further demonstrating that the sustained presence of TLR-mediated inflammation leads to EFR polarization and expansion.

Ag+LPS Boosted mice had the highest levels of serum anti-influenza antibodies (**Fig. 4.3i**), demonstrating that increased EFRs correlated with enhanced antigen-specific antibody responses compared to a GC-biased response at 10 days post-priming. To determine whether the increased in IgG levels correlated with increased serum passive protective capacity, pooled serum from each boosted group was transferred to naïve animals, who were subsequently challenged with a lethal dose of influenza. Mice receiving Ag+LPS Boosted serum showed no mortality, in contrast to mice receiving Ag alone or mice that received LPS alone (**Fig. 4.4a**). Moreover, mice that received serum from Ag+LPS boosted mice were overall more protected than mice receiving serum from Ag only immunized animals, as assessed by comparing levels of weight loss (**Fig. 4.4b**).

Discussion

Together, the above data demonstrate that TLR-mediated inflammation orchestrates faster and greater antigen-specific ASC formation through EFRs, leading to the rapid generation of protective antibodies. Furthermore, the presence of antigen alone leads to expansion of antigen-specific clones primarily within the GC compartment, with serum antibody responses that are slower and less protective at early times after immunization.

Recently, it was shown that TLR adjuvants cause infiltration of the draining LN by inflammatory monocytes (14). Interestingly, this leads not only leads to global changes in the inflammatory milieu of the LN but was shown to result in sub-compartmentalization of the LN into discrete priming zones. Specifically, areas of the LN dominated by the infiltrating monocytes generated more short-lived effector T cells, while areas with little monocyte density primarily generated long-lived memory T cells. Both B and T cells express the chemokine receptor CXCR3, whose

ligands, CXCL9/10, are expressed differentially across the LN by both myeloid and stromal populations (15). During viral infection, CXCR3-expressing T cells preferentially differentiate into effectors cells, while ablation of CXCR3 led to enrichment of memory T cells (16), indicating that certain cells within the CXCL9/10-expressing population recruit antigen-specific clones and are responsible for their effector polarization. Indeed, CXCL10-expressing, LN-resident dendritic cells (DCs) have been shown to localize towards the inter-follicular regions within hours of viral immunization, forming foci of antigen “depots” where antigen-specific T cells cluster (17).

Thus, supported by on the data shown here, a map can be drawn of how inflammation is linked to generation of ASCs during an extrafollicular response. First, front-line response signals such as TLRs generate an inflammatory milieu that leads to the recruitment of additional, innate, inflammatory cells. These cells not only amplify the original inflammatory signature but help re-shape the LN to support the generation and maintenance of terminally differentiated, effector lymphocytes. Concurrently, resident, professional, antigen-presenting cells are activated through antigen capture and processing, allowing not only for antigen-presentation, but for secretion of specific chemokines. This recruits recently activated B cells or fully differentiated plasmablasts, leading to further proliferation and secretion of antibodies. Indeed, LN-resident DCs but not migratory DCs nor subcapsular macrophages were shown to be required for early antibody production (18). As inflammation draws down, these niches shrink, leading to contraction of EFRs. As antigen becomes limiting, dissolution of inter-follicular antigen depots occurs and only stable antigen:antibody complexes sequestered by follicular dendritic cells are maintained, leading to domination of the GC response within the follicles. When antigen is in surplus, but inflammation is low, EFR niches are limited, allowing only for minimal ASC maintenance by antigen-laden dendritic cells at the inter-follicular nodes.

While the above data did not directly address suppression of GC responses by inflammation, it was observed that lack of TNF-alpha signaling led to over 3 times greater GCs after influenza infection (**Fig. 2.5**), indicating that certain inflammatory signals can suppress GC

responses above a given threshold. Another possibility is that GCs in LPS-treated LNs become dominated by clones with low affinity for self-antigen, given the high inflammatory load and extensive tissue damage. This would lead to a highly competitive GC landscape compared to antigen-only immunization, where tissue damage and cell death is limited, allowing for easier occupancy of the GC niche for target antigen-specific clones. This would also explain the observed EFR dynamics. As self-reactive B cell clones are eliminated during development, antigen-specific clones with germline specificity can be drawn into the EFR and expanded without somatic hypermutation, without a high risk of inducing self-reactive antibody responses.

During the studies described here, establishment of extrafollicular antigen depots and infiltration of inflammatory effectors likely occurred and were affected by the various modalities of immunization. Repeated administration of LPS led to expanded EFRs overall and preferential entry into EFRs by HA-specific B cells. While immunization with antigen alone (plus initial LPS administration) did generate some level of HA-specific ASCs, this population was significantly smaller compared to LPS co-treatment. Whether these limited ASCs were formed or maintained by resident DCs at the interfollicular regions, or whether they were generated by GCs, was not determined here but is of interest, as this would determine whether LPS administration contributed more towards expansion of the EFR niche or the priming events. Future studies should interrogate the dynamics of LN-resident DC populations with and without TLR-mediated adjuvant, along with their dynamics upon contraction of the inflammatory milieu.

Supplementation of antigen with LPS boosting led to increased, influenza-specific serum antibody levels (**Fig. 4.3i**), which is the simplest explanation for improved protection compared to serum from Ag Boosted mice (**Fig. 4.4**). However, another aspect of antibodies derived from TLR-induced EFRs that should be considered is its repertoire. As LPS increased B cell survival, proliferation, and IRF4 expression either comparable to or greater than BCR stimulation alone (**Fig. 3.1**), it follows that TLR signaling widens the bottleneck for antigen-mediated activation, proliferation, and differentiation, allowing for lower-avidity clones to become ASCs and thus to

increase repertoire diversity. Indeed, mice treated with rapamycin during influenza infection saw improved protection against heterologous infections due to an increase in cross-reactive antibodies (19). Further work should address both the diversity of the EFR repertoire relative to GCs after immunization and whether the type of inflammation influences the repertoire as well.

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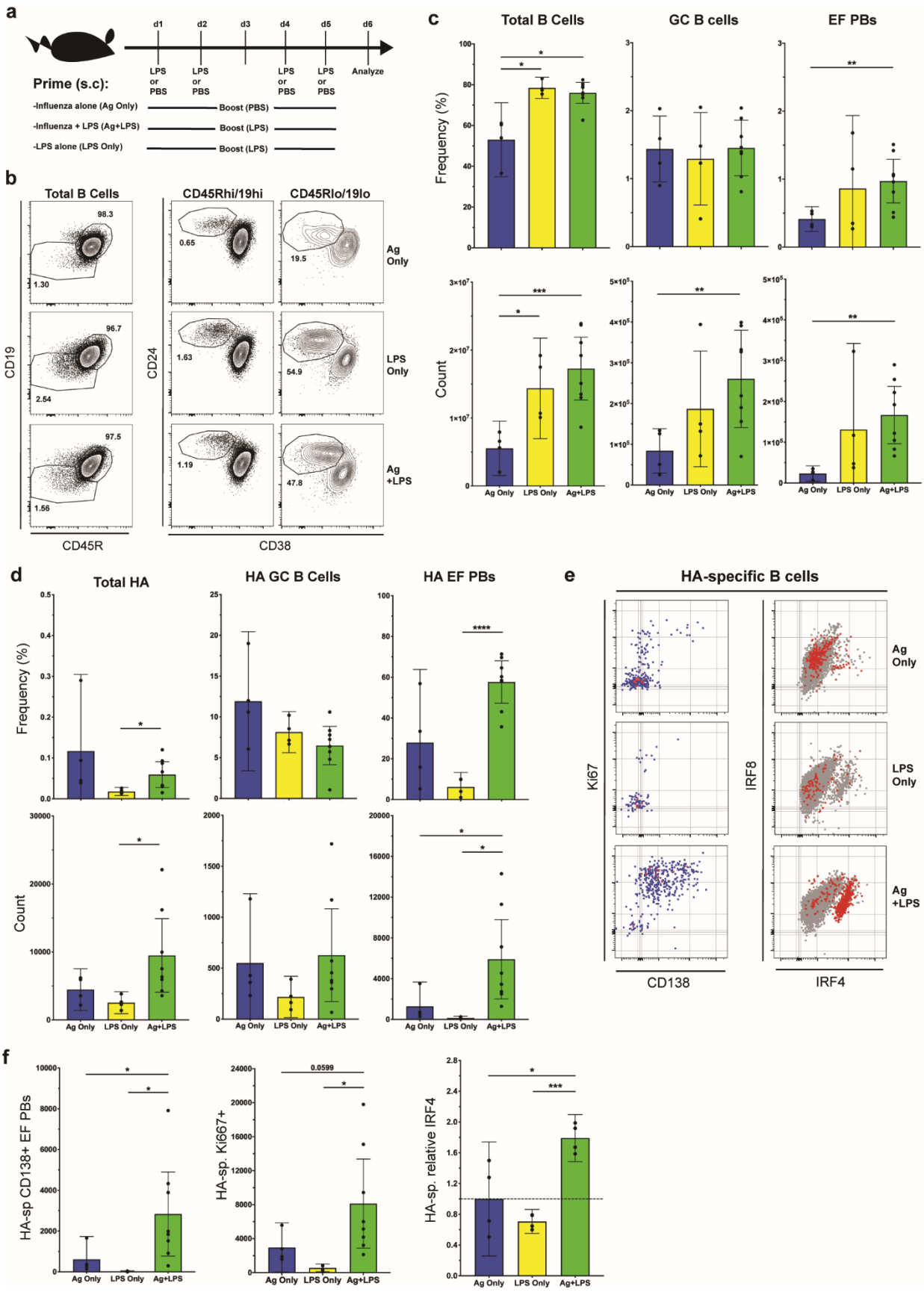
a
d1
d2
d3
d4
d5
d6
LPS or PBS
LPS or PBS
LPS or PBS
LPS or PBS
Analyze
Prime (s.c):
-Influenza alone (Ag Only)
Boost (PBS)
-Influenza + LPS (Ag+LPS)
Boost (LPS)
-LPS alone (LPS Only)
Boost (LPS)
b
Total B Cells
CD45Rhi/19hi
CD45Rlo/19lo
98.3
1.30
0.65
19.5
Ag Only
96.7
2.54
1.63
54.9
LPS Only
97.5
1.56
1.19
47.8
Ag +LPS
CD19
CD24
CD45R
CD38
c
Total B Cells
GC B cells
EF PBs
Frequency (%)
Count
Ag Only
LPS Only
Ag+LPS
d
Total HA
HA GC B Cells
HA EF PBs
Frequency (%)
Count
Ag Only
LPS Only
Ag+LPS
e
HA-specific B cells
Ag Only
LPS Only
Ag +LPS
Ki67
CD138
IRF8
IRF4
f
HA-sp CD138+ EF PBs
HA-sp. Ki667+
0.0599
HA-sp. relative IRF4
Ag Only
LPS Only
Ag+LPS

Figure 4.1. Sustained TLR-mediated inflammation generates strong EFRs in the draining LN after immunization. (a) Experimental outline. Mice were immunized s.c. with or without influenza in alum and with or without LPS, then boosted with either LPS or PBS on days specified, followed by analysis of draining LN. **(b**) Gating for identification of pre-GC/GC and EF PBs. **(c)** Frequency (top) and total count (bottom) of major B cell subsets. **(d)** Quantification of HA-specific B cell subsets as in (c). **(e**) Flow plots of HA-specific B cells from each regimen in terms of proliferation and plasma cell differentiation (left) and IRF4 vs IRF8 signature (right, HA-sp. highlighted in red). **(f)** Quantification of HA-specific EF PBs, proliferation, and relative expression of IRF4. Graphs are representative of two experiments (n>/=4). Error bars represent 95% CI. Statistical significance determined by one-way ANOVA and unpaired Student's t-test with Welch's correction. *: $p<0.05$, **: $p<0.01$ ***: $p<0.001$, ****: $p<0.0001$.

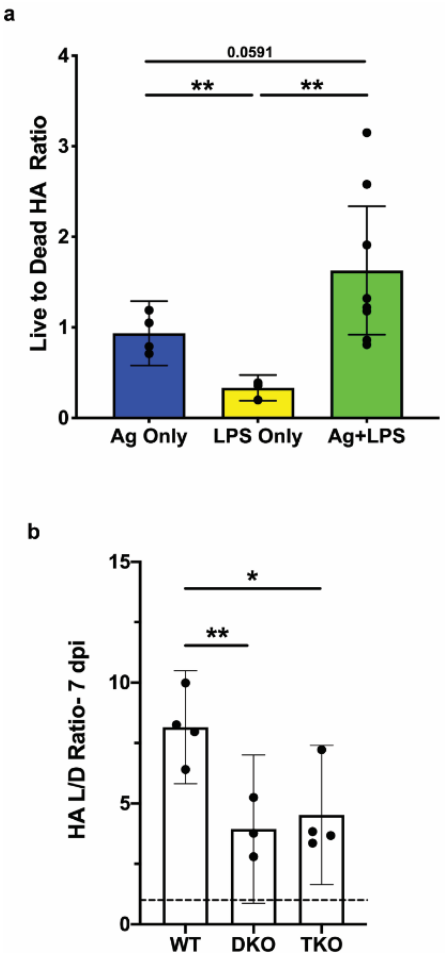

Figure 4.2. HA-specific B cell survival is modulated by TLR signals. (a) Mice were immunized s.c. with or without influenza in alum and with or without LPS, then boosted with either LPS or PBS on days specified, followed by analysis of draining LN. The ratio of live versus dead HA-specific B cell populations were assessed using dead cell dye from each treatment. (**b)** Analysis of live versus dead HA-specific B cells as in (a) but from infected mice, 7 dpi. Graphs are representative of two experiments (n>/=3). Error bars represent 95% CI, statistical significance determined by one-way ANOVA ($p<0.01$ for both a,b) and unpaired Student's t-test with Welch's correction (d,e). *: $p<0.05$ **: $p<0.01$.

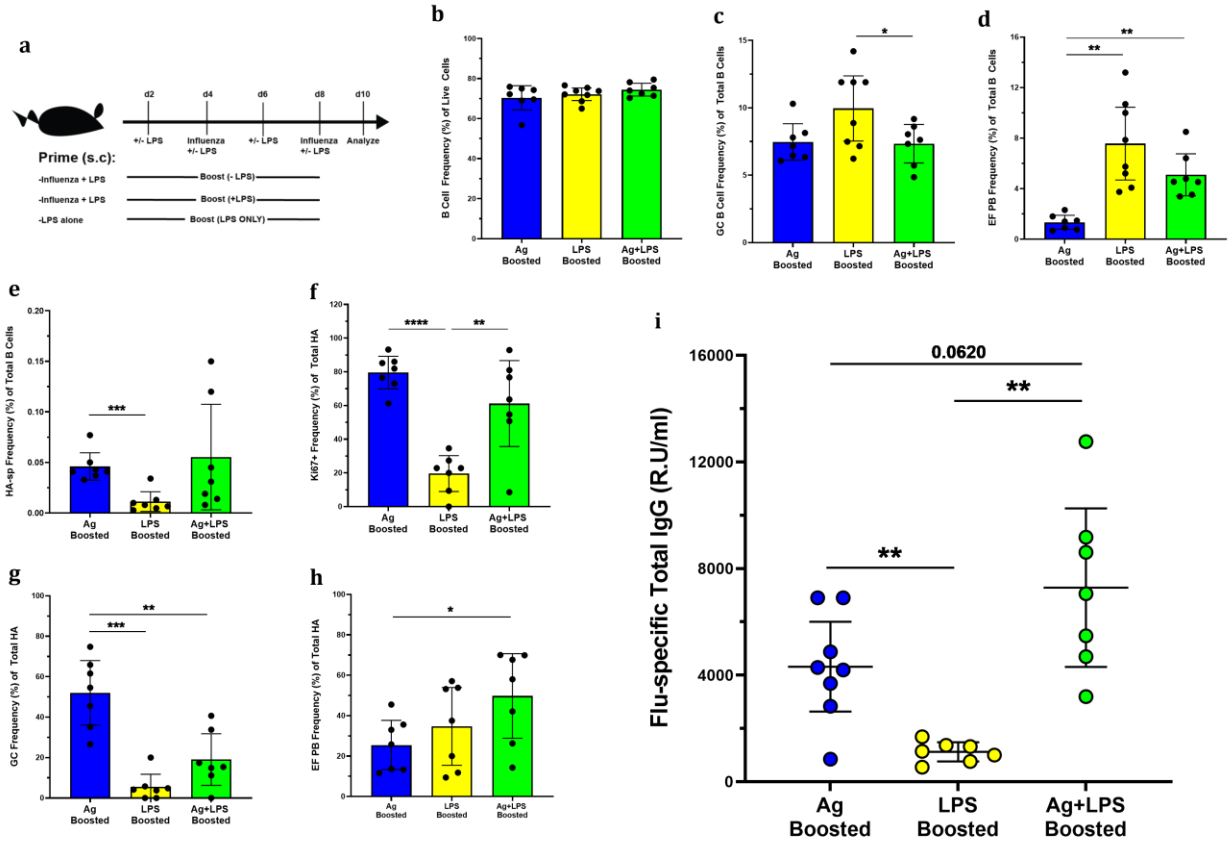

Figure 4.3. Repeated antigen exposure does not lead to biasing of HA-specific B cells towards EFR over GCs, still requires LPS to favor EFR formation. (a) Experimental outline. Mice were primed s.c. with influenza in alum and LPS, then boosted with either influenza alone (Ag Boosted) or with Influenza and LPS (Ag+LPS Boosted) on days specified. An additional group of mice was primed and boosted with LPS only (LPS Boosted). **(b-d**) Frequencies of total B cells (b), GC B cells (c), and EF PBs (d) in draining LN. **(e-g)** Frequencies of total HA B cells (e), Ki67+ HA B cells (f), GC HA B cells (g), and EF PB HAs (h). **(i**) Relative concentrations of anti-influenza serum IgG at d10 from each group. Graphs are representative of two experiments (n>/=7). Error bars represent 95% CI. Statistical significance determined by one-way ANOVA and unpaired Student's t-test with Welch's correction. *: p<0.05, **: p<0.01 ***: p<0.001, ****: p<0.0001.

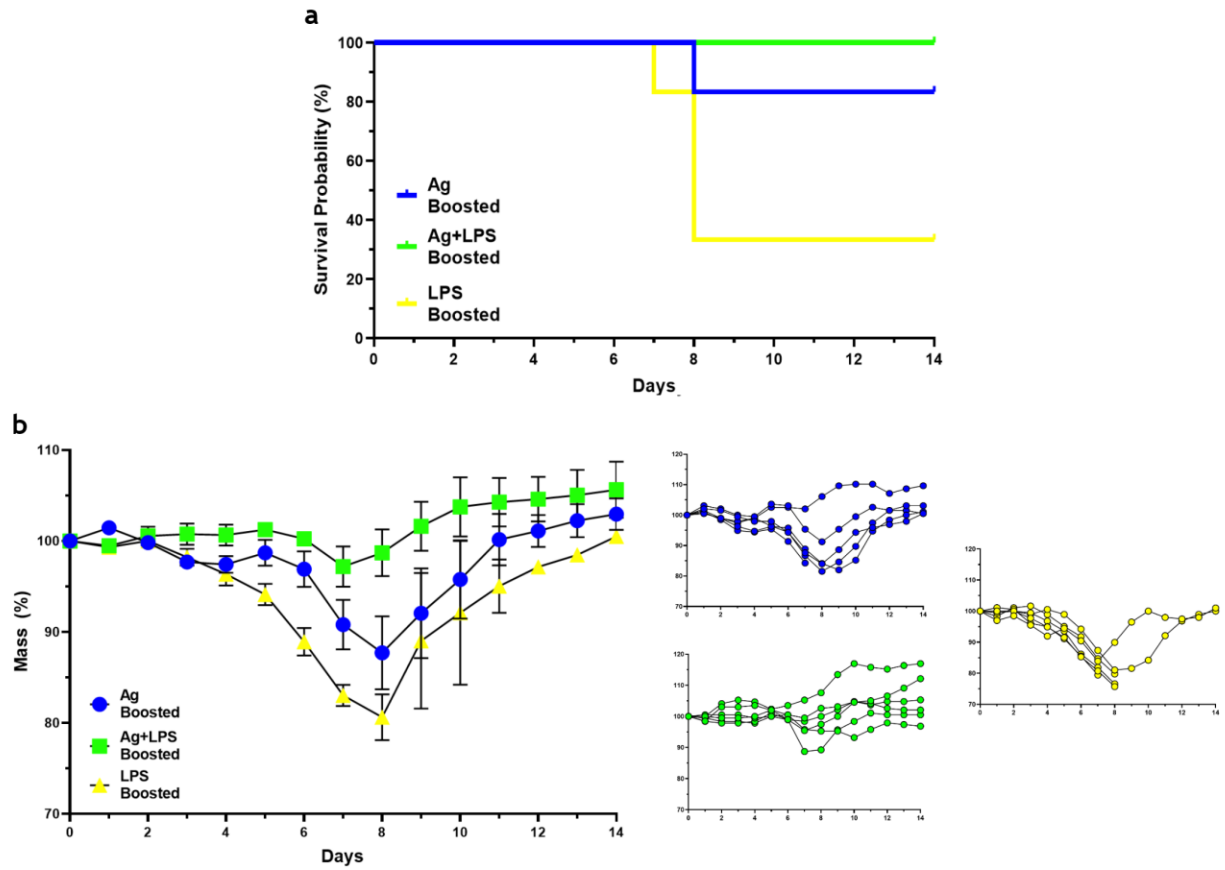

Figure 4.4. Addition of LPS to antigen boosting enhances antibody-mediated protection over boosting with antigen alone. Pooled serum from influenza-infected Ag Boosted, LPS Boosted, and Ag+LPS Boosted mice at 10 dpi was transferred to C57BL/6 mice (n=6) prior to infection with a lethal dose (100 PFU) of influenza A/PR8 the next day. **(a-b)** Survival (a) and change in body mass on average (b, left) and individually (b, right) by group over the course of infection. Error bars represent SEM.

Chapter 5

Conclusion

The current paradigm for antibody-mediated immunity generalizes it as a memory response, a ubiquitously dispersed, prevention modality that only offers protection from previously encountered pathogens or immunogenic agents. This is the basis for vaccines, where pathogen encounters are in essence simulated to allow for memory B cells and antibodies to be generated well before an actual infection. Such an approach limits antibody production to effectors of the germinal center (GC) response, only one of the three major B cell responses that collectively are multifaceted in their physiology, timing, and function. However, pathogens rapidly evolve to propogate, evade, and suppress, ever challenging host immune systems to the never ending Red Queen's Race. Additionally, disease can come not just from foreign agents, but from the host itself, as normally quiesencent cells can become tumorogenic and, even worse, metastatic, wreaking havoc on a tissue's ability to maintain homeostasis unless kept in check by a surveiling immune response. Thus, interrogation of these under-utilized B cell responses, which take part not only in immunological memory but are active also during primary challenges, is important to elucidate potential avenues for development of improved prophylatics and clinical treatments.

One less-considered B cell response come from B-1 cells, which are covered briefly in Chapter 1. Their importance lies in their innate-like triggers for non-specific, but functionally important, antibody secretion early during infection. Another population of B cells responding during primary infection are follicular B cell-derived plasmablasts formed during the extrafollicular B cell response (EFR). The studies conducted in this dissertation demonstrate that EFRs are a fully independent B cell response that offers generation of more rapid, protective antibodies. Firstly, data in Chapter 2 demonstrated that antigen-specific B cells can take a direct route to becoming an antibody-secreting cell (ASC), as the formation of actively secreting, hemagglutinin-specific ASCs during influenza infection did not require GC formation. While low levels of somatic hypermutation have been observed during long-lived EFRs (1), this is most likely a stochastic occurrence due to sustained, but not maximal, expression of IRF4

and abundant rounds of proliferation, leading to colateral expression of the IRF4-regulated activation-induced deaminase (AID) (2). Rather, the lack of requirement for GCs in EFR development comes from germline-encoded specificity. After all, T cells do not require affinity maturation of their antigen receptors to become effectors or memory cells, making the idea that all B cells absolutely require affinity maturation through GCs to properly neutralize pathogen or confer protection a puzzling notion.

Indeed, it has been demonstrated that high affinity interactions between the BCR and its cognate antigen leads to an effector fate, while lower affinity interactions confers a predispositon for the GC (3,4). This begs the question as to why GC responses dominate during non-infectious antigen delivery, i.e immunization. After all, if affinity drives polarization towards an ASC, i.e. an effector fate, then the presence of antigen alone, assuming optimal delivery, stability, etc., should result in an appreciable expansion of high affinity clones into the EFR. However, the data presented here has shown how inflammation plays a critical role in priming of the adaptive immune response, and more specifically the B cell response, in kinetics, magnitude and quality. This is articulated both in an intrinsic manner, where B cells require a functional Toll-like receptor (TLR) signaling axis either through MyD88/TRIF or TLR2/4/Unc93b for optimal activation of the NF-kB c-Rel:IRF4 pathway (**Fig 5.**1**, top**), or an extrinsic manner, where TLR-mediated inflammation drives expansion of antigen-specific B cells into the EFR over the GC (**Fig. 5.1, bottom**).

Chapter 3 addressed the intrinsic aspect of TLR requirments in B cells for functional antigen-mediated activation. TLR-null B cells showed significant reductions in viability *in vitro* in a B cell receptor (BCR) stimulation dose-dependent manner compared to wild type B cells, suggesting that the BCR signals through the TLR axis to upregulate genes important for survival. This can also be said for the induction of cell cycle entry, as TLR-null B cells did not proliferate in response to BCR stimulation, even in the presence of B cell help factors CD40L and BAFF. While many readouts of BCR signaling appeared nominal in TLR-null B cells,

nuclear localization of NF-kB c-Rel was partially compromised, and was severely reduced in expression, along with its target, IRF4, relative to wild type B cells.

Given these severe *in vitro* defects , it would be expected that EFR are completely abrogated in TLR-null mice, since this population is highly proliferative. While there were significant reductions in EFR survival, kinetics, and the protectice capacity of the antibodies they produced, EFRs not only persisted but were of greater magnitude at later timepoints in MyD88/TRIF double knockout mice. These findings indicate that there are signaling axes capable of compensating for TLR signaling either singularly or in aggregate, and thus that EFRs can be formed under many different inflammatory conditions caused by distinct pathogen challenges or tissue environments. As shown here, EFRs against influenza virus infection generated IgGs of multiple isotypes. IgG2c dominated the response, but other isotypes were present nonetheless, indicating switching of these effectors within discrete inflammatory niches of the mediastinal lymph node. It would thus be of interest to gauge what, if any, effector cytokines are secreted by EFR-plasmablasts of differing IgG isotypes. This would allow further inference of the local inflammatory milieu and, given its significant size and presence during primary influenza infection, assessment of the possible contribution of the EFR to the cytokine network as a whole.

While EFR appear to form and expand under a variety of inflammatory cues, they cannot overcome an outright dearth of inflammation, as seen during immunization. As demonstrated in Chapter 4, even repeated antigen exposure, which allowed for increased occurrences of antigen:BCR interactions by previously primed B cells, antigen-specific EFRs were several-fold smaller than antigen-specific GC populations. Only when the TLR4 agonist LPS was continusouly administered over the immunization timecourse, both in the single and the repeat antigen-inoculation models, did antigen-specific B cells favor EFRs over GCs. Given the affinity model for plasma cell generation, it is unlikely that high-affinity clones found within EFRs after LPS boosting are participating in GC responses, unless a lack of LPS somehow leads to lower

avidity interactions of intrinsically high-affinity B cells, which must be considered unlikely. Rather, LPS boosting likely allowed for entry of the highest affinity clones into the EFR, along with clones normally destined for the GC in the absence of LPS-mediated inflammation. This would coincide with clones normally of too weak affinity to enter the GC under LPS-boosted conditions. In this way, sustained TLR stimulation not only increases antigen-specific antibody levels overall, but would drastically expand the antigen-specific repertoire. This would be incredibly advantageous for generating antibodies against cross-reactive or non-mutating viral epitopes, which are generally non-immunogenic.

The studies presented here were extensive, yet there are still important questions remaining. While specific iniatiators of EFRs were identified and modeled, what happens to EFR-associated plasmablasts after virus clearance is unclear. Presumably, EF niches collapse without the inflammatory signals necessary to sustain them, leading to a withdrawal of B cell help factors and subsequent plasmablast death. However, ASCs of the C12 idiotype, BCRs identified by specific VDJ recombinations, that are found within EFRs but not GCs after primary influenza challenge, could be found in the lungs 14 days post-infection (5). This indciates that either EFR-plasmablasts may migrate out of the mediastinal lymph nodes and become resident in peripheral tissues, or that EFRs are formed within the lungs after virus clearance. Further work to elucidate the source of these plasmablasts would expand our understanding of both EFR dynamics and the process of establishing tissue-resident B cells.

As alluded to above, generation of EFRs may overall expand the antibody repertoire against a given antigen or pathogen, presumbly through enhancing weak, BCR-mediated IRF4 upreglation through TLR signaling, or through expanding EFR niches to allow for expansion of plasmablasts specific for non-immunodominant epitopes. This would suggest that EFR-derived antibodies may improve crossreactivity against multiple virus strains. Passive protection studies in a heterosubtypic model of influenza infection would therefore be informative in assessing EFR-derived antibodies' cross-protective potential.

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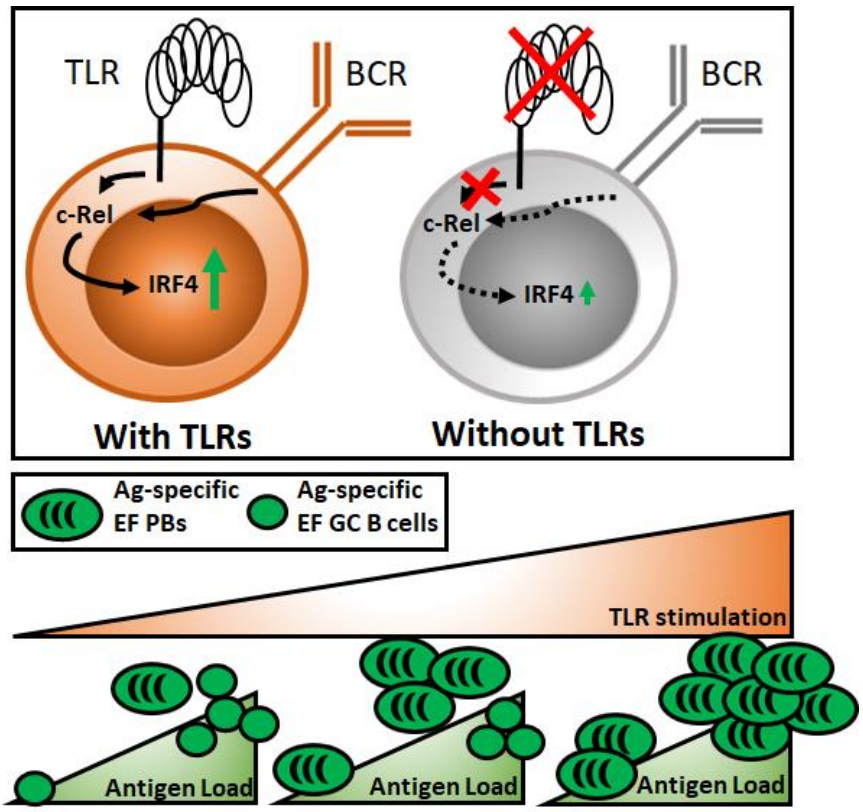

Figure 5.1. Model of TLR-mediated induction of B cell differentiation and EFRs. (Top) With a functional TLR axis either through MyD88/TRIF or TLR2/4/unc93b, BCR stimulation leads to efficient c-Rel nuclear localization and sustained upregulation of IRF4. Without a functional TLR axis, BCR signaling leads to delayed c-Rel localization, lack of long-term c-Rel expression, and a lack of sustained IRF4 induction and upregulation. (Bottom) During infection or immunization, TLR agonism or an equivalent aggregate of inflammatory stimuli polarizes antigen-specific B cells towards EFR and away from the GC response when antigen is limiting or in excess, resulting in early and protective antigen-specific antibody generation.