Rheumatoid Factor B Cell Memory Leads to Rapid, Switched Antibody-Forming Cell Responses

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Rheumatoid Factor B Cell Memory Leads to Rapid, Switched Antibody-Forming Cell Responses

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B cells are critical in the initiation and maintenance of lupus. Autoreactive B cells clonally expand, isotype switch, and mutate—properties associated with memory B cells (MBCs), which are typically generated via germinal centers. The development and functions of autoreactive MBCs in lupus are poorly understood. Moreover, mounting evidence implicates the extrafollicular (EF) response in the generation of switched and mutated autoantibodies that are driven by BCR and TLR corecognition, raising the question of whether MBCs are generated in this context. In this study, we investigated autoreactive MBC generation associated with this type of response. We transferred B cells from AM14 site-directed BCR transgenic mice into nontransgenic normal recipients and elicited an EF response with anti-chromatin Ab, as in prior studies. By following the fate of the stimulated cells at late time points, we found that AM14 B cells persisted at increased frequency for up to 7 wk. Furthermore, these cells had divided in response to Ag but were subsequently quiescent, with a subset expressing the memory marker CD73. These cells engendered rapid, isotype-switched secondary plasmablast responses upon restimulation. Both memory and rapid secondary responses required T cell help to develop, emphasizing the need for T–B collaboration for long-term self-reactivity. Thus, using this model system, we show that the EF response generated persistent and functional MBCs that share some, but not all, of the characteristics of traditional MBCs. Such cells could play a role in chronic or flaring autoimmune disease.

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inclusive definition of memory requires only Ag exposure with subsequent longevity and quiescence, but it does not necessarily require a GC or T cell help.

As noted, in several mouse models of lupus, anti-nuclear and rheumatoid factor (RF) B cell activation is largely TLR driven, GC independent, and EF localized (3–5). In such models, T cells certainly play a role, but they are not essential for isotype switch, mutation, or differentiation to AFCs (5, 33). This type of activation is found in autoimmune-prone mice, including MRL. *Fas* 

$\beta^+$, B cell–activating factor of the TNF family transgenic (Tg), and NZB/W (1, 2, 4, 34, 35). Although short-lived plasmablasts are the primary cells thought to arise from an EF response (36), it is unclear whether this response could generate MBCs. Thus, with respect to autoimmune disease pathogenesis, the questions remain what contribution naïve or MBCs make in giving rise to short-lived plasmablasts and/or to GCs.

Because memory formation is typically thought to require resolution of the response and separation of specific cells from stimulatory Ag, whether or how MBCs form in autoimmunity remains unclear, because autoimmunity is a chronic disease with a continuous supply of self-Ag. However, SLE can be relapsing and remitting (37), thus potentially providing intervals of lower self-Ag and a less inflammatory environment, allowing for B cells to rest prior to reactivation. Indeed, there is evidence of autoreactive MBCs in patients with autoimmune disease. Identified in humans by the widely used marker CD27, MBCs have been found with autoreactive specificity by virtue of carrying the 9G4 idiotype in SLE patients (9, 10). Furthermore, populations of CD27$^+$ MBCs that have V region somatic mutations have been identified in SLE patients (38), albeit at a similar frequency to normal controls and that have V region somatic mutations have been identified in SLE (1, 2, 59, 60). However, similar to the situation in human SLE patients, the history of spontaneously activated 4-44$^+$ AM14 B cells cannot be assessed in aged diseased mice due to chronic presence of the autoantigen.

An advantage of AM14 B cells is that they remain ignorant of their self-Ag in nonautoimmune-prone mice (61). However, when nonautoimmune-prone AM14 mice are challenged with TLR ligand–containing anti-chromatin Abs, B cell–intrinsic MyD88 drives differentiation of AM14 B cells to plasmablasts at the histologically identical EF site, but it does not drive detectable GC responses (6). Interestingly, this response proceeds in the absence of T cells, although T cells can support the response (5, 33). Furthermore, AM14 B cells transferred into non-Tg recipients undergo a similar response, which allows us to define the history of a finite 4-44$^+$ population in vivo following acute activation with anti-chromatin Ab (33). We used this system in this study to test whether autoreactive MBCs persist following an EF response and, if so, whether their development and function require T cells.

### Materials and Methods

#### Mice

AM14 sd-Tg mice were generated as previously described and backcrossed for at least eight generations to the BALB/cj strain (59). BALB/cj recipients were purchased from The Jackson Laboratory. D011.10$^{+/+}$ Tcr$\alpha^{-/-}$ BALB/c (D011.10) mice were obtained from Dr. Kim Bottomly (Yale University) (62).

#### B cell isolation and adoptive transfer

AM14 sd-Tg B cells were isolated from splenocytes using the EasySep Mouse B Cell Enrichment Kit (StemCell Technologies), following the manufacturer’s instructions, yielding $\geq95\%$ purity, as determined by flow cytometry. Three million AM14 sd-Tg B cells/mouse were injected i.v. in sterile PBS.

#### Ascites preparation and immunization

*Rag$^{2-/-}$* BALB/c mice were injected with pristane (Sigma) prior to i.p. injection of 10 million PL2-3 hybridoma cells (54) in sterile PBS. Ascites was collected after 1 wk, sterile filtered, and quantitated using ELISA. A total of 0.5 mg PL2-3 ascites was injected on days 0, 2, and 4 following AM14 sd-Tg B cell transfer for a full primary response to generate memory and/or 2.5 d prior to sacrifice for a secondary or early primary control response.

#### CFSE labeling

B cells were labeled in 0.1% BSA in sterile PBS at a concentration of 50 million cells/ml in 10 $\mu$m CFSE (Invitrogen) for 10 min at 37°C protected from light.

#### Flow cytometry

Abs were purified in the laboratory, as previously described (58), unless purchased as indicated. Single-cell suspensions of splenocytes were blocked using clone 24G2, and dead cells were excluded using ethidium monoazide (Invitrogen). Cells were fixed using 1% paraformaldehyde and permeabilized using 1× Perm/Wash (BD) with rat serum (US Biological). The following Abs were used: 4-44 biotin, 4-44 Alexa Fluor 647, anti-CD73 PE (TV/23; BD), and anti-Ki67 FITC (SP6; Abcam). Data were collected using an LSR II (BD) and analyzed using FlowJo software (TreeStar). Doubles were excluded during analysis.

#### ELISPOT assay

Immuron 4 plates were coated with polyclonal goat anti-mouse anti-IgG2a or anti-IgM (Southern Biotech). Splenocytes were incubated for 5–6 h at

H chain Tg and site directed (sd)-Tg mice, in which B cells, paired with an endogenously derived Vxk8 L chain, are specific for IgG2a$^\alpha$ (58). This pairing can be detected by the anti-idiotype Ab 4-44, allowing specific tracking of RF B cells. 4-44$^+$ AM14 B cells spontaneously differentiate to plasmablasts that mutate and switch at the EF site, only on autoimmune-prone genetic backgrounds and only in the presence of the self-Ag IgG2a$^\alpha$ (1, 2, 59, 60). To track a population of RF B cells, we and other investigators used AM14 BCR and molecular mechanisms in vivo. This can be approached in patients, we cannot determine their history or determine cellular activation is found in autoimmune-prone mice, including MRL.
37°C, as previously described (6). AFCs were detected using 4-44 biotin, streptavidin alkaline phosphatase (Invitrogen), and 5-bromo-4-chloro-3-indolyl-phosphate (AMRESCO) in agarose. AFCs were counted using a dissecting microscope.

Sequencing

Fixed and permeabilized naive 4-44+ and memory 4-44+ CD73+ cells were sorted using a FACSaria (BD). Sequences were obtained as previously described (1). Briefly, genomic DNA was obtained using proteinase K digestion. Following inactivation at 95°C, nested PCRs were performed using PfU Ultra II (Stratagene). Sequences were isolated using the Zero Blunt Topo Cloning Kit for Sequencing (Invitrogen) and were analyzed by the Keck Facility at Yale University. Sequences were aligned using MegAlign software (Lasergene).

Results

Anti-chromatin Abs drive resting long-lived RF B cells

To investigate whether the EF AM14 B cell response to PL2-3 anti-chromatin Abs could be long-lived, persist, and provide functional memory, we transferred AM14 B cells to BALB/c recipients. In this way, no new naive AM14 B cells could emerge to potentially confound the analysis. Additionally, CFSE labeling was used to track divisions that had occurred following Ag-specific activation with PL2-3. We identified the transferred cells of interest and distinguished authentic rare B cells (4-44+, Fig. 1A, upper middle and upper right panels) from background staining (Fig. 1A, upper left panel) by staining both surface and intracellular compartments using 4-44 labeled with different fluorochromes (63). Intracellular, as well as surface staining, was used to gain more specificity and to reduce background of cells with surface staining of cytophilic Ab, because only authentic AM14 B cells have both surface and intracellular 4-44+ Ig. CFSE-labeled 4-44+ cells transferred into hosts given PL2-3 were uniformly CFSE− at week 4 after activation by PL2-3 (Fig. 1B), in agreement with earlier findings that 99% of 4-44+ cells had fully diluted CFSE by day 6 (33). Of note, CFSE[high] 4-44+ cells in unimmunized mice were detectable, indicating that they had not divided (Fig. 1A, lower panels). Just 0.002% of live cells were 4-44+ and CFSE− in the naive mice 4 wk after transfer, similar to our previous findings at day 6 (33). Background from recipient cells is impossible to exclude completely when analyzing rare transferred cell populations; these are very likely the source of this population, although we cannot exclude that some homeostatic proliferation may have occurred. Regardless, there is a striking difference between PL2-3–treated and nontreated recipients in CFSE dilution (Fig. 1B). Virtually all 4-44+ cells exposed to PL2-3 underwent at least seven divisions.

If the Ag-exposed cells remaining at 4 wk posttransfer were bona fide memory cells, they should be in a resting state. However, some residual plasmablasts were present at 4 wk posttransfer (data not shown). Because we sought to analyze a population of pure MBCs, we increased the resting period to 7 wk. To determine whether this time point was most appropriate for assessing resting memory, we transferred CFSE-labeled AM14 B cells into mice that were naive, that were given PL2-3 7 wk prior to transfer, or that were given PL2-3 on the day of the cell transfer. As expected, transferred cells proliferated in response to PL2-3 given at the time of transfer. However, 7 wk following PL2-3 immunization there was not sufficient Ag remaining to induce any detectable proliferation (Fig. 2A, 2B). Thus, although 4-44+ cells were detectable 4 wk posttransfer and challenge, we determined that 7 wk after PL2-3 administration would be a more appropriate time point to study the putative memory and recall responses in subsequent experiments, because we could document that there was no physiologically stimulatory Ag remaining from primary immunization by this time.

To determine whether AM14 B cells immunized 7 wk earlier were indeed resting, we stained for the cell cycle marker Ki67. Nearly all of the 4-44+ cells were not Ki67+ and, thus, were not transiting the cell cycle (Fig. 2C, 2D). This was in contrast to the positive control mice, sacrificed during an early and active response to PL2-3, wherein >40% of the 4-44+ cells were Ki67+. Thus, 7 wk after immunization, 4-44+ AM14 B cells were both Ag experienced and noncycling. Therefore, we further investigated AM14 B cell persistence ≥7 wk following activation by PL2-3. In mice given AM14 B cells and PL2-3 8 wk earlier, the 4-44+ population was expanded 4-fold compared with naive controls. Both groups had populations that were above the very low background levels found in the no-cell-transfer controls (Fig 3A, upper panels, 3B, 3C). CD73 has been identified as a marker of MBCs in mice (17). CD73 was found on the majority of Ag-exposed 4-44+ B cells in PL2-3–treated mice in contrast to background levels within the naive 4-44+ population (Fig 3A, lower panels, 3D). There was >10-fold more 4-44+ CD73+ cells in PL2-3–immunized mice compared with naive mice (Fig. 3E). Thus, Ag-exposed but noncycling AM14 B cells persist for many weeks following activation by PL2-3 and express a marker typically found on classical MBCs.

One hallmark of B cell activation is affinity maturation, as reflected by somatic hypermutations of the BCR. However, it should be noted that mutation is largely absent in some subsets of GC-dependent MBCs (13, 17, 64), as well as in MBCs generated in mice that could not form GCs (27). However, somatic hypermutation was found at the EF site in aged AM14 MRL.Fas−/− mice during ongoing autoimmunity (1), as well as during the acute PL2-3–induced response (6). To test whether long-lived AM14 B cells
gained mutations during activation, we sorted 4-44+ cells from naïve mice and 4-44+ CD73+ cells from PL2-3–immunized mice 8 wk postimmunization. The results reflect two independent sorts, with sequences obtained from three naïve mice and six memory mice total. Sequencing the V\text{k}8 L chain from these sorted cells revealed only a very low level of mutation that did not differ significantly between naïve 4-44+ B cells and Ag-exposed 4-44+ CD73+ B cells. In both naïve and memory 4-44+ cells, 12.5% of sequences were mutated. In naïve cells, 3 sequences were mutated out of 24 analyzed, and all were bearing only one mutation. In memory cells, 6 sequences were mutated out of 48 analyzed. Of these, five sequences had one mutation, whereas the remaining sequence had four mutations.

RF B cell memory and rapid secondary responses are T dependent

The primary response of transferred AM14 B cells to anti-chromatin Abs is present, but reduced in magnitude, when T cells are restricted to an irrelevant monoclonal population, such as that found in the DO11.10 mouse (33). To test whether the persistence of Ag-experienced AM14 B cells is affected by the quality of T cell help, we transferred AM14 sd-Tg B cells to either wild type BALB/cJ (WT) mice or DO11.10 mice, gave PL2-3, and analyzed splenocytes 7 wk later.

In DO11.10 recipients there was no significant expansion of 4-44+ cells (Fig. 4A, 4B), nor was there a significant increase in CD73+ 4-44+ cells (Fig. 4C, 4D). WT recipients exhibited such increases upon immunization as expected, and the numbers and frequencies of 4-44+ and CD73+ 4-44+ cells were significantly higher than were those in DO11.10 recipients. Thus, Ag-specific T cell help is required to support full development of an ex-
panded long-lived 4-44⁺ population following activation by anti-chromatin Ab.

In addition to persistence, functional specific B cell memory implies a capacity for rapid secondary responses. Hence, it was possible that, even in the absence of significant expansion, residual AM14 B cells in immune DO11.10 mice were qualitatively altered. To test this, we rechallenged DO11.10 or WT mice that had received AM14 B cells and PL2-3 7 wk prior and analyzed responses at day 2.5 (Fig. 5A). Critically, this design also allowed us to determine whether AM14 MBCs formed in a WT background could mount accelerated and qualitatively different secondary responses. Strikingly, only 2.5 d following secondary PL2-3 immunization, a 4-44⁺ AFC response had emerged in WT recipients, which consisted of both IgM and IgG2a AFCs (Fig. 5B, 5C); this was not found in DO11.10 recipients. The rapid emergence of AFCs at day 2.5 is remarkable because primary EF plasmablast responses are expected no earlier than day 3.5 (65). To establish a direct comparison between memory and naive responses, we transferred AM14 B cells to an additional group of WT recipients, such that the precursor frequency was 2-fold greater than that in the memory mice. However, at the time point analyzed (day 2.5), these “new primary” control animals were only starting to make a small 4-44⁺ IgM AFC response and barely made a 4-44⁺ IgG2a AFC response. In both cases, the mean number of AFCs detected per million splenocytes was <2. Notably, the secondary 4-44⁺ IgM AFC response was 30-fold greater compared with the new primary response after normalization for precursor frequency.

The day-2.5 secondary response in WT mice was also associated with a 4-fold increase in the frequency of the 4-44⁺ population (compare Fig. 4A “PL2-3” black bar, which shows the frequency of 4-44⁺ cells in immune mice prior to reimmunization, with Fig. 5D “secondary” black bar). Similarly, expansion in the WT new primary response was 3-fold (Fig. 5D, hatched versus gray bars); therefore, proliferative capacity during the primary response and secondary response was comparable. This expansion of the 4-44⁺ population in a recall response was TD, because expansion in DO11.10 mice following the secondary treatment with PL2-3 was 10-fold less than that found in WT mice (Fig. 5D). Thus, we find that autoreactive B cell memory persistence and function are T dependent; further, autoreactive MBCs that developed in this system have been reprogrammed compared with their naive counterparts to differentiate rapidly to both IgM and IgG2a plasmablasts.

Discussion

In this article we defined the long-term kinetics of an EF RF response, taking advantage of the well-characterized AM14 system...
(1, 2, 33, 58, 59). RF B cells in the natural setting of murine lupus (66–68), as well as in this experimental system (5), are stimulated by immune complexes that contain nucleic acids; these responses are TLR7/TLR9 dependent. As such, the RF system serves as a model for antinuclear responses that has the experimen-
tal advantage that self-Ag can be controlled and introduced.
Indeed, the RF system was the first to call attention to the TLR-
dependent nature of antinuclear responses (69). Since this initial
demonstration, multiple lines of evidence have pointed to the
role of TLR/BCR coligation in the stimulation of both RF and
anti-DNA/RNA B cells (3, 66, 70). Hence, the study of RF B
cells provides insight into ANA clones through its activation
by anti-chromatin Ab.

Despite the prior notion that such responses were inherently
short-lived, we found that, in response to anti-chromatin Ab, AM14
RF B cells can persist beyond the primary response and are
functional at 7 wk postimmunization. Thus, the EF response is
temporally and functionally more complex than previously rec-
ognized. The most striking and important finding is that AM14 RF
B cells that persist after an initial EF response provide bona fide
functional memory, because they are able to differentiate to both
IgM and IgG AFCs more rapidly and robustly than their coun-
terparts in a primary response. Additionally, they show qualities
of memory, in that they are marked by CD73, and are resting, as
determined by lack of Ki67 staining. However, it is important to
point out that these MBCs differ from classic GC-derived MBCs
in lacking substantial V region somatic mutation. Thus, the EF
response does generate a form of memory. We further suggest that
in situations in which GC B cells also generate more classical
MBCs, whether in normal immunity or autoimmunity, the EF
response is also likely creating MBCs, thus contributing a different
quality of MBCs to the total memory compartment; this notion is
supported by studies of the immune response to the hapten group
4-hydroxy-3-nitrophenyl (23).

In the absence of Ag-specific T cells, AM14 B cells can make
both IgM and isotype-switched primary AFC responses, albeit of
a somewhat reduced magnitude (33). In contrast, absence of Ag-
specific T cells leads to an essentially complete loss of AM14
MBC formation and response, in contrast to certain types of B
cell memory that can be generated in a T-independent fashion
(28), this requirement of T cells for development and function of
EF-derived memory is evocative of previously published work
showing primary-switched Ab secretion, but deficiency in mem-
ory responses, if GCs or T cell help was absent (21, 24, 27). Thus,
in determining whether MBCs will persist following activation,
the site of activation may not be as critical as the availability of
T cell help.

These findings suggest that autoreactive B cell memory and
rapid AFC differentiation could contribute to SLE progression,
because autoreactive MBCs differentiate to secrete Ab faster than
do their naive counterparts. Because disease can be relapsing
and remitting, flares could be initiated by reactivation of memory
and recall AFC responses, instead of or in addition to activation of
naive precursors. Indeed, autoreactive CD27+ MBCs were char-
acterized in SLE and RA patients (9, 10), and their presence is
correlated with relapse (42–45).

Although both switched and unswitched MBC populations have
been studied by several groups (12, 13, 15–17, 71, 72), it is unclear
which classes of MBCs contribute to autoimmunity. The MBCs
that we identified from AM14 B cells in response to anti-chro-
matin Ab are largely unswitched (data not shown) and unmutated,
whereas MBCs studied in patients can be switched and mutated.
Three lines of reasoning suggest that both of these populations
are relevant to disease. First, the presence of switched, mutated MBCs
in human patients does not rule out the possibility of unswitched,
unmutated memory. In fact, unswitched MBCs have been char-
acterized in SLE patients (10).

Second, the MBCs that we studied resulted from a single acute
challenge on a nonautoimmune-prone strain. In contrast, MBCs
generated during autoimmunity are either the result of a chronic
response or serial acute responses. This concept is supported by the
finding that in the first week, on a genuine autoimmune-prone
strain, MRL.Fas80, AM14 B cells accumulated more mutations
compared with their counterparts in BALB/c mice (6). It is possi-
ble and even likely that multiple rounds of stimulation of the
unswitched population that we observed would result in persistent
switched, mutated MBCs. Supporting this notion, upon rechal-
lenge there was a very rapid switch to IgG2a of a large proportion
of putative IgM+ precursors; the secondary response at day 2.5
was ~40% IgG2a. In this regard, the switched memory re-
response seen in BALB/c mice, as would also be expected in au-
toimmune mice, suggests that a major difference between the two
environments is the exposure to Ag and possibly other inflam-
atory signals in the latter, rather than any intrinsic differences
in the B cells, although such differences cannot be ruled out.

Third, the specificity of MBCs identified so far has not been well
defined. In one pioneering study, the frequency of polyreactive or
HEp2 ELISA–reactive IgG MBCs did not differ between four patients
and controls (46). This suggests that the IgG memory compartment
may not include some classic autoreactive specificities;
these autoreactive specificities could instead be limited to the
IgM memory compartment or may be rare among MBCs. How-
ever, anti-Ro52/La–type specificities were detected in one patient,
suggesting that more classical IgG memory can be generated at least
occasionally and for some specificities (46). This study did
not examine the prevalence of RF. It will be interesting to learn
whether unswitched, unmutated MBC clones can be identified in
patients or even murine lupus models, particularly early in disease.

Initiation and primary differentiation of AM14 B cells in re-
ponse to anti-chromatin Ab can occur in the absence of T cell help
(5, 33). Similarly, autoimmunity driven by overexpression of
B cell–activating factor of the TNF family does not require T cells
(4), nor are T cells needed to drive loss of self-tolerance by anti-
DNA B cells in B6 mice (73). TLR7-dependent activation and
differentiation of RNA-specific B cells in B6 mice also do not
require T cells, because they occur unimpeded on a RAG-deficient
background (3). Nonetheless, T cells were still critical for the
generation and function of AM14 MBCs, even though these
B cells were largely unswitched and unmutated. The T depen-
dence of autoreactive MBC formation is yet another dimension of
the cooperative B–T interactions that take place at many steps of
lupus pathogenesis (74–85). What emerges from these and other
studies is a model in which TLR and BCR signals drive initial
autoreactive B cell activation independent of T cells, whereas
T cells and T–B interaction subsequently become critical for op-
timal B cell expansion (33). It remains unknown whether quali-
atively different MBC responses depend on intrinsic changes in
such cells, are programmed by memory T cells, or both. In this
study, we extended prior knowledge by showing that T cells are
also required for development and reactivation of long-lived au-
toreactive MBCs that are generated via an EF pathway.

The investigation of different immunization scenarios has re-
vealed plasticity in the roles of both GCs and T cells in B cell mem-
ory formation, in contrast to the orthodox view that B cell memory
is strictly TD and GC dependent. In our case, B cell memory
elicited by chromatin-containing immune complexes was GC in-
dependent but TD. In what ways might this type of immunogen be
distinct from others? Most likely, the defining features of nucleic
acid–containing self-Ags that dominate in lupus are the ability to costimulate endosomally expressed TLRs, provide strong BCR cross-linking, and associate with proteins that could contain T cell epitopes. Thus, this type of Ag combines features of TI-1, TI-2, and TD Ags. Taken together, this work and prior studies of the AM14 response (5, 33, 59) suggest that this type of Ag functions as TI for initiation but as TD for full development of the B cell response, without eliciting a GC response. In a similar vein, Foote and Kearney (29) demonstrated that peritoneal B1b cells can stably expand following Enterobacter cloaceae immunization; however, it was not tested whether this was GC dependent or required T cells, even if the nominal Ag dextran is considered a TI Ag. Our findings are also in keeping with data from an elegant system devised by Ecll-Dorn and Batista (86), in which they coupled beads with both protein Ag and CpG DNA, simulating an Ag similar to DNA-containing immune complexes; such Ags drove an exclusively EF plasmablast response, although memory formation was not investigated. By using a defined model system with direct relevance to the activation of autoreactive B cells in lupus, our studies connect basic insights into the pathways of MBC formation with the pathogenesis of autoantibody generation and disease. These findings should form a basis for further investigation of how autoreactive B cell memory progresses during chronic disease.

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Disclosures

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References


