Published in final edited form as:

Immunol Rev. 2019 March ; 288(1): 136–148. doi:10.1111/imr.12741.

Extrafollicular responses in humans and SLE

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Summary

Chronic autoimmune diseases, and in particular Systemic Lupus Erythematosus (SLE), are endowed with a long-standing autoreactive B cell compartment that is presumed to reactivate periodically leading to the generation of new bursts of pathogenic antibody-secreting cells (ASC). Moreover, pathogenic autoantibodies are typically characterized by a high load of somatic hypermutation and in some cases are highly stable even in the context of prolonged B cell depletion. Long-lived, highly mutated antibodies are typically generated through T cell-dependent germinal center (GC) reactions. Accordingly, an important role for GC reactions in the generation of pathogenic autoreactivity has been postulated in SLE. Nevertheless, pathogenic autoantibodies and autoimmune disease can be generated through B cell extrafollicular (EF) reactions in multiple mouse models and human SLE flares are characterized by the expansion of naïve-derived activated effector B cells of extrafollicular phenotype. In this review, we will discuss the properties of the EF B cell pathway, its relationship to other effector B cell populations, its role in autoimmune diseases and its contribution to human SLE. Further, we discuss the relationship of EF B cells with Age-Associated B cells (ABCs), a TLR-7-driven B cell population that mediates murine autoimmune and anti-viral responses.

Keywords

extrafollicular; B cells; DN2; ABC; SLE; plasma cells

The initiation of the B cell response at the T-B border

Naïve B cells, similar to naïve T cells, routinely traffic between secondary lymphoid organs (SLOs) in an attempt to maximize clonal exposure to diverse antigen sets from various anatomical geographies. Upon arrival in an SLO, B cells rapidly upregulate the chemokine

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Conflict of Interest Statement

The authors declare no conflicts of interest.

receptor CXCR5, which drives them into the B cell follicles via a CXCL13 gradient maintained by the stroma(1–3). Through various mechanisms(4), soluble antigen is deposited and maintained on the underlying stromal network(5) where it can be encountered by individual B cell clones on their ‘random walk’ through the follicle. B cells that find their cognate antigen rapidly upregulate CD69 – an inhibitor of the lymphoid egress, mediating receptor S1P1 and resulting in SLO retention(6). With similar kinetics, activated B cells upregulate the Epstein–Barr virus-induced molecule 2 (EBI2) resulting in trafficking along oxysterol gradients to the outer-follicular region near the T-B border. The slower upregulation of CCR7 on the cell surface, alongside the continued expression of CXCR5 pushes activated B cells the final distance to the T-B border to await T cell help(7).

While B cell responses develop in the follicle, dendritic cells (DCs), and other innate populations are tasked with antigen capture, processing, and presentation in the paracortical region. Having migrated from peripheral tissue, or having captured antigen directly within the lymphoid organ itself, DCs interact with naïve T cells initiating cell-mediated immunity. Similar to the B cell compartment, a fraction of activated T cells will change their chemokine expression profile resulting in directional migration. Through the reduction in CCR7 expression, and increase in CXCR5, Th cells are coaxed from the paracortical region to the T-B border where they will provide help for the ensuing humoral response(8). Interestingly, recent studies have suggested that other signals may fine-tune these migration patterns such that antigen, DCs, T cells, and B cells are efficiently localized to interact in common microenvironments within hours of antigen arrival(9).

Although interactions at the T-B border are often characterized as a singular event, migration from the initial T-B interaction site to the final differentiation site takes place in a two-step process. First, CCR7 is slowly eliminated from the B cell surface resulting in an EBI2 and CXCR5-driven relocation to the interfollicular region(10). In contrast to the fleeting T-B interactions at the T-B border, interactions in the interfollicular region are longer lasting. It remains unclear if terminal differentiation decisions are made prior to this phase, however it is now evident that the differentiation between germinal center, and extrafollicular-destined B cells can be observed in this phase of the activation process through their expression of EBI2 (Figure 1). A loss of EBI2 at the interfollicular region suggests that the activated B cell has been selected for GC incorporation, while a persistence of the receptor indicates integration into the extrafollicular(EF) pathway (11). In addition to the EBI2 loss, cells selected for GC incorporation undergo several rounds of rapid division before migrating back into the follicle to participate in the ensuing GC response(12). Recent work has highlighted the role that restricted T cell access has on the entry of activated B cells into the growing germinal center response at this stage, and selection of individual clones for incorporation seems dependent on (at least) receptor affinity and relative access to antigen(13).

Germinal Center Response

The germinal center response is often described as a microcosm of Darwinian natural selection. In the GC environment B cells compete for limited survival resources, and through rapid rounds of division, mutation, and selection, those with the highest fitness (ie. BCR

affinity) emerge as the foundation of the ensuing humoral immune response. Cells selected for germinal center inclusion at the T-B border, having already gone through a round of division, are guided deep into the follicle through the lost expression of EBI2 where they cluster and form the nascent GC. The division/mutation and selection processes are carried out in distinct anatomical regions of the GC termed the 'dark' and 'light' (LZ) zones. Depending on the strength of danger signaling, B cells cycle between the two zones from a few days to several months(14) through a chemokine-dependent trafficking process described in detail elsewhere(15).

The source of receptor diversity of the GC reaction comes both from a diverse naïve repertoire as well as the accumulation of receptor mutations by cells dividing in the dark zone due to the high expression of activation-induced cytidine deaminase (AID) and the error-prone DNA polymerase 'eta'(16). Recently, it has become clear that the number of divisions (and thus the number of accumulated mutations) that an individual clone will undergo in any given cycle is directly defined by the amount of T cell help acquired either at the T-B border, or in the LZ (17). Upon completion of the division/mutation program, the resulting mutated and expanded clones are redirected into the light zone for selection.

Perhaps counter-intuitively, the role of direct BCR signaling through antigen binding in the selection process remains slightly unclear while the acquisition of T cell help has emerged as the dominant force in clonal selection. The last decade has identified CXCR5+ BCL-6+ PD-1+ T follicular helper cells (Tfh cells) as the major mediators GC B cell response, providing key cytokines and survival signals ultimately contributing to multiple facets of the GC response including cell survival, isotype switching, and division. While interactions between LZ GC B cells and Tfh cells are usually short in duration, feed-forward signaling through CD40L and ICOSL by T and B cells, respectively, leads to cellular entanglement where LZ GC B cells gain access to high levels of the pro-survival factor BAFF, as well as helper cytokines IL-4 and IL-21(18). Interestingly, the level of peptide-MHC presented on the B cell surface (particularly in relation to other B cells in the local environment) has been shown to dictate the quantity of T cell help received by LZ B cells, and likely helps drive the LZ selection process(19). Ultimately, through receptor engagement and T cell help, GC B cells are directed either back into the dark zone for further rounds of division/SHM, exit the GC to as memory or plasma cells, or die due to a failure to acquire sufficient pro-survival signaling.

Extra-follicular Response

T-Independent Responses

While the germinal center has remained the focus of humoral immune research for decades, it has long been understood that the GC reaction is not the sole contributor to humoral immunity. Early work identified classes of highly repetitive antigens, T-Independent 2 (TI-2) antigens that resulted in rapid antibody secretion even in the absence of T cell help(20, 21). TI-2 antigens, often highly repetitive polysaccharides, were extensively described as B cell responses occurring outside of the follicle (extrafollicular) resulting in prompt and robust primary responses, and the potential for long term immunity(22). Despite the importance of this humoral arm, true B cell memory often fails to develop in responses to TI-2 antigens

often resulting in a lack of interest from the field and the relegation of extrafollicular responses to the fringes of rheumatology and vaccinology. This view of EF responses, however, fails to capture their complexity and results in an incomplete picture of humoral immune progression.

Although most early work focused on TI-2 responses exhibited failure to develop B cell memory, it has been known for decades that memory can be elicited from TI-2 antigens such as dextrans (23) or LPS(24). More recent work has confirmed these findings definitively, identifying a distinct T-I B cell memory pool that expands upon antigen prime, and is carefully regulated through antigen-specific IgG feedback(25). These findings, in addition to the more recent understanding that even T-dependent antigen responses can occur in extrafollicular space (described later), demand that more attention be paid to the non-GC responses that almost certainly play a critical role in humoral immunity in both mice and men.

T-Dependent Responses

The interactions between T cells and B cells following initial antigen stimulation seem critical in determining the fate of an individual B cell clone. Classically, integration into GC or EF responses has been largely considered a question of affinity(26), although more recent work has suggested that T-cell interactions (in T-dependent responses) and innate-sensor signaling also play a significant role. While BCL-6+ PD-1+ Tfh cells are indispensable for GC responses (see above), it has become clear that an early population of BCL-6+ PD-1- T cells arrive at the T-B border within hours of antigen stimulation, and are critical for the establishment of a T-dependent EF response(27). Through the production of IL-21, Lee et al. have demonstrated that these pre-GC Tfh cells are critical for the class-switch recombination that occurs early in blasting EF B cells(27) in SLOs, although this may not be true for all TI-2 antigens. Outside of SLOs, peripheral helper T cells have been recently characterized as supporting EF responses in inflamed tissues such as those present in rheumatoid arthritis(28).

Unlike GC B cells, B cells fated to the extrafollicular pathway maintain their EBI2 expression and increase expression of the chemokine receptor CXCR4(7, 29, 30). This combination of receptor expression drives B cells out of the follicle environment, ultimately localizing in the bridging channels of the spleen, or the medullary cords of the lymph nodes as early as 24hours following activation. In the spleen, this localization places emerging extrafollicular responses in the vicinity of marginal-zone resident dendritic cells, shown to help drive T-dependent extrafollicular response by efficiently priming B cells for antigen presentation(31). In these environments, activated B cells differentiate directly into blasting B cells, and eventually into antibody-secreting B plasmablasts – bypassing the traditional GC(32). They are class-switch recombination competent, are capable of sustaining long-term response under some conditions, and have been shown to be critical in the early control of infection prior to the establishment of the ‘traditional’ humoral response(25, 33). It is also worth noting that this extrafollicular localization places these emerging responses in close proximity to the secondary lymphoid organ vasculature, perhaps providing a streamlined avenue for efficient contribution to the systemic antibody response(34).

Another hallmark of extrafollicular responses is the ubiquitous upregulation of the plasma cell differentiation factor BLIMP-1. While germinal center B cells may eventually express BLIMP-1 as they exit the germinal center as plasma cells, extrafollicular B cells can be seen upregulating the transcription factor within 18h of initial stimulation, resulting in a direct suppression of the B cell transcription factor PAX5 which is required for germinal center formation(35). In addition to repressing a GC phenotype, expression of BLIMP-1 has now been shown to be crucial for antibody production, and the regulation of the unfolded protein response(36). In this way, cells that have been fated to the extrafollicular pathway can be viewed as having locked in a plasma-cell fate within hours of antigen arrival, and B-cell stimulation. Although the plasma cells derived from this pathway are considered to be low affinity (due to a lack of SHM and selection) and short-lived, recent study into the pathways leading to extrafollicular response, and our own work in human B cells suggest that this may not always be the case. In fact, contribution of this pathway into human disease may help account for the long-lived plasma cell responses commonly seen in systemic lupus erythematosus (SLE) and lupus nephritis. In subsequent sections, we will dissect the variety of circumstances under which these extrafollicular responses are generated and the role of these responses in the control, or propagation of human disease.

Extrafollicular B cells in protective and pathogenic responses

Certain murine infection models have decisively shown that particular bacteria have developed the ability to modulate GC response kinetics and induce an extrafollicularly dominated B cell response (37–39). This phenomenon is perhaps best illustrated by *Salmonella typhimurium* infection which in addition to a robust and persistent (~5 weeks) EF plasmablast responses, also induces significantly delayed GC development (33). On the surface, these findings would suggest a persistent, non-specific expansion due to pathogen-associated molecular pattern recognition (e.g. TLR activation), yet the response was determined to be T-cell dependent; focused on outer membrane bacterial proteins; and highly class switched. Even though GC responses did eventually arise when the bacterial load was low, and high affinity antibodies eventually developed, CD154/CD40L-deficient mice were shown to control the infection as well as wild-type suggesting no reliance on GCs. Importantly, this model demonstrated that somatic hypermutation and affinity maturation can be also achieved through EF responses (37). Delayed and diminished GC responses/kinetics with a powerful and critical early EF response are also present in other bacterial models of *Borrelia burgdorferi* and *Ehrlichia muris* (38, 39).

Certain parasitic protozoan infections also present with strong EF responses responsible for overwhelming plasmacytosis within secondary lymphoid tissues as illustrated by murine *Trypanosoma cruzi* (Chagas' disease) infections (40). A similar extended plasmablast expansion is present in severe canine infections of *Leishmania infantum* (41).

Additionally, viruses also have lessons to teach in regards to EF B cell responses. In the context of retroviral infection, EF responses promote complex outcomes of potential significance for disease pathogenesis. Within a mouse mammary tumor virus (MMTV) model, early antigen acquisition by B cells and EF plasmablasts are key mediators of retroviral dissemination throughout both lymphoid and non-lymphoid tissues (42) and

indicate a deleterious effect of the EF arm within the context of anti-retroviral B cell responses. Non-retrovirus viral infections can also contribute interesting avenues for the pathogenic skewing of B cell activation towards an EF fate. Thus, human herpesvirus-8/HHV-8, especially in the context of HIV co-infection, has been shown to cause plasmablastic-multicentric Castleman's disease, a lymphoproliferative disorder characterized by the polyclonal production of extrafollicularly derived plasmablast-like B cells (43). Additionally, LMP1 and EBNA2 proteins derived from the Epstein-Barr virus have been shown to interfere with TCL1 and/or BCL6 expression, both representing key signaling molecules in the GC induction and maintenance pathway, fundamentally skewing responses to an EF default (44, 45). These observations are of particular interest for SLE given the proposed causal role of EBV and the recent demonstrations that EBNA2 and associated transcription factors occupy a substantial fraction of autoimmune risk loci associated with SLE and other human autoimmune diseases (46). Moreover, these studies identified B cells as a likely site of action of EBNA2.

Extrafollicular B cell reactions in SLE models

During the EF expansion of an early pathogen response, high affinity B cells situate along the T-B border and red pulp and begin to differentiate and proliferate through BCR mediated signaling (47, 48). T cell help can be given through Tfh-like cells with costimulatory activity through CD40L and ICOS, as well as IL-21 production, but in cases where BCL6 is eliminated (as well as GC responses) memory B cells still form suggesting Tfh help is not obligatory for EF memory B cell formation (27, 47–49). Additionally, class switch recombination and SHM are also engaged in EF responses which allows for further affinity maturation outside follicular responses (37).

As best illustrated by anti-DNA antibodies, pathogenic autoantibodies are typically class switched and display high levels of somatic hypermutation and affinity maturation (50). Accordingly, it has been assumed that such autoantibodies could only derive from GC reactions. This assumption however has been refuted by a number of studies initiated with the demonstration that autoreactive B cells and PB develop in the T cell areas in MRL.FAS^{lpr} mice (51) an important animal model for our understanding of EF activation in SLE. Within this genetic background, in mice expressing the AM14 transgenic rheumatoid factor (RF) almost all RF+ B cells concentrate within splenic areas devoid of follicular DC co-localization (52). Interestingly, these RF+ B cells co-localized with CD11c-high DCs, were shown to be strongly proliferative, and could efficiently produce antibody. Moreover, these autoreactive B cells experienced class switch and underwent significant SHM and clonal expansion outside the GC (53). Further characterization of this model revealed that although T cells were technically expendable for the activation of the EF RF+ response, the presence of T cell derived CD40L and IL-21 signals, but not ICOS, synergistically enhanced the EF RF+ blast formation (54). Nevertheless, SLE and Sjogren's-like autoimmune manifestations with expansion of EF autoreactive B cells also develop in T cell-deficient BAFF-transgenic mice (55).

Autoreactive B cell activation outside the GC has also been demonstrated for anti-DNA antibodies in the MRL/lpr background (56). Given however, that the *lpr* defect (CD95/Fas-

deficiency) is an uncommon/unknown causal feature of SLE (57) and more an accelerator of underlying autoimmunity, other studies have addressed the AM14-transgene function within murine lupus models more closely associated with human SLE based on genetic susceptibilities. Utilizing the B6.*Sle1.Sle2.Sle3* model EF responses were again shown to be the dominant pathway for blast formation in RF+ responses (58). However, this study shows in contrast that B6.AM14-transgenic control mice were not able to form RF+ antibody secreting cells, suggesting genetic background/susceptibility may be a critical player. A major role for EF reactions in the generation of pathogenic autoreactive B cells has also been established in other mouse models, including the NZB/W and graft-versus-host models of lupus-like autoimmunity (59, 60).

It is important to note that, concordant with human SLE factors, murine lupus can be induced by dysregulation of pathways known to contribute to EF responses. Thus, both BAFF and APRIL are highly linked to SLE pathogenesis and can lead to T cell independent class switching and AID expression (61, 62). Similarly, TLR signaling, predominantly through TLR9 and TLR7, is a strong additive signal for the induction of EF responses (61, 63, 64). CpG DNA stimulation can induce somatic hypermutation and class switching in a T cell independent fashion and is synergistic with BCR activation. These mechanisms are used by MRL/lpr and NZM congenic AM14-transgenic mice to induce powerful immune complex-mediated activation of B cells and anti-chromatin autoantibody production (54, 58, 65, 66). Moreover, TLR7 over-expression leads to or accelerates lupus-like disease in multiple mouse strains, and TLR7 gene copy number or polymorphisms are associated with susceptibility to human SLE (67–69). Importantly, over-expression of TLR7 on B cells leads to the extrafollicular expansion of autoreactive anti-RNA transitional B cells in an interferon (IFN)-independent fashion (70). These cells undergo class switch recombination and their TLR7-driven proliferation was linked to the expression of AID and T-bet.

Of note, the role of T-bet in the regulation of autoreactive B cells and autoimmune disease has gained prominence over the last decade and offers important lessons in the participation of EF reactions. Indeed, the T-bet transcription factor, initially recognized for its critical role in IFN γ -induced T_H1 fate commitment, is also responsible for IgG2a class switch recombination production and its over-expression may lead to the generation of pathogenic autoantibodies (71). Interestingly, T-bet can also be directly induced by TLR9 and TLR7 stimulation of B cells (72, 73), and TLR7 stimulation of transgenic B cells that recognize nucleic acids can break B cell tolerance of anergic B cells and induce lupus-like disease in a non-autoimmune background through the expansion of T-bet-expressing extrafollicular B cells (74). More recent studies have defined the expression of T-bet on a particular subset of B cells that accumulate with age in normal mice (Age-Associated B cells or ABCs) but are prematurely expanded in autoimmune mice (75, 76). These studies have established a critical role for TLR7 stimulation of ABCs in viral clearance as well as in the induction of autoimmune disease. Interestingly, autoimmune ABCs accumulate outside the B cell follicle at the T-B border (77) and extrafollicular accumulation of ABC has also been documented in mice deficient of the SWEF family members SWAP-70 and DEF6 (the latter representing a human risk allelic variant for SLE) (78, 79). In this model, SWEF double knockout female mice develop a lupus like autoimmune disease characterized by an expansion of IgG2c autoantibody-producing CD11c high, T-bet high B cells. It is important to note however that

TLR7-induced ABCs can also contribute to autoantibody generation and clinical autoimmunity through the formation of GC in a B cell-intrinsic T-bet-dependent fashion (76). Other studies however suggest that the defining ABC transcription factor T-bet may not be required for the generation of autoimmune GC induced through IFN γ stimulation, a process that instead seems to depend of the B cell-intrinsic expression of BCL6 (80). In contrast, in the SWEF-deficient model, DEF6 and SWAP-70 inhibit the differentiation of CD11c high cells by preventing IL-21 driven targeting of IRF5 and subsequently T-bet to a subset of genes highly expressed in CD11c high cells (79). Importantly, reduced IRF5 expression both eliminated the expansion of CD11c high cells and ameliorated autoimmune disease by reducing autoantibodies and kidney damage. Both *DEF6* and *IRF5* have lupus risk variants and a similar dysregulation of CD11c high B cell development, and may be partly responsible for the expansion of pathogenic effector B cells in human SLE as further discussed below.

Additional insight into the participation of the EF response in systemic autoimmunity and into B cell intrinsic control mechanisms are provided by highly informative studies of Ets1, a transcription factor that negatively regulates PC differentiation by interfering with BLIMP1 activity (81). Interestingly, Ets1, whose hypomorphic variants confer increased risk for human SLE, has been shown to downregulate hyperresponsiveness and plasma cell differentiation in response to TLR9 stimulations, and its deficiency leads to systemic autoimmunity in mice (67–69). Mechanistically, Ets1 levels sufficient to prevent plasma cell differentiation in response to BCR or TLR stimulation are maintained through the Lyn-CD22 inhibitory pathway (82). In keeping with its regulatory function, Ets1 activity has been shown to be required to maintain B cell tolerance and its deficiency leads to autoimmune disease through the accumulation of autoreactive extrafollicular plasmablasts (83).

Characterization of human extra-follicular B cell responses

As previously discussed, an EF response could be operationally defined as the early phase of naïve B cell activation leading to the generation of short-lived PB outside the B cell follicles. Activated naïve B cells can generate germinal center reactions within the B cell follicle leading to the eventual formation of long-lived memory cells and PC. Whether GC are formed by the same activated naïve B cells that contribute to the EF response, possibly through asymmetric division (84) remains unknown. From an experimental standpoint, assessing the participation of EF responses in human SLE and human immune responses in general has been limited by lack of identifying markers for the cellular components of this pathway. In turn, this limitation stems at least in part from lack of access to lymphoid tissue in the study of SLE or more in general in subjects experiencing an acute immune response, in the setting which extrafollicular responses would be expected to occur. Nevertheless, tissue studies offer some insights into the nature of EF B cells. Thus, monocytoid (85) and tissue-based atypical memory B cells (86, 87) accumulate within the subepithelial regions of human tonsils and lymph nodes. Monocytoid B cells are CD19+ cells that morphologically resemble monocytes, and are found adjacent to sinusoidal spaces in lymph nodes during lymphadenitis, often during HIV or toxoplasma infection. These cells lack expression of memory (CD27) and germinal center (BCL6) markers, and several lines of evidence suggest

that monocytoïd B cells may be undergoing extrafollicular activation and differentiation which include: active proliferation, expression of AID/*AICDA* and the presence of a substantial population of class switched cells that express mutated IgG transcripts that appeared to be locally generated (85, 88). These observations support the idea of ongoing class switching and SHM outside of follicles, a concept firmly established in elegant studies of the human tonsil demonstrating that abundant B cells, characteristic of the crypt epithelium, express AID and undergo class switch recombination to IgG and IgA upon stimulation from BAFF and IL-10 produced by epithelial cells activated through TLR stimulation by bacterial products (89). Importantly, AID induction and class-switch recombination can also be induced through innate stimulation by BAFF/BLyS and APRIL produced by DC independently of CD40 (62).

Notwithstanding its obvious limitations, as further discussed below, the peripheral blood is a highly informative compartment for the understanding of human B cell heterogeneity and homeostasis. In steady-state, blood studies identify a small fraction of circulating transitional cells and a predominance of resting naïve B cells together with a variable frequency of different types of CD27+ memory cells and low numbers of circulating PB. The systemic nature of SLE, a chronic autoimmune disease characterized by acute exacerbations punctuated by large expansions of circulating PB, provides a unique opportunity to identify B cell populations that participate in acute immune responses as well as activated memory compartments (90–93). Indeed, blood studies in SLE have proven highly informative for this purpose and have provided critical information for our understanding of the human EF activation pathway and its participation in SLE. Experimentally, these goals were accomplished by taking advantage of a comprehensive B cell Immunomics approach to: 1) Identify B cell subsets abnormally expanded during acute SLE flares; and 2) to establish a developmental link between B cell subsets of interest through deep sequencing of the BCR repertoire and integrated transcriptional and epigenetic analysis (94–96).

Following this approach, we first demonstrated that acute SLE flares are characterized by the expansion of a new population of activated naïve cells (aNAV) characterized by a IgD+ CD27- Mitotracker Green+ phenotype and downregulation of CD21 and CD23 (94). Moreover, using a combination of Next Generation Sequencing of the BCR repertoire and proteomics characterization of serum autoantibodies, we demonstrated that aNAV cells contribute to a major fraction of circulating ASC. These naïve-derived ASC in turn produced some of the dominant serum autoantibodies, a feature confirmed not only by quantitative proteomics of affinity-purified autoantibodies but also by single cells analysis of ASC. Additionally, the generation of monoclonal antibodies demonstrated that naïve-derived, unmutated antibodies could display substantial SLE-associated autoreactivity. Combined, our findings indicated that direct differentiation of naïve B cells could generate autoreactive ASC without the need for GC-dependent SHM.

More recently, we have expanded the characterization of the cellular components of the human EF B cell pathway through the identification of a TLR7-induced B cell effector population that derives from aNAV cells and is poised to differentiate into ASC in active SLE patients (96). These effector cells have the class switched IgD-, CD27- double negative (DN) phenotype we had previously shown to be displayed by a minority of B cells in healthy

individuals but a much larger fraction in patients with active SLE (97). DN B cells can be split into two major fractions: DN1 cells, the dominant fraction in HC, with a CXCR5+, CD21+ phenotype; and DN2 cells, the dominant fraction in active SLE, with a CXCR5-, CD21- phenotype. DN2 cells are also characterized by high expression of CD11c and T-bet, two central markers of murine ABCs.

The combination of BCR deep sequencing and molecular analysis established substantial clonal relationships between aNAV, DN2 and ASC and demonstrated close transcriptional similarity between aNAV and DN2 with only 42 differentially expressed genes (DEG) between these two populations. In keeping with this transcriptional similarity, with the exception of IgD, aNAV and DN2 cells share virtually all defining surface markers (including downregulation of CXCR5, CD21 and CD23 and high levels of CD19, CD11c, FcRL5, SLAMF7, CD22 and PD-1), and critical transcription factors (TF) including high levels of T-BET. In contrast, while only 20 DEG separated DN1 and CD27+ class-switched memory cells (SWM), over 700 DEG separate DN2 from resting naïve cells, DN1 and SWM cells. From a functional standpoint, DN2 cells both in healthy subjects and SLE display a transcriptional landscape and functional properties consistent with an effector population poised to differentiate into ASC with little cell division upon stimulation through TLR7 and IL-21 (data not shown). Of note, robust DN2 generation and ASC differentiation can be induced from resting naïve B cells and in a more vigorous fashion from aNAV cells.

The transcriptional landscape of DN2 and aNAV is distinctive from naïve and memory B cells owing to their expression of high levels of the key T_H1 transcription factor *TBX21*/T-bet which is necessary for effector function in both IFN γ producing CD4+ T cells (98) and cytotoxic CD8+ T cells. At least in the mouse, B cell intrinsic T-bet expression is also necessary for the production of IgG2A and effective anti-viral humoral immunity (99, 100). DN2 cells also express high amounts of *ZEB2*, a TF that acts cooperatively with T-bet to promote effector differentiation and inhibit memory differentiation in cytotoxic T cells (101, 102). Importantly, despite sharing IL-21 receptor expression with naïve cells, only naïve cells express high levels of several repressors of effector differentiation including *BACH2* which can directly repress *PRDM1*/BLIMP1 (103), and *FOXO1* which inhibits T-bet mediated effector function in CD8 T cells (104). In distinction to DN2 cells, SWM and DN1 express high levels of TCF7 a TF that is repressed by T-bet/Zeb2, promotes central memory differentiation and inhibits effector differentiation (102). In addition to the T-Bet/Zeb2-regulated network, DN2 cells are characterized by high levels of expression of IRF4 and IRF4-regulated genes, in particular of IRF4 target genes enriched in plasma cells. The balance of IRF4 and IRF8 determines bifurcating B cell fates with high IRF8:IRF4 ratios promoting germinal center differentiation and high IRF4:IRF8 ratios favoring plasma cell differentiation (105). Consistent with their commitment to an effector plasma cell fate, DN2 and aNAV cells have the lowest IRF8/IRF4 ratio of any B cell subset. In addition, these two populations express the lowest levels of *ETS1*, which as stated above is a TF that inhibits PC differentiation (68) and whose hypomorphic alleles are associated with increased SLE risk (69). As previously discussed, *Ets1* deficiency induces lupus-like disease in mice through the extrafollicular accumulation of autoreactive plasma cells. Interestingly, in contrast to the GC fate induced by the association of IRF4 with *Ets1* through the TF PU-1 and SpiB, high IRF4 levels in an *Ets1*-independent fashion shifts IRF4 genomic targeting to lower affinity

Interferon Sequence Responsive Elements (ISRE) motifs that are enriched in genes involved in plasma cell differentiation (106). Consistent with the known kinetics of the EF response, Ets1-independent IRF4 targeting which would be predicted by the transcriptional profile of DN2 cells, has been shown to induce preferential differentiation into short-lived plasmablast (106).

Integration of extrafollicular B cells into current human B cell classifications. Relationship to Age-Associated B cells (ABC) and other T-bet⁺ B cells

Conventional classifications of human peripheral B cells are based on the ability of a few surface markers to discriminate transitional, naïve, memory and ASC (both plasmablasts and plasma cells) among CD19⁺ cells. The combination of these markers, typically comprising IgD, IgM, CD27, CD38 and CD24, also allow discrimination of several CD27⁺ memory populations including: IgD⁺/IgM⁺ unswitched cells, IgM-only pre-switch cells, class-switched cells predominantly comprised of IgG⁺ or IgA⁺ cells, and very small fractions of IgD-only and IgE⁺ cells. In steady-state healthy subjects, the majority of memory cells display a resting phenotype with expression of CD21 and absence of conventional activation markers such as CD80, CD86 and CD95. In contrast, in the context of persistent antigenic stimulation in either active SLE or chronic infections like HIV or malaria, the peripheral blood contains a substantially enlarged fraction of activated memory cells characterized by loss of CD21 and upregulation of CD80/CD86 and/or CD95 (91, 107, 108). Cycling, antigen-specific activated memory cells marked by upregulation of CD71 can also be observed during the early response to vaccination (109). Finally, chronic HIV (107) and malaria (108) infections are characterized by large expansions of so-called atypical memory cells usually defined and enumerated on the basis of a CD27⁻ CD21⁻ phenotype. In these conditions at least, but not in SLE, atypical memory cells are hyporesponsive to BCR stimulation owing at least in part to the activity of the FcRL4 inhibitory receptor.

In addition to these well-recognized populations, the prominent role of ABC cells in murine autoimmune and anti-viral responses previously described in this review has created significant impetus for the recognition of a human ABC equivalent. While initial human studies guided by the expression of CD11c failed to detect ABC expansions in SLE (110), elevated frequencies of putative ABC-like cells have been postulated in a number of subsequent studies including in HIV infection (111), SLE (112), post-influenza vaccination (113) and in cancer patients treated with checkpoint inhibitors in whom the expansion of these cells correlated with the risk of developing autoimmune complications (114). It should be noted however that the markers used to define these populations have been rather variable and usually dependent on the expression of CD11c or the absence of CD21 with the latter representing a feature shared by activated B cells in multiple compartments and also postulated to identify a subset of anergic B cells (115). It is also important to note that in essentially all human studies (110, 111, 113, 114), ABC-like cells were shown or postulated to represent CD27⁺ activated memory B cells. In contrast, our own findings clearly document that while all the major B cells compartments (naïve, memory and DN cells), contain a fraction of cells that could qualify as ABC on the basis of their expression of

increased levels of CD11c and T-bet and loss of CD21, it is the DN population and in particular the DN2 fraction that contains the largest frequency of ABC-like cells. Similarly, our analyses document that at least in SLE, the majority of ABC-like cells with the highest expression levels of CD11c, T-bet and FcRL5 (another helpful marker for this population) are contained within the CD27- CD21- activated naïve and DN2 populations (Figure 2). Accordingly, combined with the absence of CXCR5 in these cells and the extrafollicular localization of ABC in several mouse models, we propose that the human equivalent is predominantly represented by the extrafollicular B cell response.

Perhaps more importantly, we contend that the preponderance of evidence supports the notion that loss of CD21 and upregulation of CD11c and T-bet may be a common feature of human B cell activation in multiple compartments, and it may therefore be misleading to use these features in isolation of other markers to adjudicate labels that confer the connotation of separate developmental lineages. Additional studies will be required to elucidate whether the ABC-like phenotype does indeed identify merely an activation component within all B cell populations or indicate a separate differentiation fate with specialized function.

Extrafollicular B cell responses in human SLE. Induction mechanisms and significance

As previously discussed, active SLE is characterized by dramatic expansions of interconnected aNAV, DN2 cells and plasmablasts; a picture consistent with enhanced activation of EF B cell responses. Moreover, expanded EF clones contribute substantially to the contemporaneous SLE serum autoantibody repertoire during disease flares. Therefore, the combined evidence supports a pathogenic role for this pathway. Accordingly, understanding the mechanisms that underpin EF B cell activation in SLE should contribute substantially to elucidate disease pathogenesis and to identify new immunomodulatory therapeutic targets.

Our published studies provide some informative clues. Thus, despite multiple similarities in the overall nature and function of DN2 cells, important differences exist between healthy and SLE subjects. Most prominently, SLE DN2 cells display hyper-responsiveness to a number of stimuli known to contribute to SLE pathogenesis including TLR7, IL-10, IL-21 and Type-III interferon (IFN λ) but interestingly, not to type I IFN. TLR7 in particular seems to be central to the behavior of SLE DN2 cells as this type of innate responsiveness in SLE is mediated by enhanced expression of TLR7 and lower levels of inhibitors of TLR signaling, including TRAF5, the central negative TLR regulator in B cells (116). TRAF5 deficiency may also account for the inability of DN2 cells to respond to in vitro stimulation through CD40L, a pathway required for the generation of GC. Interestingly, in a small study CD40L blockade in human SLE did not decrease the large numbers of DN cells characteristic of active lupus (117). Combined, the available information suggests that DN2 cells are likely generated in a CD40L-independent and presumably GC-independent fashion.

In addition to TRAF5 deficiency, expansion of EF B cell responses in SLE may be promoted by a more generalized deficiency of inhibitory mechanisms. Thus, SLE DN2 have the lowest expression of negative regulators of NF- κ B signaling, *NFKBIA* and *TNFAIP3* (A20); and of the NOD2 regulator TRAF4. Combined with over-expression of RIG-I-like receptors

(RLRs) for dsRNA and DNA sensors, it seems likely that SLE DN2 B cells may be dysregulated through exaggerated innate stimulation coupled to defective negative regulation of effector pro-inflammatory pathways. Of particular significance, A20 hypomorphic variants contribute to genetic risk for SLE (118) and A20-deficiency induces B cell hyper-responsiveness, autoantibody production and lupus-like disease (119). Defective negative regulation of B cell activation and differentiation as a contributor to enhanced EF responses in SLE is also indicated by both lack of expression and defective function of surface inhibitory receptors. The former situation is illustrated by the lack of expression on SLE DN2 cells of FcRL4, which accounts for the lack of BCR responsiveness of DN2-like exhausted B cells characteristic of HIV viremia and malaria. In turn, defective function is illustrated by the lack of expression of Ets-1, whose levels are regulated by CD22 activity in response to BCR stimulation (82).

Mechanistically, EF responses may contribute to disease in SLE in different ways including their previously discussed contribution to the systemic generation of pathogenic autoantibodies, a property documented by the ability of DN2 cells to produce anti-RNA and anti-RNA-binding protein autoantibodies including anti-Ro and anti-Smith antibodies. Moreover, DN2 cells are likely to contribute to local production of autoantibodies in lupus nephritis. Indeed, DN2-like CD11c+ bright B cells are found in the kidney of lupus nephritis patients and can make up the majority of B cells in tissue infiltrates (112). Importantly, tubular-interstitial inflammation with a major component of B cell and plasma cell infiltration is common in lupus nephritis and correlates with poor kidney outcome. However, clearly organized ectopic GC are only present in a minority (6%) of lupus nephritis patients (120). Therefore, it is likely that kidney infiltrating B cells and PC are generated in situ in a GC-independent fashion. It should be noted however, that systemically, naïve cells, may be particularly adept to generating new GC (121, 122) and that the formation of spontaneous GC that induce systemic autoimmunity requires B cell-intrinsic TLR7 signaling (123, 124). Accordingly, even in the context of long-established autoimmune memory, ongoing recruitment of activated naïve B cells may represent a major pathogenic event in lupus flares. This may occur not only through the generation of autoreactive effector responses, but also through the generation of new GC, and the associated downstream consequences including the spread of autoimmune memory and enhancement of the long-lived plasma cell compartment.

Conclusion

In summary, there is ample experimental evidence in animal models to support an important contribution of EF B cell responses to both protective and pathogenic situation and are of particular relevance to autoimmune diseases. The identification of the cellular components of the human EF compartment should enable investigators to delineate the contribution of this pathway to different types of immune responses and their participation in different phases of autoimmune diseases in general and of SLE in particular. In turn, precise studies of the inducers and molecular programs responsible for the activation of the EF pathway should be invaluable to inform new regulatory strategies to either dampen or activate this pathway as needed for the desired immunological outcome (i.e., enhancement of vaccine responses or suppression of autoimmune responses). Finally, the study of the early,

unmutated cellular components of the EF pathway and their ASC progeny should allow investigators to identify the original triggering self-antigens responsible for the initial recruitment of B cells into pathogenic autoimmune responses.

Acknowledgements

We would like to thank all members of both the Sanz and Lee laboratories for their critical discussion and proofing of the manuscript. Funding support was provided by the National Institutes of Health (Sanz: U19-AI110483 Autoimmunity Center of Excellence, R37-AI049660; Lee: P01-AI078907, R01-AI121252, and U19-AI109962.; Sanz/Lee: P01-AI125180; Woodruff: T32-DK007656) and the United States-Israel Binational Science Foundation (Sanz: BSF-2013432)

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Figure 1. Pathways of B cell activation. Cartoon of three known pathways of B cell activation and effector differentiation. Differential features of T-I and T-D EF responses are summarized from animal studies and remained to be ascertained in human responses. Murine T-independent responses (left, purple) result in direct conversion of naïve B cells into Ab secreting cells within days of stimulation. T-dependent, EF responses (middle, blue) go through a series of stages before terminal differentiation, and show higher levels of clonal expansion, CSR, and memory formation than T-independent responses. The cartoon depicts the proposed cellular components of the human EF differentiation pathway driven by T-cell cytokines IL-21 and IFN γ in a TLR7-driven fashion (96). GC responses (right, red) result in

the highest levels of affinity maturation, memory formation, and LLPC development, but are slower to develop.

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Figure 2.

Comparative B cell phenotypes and T-bet expression in peripheral B cell subsets. Top left contour plots: Flow cytometry characterization of T-bet⁻ and T-bet⁺ B cells within SLE. The vast majority of T-bet⁺ cells concentrate within the DN (IgD⁻ CD27⁻), and naïve (IgD⁺ CD27⁻) populations. In turn, T-bet⁺ DN and naïve cells universally express a CXCR5⁻ CD11c^{high} (CD21^{low}; not shown), phenotype, characteristic of DN2 and activated naïve (aNAV). A small cell fraction of a similar T-bet⁺ CXCR5⁻ CD11c⁺ (CD21^{low}; not shown) phenotype can also be identified within the SWM compartment. Top right histograms:

Comparative B cell subset histogram profiles utilizing resting naïve (rNAV) or DN2 B cells as references conclusively reveals that dominant T-bet expression resides in aNAV and DN2 B cell fractions. SWM- (CD27+ IgD- CXCR5- CD11c+) and to a lesser extent SWM+ (CD27+ IgD- CXCR5+ CD11c-) have higher T-bet expression than rNAV but much less than aNAV and DN2.

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