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Short-Lived Plasmablasts Dominate the Early Spontaneous Rheumatoid Factor Response: Differentiation Pathways, Hypermutating Cell Types, and Affinity Maturation Outside the Germinal Center

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We used a newly validated approach to identify the initiation of an autoantibody response to identify the sites and cell differentiation pathways at early and late stages of the rheumatoid factor response. The autoimmune response is mainly comprised of rapidly turning over plasmablasts that, according to BrdU labeling, TUNEL, and hypermutation data, derive from an activated B cell precursor. Surprisingly, few long-lived plasma cells were generated. The response most likely initiates at the splenic T-B zone border and continues in the marginal sinus bridging channels. Both activated B cells and plasmablasts harbor V gene mutations; large numbers of mutations in mice with long-standing response indicate that despite the rapid turnover of responding cells, clones can persist for many weeks. These studies provide insights into the unique nature of an ongoing autoimmune response and may be a model for understanding the response to therapies such as B cell depletion. The Journal of Immunology, 2005, 174: 6879–6887.

Systemic autoimmune diseases are characterized by activation of autoreactive B cells that leads both to autoantibody (autoAb) production and T cell activation (1). Whether this activation occurs because of a failure of central tolerance (2–6), anergy (7, 8), or other mechanisms of peripheral tolerance (9–13), or is due to activation of ignorant B cells (14, 15), it represents a functional loss of tolerance. After the initial loss of tolerance, tissue destruction ensues, which in turn promotes further inflammation, more extensive loss of self-tolerance, and ultimately symptoms of autoimmunity. In addition, the self-epitopes targeted early in the response may differ from those targeted later, a phenomenon known as epitope spreading. Thus, early and late stages of autoimmunity may differ in the subsets of autoreactive cells that are activated, sites of activation, and the types of effector cell that result. Immune dysregulation at the end stage of disease could be of a different nature from that which occurred during the initial loss of self-tolerance. However, because the onset of spontaneous systemic autoimmunity is not predictable from individual to individual, it has been difficult to study early events in the loss of self-tolerance and determine how they evolve into more established disease with time.

Ig-transgenic (Ig-Tg) mouse models have been instrumental in studies of normal B cell self-tolerance and its loss during disease (2–5, 8). Particularly interesting in this regard are models that focus on disease-related autoantigens, which have demonstrated a variety of tolerance mechanisms and in selected cases escape from these in the context of a disease-prone genetic background (5, 8, 11, 12, 16, 17). We have been focusing on the AM14 system, an Ig-Tg model with IgG-specific (rheumatoid factor (RF)) B cells (14, 15, 18). RF autoAbs are found at high levels in rheumatoid arthritis, Sjogren’s syndrome, in some patients with systemic lupus erythematosus (SLE) (19), as well as in Fas-deficient lpr mice (20, 21). In AM14 Ig-Tg mice, the H chain confers specificity for the Fc portion of IgG2a when paired with an endogenous or Tg-encoded Vx8 L chain (22). AM14 binds only to IgG2a of the ‘a’ allele; thus, in IgH congenic mice, RF B cells can be studied in the presence or absence (i.e., in IgHb mice) of a defined and measurable autoantigen. Using this model, we initially found that, in contrast to most other models, the RF B cells develop normally and are immunocompetent in normal IgHb mice (23), a phenotype referred to as clonal ignorance.

To determine how RF B cells become activated on an autoimmune-prone background, we crossed the AM14 Tg onto the MRL/lpr strain (24). RF B cells in spleens of older H chain Ig-Tg MRL/lpr mice form clusters at the T zone-red pulp borders and undergo somatic hypermutation at this site; they are only very rarely found in germinal centers (GCs) (15). The activation of autoreactive B cells required the presence of the nominal autoantigen and did not occur on the IgHb background. Interestingly, there was great mouse to mouse variability in the onset and extent of RF B cell Ab-forming cell (AFC) induction. This is similar to the stochastic onset of appearance of various autoantibodies in non-Tg MRL/lpr and other autoimmune-prone mice (25). Such variability in the onset and nature of disease has confounded efforts to identify the earliest phases of autoreactive B cell activation. Consequently, whereas there are a number of studies of the autoimmune response in our system and others, these data mainly reflect the later stages of established autoimmunity.

To understand the initiating events in the loss of B cell tolerance, we would like to identify the site(s) of initial B cell activation in secondary lymphoid organs such as the spleen; for example,
does RF B cell activation occur at the T-B border or the marginal sinus-bridging channel? In addition, we would like to know whether induction of autoimmunity occurs at other secondary lymphoid sites and whether it is synchronous with activation in the spleen. Finally, it is important to define the cell types and differentiation states found during initial RF B cell activation as well as how the response evolves over time. Recently, we developed a system to identify which mice were at the early stages of initial RF B cell activation based on the appearance of activated RF B cells in the peripheral blood (53). In the present study, we use this system to address some of the questions defined above concerning the sites, tissues, and cell types involved in the early phases of a spontaneous autoimmune response and how these evolve with time.

Materials and Methods

Mice

The AM14 H Tg is a conventional IgM-only Tg. AM14 H Tg mice (22) were backcrossed at least 10 generations onto the MRL/lpr background. All mice were housed under specific pathogen-free conditions.

Identification of converted mice and FACS analysis

Mice that had initiated an RF B cell AFC response in the spleen (“converted mice”) were identified by FACS on PBL, detecting RF B cells using the 4-44 anti-Id Ab, as described.2 Splenocytes were prepared as described (18). FACS analysis of PBL and splenocytes was performed as described (18). Propidium iodide was used to exclude dead cells. For detection of intracellular Ab, membrane-stained cells were resuspended in 1% parformaldehyde solution/PBS for 20 min on ice. Following fixation, the cells were washed in FACS buffer and resuspended in permeabilization buffer (PBS/0.3% saponin/1% BSA/0.05% sodium azide) at 4°C. After 2 h, the cells were washed and resuspended in labeled 4-44 Ab, diluted in permeabilization buffer with 10% rat serum, and incubated overnight at 4°C. Cells were then washed in permeabilization buffer followed by FACS buffer and analyzed by FACS. Eithdium monoazide bromide (Molecular Probes) was used to exclude dead cells in procedures involving fixation (26). Unfixed cells were incubated for 15 min in the dark with ethidium monoazide bromide, exposed to light for 10 min, then washed before fixation.

Reagents

Abs were prepared in our laboratory as described (22): 4-44-biotin (anti-Id), 4-44-FITC, 4-44- Alexa Fluor 647, 4-44-Alexa Fluor 488, 30H12-biotin (anti-CD20.2), C363–29B-FITC (anti-CD3), RA3–6B2–FITC (anti-B220), anti-CD23–FITC and PE, anti-CD80–PE, anti-CD86–PE, anti-I-Ak–FITC, anti-CD90.2, C363–29B-FITC (anti-CD3), RA3–6B2–FITC (anti-B220), and goat anti-rabbit Alexa Fluor 633 (Molecular Probes) were used as secondary reagents for stromal cell-derived factor-1-Fc and anti-CXCR5, and streptavidin-PE (Molecular Probes) were used to detect biotinylated reagents. Anti-human IgG–PE (Jackson ImmunoResearch Laboratories) and streptavidin–PE (Molecular Probes) were used to detect biotinylated reagents. B220, CD154, CD19, CD20, CD80, CD86, and IgM were detected with PE-conjugated Abs prepared in our laboratory as described (22) were: 4-44-biotin (anti-Id Ab, as described.4 Splenocytes were prepared as described (18). FACS analysis of PBL and splenocytes was performed as described (18).

Histology

Sections were prepared and stained as described (27). MOPA-FITC staining was amplified using anti-FITC Alexa Fluor 488 (Molecular Probes) as a secondary. Nuclei were identified with 4’,6-diamidino-2-phenylindole (Molecular Probes). Fluorescent images were captured on an Olympus BX-40 microscope using a SPOT-RT Slider (Scanalytics) digital camera.

Cell proliferation and apoptosis assays

Mice were given an i.p. injection of 2 mg of BrdU (Sigma-Aldrich) in PBS at 2, 12, 24, or 72 h before sacrifice. Splenocytes were first membrane-stained, and BrdU labeling was detected as previously described (28). Apoptosis was detected with the CaspGLOW fluorescein active caspase staining kit (Biovision).

Results

The early splenic response in converted AM14 MRL/lpr mice is marked by the appearance of a CD22low population of 4-44+ B cells

We recently showed (53) that the appearance of Id 4-44+ RF B cells in PBL correlated strongly with the presence of elevated numbers of RF AFC in the spleen and conversely that the absence of such cells in PBL predicted the presence of only very low numbers of RF AFC in spleen. From these data we reasoned that the initial appearance of RF B cells in PBL marked the onset of the initial RF autoimmune response in the spleen, a phenomenon we termed “conversion.” We therefore used this approach to identify early converts and then study the nature of the response.

In early converts, 4-44+ cells accumulated and mutated primarily at the T zone-red pulp border (Fig. 1A) (15), similar to aged IgH MRL/lpr mice (15). We used FACS and immunohistology to understand the origin and identity of these unusual mutating cells. 4-44+ cells from IgH nonconverted IgH mice made up 1–2% of the splenic population and were uniformly CD22high (Fig. 1B).

In recently converted mice, there was a significant expansion of a novel 4-44+ CD22low population (compare upper left quadrants in Fig. 1, B–D, summarized in Fig. 1E). This population of cells expanded further in mice that had converted at least 2 wk ago (Fig. 1E). In contrast, the CD22high population rarely expanded at any stage of conversion (Fig. 1, B–E). The CD22high population was B220+, CR1low+, CD23low, CD44+, CD80-high, CD86-high, class II-high, CCR5high, and CXCR4high (Fig. 1, F–N). The expression patterns of CXCR4 and CXCR5 are consistent with localization of the CD22high population outside of follicles and in the marginal sinus-bridging channels (30). The CD22high population in both converts and nonconverts was B220high, CR1-high, CD23 variable, class II-high, CCR5-high, and CXCR4-low (Fig. 1, F–N). These markers, particularly the chemokine receptors, are consistent with a follicular localization (30). In converted mice, a fraction of the CD22high population reproducibly demonstrated increased expression of the activation markers CD44, CD80 and,
a slight degree, CD86. This can be seen in Fig. 1, comparing the blue, thin solid line to the dashed green one. Note that the CD22<sup>high</sup> cells from nonconverted mice (Fig. 1) lack the population of cells with higher expression of these markers; this population is also lacking in IgH<sup>b</sup> mice (not shown).

**CD22<sub>low</sub> cells are AFCs that reside at the T zone red pulp border and maintain normal levels of surface Ig**

Consistent with the FACS results, immunohistochemical analysis of frozen sections from converted mice showed that most of the 4-44<sup>+</sup> cells in the bridging channels were CD22<sup>low</sup>/syndecan<sup>high</sup> (Fig. 2, A–G). At this site, there were also smaller numbers of CD22<sup>high</sup> (Fig. 2C) as well as syndecan-negative cells (Fig. 2G). Conversely, most of the 4-44<sup>+</sup> cells in the follicles were CD22<sup>low</sup> (Fig. 2, B and D). FACS analysis confirmed that most CD22<sup>low</sup> cells were expressing high levels of syndecan (Fig. 2H, red line) in contrast to CD22<sup>high</sup> cells, which were syndecan negative (Fig. 2H, blue and green lines). The FACS-sorted CD22<sup>low</sup> population was responsible for virtually all of the Ab secretion (17% of 4-44<sup>+</sup> CD22<sup>low</sup> cells formed ELISpots vs only 0.33% of 4-44<sup>+</sup> CD22<sup>high</sup> cells), demonstrating that CD22<sup>low</sup> cells were indeed AFCs, consistent with the syndecan staining and their appearance on cytospin (Fig. 2, L and M).

**Surprisingly, although the CD22<sup>low</sup> cells were AFCs, they were not classical plasma cells.** They had normal levels of expression of surface Ig and class II, unlike terminally differentiated plasma cells, which lack surface Ig and class II (31, 32). Rather, these AFCs had features more consistent with plasmablasts, given their persistent expression of activation markers and surface Ig. In fact, these plasmablast-like cells were the dominant population, as very few sIg<sub>lo/neg</sub> classical plasma cells could be identified in converted mice. Rather, although most of the cells with high intracellular Ig staining (a marker of both plasma cells and plasmablasts, but not resting or activated B cells) were CD22<sup>low</sup> (Fig. 2M), they had normal levels of surface Ig (Fig. 2, K and L; compare yellow R6 gate with green and red R4 and R5 gates in Fig. 2L).

**CD22<sup>low</sup> cells with a similar, but not identical, FACS profile are found in PBL**

We wondered whether either of the two populations of RF B cells in the spleens of converted mice corresponded to the 4-44<sup>+</sup> cells that signify conversion in blood. FACS analysis showed that RF<sup>+</sup> B cells in blood of converted mice (Fig. 3, solid line) are indeed similar to the CD22<sup>low</sup> subset in that most have down-regulated CD22, as well as CR1/2 (Fig. 3, B and C). Also like the cells in spleen, 4-44<sup>+</sup> cells among PBL had increased levels of CD86 and...
FIGURE 2. Location and identity of CD22low and CD22high RF B cells. A–D, Splenic follicle stained for Thy1.2 (red) and CD22 (blue) (A) and CD22 (blue) and 4-44 (red) (B). Most of the CD22high cells reside in the follicles and most of the CD22low 4-44+ cells are at the edge of the T zone and red pulp. Boxed areas are enlarged in C and D. There are numerous scattered 4-44+/CD22+ cells at the T zone red pulp border (C); there are 4-44+/CD22high follicular cells that stain more weakly than extrafollicular CD22low cells (D) since they lack the cytoplasmic Ig (see I and J). E–G, Dark-staining 4-44+ cells costain with syndecan. E, 4-44 (red) and CD3 (blue); F, syndecan (red) and CD3 (blue); G, 4-44 (red) and syndecan (blue). In G, note the doubly stained, almost black 4-44+/syndecan+ cells as well as scattered cells staining with only one or the other marker. H, 4-44+/CD22low cells (thick red) from recent converts are syndecan− and all of the 4-44+/CD22high cells (thin blue) are syndecan+, regardless of conversion status. As a control, 4-44+/CD22high cells from a young nonconverted mouse (green dashed) are shown. I–J, Cytospins of sorted CD22low/4-44+/CD22high (I) and CD22high/4-44+ splenocytes (J) demonstrating strong cytoplasmic staining in all of the CD22low cells. Green = 4-44 and blue = 4′,6′-diamidino-2-phenylindole. K–M, Most of the 4-44+/CD22low AFCs in converted mice have normal surface Ig levels, and very few resemble true plasma cells. Plots are gated on cells that were alive before fixation. Very few cells in the nonconverted mouse (K) have intracellular 4-44+ staining. Plasma cells are expected to have bright intracellular staining but weak surface staining (R6, orange); few were seen in the converted mouse (L), although membrane 4-44+ cells with intermediate (R4) and high (R5) intracellular Ig were present. M, CD22 expression in the 4-44+ populations gated in the plot of the converted mouse in L. Nearly all of the cells with elevated intracellular staining (R4–6) are CD22low, and all of the cells with very low intracellular staining (R3) are CD22high. Were very bright for CD44 (Fig. 3, E and F) and had down-regulated MHC class II molecules (Fig. 3D), perhaps to an even greater extent than splenic CD22low cells (Fig. 1L). However, unlike their CD22low counterparts in spleen, the cells in PBL were homogenously positive for B220 (Fig. 3A), which was variably down-regulated on CD22low cells in spleen (Fig. 1F). Interestingly, the cells were syndecan-negative (not shown), in contrast to the splenic counterparts (Fig. 2, F–H). Thus, RF+ cells in PBL were
clearly activated and had some features of plasmablasts. The lack of syndecan, which is not uniformly expressed on plasmacytes (33, 34), may reflect the migratory nature of the PBL cells, as syndecan is an adhesion molecule and when expressed may prevent migration into blood. The exact significance and origin of the PBL cells remains to be worked out; it will be of interest as these cells do resemble cells found in PBL of SLE patients with active disease (34). To begin to address this, we have performed limited V/H sequence analysis of cells sorted from PBL. We could readily recover V/H8 sequences even from small numbers of such cells, confirming their identity. More importantly, these cells did contain mutations (average of 2 mutations per sequence), establishing that these B cells circulate in response to autoantigen-driven stimulation and that they derive from a compartment of B cells undergoing mutation or that had mutated in the past. Such precursors are presumably in the spleen, given that their appearance only correlated with the activation of RF B cells in spleen and not LN (53).

**Both CD22 low and CD22 high cells in spleen are actively proliferating, but CD22 low cells have higher rates of apoptosis**

The AFC/CD22low populations in PBL and spleen resembled plasmablasts more than classical plasma cells. Plasmablasts are dividing cells, and in our view, cell division definitively separates non-dividing plasma cells from plasmablasts (35). To determine whether the CD22low population was dividing, mice were given BrdU and sacrificed at various intervals. After 2 h of labeling, both CD22low and CD22high cells in converted mice had similar proportions (10–20%) of BrdU-labeled, 4-44+ cells (Fig. 4A). Appearance of labeled cells after only 2 h of BrdU exposure indicates active proliferation and further establishes the CD22low cells as plasmablasts. BrdU-labeling also demonstrated that the CD22high population in converted mice was activated and proliferating, as the proportion of BrdU+ cells was substantially higher among CD22high cells of converts than of nonconverted or IgHb mice. However, unlike the CD22low cells, only a small percentage of CD22high cells remained BrdU+ after 24 h (Fig. 4A). Since the initial proliferation rate in both populations is similar, the CD22high cells either must die faster than the CD22low cells or differentiate quickly into CD22low cells once activated. To distinguish these possibilities, we measured apoptosis using TUNEL. If the more rapid loss of BrdU label from the CD22high population were due to apoptosis, we would have expected a greater frequency of TUNEL-positive cells in this population than in the CD22low one. However, on average, 38.4% of the CD22low cells were undergoing apoptosis directly ex vivo, whereas only 22.2% of the CD22high cells were undergoing apoptosis (Fig. 4B). This degree of apoptosis was associated with activation, because only 7.8% and 4.1% of CD22high cells from IgHb mice and IgHb non-converts, respectively, were apoptotic. It thus seems likely that the more rapid loss of label from the CD22high population must be attributable to differentiation to CD22low cells. Therefore, CD22low cells derive from CD22high cells as well as from self-renewal. The apparent slower decay of BrdU+ CD22low cells at later points following the pulse label (Fig. 4A) is also consistent with this interpretation since labeled CD22high cells would be differentiating into labeled CD22low cells, thus replenishing the latter compartment. Attempts to further investigate this in cell transfer experiments were unsuccessful due to limited cell recoveries and a propensity of CD22low cells to die ex vivo during purification.

**Somatic mutation in CD22 low and CD22 high cells**

Since there were two populations of proliferating RF B cells at the T zone-red pulp border, neither of which is classically thought to undergo somatic hypermutation, we wanted to determine which of these cell types was actively mutating. To determine mutational content in the two populations, we FACS-sorted both, sequenced...
the endogenous Vκ8 L chain, and calculated the average number of mutations/sequence (Fig. 4C). In recent converts, CD22<sup>low</sup> cells had on average twice as many mutations as their CD22<sup>high</sup> counterparts (p = 0.004). However, this difference is accounted for by a higher fraction of unmutated sequences in the CD22<sup>high</sup> cells, as shown in the histogram of the distribution of the number of mutations/sequence (Fig. 4D); considering only the mutated sequences, the frequency of mutations is similar in the two populations (CD22<sup>low</sup> = 2.5 ± 0.3 mutations, CD22<sup>high</sup> = 2.0 ± 0.2 mutations, p = 0.22).

Interestingly, the average number of mutations increases significantly in old converts (Fig. 4C, p = 0.03). Among these more-mutated sequences, a clear bimodal distribution emerges in those from the CD22<sup>high</sup> population (Fig. 4E). The bimodal distribution could be explained if the CD22<sup>high</sup> population includes both activated (mutating) and resting (unmutated) cells, consistent with the phenotypic heterogeneity of the CD22<sup>high</sup> population described above (see Fig. 1, H–J). Since both populations contain mutations, then either both are mutating or only one is mutating and it then differentiates into the other. Assuming that the CD22<sup>high</sup> cells differentiate into the CD22<sup>low</sup> cells, as suggested by the BrdU-labeling kinetics and TUNEL<sup>+</sup> frequencies, then the CD22<sup>high</sup> population must be mutating. However, this explanation does not exclude that the CD22<sup>low</sup> cells are also mutating. Some ongoing mutation in the CD22<sup>low</sup> population is quite plausible, since it is clearly undergoing active cell division; in fact, a proportion of the CD22<sup>low</sup> sequences has more mutations than any of the CD22<sup>high</sup> sequences (Fig. 4, D and E), suggesting that some additional mutation can take place among CD22<sup>low</sup> cells.

**Affinity maturation of the RF response**

Somatic mutations can lead to higher affinity Abs and these can be selected for during Ag-driven immune responses (36, 37). There is evidence that similar selection can lead to higher affinity autoantibodies to DNA, as determined in hybridoma studies (38, 39). However, there is little evidence for affinity maturation of RF B cells (40), although elevated R/S ratios in CDRs appeared to reflect selection in one study (41). To determine whether the response occurring in the spleen of converted AM14 H Tg MRL/lpr mice led to the selection of higher affinity RF B cells, we used an ELISA assay as previously described (29) that can determine relative affinity of RF B cells. When the target substrate on the ELISA plate is diluted in concentration, the binding of lower affinity RF is reduced before that of higher affinity RF. A similar assay has been described to detect relative affinity of nitrophenyl-specific Ab based on the reduced density of hapten using differentially haptenated substrate (42). We tested two different sources of RF. First, we recovered three 4-44<sup>-</sup>/RF hybridomas from a fusion of spontaneously activated B cells from the spleen of a converted mouse. These RFs expressed Vκ8 light chains (data not shown). Notably, these hybridomas had apparently higher affinities than the germline AM14 IgM Ab transfectoma 400μ23 (14) (Fig. 5A and data not shown). The higher affinities are indicated by the stronger relative binding at low densities of IgG2a target Ag. We also cultured whole splenocytes from converted mice overnight to allow the resident plasmablasts to secrete RF into the medium. These supernatants were then tested in the differential substrate assay (Fig. 5B). Again, supernatants demonstrated higher affinity than the...
germline. Together, these two experiments indicate that the process of RF B cell activation by autoantigen selects for higher affinity RF B cells.

Discussion

Systemic autoimmunity is a complex process involving initial loss of B and T cell tolerance followed by engagement of multiple effector functions that lead to tissue destruction. The initial inflammation undoubtedly leads to subsequent activation of further waves of autoreactive lymphocytes and repetition of the cycle until a state of chronic inflammation ensues. Most studies of humans and mice have only been able to identify and characterize disease at this late stage. In part, this is because early stages of disease may be asymptomatic and because stochastic or environmental events play a major role in determining the onset and nature of autoimmunity—even identical twins are not always concordant, and inbred mice in the same cage can have markedly different kinetics and features of autoimmunity.

It is critical to obtain more information about the nature of early events in the process. This has been very difficult precisely because the environmental and stochastic factors that incite autoimmunity are not under experimental control, and thus it is difficult to know at what stage of disease any individual is at a given age or time point. We previously devised and validated a method to identify the onset of the RF B cell response in a cohort of autoimmune-prone mice carrying a Tg for the H chain of an RF autoantibody. These mice are destined to make an RF autoantibody response, but the time of onset varies over a period of several months (53). We also showed in this system that older mice that had made an RF response demonstrated a predominant extrafollicular Ab-forming cell response without an RF GC response. These extrafollicular B cells were quite unusual in that they were undergoing somatic hypermutation but were not further characterized (15).

B cell subpopulations that develop during the RF response

The major goal of the present work was to define the populations that were participating in the early splenic RF response, taking advantage of the ability to identify the onset of the RF response in individual animals, a process we termed conversion. We found that there were both CD22<sup>high</sup> plasmablasts and CD22<sup>high</sup>-activated B cells in converted mice. These populations were only found in mice with ongoing autoimmunity. We were surprised to clearly identify a plasmablast—as opposed to a plasma cell—as the dominant producer of RF autoAb. The normal plasmablasts that dominate early immune responses to foreign Ags are not completely characterized; however, the population we observed seemed to resemble the normal population, as evidenced by high expression of CD44 and syndecan and low expression of CD22 (43, 44). Despite this, it remains possible that there are subtle differences between plasmablasts secreting RF autoantibody in autoimmune-prone mice and those responding to foreign Ag in normal mice. An even more complete characterization of both populations is required.

The identification of the AFC as a plasmablast has important implications. These plasmablasts have characteristics that would allow presentation of Ag to T cells and thus may be active in this role. Further, plasmablasts would be expected to respond quite differently to certain therapeutic interventions; for example, their surface phenotype including the lack of typical B cell markers like CD20 and CD22 is important if one is designing Ab-mediated therapy to eliminate AFCs. Plasmablasts can interact with dendritic cells (DCs) and may require factors from DCs for their survival (45, 46). Indeed, we previously showed that RF AFCs were in contact with CD11c<sup>+</sup> DCs in the spleens of converted mice (15). If these interactions are important for plasmablast survival then disrupting them could represent a therapeutic strategy. For example, plasmablasts in vitro respond to BAFF (46), a myeloid product (47), and this could explain the efficacy of inhibition of BAFF/APRIL in vivo in MRL/lpr mice (48). The abnormal regulation of AFCs in NZM22410 mice (49) further highlights the importance of understanding the genesis of AFCs in autoimmunity. The present study makes an important contribution by identifying the key role of the plasmablast and defining its phenotype and relationship to an activated B cell precursor.

![FIGURE 5.](image-url) RF AFCs generated in the spontaneous response have higher affinity than the germline AM14 HL combination. Low affinity RF loses binding when the Ag coating is low density (29). This is seen by comparing 20.8.3 (solid line, round symbols in A and B), a very high affinity RF clone, and 400t<sub>μ</sub>23 (light line, squares in A and B), the original AM14 low affinity unmutated RF clone. y-axis shows binding relative to maximal binding for each sample to normalize for different starting concentrations of samples. The x-axis represents the concentration of IgG2a target bound to the plate. Note that with decreasing target Ag the binding of high affinity 20.8.3 is relatively unaffected whereas most of the binding by low affinity 400t<sub>μ</sub>23 is lost. A, relative binding to varying concentrations of coated IgG2a target for two Id<sup>+</sup> RF hybridomas (dashed lines) made by fusion of spleens from a converted H Tg MRL/lpr mouse as described in Materials and Methods. At concentrations of IgG2a<sub>a</sub> &lt;0.5 μg/ml, these hybridoma RFs show increased IgG2a<sub>a</sub> binding relative to 400t<sub>μ</sub>23. The unusual prozone effect for 15C was a reproducible observation. B, Splenocytes from three aged H MRL/lpr mice that had high numbers of id<sup>+</sup> ELISpots (dashed lines, diamonds) were cultured overnight and supernatants assessed by ELISA as shown in A. The solid thin line (triangles) represents supernatant from spleens of double HL Tg BALB/c mice in which the Tg L chain is unable to mutate. Like 400t<sub>μ</sub>23, binding by this supernatant falls off at the lower concentrations of coated target. In contrast, note that the titration curves for splenocyte supernatants from the H MRL/lpr mice approach that of the high affinity 20.8.3 clone.
A recent report presented a characterization of AFCs in the spleen of autoimmune New Zealand Black/White mice (54). Although this study did not determine the stage of disease we did here or focus on RF B cells, they did find a substantial proportion of short-lived plasmablasts. The authors highlighted the finding of long-lived plasma cells seen in their model, which were not observed in ours. Whether this represents a difference in strain, specificity, stage of disease, or methodology is unclear. Nonetheless, the presence of short-lived plasmablasts is a common feature.

Cell death and division in the autoimmune B cell response

Analysis of cell division and death revealed an ongoing, highly dynamic process of autoimmunity. Both the CD22high and CD22low populations were undergoing very rapid cell division and apoptosis. The revelation that the autoimmune reaction in the spleen is so dynamic, if it parallels the human situation, could explain the efficacy of cytotoxic and anti-proliferative agents in SLE as well as the rapid time course of such responses. Emerging data from humans treated with anti-CD20 to deplete CD20pos B cells (but presumably not CD20neg plasmablasts or plasma cells directly) demonstrate that some but not all autoantibody titers fall relatively quickly (50, 51). This could be explained if the plasmablast population were being renewed by a proliferating CD20pos B cells (this study).4 CD22high activated B cells are very likely the precursors of CD22low plasmablasts. This event is accompanied by the appearance of a plasmablast-like cell in the PBL. The entire process is highly dynamic, particularly in the plasmablast compartment. CD22high possibly CD22low cells are undergoing somatic hypermutation as well as selection for higher affinity. The response is progressive and includes at least some clones that live for a long period.

The extent of mutation in the two populations provides insight into the dynamic nature of the response. This is based in part on the concept that the number of mutations in a given cell serves as a time clock for the number of cell divisions it has undergone. Indeed, sequences isolated from mice converted for long periods contain more mutations than those of recent converts (Fig. 4, D and E). This indicates that there are some relatively long-lived clones of RF B cells. In fact, some RF B cells have as many as 15 mutations in their Vκ region alone. Assuming a rate of 0.25 mutations/V region/division (15, 52), in these clones there were 60 or more divisions during the phase of active somatic hypermutation. BrdU labeling indicates a division rate of at least every 12 h, meaning that some clones survive for ≥4 wk in the actively mutating state.

Thus, despite active proliferation and cell death among the plasmablast fraction, there must be a precursor population—most likely the activated CD22high cells—that can provide for clonal longevity through many cell divisions.

Finally, we addressed whether mutation during the extrafollicular RF response is accompanied by selection. Using hydridomas as well as assay of polyclonal supernatants collected from splenocytes of converted mice, we showed that much of the secreted RF has a higher affinity than the germline AM14 Ab. This is consistent with affinity maturation due to somatic hypermutation. Supporting this interpretation is the finding of certain recurrent replacement mutations in CDRs in our collection of V region sequences (Ref. 15 and unpublished data). Thus, we have now shown that both mutation and selection occur outside the GC in the spontaneous RF response in MRL/lpr mice.

Conclusion

We have used the RF Tg system to develop a detailed picture of both the onset and evolution of a model autoantibody response in the spleen. Like the established response (15), the early response does not include GCs. Instead, it begins as scattered foci of extrafollicular sites of proliferation and differentiation of RF B cells (this study).2 CD22high activated B cells are very likely the precursors of CD22low plasmablasts. This event is accompanied by the appearance of a plasmablast-like cell in the PBL. The entire process is highly dynamic, particularly in the plasmablast compartment. CD22low and possibly CD22low cells are undergoing somatic hypermutation as well as selection for higher affinity. The response is progressive and includes at least some clones that live for a long period.

This picture of a dynamic ongoing response has implications for our understanding of spontaneous systemic autoimmune disease and strategies for its treatment. In future work, we would like to determine whether this scenario applies to other autoAbs and other murine models of autoimmunity as well as human disease. Given the common autoAb profiles of MRL/lpr mice and human disease, we expect that our findings will be generalizable; however, even potential differences will be interesting because they will probably reflect the range of clinical disease and autoAb profiles that vary greatly among patients with SLE.

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Disclosures

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References


