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Journal Title: Immunological Reviews
Volume: Volume 284, Number 1
Publisher: Wiley: 12 months | 2018-07-01, Pages 120-131
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1111/imr.12660
Permanent URL: https://pid.emory.edu/ark:/25593/tvbrm

Final published version: http://dx.doi.org/10.1111/imr.12660

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Accessed November 20, 2019 12:33 PM EST
Understanding B-cell activation and autoantibody repertoire selection in systemic lupus erythematosus: A B-cell immunomics approach

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Abstract

Understanding antibody repertoires and in particular, the properties and fates of B cells expressing potentially pathogenic antibodies is critical to define the mechanisms underlying multiple immunological diseases including autoimmune and allergic conditions as well as transplant rejection. Moreover, an integrated knowledge of the antibody repertoires expressed by B cells and plasma cells (PC) of different functional properties and longevity is essential to develop new therapeutic strategies, better biomarkers for disease segmentation, and new assays to measure restoration of B cell tolerance or, at least, of normal B cell homeostasis. Reaching these goals, however, will require a more precise phenotypic, functional and molecular definition of B-cell and PC populations, and a comprehensive analysis of the antigenic reactivity of the antibodies they express. While traditionally hampered by technical and ethical limitations in human experimentation, new technological advances currently enable investigators to address these questions in a comprehensive fashion. In this review, we shall discuss these concepts as they apply to the study of Systemic Lupus Erythematosus.

Introduction

In systemic lupus erythematosus (SLE), it is widely accepted that breach of B-cell tolerance and abnormal activation represent critical steps in the initiation of the pathogenic cascade leading to clinical disease (1–3). These areas of study are still poorly understood in humans, though, due to experimental limitations and complexities in probing the extreme diversity of the human B-cell repertoire. However, modern interrogation of immune repertoires through sequencing and proteomic technologies offer new approaches to understanding the dynamics of protective and pathogenic immune responses (4, 5). Adaptive immune receptor repertoire sequencing (AIRR-seq), a method of using high-throughput sequencing to examine T-cell
receptor and/or immunoglobulin (Ig) repertoires, in particular, can be applied to unfraccionated populations, specific immune cell subsets, and single cells. This approach, and its ability to characterize the diversity, clonal overlap, and maturation of immune repertoires, represents a profoundly useful tool to analyze immune responses in SLE and other autoimmune disorders. Integration of AIRR-seq with other novel immune-profiling techniques provides potential to segment patient populations, predict disease outcomes and monitor responses to therapy.

In this review, we will discuss current experimental approaches to the study of human B-cell activation, differentiation, and self-tolerance in SLE within the context of immune-profiling and repertoire sequencing, but with a primary focus on work from our lab and its overall contribution to the field. We describe the power of AIRR-seq and integrated transcriptional and epigenetic analyses to identify novel B-cell populations and catalogue conventional and newly defined cell populations within different B-cell differentiation pathways. In this context, we will discuss data derived in our laboratory from a multitude of SLE patients and from other autoimmune diseases, as well as healthy vaccinated individuals. We will demonstrate distinctive repertoire features of SLE antibody secreting cells (ASC), including higher diversity and lower rate of somatic hypermutation (SHM) relative to the ASC expanded in healthy subjects in response to recall immunizations. Combined with extensive phenotypic and molecular analyses, our data indicate ongoing recruitment of newly activated B cells possessing a general naive B-cell phenotype (hereinafter referred to as recently activated Naive; aNAV) B cells, possibly through extra-follicular pathways and/or early germinal center (GC) reactions during SLE flares. Finally, we shall review the application of AIRR-seq to understanding the selection of different VH4–34-encoded autoreactivities.

Defining B-cell tolerance through repertoire studies in human autoimmunity

Defective B-cell tolerance for self-antigens, leading to the generation of pathogenic, isotype-switched autoantibodies, is at the center of multiple human autoimmune diseases and is critical, in particular, for the development of SLE. Indeed, SLE is a quintessential systemic autoimmune disease characterized by high abundance of the most diverse array of autoantibodies among any human autoimmune condition (6). Such promiscuity points to generalized defects in B-cell regulation underlying a proclivity to generate productive responses to multiple self-antigens, rather than a narrow B-cell dysregulation induced by a particularly immunogenic antigen. This scenario is supported by the concentration of disease risk alleles on B-cell signaling pathways (7, 8). In contrast to SLE, the autoimmune response is restricted to one or a few antigens in many other autoimmune diseases such as pemphigus, myasthenia gravis, type 1 diabetes, and myositis with other systemic conditions such as Sjogren’s, Systemic Sclerosis and even Rheumatoid Arthritis falling somewhere in between in terms of the diversity of their antigenic targets. It is important to note, however, that despite decades of research and progress in our understanding of SLE and other systemic autoimmune diseases, with the possible exception of Rheumatoid Arthritis, little is still known regarding the nature of triggering, selecting and target antigens (possibly different), the nature of the responding cells that mediate pathogenic responses, and the properties and
complexity of the antibody repertoires mediating disease. Also unknown is whether the same cells and antibodies mediate immune-complex induced systemic lupus manifestations and local inflammation in target tissues.

Central questions and challenges in understanding autoimmune B-cell responses in SLE

Human B-cell tolerance is enforced through different mechanisms, prominently including deletion, anergy, and receptor editing (1). These mechanisms operate at multiple checkpoints during early bone marrow (BM) B-cell development and lead to the establishment in healthy subjects of a mature naïve B-cell compartment of decreased autoreactivity relative to the primary, unselected antibody repertoire expressed by newly emerging immature B cells (9). Yet, even in healthy subjects the mature naïve B-cell compartment is still endowed with a significant degree of autoreactivity, thereby imposing the need for additional peripheral tolerance checkpoints (10). Such late checkpoints are enforced throughout B-cell activation and differentiation into ASC (11, 12). Particularly important is to avoid differentiation into long-lived plasma cells (LLPC), that survive indefinitely within the BM microenvironment in a B-cell autonomous fashion and are typically refractory to existing therapies including B-cell depletion.

Thus far, the experimental approach to understanding defective checkpoints in human autoimmunity has relied upon measuring the frequency of autoreactivity using monoclonal antibodies (mAb) generated from single cells sorted from B-cell populations representing distinct stages of differentiation/activation and purified according to accepted surface markers (9). This approach has defined substantial contraction of autoreactivity during maturation of transitional cells into naïve B cells only for the same level of autoreactivity to be restored into the isotype switched memory compartment through SHM during the GC reaction (11). Subsequently, autoreactive memory cells are limited in their ability to differentiate into BM LLPC at late peripheral checkpoints (12). While highly informative, these approaches have suffered from low throughput (the analysis being limited to a few hundred cells) and are limited to general ANA (anti-nuclear antibodies) and DNA. While still limited to global ANA reactivity, much higher throughput has been recently achieved by a flow-based assay that documented progressive attrition of ANA-reactive B cells through peripheral checkpoints, resulting in a very low frequency within the isotype switched memory compartment. Interestingly, however, SLE subjects were able to enforce the same level of ANA censoring as healthy controls with the notable exception of the anergic naïve compartment which contained higher levels of autoreactive B cells in healthy subjects (13). Finally, a combination of flow cytometry, next generation sequencing (NGS), and single cell mAb analyses has been used to understand the fate of intrinsically autoreactive 9G4+ B cells expressing autoantibodies encoded by VH4–34 (1). These studies identified GC censoring as an important tolerance mechanism either through deletion or mutation away from the germline-encoded autoreactivity (14, 15). These mechanisms are defective in SLE thereby explaining the high levels of serum 9G4 autoantibodies observed in SLE in a disease-specific fashion (14, 15).
B-cell and antibody repertoires in SLE

As discussed, the subversion of immunological tolerance underpins the development of autoimmune disorders. Thus, tolerance breakdown enables clonal expansion and affinity maturation of B cells reacting with self-antigens, ultimately leading to positive selection of pathogenic B cells and plasma cells (PC). In turn, the generation of pathogenic autoantibodies cause tissue damage either through inflammatory immune complexes or in situ binding to tissue antigens. These processes evolve in an antigen-driven fashion through critical molecular changes that include isotype switching from IgM to more pathogenic IgG subclasses and SHM. While the molecular basis of these processes is well understood, a precise delineation of their participation in human autoimmunity has been hampered by experimental limitations with most studies restricted to a relatively low number of mAb at a single point in time. Moreover, studies have centered on pre-determined self-antigens believed to represent disease targets such as DNA in SLE (16). Of note, however, the actual nature of the antigens that trigger the initial tolerance breakdown in naïve B cells remains to be clarified (17). Different studies have established that the unmutated germline version of high-affinity IgG autoantibodies of known specificity generally loses autoreactivity (10). This observation is consistent with sequential selection of pathogenic clones that starts with an unrecognized self-antigen (or cross-reactive foreign antigen), followed by selection against a separate antigen (18). A non-mutually exclusive alternative model would implicate a generalized process of naïve B-cell activation in SLE and stochastic acquisition of pathogenic autoreactivity through SHM and selection by self-antigen within the GC, an environment specialized in prolonged antigen presentation and cyclical clonal expansion which is rich in self-antigens in part due to high levels of apoptosis and defective apoptotic cell clearance. Another possibility, described in the mouse anti-DNA response (19, 20), is that after being first stimulated by a triggering antigen, autoreactive B cells would subsequently acquire a separate autoreactivity against a different self-antigen (target antigen), through SHM either in the GC (21) or in the local environment of target tissues such as the kidney in patients with lupus nephritis (22). Indeed, this process could also help explain the well-documented phenomenon of epitope spreading responsible for the progressive addition of antigenic targets leading to disease development and progression (23). Our work, reviewed below, suggests that an ongoing incorporation of new naïve B-cell clones into the autoreactive repertoire is central to the pathogenesis of SLE.

Integrating repertoire analysis into a B-cell immunomics approach to SLE

Possibly contributing to the explanation of diverging results in tolerance studies, current experimental approaches suffer from previously discussed limitations including low throughput and a relatively narrow scope of the autoreactivity tested. There is, therefore, a major need for high throughput single cell testing of a large number of autoreactivities. Extant studies and current approaches are also hampered by the use of B-cell classifications based on a limited set of surface phenotypic markers. However, current classifications are inconsistently used in different studies and fail to recognize heterogeneity within predefined populations. The latter problem is due to sharing of phenotypic classifiers across different populations, or the presence of activated or anergic cells within larger parental subsets. Through immune profiling and repertoire analysis, our group illustrated an example of this
limitation by highlighting the heterogeneity of IgD+CD27− naïve cells under certain disease states (4). This problem highlights the need for a more precise classification of human B cells based on an integrated understanding of their cellular, molecular and repertoire properties. Moreover, if we are to understand the significance of increased autoreactivity in a given B-cell population, it will be imperative to match their repertoire with the effector repertoires expressed by PC, and the ultimate effector compartment represented by serum autoantibodies. Finally, it is important to understand the origin of autoreactive PC that infiltrate target organs such as the kidneys in lupus nephritis (24–26). To that end, the antibody repertoire of tissue-resident PC needs to be characterized and matched with the one expressed by systemic cells, and clones shared between the two compartments should be then analyzed for evidence of affinity maturation and/or a shift in autoreactivity in order to understand maturation and antigenic targets within the kidney. Already some groups have begun to lay a foundation for these studies by analyzing the clonal makeup and connectivity of B cells from various tissues in the human body with remarkable results (27, 28).

Addressing these questions requires a comprehensive experimental toolbox capable of integrating cellular and molecular analyses with a detailed characterization of the antigenic targets recognized by different antibody repertoires. Ideally, experimental assays should be high throughput to allow the study of large numbers of cells and antibodies from multiple samples and individuals. In our current experimental approach, summarized in Fig. 1, mononuclear cells (PBMC) and serum obtained from peripheral blood samples (PBL) are used to obtain a comprehensive picture of B-cell homeostasis. In addition, the complexity and properties of cellular antibody repertoires and serum autoantibodies of predefined specificity are determined, and the repertoire connectivity between distinct cellular compartments is assessed. Despite more limited availability, we are also able to frequently obtain research kidney biopsy cores in patients with lupus nephritis and BM aspirates from both healthy donors and SLE patients. In a typical sequence of events, PBMC are analyzed using multidimensional flow cytometry after staining for 12–16 surface and intracellular markers. This approach identifies quantitative abnormalities of B-cell homeostasis between SLE, HC, and other autoimmune diseases (25, 26, 29, 30). Moreover, through the use of an increasing number of agnostic automated analytical methods (31, 32), cytometry analysis has the power to identify unheralded populations characteristic of different disease conditions. Similar multidimensional datasets can be obtained through other cytometry methods including CyTOF and Chip-Cytometry (33, 34). Cytometry data can be also used to identify B-cell fingerprints that integrate all B-cell populations and are able to separate SLE patients into multiple clusters with distinct clinical and immunological features (Wei et al, manuscript in preparation) (25, 26, 31). Once B-cell subsets of interest are identified, the corresponding cells are sorted to high purity and RNA and DNA are dual-extracted for molecular analysis and for antibody repertoire studies. The latter is accomplished through AIRR-seq, which is coupled with high-resolution proteomics of serum immunoglobulin (Ig-seq), to obtain a quantitative map of antibody repertoires at the individual clonotype level (35, 36). As further discussed below, AIRR-seq can also be performed with ultra-high throughput at the single cell level while pairing the antibody heavy and light chains originally expressed. Accordingly, this approach permits the generation of mAb reflecting the actual repertoire. In addition to AIRR-seq, RNA is used for transcriptional analysis using
RNA-Seq while genomic DNA is used for epigenetic analysis, cellular quantification, and also stored for future genetic analysis. In our work, performed in collaboration with Drs. Jerry Boss and Chris Scharer of the Department of Microbiology and Immunology at Emory University under the aegis of the NIAID Autoimmunity Centers of Excellence, epigenetic analysis is accomplished by a combination of the following assays: ATAC-Seq (chromatin accessibility) (37); dual enzyme Reduced Representation Bisulfite Sequencing (CpG methylation); and whenever allowed by cell numbers, CHIP-seq (histone mapping). Currently, with the exception of histone mapping which requires large numbers of cells for each anti-histone-specific assay, robust and informative AIRR-seq, as well as transcriptional and epigenetic analyses, can be achieved with as few as 1,500–2,000 cells. Of course, repertoire analysis benefits greatly from sequencing as many cells as possible in order to obtain the best representation of diversity and clonality within any B-cell population.

The integration of transcriptional and epigenetic data provides a comprehensive molecular landscape of different B-cell subsets that can then be used for multiple purposes including: validating the identity of pre-defined subsets with different functional properties including activation, anergy, exhaustion and antibody secretion; discovering unexpected features which may in turn lead to changes in B-cell classification; identification of novel markers; definition of intrinsic and extrinsic pathways of B-cell activation that may differ between subsets and disease conditions; establishing developmental relationships between populations; and defining subsets that are actively repressed (important for tolerance and transplantation) or conversely, poised for activation despite the absence of conventional surface or transcriptional markers. These layers of information provide a powerful context for the interpretation of repertoire data and enhance its ability to order clonally-related cells within developmental pathways. Representing a new frontier in immunological research, recently developed technologies now permit us to address these issues at the single cell level, at least when it comes to repertoire and transcriptional analysis and possibly ATAC-seq. From a repertoire standpoint, ultra-high throughput analysis of Ig repertoire can be combined with large scale generation of mAbs using emulsion-based PCR of thousands to millions of single cells to sequence heavy and light chain combinations through linkage PCR or the use of barcode bearing beads or hydrogels (38, 39). Some such platforms, including the 10× Genomics Chromium system, can also use total RNA sequencing information alongside targeted enrichment of Ig transcripts to provide a robust method to analyze gene expression on the same cells from which paired Ig chain information is obtained. Thus, it is currently feasible to obtain large scale simultaneous assessment of Ig repertoire; pairing of antibody heavy and light chain information; expression of antibody repertoires; detection of heterogeneity in B-cells and PC populations; and understanding of the activation, differentiation and survival programs of individual cells expressing antibodies of interest. The latter goal can be greatly facilitated by pre-selecting antigen-specific B cells using flow cytometry or magnetic enrichment. Alternatively, global B-cell populations can be interrogated using single-cell antibody cloning coupled with expression of the repertoire as a yeast-display library that can then be screened for antigen specificity by flow cytometry (40).
Understanding the cellular basis of autoantibody generation in human SLE

As previously discussed, a central question in SLE is understanding the antigenic and molecular events leading to B-cell activation, breakdown of tolerance, and differentiation either into long-lived memory cells and PC or short-lived plasmablasts (PB). As also previously indicated, most extant studies in human SLE have been limited in scope without extensive repertoire analysis. We have started to address these issues through the combined B-cell immunomics approach described earlier in this review to understand the properties and cellular origin of the ASC and their contribution to changes in serum autoantibodies. During lupus flares, large numbers of newly generated, proliferative ASC are released into the periphery after being generated in secondary lymphoid tissues through either antigen specific or polyclonal stimulation (41), a process seemingly similar to vaccine recall ASC responses that peak 6–10 days after immunization (42). Through the use of single cell experiments, it has been shown that post-vaccination ASC are oligoclonal in nature with up to 80% being antigen-specific, a 100- to 800-fold enrichment from pre-immunization levels. Multiple studies indicate that these changes result from activation of pre-existing antigen-specific memory cells (43).

Studies conducted by our group revealed a very different picture of ASC in acute SLE through the comparative study of patients experiencing SLE flares and healthy volunteers who received vaccination against tetanus or flu a week prior. ASC, including CD138+ and CD138− fractions, were sorted in large numbers and analyzed via AIRR-seq for in-depth analysis of Ig repertoire properties including heavy-chain variable region (VH) diversity and isotype utilization, degree of SHM, clonality and connectivity with the Ig repertoire expressed by naïve and memory cells purified from the same blood sample. In addition, repertoire autoreactivity was ascertained by ELISPOT analysis of unstimulated ASC and of memory cells induced to differentiate into ASC. Autoreactivity was also measured for selected recombinant mAb generated from ASC and other B-cell compartments using single cell PCR amplification.

Consistent with the existence of a pre-formed memory compartment, isotype-switched autoantibodies are present in SLE serum for several years prior to disease onset (1). Yet, in contrast to memory vaccine responses, ASC in flaring SLE were largely polyclonal and expressed a highly diverse VH repertoire. Nevertheless, we also identified a number of substantial clonal expansions in SLE ASC that predominantly expressed VH4–34 (4). The functional consequence and disease relevance of these clonal expansions was demonstrated by matching their Ig sequences with the most abundant VH4–34-encoded autoantibodies detected in the same patient’s serum by mass spectrometry analysis of 9G4+ IgG fractions. Other remarkable features of SLE ASC included a significantly lower rate of SHM compared to both memory cells from the same patients and to memory cells and post-vaccination ASC in healthy individuals. Importantly, in addition to lower overall rates of SHM, lupus ASC also contained a significant fraction (15–50%), of cells with no or very low mutation. Of note, circulating ASC during SLE flares (whether CD138− or CD138+), are universally proliferative, thereby suggesting recent derivation from newly-activated B cells and arguing against a major contribution of pre-formed PC displaced from BM niches.

Consistent with recent generation, the Ig repertoire in SLE was highly connected between
the CD138+ and CD138− ASC fractions, and between ASC populations and the CD19+ B-cell compartment. Notably, SLE ASC were clonally connected not only to memory B cells but also to a substantial degree, naïve B cells. In contrast, post-vaccination ASC in healthy individuals were largely connected to memory cells only.

Combined, SHM and repertoire connectivity data indicate that, despite the presence of an autoreactive memory compartment, newly aNAV B cells have a major contribution to the formation of autoreactive ASC during SLE flares. This conclusion was also supported by the demonstration that unmutated VH4–34 mAbs generated from ASC and naïve B cells displayed substantial ANA autoreactivity, as well as binding to other lupus antigens including dsDNA, chromatin, Ro, and ribosomal P proteins.

A polyclonal ASC repertoire could be explained either by generalized bystander B-cell activation or, alternatively, by antigen-driven activation of large numbers of clones responding to multiple epitopes displayed by the many autoantigens known to be the targets of lupus autoantibodies (23). However, the aggregate of our studies strongly favors the former possibility. In particular, in the absence of recent immunization or infection, SLE patients had a significantly increased number of anti-flu ASC that matched the frequency of flu-specific memory cells within each patient. In contrast, only a relatively small fraction of ASC (<20%), produced SLE-associated autoantibodies including anti-dsDNA, anti-Ro, anti-Sm, and 9G4+ antibodies. A combination of the two mechanisms may make more sense than either in isolation, though. Generalized B-cell activation mediated by polyclonal signal 3 provided by cytokines known to be abundant in SLE and to impact B-cell activation and GC survival (Type I and II IFN, BAFF/APRIL< IL-21, IL-17, IL-10 and IL-6) (44–48), could induce bystander stimulation while also promoting clonal expansion of antigen-specific autoreactive cells receiving BCR-transduced signal 1 imparted by SLE-associated antigens (49, 50). This possibility is supported by the predominance of VH4–34 clonal expansions consistently found at the top of the ASC repertoire during SLE flares and by the dominance among autoreactive cells of 9G4 ASC responses by ELISPOT.

Irrespective of the relative contribution of either mechanism, the high degree of memory and ASC polyclonality found in our studies argues against a progressive accumulation of dominant autoreactive B-cell clones in SLE. The production of IgG autoantibodies several years before clinical diagnosis (51) strongly indicates that in patients with well-established disease like the ones in our studies, autoreactive B-cell responses must have had the opportunity to mature for well over 10 years through constant or frequent episodic exposure to immunogenic autoantigens. According to conventional models of recall B-cell responses established in mouse models through limited cycles of immunization, a memory-derived, antigen-driven model of chronic autoimmune responses would predict the emergence of dominant clones, a tenet also supported by the nature of autoantibody responses in the lupus-like lpr mouse model (52, 53). Our results, however, are more in keeping with progressive enlargement of the autoreactive repertoire through a combination of polyclonal memory B-cell activation that would appear to dilute the emergence of dominant clones with further dilution created by ongoing recruitment of naïve cells into the memory and effector B-cell compartment. A more conclusive answer regarding the emergence of dominant clones versus the progressive enlargement of the autoimmune repertoire will require systematic
longitudinal studies of antigen-specific memory and ASC cells, a rather difficult study which will be facilitated by the ability to identify SLE-specific autoreactivities with the 9G4 idiotype and will be discussed further below.

**Clonal Persistence in SLE: Immunological Implications**

The previously described ASC repertoire in SLE is characterized by limited clonal dominance, low mutation frequency, and clonal relatedness to recently activated B cells. While these features would seemingly indicate a high clonal-turnover within the ASC compartment in SLE, evidence suggests a very long persistence of at least a portion of B-cell and ASC clones in the circulation of SLE patients (4). These results were in sharp contrast to normal responses in the context of limited antigenic stimulation where PB are short-lived and undergo apoptosis in just a few days (54). More recently, we have preliminary data in at least one patient, documenting the persistence of clonally-related circulating ASC through multiple sampling time points over 5 years. In this particular case, Ig transcripts were amplified from single cell PBMC emulsion using the 10× Genomics platform and compared to separate samples of sorted ASC collected at various intervals over a 5 year period. Strikingly, comparing the two most distant samples, over 50% of the clones identified in the later sample could be identified in the initial ASC sample (Fig. 4). This result is all the more surprising considering sampling effects of taking only a portion of the ASC pool from multiple time points.

While it is likely that SLE PB may survive longer on the basis of intrinsic longevity and extrinsic survival factors, these observations are most likely explained by ongoing stimulation of pre-existing memory cells. Moreover, the fact that these persisting clones were initially detected in the aNAV compartment strongly supports a model where dominant antigens continue to recruit new autoreactive clones into the memory compartment and induce sustained differentiation into ASC. The preliminary results are currently being expanded upon and verified in a larger cohort, but demonstrate an intriguing aspect of the SLE ASC repertoire that deserves further examination.

**Understanding the contribution of naïve B cells to SLE**

Our previous studies demonstrated that the contribution of naïve B cells to the generation of ASC during lupus flares was accounted for by aNAV B cells, (IgD+, CD27-), with a novel phenotype characterized by high levels of CD19, retention of mitochondrial dyes and loss of CD21, CD24 and CD38. In contrast to previous reports of CD23 upregulation upon naïve B-cell activation and of increased CD23+ naïve cells in SLE (55), we observed that aNAV B cells were characterized by loss of CD23. Compared to resting, CD23+ naïve cells within the same patients, aNAV B cells contained a fraction of clonally expanded cells that were responsible for the observed repertoire connectivity with ASC. Interestingly, some aNAV B cells displayed significant levels of SHM, a feature consistent with our recent demonstration of increased of AICDA transcripts (Jenks et al. Manuscript under revision), and contained substantially higher fractions of autoreactive 9G4+ cells. In order to better understand the mechanisms involved in the abnormal homeostasis of naïve B cells in SLE, we have more recently performed a comprehensive phenotypic and molecular analysis of resting-naïve and
aNAV B cells, as well as isotype-switched memory cells, and a population of cells lacking IgD and CD27 we had originally identified as highly expanded in active SLE (double negative cells; DN) (56). These studies demonstrate that aNAV B cells are characterized by the absence of CXCR5 and the expression of high levels of CD11c and the T-bet transcription factor. Interestingly, this phenotype is exclusively shared by a fraction of DN cells (termed DN2) that accounts for the expansion of DN cells in active SLE, and contains similarities with descriptions of age-associated B cells and activated B cells published by other groups (57, 58). Transcriptional analysis confirms a very close relationship between aNAV and DN2 B cells, and functional and repertoire studies strongly suggest a developmental link between aNAV and DN2 B cells leading to the generation of autoreactive ASC, likely through extrafollicular differentiation (Jenks et al. Manuscript under revision). These studies extend previous work performed in collaboration with Drs. Scharer and Boss (Department of Microbiology and Immunology at Emory University), demonstrating a strikingly different epigenetic landscape in lupus naïve B cells than in healthy naive B cells (37). Our data indicated through accessibility analysis performed by ATAC-seq that, in contrast to healthy individuals, SLE naïve cells are characterized by an open chromatin configuration in regulatory regions of genes involved in B-cell activation and in areas containing motifs for transcription factors that regulate B-cell activation and differentiation including Jun-AP-1, IRF4, and Blimp-1. Notably, these epigenetic changes were already present in resting-naïve cells indicating that SLE B cells are poised to differentiate into ASC.

Selection of VH4–34-encoded autoantibodies in SLE

Among the more than 100 autoantibody species identified in SLE, only a selected few have >95% specificity for the disease. SLE-specific autoantibodies include anti-dsDNA, anti-Smith, anti-ribosomal P, and antibodies encoded by the VH4–34 gene segment (VH4–34 antibodies). In the unmutated germline configuration, VH4–34 antibodies are recognized by the 9G4 rat anti-human idiotype mAb which binds a hydrophobic patch (HP) within the framework-1 region (FR1). This HP is formed by two discontinuous amino acid stretches (Q^6W^7 and A^23V^24Y^25) (14, 59–61), and can be disrupted by SHM thereby abolishing recognition by 9G4. Accordingly, the VH4–34 repertoire of B cells and antibodies includes 9G4+ and 9G4− members, an important distinction that can only be established through global sequencing since the analysis of 9G4+ fractions will fail to identify members with HP mutation. As discussed below, this feature bears significant connotations for our understanding of tolerance and selection of different autoreactivities in SLE. Of note, 9G4+ VH4–34 autoantibodies are greatly elevated in the serum of about 50% of all SLE patients and up to 75% of active patients, accounting for 10–40% of total IgG and a substantial fraction of anti-dsDNA antibodies (15, 62). In addition, we have shown that VH4–34 antibodies contribute the majority of antibodies against apoptotic cells, a major source of self-antigens in SLE. Elevated serum titers of 9G4 antibodies correlate with disease activity, lymphopenia and nephritis. Interestingly, we have shown that while 9G4 serum titers decrease weeks after rituximab-induced B-cell depletion, normalization may take significantly longer, a behavior suggesting that these autoantibodies are produced by both short-lived and long-lived PC. Our work provides a cellular explanation for the serological
findings. Thus, we initially showed that SLE is characterized by defective censoring of 9G4 B cells in the GC, the main checkpoint in healthy subjects. In turn, GC expansion would account for the accumulation of 9G4 memory cells and LLPC (14). In addition, as discussed above, we have demonstrated that 9G4 B cells have a major contribution to the generation of newly generated PB through the activation of naïve cells already endowed with SLE-associated autoreactivity in the absence of SHM. This feature would explain the elimination of a fraction of 9G4 antibodies shortly after treatment with rituximab (63, 64).

Given its clinical relevance, the analysis of the VH4–34 repertoire represents a powerful experimental system for the study of the breakdown of B-cell tolerance and the selection of pathogenic autoantibodies in SLE in a disease-specific fashion. Its uniqueness is determined by a fascinating correlation between structural and immunological properties. Thus, VH4–34 antibodies are intrinsically autoreactive owing to the expression of the FR1 HP that is also responsible for the expression of the 9G4 idiotype. Multiple studies have shown that the HP determines binding to N-acetyl-lactosamine epitopes present in widely expressed glycoproteins in a fashion that is largely independent of the heavy-chain complementarity determining region 3 (HCDR3) and associated light chains. Self-antigens recognized in this fashion by VH4–34 antibodies prominently include the I/i blood group antigens and specific glycoforms of CD45/B220. The former autoreactivity accounts for the pathogenic IgM cold-agglutinins generated during acute EBV and Mycoplasma pneumoniae infections. Strikingly, VH4–34 antibodies account for essentially 100% of all anti-I/i cold agglutinins thus far analyzed. In turn, the anti-B220 autoreactivity of 9G4 antibodies mediates their ability to bind to human and mouse B cells and to induce lymphotoxicity. Building on previous work from other groups, our detailed structural analysis of monoclonal 9G4 antibodies derived from different B-cell compartments in SLE patients and healthy individuals has clearly defined two major types of VH4–34-encoded autoreactivity determined by the presence or absence of the HP. As expected, the expression of the 9G4 idiotype as well as anti-B-cell autoreactivity is fully dependent on the preservation of the HP. In contrast, HP-independent autoreactivity includes binding to apoptotic cells and dsDNA. Consistent with previous work, the HCDR3 can contribute to HP-independent binding and individual light chains may modify the autoreactivity of selected antibodies in both the HP-dependent and HP-independent groups.

The structural correlates provide a unique opportunity to use large scale repertoire sequencing strategies to track the antigenic drivers of selection and functional significance of the 9G4+ and 9G4− components of the VH4–34 repertoire. It also allows us to ascertain the contribution to physiological B-cell tolerance of clonal redemption, a model originally noted and proposed by Zheng et al. (65) and elegantly expanded upon by Reed et al. (66). In this mechanism, SHM would eliminate germline-encoded VH4–34 autoreactivity, thereby allowing the selection of protective VH4–34 antibodies in response to vaccination or infection without associated autoimmunity. Proof-of-principle for clonal redemption has been recently provided through the analysis of selected VH4–34-encoded anti-influenza mAbs lacking the HP. These mAb regained binding to RBC I antigens, the canonical VH4–34 autoreactivity, upon reversion in vitro to the germline sequence. We have recently analyzed our dataset of VH4–34-encoded unique sequences obtained by NGS from healthy individuals and SLE (N=242,936 and 470,731, respectively), in order to determine the
frequency of clonal redemption in the isotype switched memory compartment of healthy subjects and SLE patients. The same analysis was performed for ASC present in the circulation of healthy individuals seven days after vaccination and during SLE flares (n = 16 healthy, 29 SLE). Consistent with clonal redemption, our data indicate that relative to SLE, memory cells and ASC from healthy subjects contain a significantly lower fraction of HP+ VH4–34 sequences (Fig. 5). However, the HP, and therefore the potential for HP-dependent autoreactivity, are still retained in an average of 50% of healthy memory cells indicating that additional tolerance mechanisms may be operative. Indeed, late tolerance checkpoints have been proposed to prevent the differentiation of autoreactive memory cells into autoantibody-producing PC. This possibility, however, seems diminished by our finding that the frequency of HP+ VH4–34 sequences remains unchanged in ASC triggered by vaccination of healthy individuals. Though, it is formally possible that ASC derived from autoreactive VH4–34 memory cells might not differentiate into LLPC, thereby diminishing their pathogenic significance. Alternatively, it is also possible that autoreactive VH4–34 memory clones may have been redeemed by light chain editors, where certain light chains modify binding properties of the heavy chain (67, 68), or the use of particular HCDR3s. Ultimately, the contribution of different mechanisms to prevent VH4–34-mediated autoimmunity will have to be assessed by high-throughput analysis of single memory cells and PC obtained from peripheral and BM compartments. Such studies are underway in our laboratory using the approaches illustrated in Fig. 1.

Perhaps not surprisingly, our NGS work also indicates that clonal redemption is less effective in SLE with as much as 75% of VH4–34 memory cells and ASC retaining the HP. These data are quite consistent with our previous single-cell analysis showing preservation of the HP in the majority (average 75%) of SLE memory cells (69). Nevertheless, it is important to consider that in the absence of the HP, VH4–34 B cells can also react with other important SLE autoantigens including dsDNA, Smith, and apoptotic cells. Therefore, we believe that our data indicate a role for selective pressure to retain this germline-encoded region even in the presence of extensive SHM. As before, extensive single-cell analysis will be required to clarify this important question and overall, to ascertain whether the selection of pathogenic VH4–34 clones in SLE is mostly driven by affinity maturation against the original antigen or, instead, involves diversification through selection of mutated clones by separate antigens.

Conclusions

The breakdown of immunological tolerance and subsequent selection of pathogenic autoreactive B-cell clones is likely to depend on a complex interplay between intrinsic B-cell properties determined genetically and epigenetically, and multiple self-antigens. The actual nature of the self-antigens involved in the triggering and expansion of pathogenic clones and the targeting of diseased tissues remain to be determined. Similarly, the role of different B-cell compartments and pathways of B-cell activation remain to be fully elucidated. Improving understanding of human B-cell diversity and the development of powerful technologies, allow large scale interrogation of the properties of the B-cell repertoire expressed by distinct B-cell and PC populations carefully defined by discriminating phenotypic markers. The integration of repertoire studies and the molecular programs
characteristic of different populations will provide critical answers and enhance our ability to predict disease development and outcome, understand disease heterogeneity, and design new treatments targeted to the peculiarities of disease segments and individual patients.

Acknowledgments

This work was supported by the NIH and National Institute of Allergy and Infectious Diseases (NIAID) awards P01AI125180 and R37AI049660, and through the Emory Autoimmunity Center of Excellence U19 AI110483. The authors would also like to acknowledge the support of the Lowance Center for Human Immunology, the Lowance Immune Profiling Program, and Children’s Healthcare of Atlanta and Emory University’s Pediatric Flow Cytometry Core.

References


Cells are collected from either peripheral blood, bone marrow, or tissue. The cells are then phenotyped using a multi-color flow cytometry panel designed to identify major B-cell populations and subsequently sorted into various subsets via fluorescence-activated cell sorting (FACS) before RNA and DNA is extracted. Transcriptome, epigenetic, and repertoire analysis are performed to determine gene expression and epigenetic profiles of the cells. In some cases, emulsion-based barcoding is conducted to allow single-cell gene expression and paired heavy and light chain analysis on individual cells. At the same time, serum can be collected for mass spectrometry analysis and comparison to repertoire data to determine representation of antibodies in the serum. Finally, paired-chain sequences can be synthesized and tested for reactivity against various antigens.
Figure 2.
Germinal center and extra-follicular pathways of antibody secreting cell (ASC) generation are shown. Repertoire and immune profiling studies allowed the identification of a novel population of recently activated B cells still expressing a naive B cell phenotype. These cells are clonally related to ASCs in circulation, display a small amount of somatic hypermutation, some clonal expansion, and their presence was dependent on disease activity. A similarly related subset of CD27-IgD− cells (DN) are possibly an isotype-switched version of the activated naive cells and show more mutation and even more relatedness to ASCs.
Figure 3.
a) Mean mutation frequencies of clones identified in Naive, Isotype Unswitched Memory (UnSw), Isotype Switched Memory (SwM), and Antibody Secreting Cells (ASC) show a significantly lower frequency of somatic mutation in ASC collected from SLE subjects. b) Sequences with less than 3 total mutations in the variable (V) region were identified in SwM and ASC populations and plotted as a percent of total sequences. (n = 16 healthy naive; 14 SLE naive; 4 healthy UnSw; 6 SLE UnSw; 16 healthy SwM; 29 SLE SwM; 14 healthy ASC; 11 SLE ASC; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 via Wilcoxon signed rank test)
Heavy chain immunoglobulin transcript sequencing was conducted on an SLE subject in April of 2013 and then again using 10× Genomics’ emulsion-based paired chain sequencing method on a separate January of 2018 sample collection. In this figure, clones from both longitudinal samples and a sample from a separate SLE subject were individually size-ranked and plotted along the outer track. Clones matching in multiple samples were then connected by the inner lines. 52.3% of identified clones in the 2018 sample were also found in the sample from 2013, suggesting that a large percentage of clones persist for at least 5 years in SLE subjects. The second SLE subject was found to only have 1 clone in common with the 2013 collection of the first subject and none with the 2018 collection. Clones in this figure were identified by 100% HCDR3 match, same variable region (V), and same joining region (J).
Figure 5.
Mutations in the hydrophobic patch (HP) of VH4–34 sequences were determined by identifying non-silent mutations in AA23–25 (AVY). Percent of VH4–34 sequences without HP mutations are plotted for each B-cell population in SLE and healthy subjects. (n = 16 healthy naïve; 14 SLE naïve; 4 healthy UnSw; 6 SLE UnSw; 16 healthy SwM; 29 SLE SwM; 14 healthy ASC; 11 SLE ASC; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 via Wilcoxon signed rank test)