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B-Cell Activation and Tolerance Mediated by B-Cell Receptor, Toll-Like Receptor, and Survival Signal Crosstalk in SLE Pathogenesis

PRINCIPAL INVESTIGATOR:
Michael P. Cancro

CONTRACTING ORGANIZATION:
University of Pennsylvania
Philadelphia, PA 19104-6205

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B-Cell Activation and Tolerance Mediated by B-Cell Receptor, Toll-Like Receptor, and Survival Signal Crosstalk in SLE Pathogenesis

**5. AUTHOR(S)**
Michael P. Cancro, Jean L. Scholz

**E-Mail:** cancro@mail.med.upenn.edu

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Pennsylvania
3451 Walnut St.
Philadelphia, PA 19104-6205

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
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**14. ABSTRACT**
We previously found that B cell receptor (BCR)-delivered TLR9 agonists initiate a response involving proliferation followed by abrupt cell death; furthermore, responding cells are rescued by survival cytokines. We posited this as a normal immune response-limiting mechanism that, if thwarted, may lead to persistence of self-reactive antibody-secreting cells. With this proposal we seek to characterize the pathways leading to post-proliferative death and rescue, and to determine how different forms of rescue lead to alternative differentiation outcomes. Accordingly, the most significant finding during the first year research period is that in the context of BCR-delivered TLR9 signals, IL-21 promotes and IL-4 opposes the T-bet+CD11c+ “ABC” (age-associated B cell) fate that is associated with humoral autoimmune disease.

**15. SUBJECT TERMS**
regulation of B cell responses; TLR7/9 agonists

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1. INTRODUCTION: This grant is based on our observation that BCR-delivered TLR9 agonists initiate a self-limiting response involving proliferation and differentiation, followed by abrupt cell death, but that B cells can be rescued from death and directed towards effector fates by survival and differentiation mediators. The planned and ongoing studies are thus to investigate the outcomes of this rescue and differentiation in both murine and human cells – with emphasis on how different forms of rescue lead to alternative fates associated with autoimmune disease.

2. KEYWORDS: B cell, T-bet, Autoimmunity, SLE.

3. ACCOMPLISHMENTS:

What were the major goals of the project? The goals of the program are to detail the pathways mediating post-proliferative death, rescue, and differentiation of each peripheral B cell subset in mice and humans, and to assess how alternative forms of rescue mediate distinct differentiative outcomes.

What was accomplished under these goals? We have made substantial progress towards these related aims. In our studies to date, we have found that rescue can be achieved by costimulation and by TFH cytokines, including IFN gamma and IL21, but that the downstream pathways and transcriptional programs initiated differ. We have also found that in either of these circumstances, and in contrast to what is observed when survival cytokine BlyS alone mediates rescue, cells adopt the characteristics of B cell effector subsets recently described by our laboratory as “age-associated B cells” (ABCs). These cells are of interest because ABCs emerge much earlier in autoimmune-prone strains, and are enriched for autoreactive and polyreactive BCR specificities. Further, ABCs are preferentially activated by TLR7 and TLR9 agonists. Current findings in toto suggest ABCs are antigen-experienced B cells characterized by a T-bet driven transcriptional program, inasmuch as all of them, despite heterogeneity in other aspects, express antigen presenting cells, and tend to secrete IL10 and IFN-gamma on activation.

IL21 and IL4 reciprocally regulate the T-bet+ and CD11c+ “ABC” fate in the context of TLR9 and TLR7 signals. We have discovered that IL21 directs adoption of the ABC fate in the context of TLR9 or TLR7 stimulation, but can be blocked by IL4. These findings are summarized in Figs. 1 and 2 and discussed below.

![Fig. 1](image1.png) **Fig. 1:** IL21 & IL4 reciprocally regulate T-bet in STIC9 stimulated B cells. Splenic CD23+ B cells were cultured in the conditions shown for 72h, harvested and assessed for Tbx21 message (upper) & T-bet protein (lower panel).

![Fig. 2](image2.png) **Fig. 2:** IL21 and IL4 reciprocally regulate the T-bet+ CD11c+ fate in the context of TLR7 or TLR9 signals. Splenic CD23+ B cells from IL21 KO, UNC93b KO, or WT mice were loaded with CFSE (green) or VCT (violet) dyes respectively, co-cultured with the stimuli shown for 48h, and harvested and cytofluorimetrically assessed for dye dilution as well as T-bet and CD11c.

The results shown in Fig. 1 reveal that in the context of BCR-delivered TLR9 stimulation, IL21 upregulates T-bet, whereas IL4 blocks this effect. In the second set of studies (Fig. 2), WT and either IL21R or UNC93B KO mice were co-cultured and stimulated with either TLR7 or TLR9 agonists, along with various combinations of IL21 and IL4 or IFN-gamma. Similar results are obtained with either TLR9 or TLR7 agonists in each KO/WT co-culture, so one example of each is shown. Several conclusions can be drawn from these data. The WT cells reveal that: (i) IL21, in the context of either TLR9 or TLR7 stimulation, drives the T-bet+CD11c+ fate; (ii) IFN-gamma, while capable of driving T-bet+ differentiation as previously reported, does not induce CD11c; (iii) IL4 blocks the IL21-driven T-bet+CD11c+ fate, but not IFN-gamma mediated T-bet induction. The KO cells in each co-culture (green) indicate that B cell intrinsic IL21 and TLR signals are necessary and that trans effects are not responsible, since in both cases only the WT cells responded by T-bet and CD11c upregulation.

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In a second set of representative experiments, we interrogated these forms of rescue for their ability to foster different antibody isotype switching characteristic of ABCs. These data are shown in Figure 3 and discussed below.

Several conclusions can be drawn from these data: (i) **Fig. 3A** shows that both IL21 and IL4 rescue FO B cells from TLR9 driven post-proliferative death, although neither is as effective as BLyS or CD40 ligation. This is interesting from the standpoint of the prior iteration of this grant, as reviewer #2 had prompted us to wonder whether the rescue per se might be separate from promoting the AB C fate, and this seems to be at least in part true; (ii) **Fig. 3A** also shows that CD40 costimulation with either IL4 or IL21 yields nearly complete rescue; (iii) Figs. 3 B & C show that IL21 in this context promotes T-bet expression and a change in isotype switching from predominantly IgG1 to IgG2a/c.

We have performed an initial transcriptional analysis of this type, comparing the transcriptional profile generated by IL21 versus IFN-gamma in the context of TLR9 signals, and the top 50 genes that differ between stimuli or genotype are shown in **Fig. 4** (N.B.: These are relative within-gene differences, not absolute expression levels, so only within-gene comparisons can be made). These data are preliminary, but they make some potentially interesting points. First, there are genes whose upregulation clearly relies on T-bet expression, as well as those that do not, for each cytokine. Second, the IFN-gamma vs. IL21 driven profiles differ, suggesting that establishing T-bet+ B cells by different routes may produce different types of effectors. Third, T-bet appears to have both positive and negative regulatory functions in these conditions.

![Fig. 3: IL21 and IL4 reciprocally regulate adoption of the ABC phenotype in STIC9 stimulated cells. A) FO B cells were cultured in the presence of the indicated additions, and monitored for division and survival at 72h. B) Supernates were assayed by ELISA for total Ig and Ig isotype following culture for 5d in the conditions indicated in the boxed legend. C) T-bet expression was assessed cytofluorimetrically after 72h of culture with STIC9 and the indicated additions. Open histograms are WT B cells; closed histograms are staining control Tbx21 KO B cells.](image)

**Fig. 4: T-bet influences transcription patterns downstream of TLR9 plus IL21 or IFN-γ.** FO B cells from WT or Tbx21 KO mice were stimulated with CpG and either IL21 or IFN-γ for 20h, then RNA was prepared and analyzed using Illumina BeadChip mouse ref8-v2 array. This comparison assesses within-gene expression differences.
What opportunities for training and professional development has the project provided? Although training is not a goal of the project per se, studies have helped serve as a research training vehicle for a graduate trainee (M. Naradikian) whose primary support is from a T32 grant but who receives a small portion of his graduate stipend from this project.

How were the results disseminated to communities of interest? The work under this award has contributed to several research papers or reviews/commentaries in peer reviewed journals (see below). In addition, aspects of the work were presented at the 2015 Keystone B cell meeting (in separate talks from Dr. Cancro and Mr. Naradikian). In addition, Dr. Cancro has presented aspects of the work during invited seminars or plenary talks at academic and research institutions or international meetings in the past year, including Yale University, Stanford University, UTHSC San Antonio, Univ. of Toronto, Emory University, The American Society for Transplantation meeting, and several others.

What do you plan to do during the next reporting period to accomplish the goals?

Ongoing studies will now extend these findings to additional B cell subsets in both mice and humans, as well as to B cells in SLE patients. Based on our inhibitor and transcriptional analyses, we will further interrogate the downstream intracellular pathways that mediate rescue and fate adoption, as well as the transcriptional profile characterizing these cells.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The progress to date has been strong, and has uncovered an important association between certain forms of rescue – likely those mediated by TFH cytokines – with adoption of a cell fate that is associated with humoral autoimmunity. This may lead to an understanding of the origin of B cells responsible for producing detrimental antibodies in autoimmune diseases, particularly Lupus and related diseases.

What was the impact on other disciplines? Nothing to report.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Nothing to report.

5. CHANGES/PROBLEMS: Nothing to report.

6. PRODUCTS

PUBLICATIONS: The following publications resulted in full or in part from this grant support.


Two additional manuscripts based on the findings described above are also currently under revision or review.

Presentations:

Keystone B cell Symposium
Website(s) or other Internet site(s) Nothing to report.

Technologies or techniques Nothing to report.

Inventions, patent applications, and/or licenses Nothing to report.

Other Products Nothing to report.

7. PARTICIPANTS AND COLLABORATING ORGANIZATIONS:

<table>
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<tr>
<th>Name</th>
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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following grants were received or renewed during the past year:

R01 AI118691-01; Cancro, Michael (PI); 02/10/15-01/31/20
Mechanistic studies of BLyS-mediated modulation in HIV-1 Env-specific antibody responses

T32 AI055428-12; Cancro, Michael (PI); 06/01/03-07/31/19
Training Program in Immune System Development and Regulation

What other organizations were involved as partners?: Nothing to report.
8. SPECIAL REPORTING REQUIREMENTS:

Quad chart: This quad chart has been updated to show progress towards goals and to illustrate the new features of our working model based on discoveries resulting from this support.

B cell activation and tolerance mediated by B cell receptor, Toll-like receptor, and survival signal crosstalk in SLE pathogenesis.

Log Number: PR130769

Study/Product Aim(s)

- **Aim 1:** We will detail the pathways mediating post-proliferative death, rescue, and differentiation of each peripheral B cell subset in mice and humans.
- **Aim 2:** We will assess how alternative forms of rescue mediate distinct differentiation outcomes.
- **Aim 3:** We will determine whether B cells from selected SLE patients are refractory to post-proliferative death mediated by TLR9 agonists, or more responsive to plasmablast or GC B cell differentiation in response to rescue signals.

Approach

We will further detail major murine and human B cell subsets that are susceptible to post-proliferative death via BCR-delivered TLR9 ligands; interrogate the signals mediating death and rescue; and determine the differentiation fates yielded by different forms of rescue. We will test whether the B cells of individuals carrying SLE risk alleles show failures in the response-limiting mechanism.

Timeline and Cost

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Estimated Budget ($K)(TDC) $400 $400 $400

Updated: Sept 2015, M.P. Cancro, Univ. of Pennsylvania

9. APPENDICES:


Nucleic Acid–Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation

Shruti Sharma,* Katharine A. Fitzgerald,* Michael P. Cancro,† and Ann Marshak-Rothstein*

Distinct families of germline-encoded pattern recognition receptors can sense both microbial and endogenous nucleic acids. These DNA and RNA sensors include endosomal TLRs and cytosolic sensors upstream of stimulator of type I IFN genes (STING) and MAVS. The existence of overlapping specificities for both foreign and self nucleic acids suggests that, under optimal conditions, the activity of these receptors is finely tuned to effectively mediate host defense yet constrain pathogenic self-reactivity. This equilibrium becomes disrupted with the loss of either TLR9 or STING. To maintain immune protection, this loss can be counterbalanced by the elevated response of an alternative receptor(s). Unfortunately, this adjustment can lead to an increased risk for the development of systemic autoimmunity, as evidenced by the exacerbated clinical disease manifestations of TLR9-deficient and STING-deficient autoimmune-prone mice. These studies underscore the delicate balance normally maintained by tonic signals that prevent unchecked immune responses to nucleic acids released during infections and cellular duress or death. *The Journal of Immunology, 2015, 195: 3507–3512.

Nucleic acids (NAs) are the principal means of information transfer in most organisms. The conveyance of information from DNA (nuclear) to RNA (cytosolic) in eukaryotic cells relies on the precise segregation of NAs into appropriate nuclear, endosomal, and cytosolic compartments. These processes are highly systematized, actively maintained, and closely monitored by intrinsic NA sensors. This strict regulation of endogenous NAs allows abrupt shifts in the quantity and quality of NAs to serve as surrogate indicators of microbial infection that, in turn, initiate host defense mechanisms. However, because these sensors also detect endogenous NAs, inappropriate accumulation of these self-derived molecules can also provoke host responses, in some cases fostering autoimmunity and autoinflammation. Accordingly, the responses elicited by NA sensors must be programmed to optimize host defense, as well as to properly constrain responses to self-NAs. Further, because most microbes can engage multiple NA sensors, regulatory cross-talk likely exists to integrate the aggregate of signals generated by individual sensors. We propose that, under homeostatic conditions, these NA-sensing regulatory networks are finely tuned to the “tonic” receptor engagement levels mediated by endogenous NAs. Accordingly, the loss or inactivation of one sensor system impacts the remaining regulatory network, adjusting the calibration set point and affording heightened sensitivity to exogenous NAs. However, although such compensatory mechanisms may ensure adequate host defense, they may also confer an increased risk for the development of autoimmune responses.

In this article, we briefly review the evidence for NA sensor involvement in autoimmunity and autoinflammation and provide examples of endogenous ligands that are likely to promote these conditions. We also summarize studies that document the connection between loss of the endosomal DNA sensor TLR9, or loss of the cytosolic DNA sensor stimulator of type I IFN genes (STING), and more severe system lupus erythematosus (SLE). Potential molecular mechanisms that might account for these paradoxical observations are discussed.

Endosomal and cytosolic NA sensors contribute to autoimmunity and autoinflammation

The importance of sensing inappropriate NA accumulation emerged with the identification of TLR9 as an endosomal sensor for bacterial DNA (1). Thus TLR9, as well as subsequently described RNA-specific TLRs (TLR3, TLR7, TLR8, and TLR13), clearly plays critical roles in microbial immunity (2). However, autologous DNA and RNA also activate these TLRs, so the aberrant distribution of endogenous NAs can similarly foster immune activity, including the activation of autoreactive B cells, IFN-producing plasmacytoid dendritic cells, neutrophils, and other myeloid-derived APCs (3–5). As a result, endosomal TLRs can play key roles in the initiation and progression of systemic autoimmune diseases. In fact, endosomal TLRs have been implicated in all murine models of spontaneous SLE, because autoimmune-prone mice, deficient

*Program in Innate Immunity, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and †Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

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Address correspondence and reprint requests to Dr. Ann Marshak-Rothstein, Program in Innate Immunity, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, LRB 309, Worcester, MA 01605. E-mail address: ann.rothstein@umassmed.edu

Abbreviations used in this article: ERE, endogenous retroelement; NA, nucleic acid; SLE, systemic lupus erythematosus; STING, stimulator of type I IFN genes; Treg, regulatory T cell.
in the expression of MyD88, Unc93B1, IRF5, both TLR7 and TLR9, or TLR7 alone, invariably exhibit less severe disease manifestations than do the corresponding gene-sufficient strains (6–14). Moreover, hydroxychloroquine, a drug that blocks endosome acidification and thus TLR activation, is routinely used to treat SLE patients.

The contributions of TLR7 and TLR9 are particularly clear in B cells, where TLR9-deficient autoimmune-prone mice fail to make autoantibodies reactive with dsDNA or nucleosomes, and TLR7-deficient autoimmune-prone mice lack autoantibodies against RNA or RNA-binding autoantigens found in macromolecular complexes, such as spliceosomes, nucleosomes, or ribosomes (6, 14). Conversely, elevated expression of TLR7 causes more severe disease in autoimmune-prone strains (15–18), and very high TLR7 copy number yields additional organ-specific autoinflammation (19). TLR8 also was implicated in murine SLE (20), and overexpression of human TLR8 exacerbates joint inflammation in a collagen-induced arthritis model (21). Finally, TLRs were linked to macrophage activation and the ensuing fetal cardiac fibrosis that develops in the offspring of mothers expressing autoantibodies reactive with the RNA-binding protein Ro60 (22), illustrating a role for TLRs in human autoimmune disease. There is also considerable genetic data linking TLRs to SLE. Polymorphisms in IRF5, a transcription factor downstream of both TLR7 and TLR9, were associated with SLE (23). Moreover, individuals whose cells cannot properly degrade extracellular DNA as a result of reduced expression of DNase1 or DNase1L3 are at increased risk for developing SLE (24, 25).

Nuclease deficiencies also implicated cytosolic NA sensors in systemic inflammation. For example, mutations in the cytosolic Dnase1, the cytosolic RNase RNaseH2A, or the RNA-editing enzyme ADAR1, are linked to Aicardi-Goutieres syndrome, a debilitating neuroinflammatory condition (26–29), as well as to different forms of SLE (30). In mice, Trex1 deficiency results in a type I IFN-driven systemic inflammation, causing myocarditis (31, 32) and inflammation of skeletal muscle, tongue, skin, and glandular stomach (33). Genetic deletion of murine Dnase2a, an endonuclease primarily expressed in phagolysomes or autophagosomes, results in an even stronger IFN response and embryonic lethality, at least in part due to the leakage of DNA from saturated lysosomes into the cytosol (34, 35). However, NA sensors can also activate IFN-independent pathways and, in the absence of the type I IFNR, Dnase2a deficiency leads to inflammatory arthritis and SLE-like autoimmunity (36, 37). In patient populations, single-nucleotide polymorphisms in the Dnase2a promoter region that correlate with low Dnase2a serum activity are risk factors for rheumatoid arthritis (38). Other defects in lysosome formation, storage, or function are associated with various forms of arthritis (39, 40) and illustrate the need for proper lysosomal NA degradation in the prevention of systemic inflammation.

Numerous cytosolic DNA receptors were identified recently, including cGAS and IR204 (41, 42). These sensors, or second messengers derived from these sensors, converge on the ER-associated protein STING to activate downstream pathways leading to the expression of IFN-inducible genes and proinflammatory cytokines (43). Importantly, the systemic inflammation resulting from Trex1 deficiency and the arthritis resulting from DNase2 deficiency depend on STING expression (33, 44). In addition, gain-of-function STING mutations in patient populations were recently linked to SAVI, a clinical syndrome associated with elevated type I IFN, severe vasculopathy, arthritis, pulmonary fibrosis, and, in some cases, SLE-like autoantibody production (45, 46). Thus, NA sensors orchestrate the onset or progression of chronic inflammatory diseases, in many cases driven by autologous NAs. Intriguingly, the same STING mutation, V155M, results in highly variable disease outcomes (45, 46), pointing to critical interactions between STING-dependent pathways and other genetically inherited or environmentally triggered disruptions of immunoregulatory networks.

**Endogenous NAs from diverse sources target distinct sensor systems**

The origins of autologous NA ligands are diverse and include cell-extrinsic and -intrinsic sources. For instance, the majority of autoantibodies in SLE patients, as well as related systemic autoimmune diseases, such as Sjögren’s syndrome or systemic sclerosis, bind autologous DNA- or RNA-associated protein complexes often found on dying cells or persistent apoptotic blebs (47). This cell-extrinsic apoptotic debris is normally “silent” and rapidly cleared through noninflammatory mechanisms (48, 49). However, when cell debris is not properly removed, it can be endocytosed by autoreactive BCRs and delivered to TLR-containing compartments, leading to activation, autoantibody production, immune complex formation, and amplification of the response by FcγR+ APCs (50). Moreover, failure to clear apoptotic cells may lead to secondary necrosis or other immunogenic forms of cell death (48, 49). The premise that the excessive accumulation of extracellular cell debris is the source of the endogenous TLRI ligands in SLE was explored experimentally by the development of mice that express a bovine RNase transgene. Autoimmune-prone RNase-transgenic mice are protected from TLR7-driven disease (19).

Cell-intrinsic sources of autologous NAs include transcribed and reverse-transcribed retro-elements (32), damaged genomic DNA (51), and oxidized mitochondrial DNA (52, 53). These accumulate in the cytosol, independently of receptor-mediated internalization. Endogenous retroelements (EREs) form ∼40% of the mammalian genome and have a long evolutionary history with host cells (54). Several steps in the ERE lifecycle that involve active transcription of their genomes into RNA and then reverse transcription to cDNA occur in the cytosol (54). It is at this interface of ERE replication and innate sensing where EREs are a potent source of endogenous NA damage-associated molecular patterns (32). Cytosolic nucleases, such as Trex1, RNaseH2, and a deoxyribonuclease triphosphohydrolase SAMHD1, limit the exposure of cell death (48, 49). The premise that the excessive accumulation of extracellular cell debris is the source of the endogenous TLRI ligands in SLE was explored experimentally by the development of mice that express a bovine RNase transgene. Autoimmune-prone RNase-transgenic mice are protected from TLR7-driven disease (19).

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NAs can be sensed in the lysosomal compartment by the endosomal TLRs, and the ensuing loss of lysosomal integrity due to excessive swelling or frustrated/repeated fusion events can further release their components into the cytosol, where cytosolic NA sensors can be engaged (37, 57, 58). Mitochondria are an additional major source of endogenous NA (59). Normal turnover of stressed and damaged mitochondria via autophagy results in the access of mitochondrial-derived NA (i.e., mitochondrial DNA) to endolysosomal TLRs with some regularity, and this can cause inflammation when clearance is perturbed (59). Moreover, mitochondrial instability during cellular stress or cell death can release mitochondrial DNA into cytosolic compartments and activate cytosolic NA-sensing pathways (52, 53, 60). These examples reflect the variety and overlapping sources of DNA and RNA that gain access to both endosomal and cytosolic receptors during chronic infection, autoimmunity, and autoinflammation, raising the possibility that simultaneous activation of multiple pathways may lead to persistent or fatal inflammation.

**Negative regulatory function of TLR9**

In the presence of excess ligand, NA sensors play a critical role in immune activation. However, NA sensors can also negatively regulate immune responses. For example, despite the inability to make anti-dsDNA autoantibodies, all strains of TLR9-deficient autoimmune mice produce elevated Ab titers against RNA and RNA-associated autoantigens and develop autoinflammation, raising the possibility that simultaneous activation of multiple pathways may lead to persistent or fatal inflammation. BCR/TLR9 coengagement appears to limit the survival of mature autoreactive B cells and, thus, preclude sustained Ab secretion, germinal center formation, and affinity maturation (67, 69). This strategy may allow for the production of low-affinity IgM autoantibodies that facilitate immune clearance of endogenous NA cell debris without the risk for a sustained and focused self-reactive IgG autoantibody production. It is tempting to speculate that either cell-intrinsic or -extrinsic factors capable of compromising this checkpoint might underlie some autoimmune etiology. In the context of ongoing inflammation, these inherently short-lived responses might be sustained and redirected through the receipt of additional survival or differentiation signals that could extend the duration of Ab secretion or afford GC initiation, affinity maturation, and establishment of long-lived plasma cell pools, essentially converting the negative regulatory function of TLR9 to disease-promoting activity.

**Negative regulatory function of the cytosolic DNA sensor STING**

Recent studies also revealed an unanticipated negative regulatory role for cytosolic DNA sensors. As described above, STING clearly plays a fundamental role in driving type I IFN production when triggered by excess ligand, and genetic variants are associated with human SLE. Nevertheless, STING−/− lpr/lpr mice develop more severe SLE than their STING-sufficient counterparts (70). Unexpectedly, IRF3 (the type I IFN inducing transcription factor downstream of STING) is not required for this apparent STING-mediated immune suppression, because IRF3−/− and IRF3−/− lpr/lpr mice developed comparable levels of disease (70). In line with the STING−/− lpr/lpr data, C57BL/6 STING−/− mice injected i.p. with the proinflammatory mineral oil pristane developed a more severe TLR-dependent inflammatory response than littermate STING-sufficient mice (70). This remarkable parallel between TLR9 and STING is summarized in Fig. 1.

This STING-mediated suppression may reflect the capacity of STING to control the phosphorylation of SHP1/2 and downregulate JAK1/STAT1 signaling (71). STING also was reported to directly activate STAT6 (72), and STAT6 can promote the activation of M2 (anti-inflammatory, wound healing) macrophages (73) that could ameliorate disease pathology. STING deficiency was further associated with a reduction in the number of regulatory T cells (Tregs) in STING−/− lpr/lpr secondary lymphoid organs. Apoptotic debris induces the production of IDO, an enzyme that generates tryptophan derivatives that, in turn, promote Treg differentiation (74); therefore, reduced IDO levels correspond to a loss of Treg-mediated tolerance and increased autoimmunity. Remarkably, very little IDO could be found by immunostaining of STING−/− lpr/lpr spleens compared with age-matched lpr/lpr spleens, and loss of IDO correlated with decreased Treg numbers (70). The identification of the signaling molecules and transcription factors downstream of STING that are major players in these suppressive functions remains to be determined. Potential signaling intermediates include NIK and p52, because these can suppress type I IFN responses (75). Alternatively, other IRFs, such as IRF1 or IRF5, may play a cell-specific role in modulating cytokine profiles downstream of STING, similar to their roles in RIG-I-like receptor pathways (76). It will be important to...
explore the impact of STING deficiency in additional models of systemic autoimmunity and determine whether loss of its homeostatic function results in similar increases in disease severity.

How are NA sensors calibrated?

Endosomal and cytosolic sensors serve overlapping functions in protection against infectious agents. For example, both TLR9- and STING-dependent pathways are activated by malarial parasites (77–79), and multiple endosomal TLRs play a role in murine viral immunity (80, 81). Moreover, TLR9 synergizes with TLR2 in protection against HSV-1 and other viral infections. Analogously, cGAS plays a major role in activating innate immune responses important for protection against RNA and DNA viral infections (82). These interdigitating pathways suggest that such synergy is advantageous, and perhaps necessary, for an appropriate sterilizing immune response in some of these cases. Therefore, if one arm of the innate immune system is compromised, then one might anticipate a need to bolster an alternative innate immune pathway. This adjustment requires an intrinsic calibration mechanism to establish thresholds prior to frank infection. Inasmuch as these receptors also sense endogenous ligands, an attractive possibility is that cells titrate their capacity for NA reactivity against homeostatic levels of endogenous NAs.

This premise is supported by the documented heightened response of STING−/− myeloid cells to TLR ligands compared with STING-sufficient controls. This hyperresponsiveness corresponded to a reduction in the basal expression level of a number of negative regulators of TLR signaling (e.g., A20, Nlrc3, SOCS1, and SOCS3) (70) (S. Sharma, unpublished observations). Moreover, when STING was overexpressed in the RAW264.7 macrophage line, the cells were hyporesponsive to TLR ligands and expression of negative regulators was increased compared with vector control cells (70). Thus, under homeostatic conditions, the normal turnover of NAs under noninflamed conditions appears to provide a low-level tonic signal to STING or STING-dependent sensors and, thereby, calibrate TLR-dependent responses.

In the context of host defense, this retuning of the regulatory network may enhance TLR-mediated microbial immunity in a setting where the cytosolic DNA sensors are inactive. However, the same adjustments appear to disrupt the balance that limits self-reactivity because STING−/− SLE-prone mice develop more severe disease. It is important to note that loss of one pathway is often sufficient to cause increased susceptibility to pathogens still detected by alternate pattern recognition receptor pathways, although it is not always clear whether increased pathogenicity comes from increased microbial burden or unchecked activation of the complementary pathways.

Whether TLR9 deficiency similarly impacts regulatory networks remains to be determined. However, increased TLR7 activity due to the greater availability of Unc93B1 in the absence of TLR9 essentially leads to the same outcome: greater TLR7-mediated host defense but more severe autoimmunity. Future studies need to address whether distinct sources of endogenous ligands mediate negative versus positive regulatory effects, as well as how ongoing inflammatory responses intersect these pathways. Most importantly, it will be important to understand how these pathways promote disease, as well as how they can be downregulated to most effectively manipulate these pathways therapeutically.

Conclusions

Both endosomal and cytosolic NA sensors detect autologous ligands, and the excessive accumulation of endogenous NAs

FIGURE 1. Endosomal and cytosolic sensors promote and negatively regulate systemic autoimmunity and inflammation. NA-sensing receptors detect endogenous ligands and promote autoimmunity and inflammation. Examples of the expected outcomes for loss-of-function or gain-of-function mutations that modulate the activity of these receptors are indicated by the solid arrows. However, TLR9 deficiency and STING deficiency can also lead to more severe clinical manifestations, as indicated by the dashed lines.
can promote fatal inflammation. Nonetheless, the normal turnover of endogenous NAs and their capacity to modestly engage NA sensors, even under homeostatic conditions, likely play key roles in adjusting the balance between innate immune components. For example, in the absence of TLR9 or STING, responses initiated by the remaining innate sensors are tuned up, presumably as a means to better cope with potential microbial challenge. Unfortunately, such an adjustment comes with an increased risk for poorly controlled autoimmune responses. Whether tonic signaling of NA sensors has a similar impact in human populations remains unresolved. However, the data from mouse models highlight the need for caution in the design and application of STING and TLR inhibitors for the treatment of systemic autoimmunity and/or autoinflammation, because there is the potential to perturb an equilibrium that facilitates appropriate protective immunity but guards against autoimmune pathology. A better understanding of the integrated network governing NA-sensing pathways should reveal points amenable to intervention in autoimmunity or autoinflammation.

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References


Age-Associated B Cells: A T-bet–Dependent Effector with Roles in Protective and Pathogenic Immunity

Kira Rubtsova,†*,‡, Anatoly V. Rubtsov,†*,‡, Michael P. Cancro,§ and Philippa Marrack*,†,‡,¶

A newly discovered B cell subset, age-associated B cells, expresses the transcription factor T-bet, has a unique surface phenotype, and accumulates progressively with age. Moreover, B cells with these general features are associated with viral infections and autoimmunity in both mice and humans. In this article, we review current understanding of the characteristics, origins, and functions of these cells. We also suggest that the protective versus pathogenic actions of these cells reflect appropriate versus aberrant engagement of regulatory mechanisms that control the Ab responses to nucleic acid–containing Ags. The Journal of Immunology, 2015, 195: 1933–1937.

Advancing age is accompanied by shifts in many qualitative and quantitative aspects of immune function. These changes, collectively termed immune senescence (1, 2), include blunted primary and memory immune responses, reduced vaccine efficacy, and increases in the prevalence of inflammatory and autoimmune pathologies (2–6). Although the underlying mechanisms remain unclear, a growing literature documents contributions from age-associated changes at the systemic, molecular, and cellular levels. Systemically, serum and local concentrations of inflammatory cytokines are progressively elevated in both mice and humans, yielding an overall phenomenon described as inflamming (7, 8). In addition, monoclonal gammopathies, as well as Abs reactive with chromatin and dsDNA, frequently emerge with increasing age (9–12). Finally, with a few exceptions, such as type 1 diabetes and juvenile rheumatoid arthritis, the frequency of autoimmune disease increases with age. Moreover, B cells with these general features are detected in the spleen, blood, and bone marrow and bear hallmark features of Ag-experienced cells. The emergence and characteristics of age-associated B cells

Recently, our laboratories described a phenotypically and functionally unique B cell subset that accumulates with age that we named age-associated B cells (ABCs) (22, 23). These cells display a characteristic transcriptional profile, compete homoeostatically with naive follicular (FO) and marginal zone (MZ) B cells, and bear hallmark features of Ag-experienced cells. ABCs are detected in the spleen, blood, and bone marrow and less frequently in the peritoneal cavity or lymph nodes. Detailed understanding of their relative to splenic follicles and MZs is lacking, but recently reported age-associated changes in the cells occupying MZs make these sites a potential candidate (24). Finally, ABCs are associated with appropriate humoral responses to certain classes of infectious and inflammatory stimuli, arise prematurely in autoimmune-prone mouse strains,
and may be enriched for autoreactive Ab specificities (23, 25). The origins and roles of ABCs in normal immune responses, as well as in immune senescence and autoimmunity, remain areas of intense investigation.

Although sharing many features, some heterogeneity exists among ABCs. Hao et al. (22) identified ABCs by the lack of both CD21 and CD23 expression. The frequency and the numbers of these B cells increased with the age, accounting for as much as 30% of splenic B cells in 22-mo-old mice. Further phenotypic analysis of this CD23−/CD21− ABC population revealed that they differ from MZ, FO, or B1 B cells and showed that they express several markers shared with exhausted memory B cells (26). Simultaneously, Rubtsov et al. (23) reported a population of CD11c−/CD11b+ B cells that appears in healthy aged female mice and in autoimmune-prone animals (23). These cells clearly overlapped with those reported by Hao et al. (22), because they expressed low levels of CD21 and CD23 and elevated levels of CD5, Fas, and CD138. However, in contrast to the more broadly defined cells described by Hao et al. (22), the CD11c−/CD11b+ B cells described by Rubtsov et al. (23) uniformly expressed high levels of the activation markers CD80, CD86, and MHC class II. A comparison of surface markers among the ABCs defined by Hao et al. (22) and Rubtsov et al. (23) is shown in Table I. Importantly, both groups found that ABCs accumulate with age and tend to arise earlier and more consistently in female animals. Although this surface phenotype heterogeneity remains to be fully resolved, it likely reflects alternative routes of ABC generation.

A key feature of ABCs is that they express and depend upon B cell–intrinsic expression of the transcription factor T-bet (25). Consistent with this notion, T-bet overexpression induces acquisition of the ABC phenotype (25), indicating that it acts as a master regulator of ABC character. The exact mechanism whereby T-bet promotes and maintains the ABC phenotype remains unclear, but ongoing chromatin immunoprecipitation and deep sequencing studies will likely reveal both direct and indirect effects of T-bet on characteristic ABC gene expression patterns.

As might be anticipated from their unique T-bet driven transcriptional program, ABCs differ substantially from other B cell subsets in their activation requisites, functional capacities, and survival requirements. In contrast to FO or MZ B cells, ABCs survive but respond poorly to BCR engagement. However, they proliferate robustly to stimulation with either TLR9 or TLR7 agonists, either alone or in combination with BCR ligation. Moreover, following TLR stimulation in vitro ABCs elaborate a unique spectrum of regulatory cytokines, with notably robust production of both IL-10 and IFN-γ. Recent in vivo studies have suggested that they are also an abundant source of TNF-α in vivo (27).

While most murine ABCs express IgM, they rapidly switch to IgG production after stimulation with TLR ligands (23, 25). Regardless of their source – autoimmunity, age or viral infection – ABCs are prone to IgG2a/c production (23, 25), consistent with the established role of T-bet in switching to this IgH isotype (28–32). However, the specificity of the IgG produced by ABCs differs depending on their source; ABCs obtained from autoimmune or aged mice produce autoreactive IgG, whereas ABCs from virally infected mice produce predominantly antiviral IgG (Fig. 1) (23, 25). Together, these observations imply involvement of BCR signaling during the differentiation and recruitment of B cells into the ABC subset, despite their apparently dampened response to BCR ligation alone.

In addition to Ab secretion, ABCs can serve as Ag presenters; following activation, they can produce regulatory cytokines capable of skewing the differentiation of other adaptive and innate cell subsets. For example, early studies showed that ABCs obtained from aged animals can present Ag and tend to induce Th17 polarization (22). More recent findings extend this idea and suggest that ABCs obtained from aged or autoimmune mice process and present Ag more efficiently than do other B cells (33).

The accumulation of ABCs has profound effects on the dynamics and homeostasis of peripheral B cell pools. Interestingly, ABCs express the canonical BAFF receptors BR3 and TACI, but unlike FO and MZ B cells, they do not rely on BAFF for survival. Thus, as ABCs accumulate they engender reciprocal decreases in FO B cell numbers through competition for BAFF (22). Moreover, recent studies from Riley and colleagues (27) suggest that ABCs negatively influence B-lineage commitment or development of bone marrow progenitors, implying a causal role for ABCs in the decline of B cell lymphopoiesis with age. These observations bear on reports that B lymphocyte ablation can rejuvenate B lymphopoiesis in aged individuals (34), in as much as ABCs do not reappear quickly during self-reconstitution.

It is tempting to speculate that the progressive dominance of ABCs at the expense of FO B cells impacts adaptive humoral responses, and a growing body of evidence suggests that this may be the case. For example, adoptive-transfer experiments showed that multiple aspects of T follicular helper (Tfh) cell differentiation—including those that depend upon B cell Ag presentation, such as the upregulation of IL-4 and IL-21 production—are profoundly compromised in aged mice, regardless of T cell donor age (35, 36). Thus, the outcome of cognate presentation by ABCs may differ from other APCs, failing to reinforce the Tfh cell program or directing pre-Tfh cells to alternative effector fates. In agreement with this idea, ABC presenters skew primed T cells to a Th17 fate in vitro (22).

### Table 1. Comparison of the expression of surface markers by mouse and human ABCs and exhausted human B cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mice (Ref. 22)</th>
<th>Mice (Ref. 23)</th>
<th>Humans (Ref. 23)</th>
<th>Exhausted Human B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>N/A</td>
<td>High</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B220</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>CD11c</td>
<td>1/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11b</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>CD21</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Low</td>
</tr>
<tr>
<td>CD23</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fas</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>CD138</td>
<td>N/A</td>
<td>Int</td>
<td>N/A</td>
<td>Low</td>
</tr>
<tr>
<td>CD5</td>
<td>Int</td>
<td>+</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>CD80/86</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Low</td>
<td>High</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>T-bet</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>Surface IgM</td>
<td>+</td>
<td>1/−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Surface IgG</td>
<td>Low</td>
<td>1/−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Int, intermediate; N/A, not available; −, negative; +, positive.
ABC generation in health and disease

ABCs probably arise from activation-driven differentiation. Early work ruled out the possibility that ABCs represent the product of B cell genesis in the aged microenvironment, because they do not reappear after irradiation and autoreconstitution. Instead, multiple lines of evidence now suggest that they are a normal differentiative alternative taken by naive B cells when responding to certain classes of exogenous and endogenous stimuli. Initial evidence that ABCs can arise from naive B cells was suggested by experiments in which FO B cells from young donors were transferred to replete young or old congenic hosts. One month later, the recovered donor cells that had undergone extensive division had adopted an ABC phenotype, regardless of host age (22). Although these findings showed that ABC-like cells could be derived from quiescent preimmune B cells, the activating stimuli were unclear, and the paucity of recovered cells prevented detailed functional analyses.

The early descriptions of ABCs also indicated that TLR7 and MyD88, but not IFN-αR, were required for the accumulation of ABCs (23), consistent with their being derived from events driven by activating receptors. Subsequent in vitro analyses revealed that T-bet upregulation, the hallmark of ABC generation, was induced most effectively by concomitant receipt of BCR ligation, TLR7 stimulation, and IFN-γ (25) (Fig. 2).

Taken together, these observations suggest that ABCs originate under circumstances involving Ags that engage the BCR and also contain ligands for endosomal nucleic acid sensors, which also induce a promoting cytokine milieu. If this is the case, ABCs would be expected to arise during normal adaptive responses to microbial pathogens, as well as during potentially autoreactive responses to self components, as long as this tripartite set of conditions is established. Indeed, several lines of evidence now indicate that ABCs arise and play key roles in both situations, providing clues about their emergence with age and connection with humoral autoimmunity.

ABCs in infection and immunity: B cells closely resembling ABCs arise during antiviral immune responses (25). These T-bet+ CD11c− B cells appear at the peak of the humoral immune response during infection with mouse gammaherpesvirus 68, mouse CMV, lymphocyte choriomeningitis virus, and vaccinia. B cells with very similar phenotypic and functional characteristics also were described recently in Ehrlichia muris infection (37). Importantly, ABCs derived during these responses secrete pathogen-specific IgG upon restimulation in vitro more efficiently than FO B cells from the same host, indicating recruitment of Ag-specific B cells into the ABC pool rather than nonspecific enlargement of a bystander ABC pool. Further, ABC differentiation is a critical element of the successful immune response to viral infection. Mixed bone marrow chimeras in which the B cell compartment was T-bet deficient and unable to initiate ABC differentiation displayed dramatically reduced viral-specific IgG2a/c titers, less efficient viral clearance, and higher viral burden (Fig. 1) (25). This is in agreement with prior studies indicating that IgG2a/c most effectively drives viral clearance due to its efficiency in Ab-dependent cell-mediated cytoxicity and high affinity for activating FcRs (38–41).

These findings also strengthen the idea that ABCs arise via BCR-mediated activation in the context of TLR stimulation and appropriate cytokine milieu; BCR engagement affords virus uptake and trafficking to endosomal nucleic acid sensors, whereas NK cells and T cells secrete abundant IFN-γ in response to the virus to provide the appropriate cytokine microenvironment (Fig. 2).
ABCs in autoimmunity. B cells phenotypically similar to ABCs also appear in young autoimmune-prone mice (42). Moreover, the appearance of ABCs is correlated with disease onset in several murine lupus models, including MRL<sup>pr</sup>, NZB × WF1, MER<sup>−/−</sup>, and BXSB mice.

The potential relevance of ABCs to human autoimmunity was tested by screening human PBMCs obtained from either healthy or autoimmune donors for the presence of a similar B cell subset. The results show that PBMCs from donors with some autoimmune diseases contained a high percentage of CD11c<sup>−</sup>/CD21<sup>−</sup> B cells. In addition, these human ABC-like cells, similar to their murine counterparts, expressed low levels of CD23 and high levels of CD5 and CD86. However, unlike murine ABCs, the human ABC equivalents were isotype switched (Table I) (23). Other investigators observed a similar B cell subset in the peripheral blood of autoimmune patients, but in these studies the cells were identified as CD19<sup>high</sup>/CD21<sup>low</sup> (43–46). Together with the more pronounced and reliable emergence of ABCs in female mice, these findings in toto may provide clues as to why the majority of autoimmune diseases are more frequent in females.

B cells with similar phenotype were described in HIV-viremic individuals (47) and identified as FCRL-4–expressing exhausted-like B cells. Moir et al. (47) reported that FCRL-4–expressing B cells have low levels of CD21 and high CD11c expression (refer to Table I for the comparison of exhausted B cells and ABCs). Because FCRL-4–expressing B cells (similar to ABCs) express CD11c and CXCR3, they suggested that this B cell subset is similar to exhausted T cells (48) and can be driven by the persistent viral infection.

The exact combination of events that promote self-reactive ABCs in autoimmune-prone individuals remains unclear. It is tempting to speculate that autoantigen-specific B cells engage and internalize autoantigens via their BCRs and, if these are chromatin or ribonucleoparticles, will ligate endosomal TLRs. The third prerequisite for ABC generation, INF-γ or other promoting cytokines, may be derived from TLR7 engagement in NK cells or from bystander TH1 cells. It is noteworthy that TLR7 and IFN-γ signaling are well-established factors in the etiology of humoral autoimmunity (49–55).

Support for this model comes from mixed bone marrow chimeras in which Mer<sup>−/−</sup> mice, which lack receptors for effective clearance of apoptotic debris, were reconstituted with ABCs that could be depleted by diphtheria toxin (23). Notably, ABC depletion reduced autoantibody titers in these animals (23). Also consistent with this idea, TLR7 deficiency in either Mer<sup>−/−</sup> or Nba2 mice led to the absence of ABCs and significant reductions in autoantibody titers (Fig. 1) (42). Although these findings all suggest a role for ABCs in humoral autoimmunity, further work is required to fully reveal the underlying causal associations.

ABCs accumulate with age. Although the discovery of ABCs arose from studies in aged and autoimmune-prone mice, emerging findings suggest that this unique B cell subset reflects chronic or repeated exposures to stimuli that prompt a T-bet–centered transcriptional program, and that these cells progressively accumulate throughout life, eventually displacing a substantial proportion of the preimmune B cell pool with advancing age. In this context, ABCs may represent a specialized memory B cell subset directed toward chronic or endogenous pathogenic microbes. They might also be the product of B cells that react with nucleic acid–containing autoantigens that, under normal circumstances, are beneficial for housekeeping roles, such as the clearance of apoptotic debris. However, under circumstances in which inflammatory cytokines are persistently elevated, such as in advancing age, they might expand beyond normal homeostatic limits. These possibilities are not mutually exclusive and are amenable to experimental interrogation.

However, it is not clear why the appearance of ABCs is gender biased in aged animals. Sex hormones might contribute, but there is no evidence to support this idea. The X-linked Tlr7 gene might also be involved, because some regions on the X chromosome can escape inactivation and yield to the overexpression of some X-linked genes (30). If Tlr7 is among these, at least in some cells, it might lead to consistently increased numbers of ABCs in females with age.

Conclusions

Current findings in toto suggest that ABCs are Ag-experienced B cells that are characterized by a T-bet–driven transcriptional program. Moreover, they play dichotomous roles in health and disease. ABCs are essential for effective immune responses against certain classes of infectious agents, likely reflecting the need for key effector functions mediated by IgG2a/c and inflammatory cytokines. Conversely, the sustained accumulation of ABCs can have detrimental effects, including a propensity for autoinflammatory and autoimmune pathologies. Based on the prerequisite for endosomal TLRs in ABC generation and activation, these seemingly paradoxical outcomes may reflect intricacies of the regulatory mechanisms that have evolved to control Ab responses to nucleic acid–containing Ags. Obviously, sensing pathogen-derived intracellular nucleic acids is critical to inducing immune effectors that eliminate or control such infections. We hypothesize that ABCs evolved, as a product of a specific set of B cell–activating signals, via BCR, TLR7, and IFN-γ. Therefore, we hypothesize that ABCs represent a stage of B cell activation or a differentiated effector stage and, upon further TLR7 triggering, may differentiate into Ab-secreting plasma cells.

We also suggest that evolution selected for the ABC-differentiatiative pathway, components of which are evident in viral infections, because it leads to effective antiviral humoral immunity. Despite being essential to health, the same mechanism can be triggered in response to self-Ag and thus, in rare individuals, causes damaging disease. Accordingly, interrogating the mechanisms that control ABC formation, activity, and persistence may reveal targets for intervention in both microbial pathogenesis and autoinflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

References


Endosomal TLRs play an important role in systemic autoimmune diseases, such as systemic erythematous lupus, in which DNA- and RNA-associated autoantigens activate autoreactive B cells through TLR9- and TLR7-dependent pathways. Nevertheless, TLR9-deficient autoimmune-prone mice develop more severe clinical disease, whereas TLR7-deficient and TLR7/9-double deficient autoimmune-prone mice develop less severe disease. To determine whether the regulatory activity of TLR9 is B cell intrinsic, we directly compared the functional properties of autoantigen-activated wild-type, TLR9-deficient, and TLR7-deficient B cells in an experimental system in which proliferation depends on BCR/TLR coengagement. In vitro, TLR9-deficient cells are less dependent on survival factors for a sustained proliferative response than are either wild-type or TLR7-deficient cells. The TLR9-deficient cells also preferentially differentiate toward the plasma cell lineage, as indicated by expression of CD138, sustained expression of IRF4, and other molecular markers of plasma cells. In vivo, autoantigen-activated TLR9-deficient cells give rise to greater numbers of autoantibody-producing cells. Our results identify distinct roles for TLR7 and TLR9 in the differentiation of autoreactive B cells that explain the capacity of TLR9 to limit, as well as TLR7 to promote, the clinical features of systemic erythematous lupus. The Journal of Immunology, 2015, 194: 2504–2512.
dition of RNase to the culture medium (1, 17). Stimulatory ICs include defined ligands, such as IgG2a-bound CG-rich dsDNA fragments (16, 18), as well as IgG2a autoantibodies that bind cell debris or surface-bound autoantigens, which are present in the primary B cell cultures (1, 17).

The availability of autointibodies reactive with DNA and/or RNA-associated autoantigens, together with TLR-deficient RF B cells, make it possible to directly compare the downstream effects of BCR/TLR7 and BCR/TLR9 engagement. We found that in vitro activation of RF B cells, through a mechanism dependent on the BCR and TLR7, promotes the extended survival of RF B cells and their differentiation into CD138⁺ plasma blasts. BCR/TLR7- and BCR/TLR9-activation pathways also have distinct functional outcomes in vivo, where again RF B cells activated through the BCR/TLR9 pathway, but not the BCR/TLR9 pathway, preferentially differentiate into Ab-producing cells.

Materials and Methods

**Mice**

AM14, AM14 Tlr9⁻/⁻, and AM14 Tlr7⁻/⁻ mice were described previously (13, 15, 19, 20). FcγR2b-deficient BALB/c mice and CD45.1 BALB/c mice were obtained from The Jackson Laboratory. AM14 Tlr9⁻/⁻ and AM14 Tlr7⁻/⁻ mice were intercrossed to generate AM14 Tlr7/9 double-knockout mice (Tlr7⁻/⁻ Tlr9⁻/⁻). All mice were bred and maintained at the Department of Animal Medicine of the University of Massachusetts Medical School in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

**Cell culture**

Splenic B cells were positively selected and cultured in RPMI 1640/5% heat-inactivated FCS, as described previously (15), with the following ligands—1 µg/ml Cpg 1826 (ε-oligodeoxyxynucleotide[ODN], kindly provided by Idera Pharmaceuticals), 0.1–1.0 µg/ml CL097 (Innogen), and 15 µg/ml goat anti-mouse IgM Fab'2 (Jackson ImmunoResearch) —or with the mAbs PL2-3 (1 µg/ml), PA4 (0.3 µg/ml), and BWR4 (10 µg/ml) (21–23). The ligands recognized by the mAbs are derived from cell debris generated in culture; therefore, the monoclonal autoantibodies spontaneously form ICs. Better-defined ICs were formed by combining a biotinylated CG-rich dsDNA fragment (18) with streptavidin (SA) and an IgG2a anti-SA mAb, at a final concentration of 0.5 µg/ml DNA, 0.13 µg/ml SA, and 0.5 µg/ml anti-SA mAb. In certain experiments, the BWR4 cultures were supplemented with IFN-γ (300 U/ml; PBL). B cell proliferation was assessed by [³H]thymidine incorporation at the times indicated or by fluorescent dye dilution at 72 h. B lymphocyte stimulator (BLyS), provided by Biocytex and IgG1 (Jackson ImmunoResearch). Bound Ab was detected with biotinylated-4G7 in combination with SA–PerCP–Cy5.5. IRF-4 was detected using a biotinylated mouse TLR7-specific mAb, A94 (25), in combination with SA–PE. Flow cytometric analysis was carried out using a BD FACSCanto II with FACSDiva Software (BD), and analysis was conducted with FlowJo software (TreeStar).

**Gene expression**

Total RNA was extracted using the RNeasy Mini Kit (QiAGEN). Reverse-transcribed DNA (Quanta) was analyzed by quantitative real-time PCR (qPCR) using TaqMan probes for bcl-6, pax-5, and pmdn1 (Life Technologies). Samples were normalized to GAPDH and analyzed using the ΔΔCT method. For microarrays, RNA was prepared by the TRizol method (Invitrogen), purified using RNeasy Mini columns (QiAGEN), and used on Agilent mouse 60K arrays with a control pool of B cell RNAs from all genotypes (unstimulated), which served as a reference (Cy3) for each genotype’s sample over a time course of stimulation with PL2-3 (Cy5). The Cy5/Cy3 ratio of gene expression was captured and normalized to the ratio values of the wild-type (WT) at 0 h (unstimulated) array. Gene expression data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE58756 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58756).

**ELISPOT assay**

Ab-forming cells (AFCs) were measured by ELISPOT assay. Filter plates (Millipore) were coated with Abs specific for mouse IgG2a (Southern Biotech) and IgG1 (Jackson ImmunoResearch). Bound Ab was detected using biotinylated anti-clonotype 4G7 (for IgG1) or 4-44 (for IgG2a), as well as SA–alkaline phosphatase (BD Biosciences) (12). Spots were developed with BCLP/NBT substrate (Sigma–Aldrich) and counted using an ImmunoSpot reader (CT.LL).

In vivo activation of AM14 B cells

B220-purified splenic B cells (15 × 10⁶) were injected i.v. into CD45.1 BALB/c mice on day 0, together with 50 µg PL2-3. Mice received additional i.v. injections of PL2-3 on day 3 or on days 3, 7, and 10. Spleens were harvested on day 6 or 13. To assess proliferation, purified B cells were labeled with 3.5 pM VPD450 (BD) for 5 min prior to injection.

**Statistical analyses**

Statistical analyses were conducted with GraphPad Prism6 software. Comparisons between two groups were performed with a Student t test for normally distributed data. Two-way ANOVA, including the Bonferroni posttest or the Tukey multiple-comparison test, was used for multiple-group comparisons. A p value < 0.05 was considered significant.

**Results**

Monoclonal autoantibodies can activate RF B cells through TLR7- or TLR9-dependent pathways

The monoclonal autoantibodies PL2-3, PA4, and BWR4 were reported to recognize chromatin, DNA, and RNA, respectively (21–23), and to activate RF B cells in vitro through TLR-dependant mechanisms. WT, TLR7-deficient (Tlr7⁻/⁻), TLR9-deficient (Tlr9⁻/⁻), and TLR7/9 double-deficient (Tlr7⁻/⁻ Tlr9⁻/⁻) RF B cells were stimulated with the monoclonal autoantibodies, and the responses were compared with small molecule ligands for TLR9 (Cpg ODN 1826) and TLR7 (CL097). Proliferation was determined by [³H]thymidine incorporation (Fig. 1A, left panel). The PA4 response was entirely TLR9 dependent because only TLR9-sufficient cells could respond. Tlr7⁻/⁻ and Tlr7⁻/⁻ Tlr9⁻/⁻ B cells mounted comparably low responses to BWR4 compared with WT B cells, indicating a critical role for TLR7, but not TLR9, in this response. In contrast, Tlr7⁻/⁻ and Tlr9⁻/⁻ cells responded to PL2-3 significantly better than did Tlr7⁻/⁻ Tlr9⁻/⁻ cells; the relatively modest response of the Tlr7⁻/⁻ population was further increased at day 2 (Fig. 1A, right panel). Therefore, the PA4 response is TLR9 dependent, the BWR4 response is TLR7 dependent, and the PL2-3 response can be driven by both TLR9 and TLR7. These data suggest that PL2-3 binds autoantigen-associated complexes that incorporate both DNA and RNA.

We showed previously that type I IFN markedly enhanced the initial response of RF B cells to BWR4, as detected 30 h after the addition of ligand (17). Based on the somewhat delayed PL2-3 response of Tlr9⁻/⁻ cells, we also decided to monitor the PA4 and BWR4 responses over a more extended time period. In the absence of type I IFN, the BWR4-stimulated WT B cells responded well at later time points (Fig. 1B). Notably, the TLR7-driven component of the PL2-3 response (Tlr7⁻/⁻ cells) recapitulated the BWR4 kinetics, whereas the TLR9-driven PL2-3 and PA4 responses peaked and declined at an earlier time point (Fig. 1C).
Together, these data indicate that BCR/TLR7 coengagement promotes a slightly delayed, but more sustained response, than does BCR/TLR9 coengagement. The delay may be due, at least in part, to a TLR7-dependent induction of type I IFN and subsequent upregulation of TLR7 expression (19). Further studies were performed without the addition of type I IFNs.

**RNA-associated ICs promote the prolonged survival of RF B cells**

To more precisely monitor both proliferation and death, RF WT B cells were labeled with CFSE and stimulated with the same set of ligands for 72 h. Cell division was assessed by CFSE dilution, and dead cells were identified with the cell permeable DNA stain TO-PRO-3. Both PA4 and PL2-3 BCR/TLR9 coengagement of WT B cells induced several rounds of division, followed by a synchronous postproliferative cell death (Fig. 2A, upper panels). In both cases, cells could be rescued by the addition of the B cell survival factor BlyS (Fig. 2A, lower panels). This extent of cell death was not observed in cells stimulated with either the TLR9 ligand CpG 1826 or the TLR7 ligand CL097 (Supplemental Fig. 1A), indicating that coengagement of the BCR and TLR9 resulted in a functional phenotype distinct from that elicited by TLR9 alone. Importantly, under the same conditions, BWR4-activated cells divided up to three times and remained viable, even in the absence of BlyS (Fig. 2A).

The same CFSE/TO-PRO criteria were used to monitor the 72-h response of Tlr7−/− and Tlr9−/− B cells. This analysis again showed that the PA4 response was entirely dependent on TLR9 for proliferation and on BlyS for survival, whereas the BWR4 response was TLR7 dependent and BlyS independent (Supplemental Fig. 1B). In contrast, Tlr7−/− and Tlr9−/−, but not Tlr7−/− Tlr9−/−, RF cells proliferated in response to PL2-3 (Fig. 2B). Moreover, in the absence of BlyS, Tlr9−/− cells responded better to PL2-3 than did either WT or Tlr7−/− cells (Fig. 2B, 2C). The effect of TLR9 deficiency could be recapitulated with a TLR9-specific inhibitor (Supplemental Fig. 1C); WT cells stimulated by PL2-3 in the presence of the inhibitor were less dependent on BlyS for survival (Fig. 2D). We also evaluated the response to defined ICs that incorporated a biotinylated CG-rich dsDNA fragment (18), bound by SA, and delivered to the RF BCR via an IgG2a anti-SA Ab. The response elicited by such an anti-SA/SA/Bio-DNA IC was entirely TLR9 dependent (Supplemental Fig. 1D) and recapitulated the postproliferative cell death/BlyS rescue observed for PA4- and PL2-3-activated RF B cells (Fig. 2E). Thus, TLR9 mediates a dominant proliferation/postproliferative cell death response; when the TLR9 component is eliminated, either genetically or by the use of an inhibitor, the cells that respond to PL2-3 through TLR7 maintain a more sustained response.

**RNA-associated ICs drive B cell differentiation to CD138+ plasmablasts**

To determine whether BCR/TLR9 and BCR/TLR7 engagement drive comparable programs of differentiation under conditions where both populations survive and divide, WT RF B cells were stimulated for 3 d with CpG 1826, CL097, PA4, BWR4, or PL2-3 in the presence of BlyS and then analyzed by flow cytometry for markers of B cell differentiation. Plasma cells (PCs) are defined by the surface expression of CD225, CD44hi, and CD138+ (26). Remarkably, only the BWR4-stimulated cells acquired the CD138 marker, indicative of differentiation toward the PC lineage (Fig. 3A). In contrast to these RNA ICs, cells stimulated with CL097 did not become CD138+ cells, again demonstrating the difference between ICs that bind both the BCR and TLR7 compared with small molecules that only engage TLR7.

Although these data point to a distinct functional outcome of BCR/TLR7-activated cells compared with BCR/TLR9-activated cells, under the conditions of this assay, PA4 and BWR4 both bind undefined endogenous autoantigens and, therefore, potentially could form ICs that differ in size, as well as content. Theoretically, such differences could impact the extent of BCR cross-linking and/or interactions with additional pattern recognition receptors. To address these concerns, we took advantage of the fact that PL2-3 activates B cells through both TLR9- and TLR7-dependent pathways and that the TLR9 component can be removed by using Tlr9−/− B cells, whereas the TLR7 component can be removed by using Tlr7−/− B cells. Other than TLR9 or TLR7, PL2-3 should engage the BCR and any other receptor to a comparable extent in all RF B cells. Therefore WT, Tlr7−/−, Tlr9−/−, and Tlr7−/− Tlr9−/− RF B cells were stimulated for 3 d with PL2-3 in the presence of BlyS. Importantly, and as expected, WT, Tlr7−/−, and Tlr9−/− B cells divided and survived comparably (Fig. 2C), but only PL2-3-activated Tlr9−/− B cells differentiated into CD138+ plasmablasts (Fig. 3A, 3B). CD138+ cells also were detected in cultures stimulated with PL2-3 in the presence of the TLR9 inhibitor (Fig. 3C). These data demonstrate that BCR/TLR7 engagement alone is more likely to promote cell survival and differentiation toward the PC lineage compared with BCR/TLR9 engagement alone. Furthermore, simultaneous BCR/TLR9 coengagement interferes with this process.
divided and remained alive (Data represent the mean percentage (calculated as in (A)).

FIGURE 2. RNA-containing ICs induce BLyS-independent survival. (A) CFSE-labeled RF WT B cells were stimulated with the indicated ligands in the absence (upper panels) or presence (lower panels) of 50 ng/ml BLyS for 72 h. Proliferation was measured by CFSE dilution, and cell death was measured by uptake of TO-PRO-3. The quadrants depict the following: CFSE undiluted; lower left, live divided (TO-PRO-3 diluted); upper right, dead divided cells (TO-PRO-3 undiluted); upper left, dead divided cells (TOP-RO-3 undiluted); lower right, live undivided cells (TOP-RO-3 CFSE undiluted). Division numbers are indicated by red arrows underneath the flow plots. Flow plots are representative of three independent experiments. *p < 0.05, **p ≤ 0.005, two-way ANOVA and Bonferroni posttest.

Molecular markers of plasmablast differentiation

To further verify the PC-skewed phenotype, RNA was isolated from WT B cells 4 d after BWR4, PA4, or PL2-3 activation and analyzed by qPCR for the expression of molecular markers of B cell differentiation. Consistent with the expression of CD138, BWR4-activated WT B cells expressed higher levels of Prdm1 and lower levels of bcl-6 and Pax5 (27) than did any of the populations activated by BCR/TLR9 engagement (Fig. 3D). Overall gene expression patterns were examined further by microarray analysis of PL2-3–activated WT and TLR-deficient cells. Overall, the gene expression profiles of WT and Tlr7+/− B cells were remarkably similar. Both populations only upregulated genes associated with PC differentiation at early time points, followed by downregulation of these genes at later time points (Fig. 3E). In contrast, Tlr9+/− B cells upregulated and maintained expression of the PC transcription factors Prdm1 and Irf4 (Fig. 3E) (28, 29). Moreover, expression of B cell transcription factors, such as Bcl6, Pou2f2, SpiB, and Ebf1, known targets of Prdm1, were repressed more strongly in Tlr9+/− cells at later time points (Fig. 3F) (30). Coordinate activation of PC-related markers like syndecan-1 (Sdc1, Cd138), Egr1, and Csf1 were expressed at higher levels in the absence of TLR9, whereas expression of typical B cell markers (MHCII, Cxcr5, and Cd20) was trending downward at 42 h (Fig. 3F) (30, 31). Finally, during PC differentiation, expression of BCR signaling components is lost, and cells redirect biosynthesis to increase the size and number of organelles like the endoplasmic reticulum, Golgi, and lysosomes to deal with the increased secretory load required for Ig secretion (30, 32). These changes are seen clearly in Tlr9+/− B cells, as reflected by a downward trend in the expression of BCR signaling components (Cd79a, Btk, Syk) (Fig. 3F) and increased expression of gene products required in the endoplasmic reticulum (Kdelr3, Uap1) and lysosomes (Lamp2) (Fig. 3E). Together, these data further support the idea that BCR/TLR7 coengagement in the absence of BCR/TLR9 coengagement favors differentiation of FO B cells toward the PC lineage.

Regulation by Irf4 and Irf8

Recent studies showed that high concentrations of IRF4 result in preferential binding to IFN sequence motifs that are found in the promoter regions of genes associated with PC differentiation (33). Therefore, IRF4 protein levels were monitored by flow cytometry in WT and TLR-deficient RF B cells stimulated with PA4 or PL2-3 (Fig. 4A). IRF4 protein was upregulated in all activated populations at 24 h postactivation (gray line), but high levels of IRF4 protein were only sustained at 72 h in the PL2-3–activated Tlr9+/− cells (black line). A similar trend in mRNA levels at 42 h, as detected by microarray, confirmed the sustained expression of IRF4 in PL2-3–activated Tlr9+/− cells (Fig. 4B, left panel). In addition to IRF4, the transcription factor Irf8 is important for B cell development and differentiation. However, Irf8 is upregulated in activated and germinal center B cells but is not required for PC differentiation (34). In contrast to IRF4, analysis of the microarray RNA expression levels revealed an early upregulation of Irf8 at 6 h in PL2-3–stimulated WT and Tlr7+/− cells (Fig. 4B, right panel) and less induction of Irf8 in Tlr9+/− cells. The RNA expression levels correlated with upregulation of Irf8 protein at 14 h in Tlr7+/− cells, as measured by flow cytometry (Fig. 4C). Together, the data suggest that the differentiation of...
BCR/TLR7-activated cells is determined by an IRF4-dependent regulatory network, whereas the differentiation of BCR/TLR9-activated cells is restrained by a counteracting IRF8 network early during activation. Notably, IRF8 protein levels were increased at 72 h after both BCR/TLR7 and BCR/TLR9 coengagement, which was indicative of delayed expression of IRF8 in the BCR/TLR7-activated cells. However, late expression of IRF8 did not seem to promote PC differentiation.

**TLR7 expression in WT and Tlr9<sup>−/−</sup> RF B cells**

Both TLR7 and TLR9 depend on Unc93b1 to acquire functional activity. The D34A mutant of Unc93b1 preferentially binds TLR7, and gene-targeted mice that express Unc93b1 D34A develop lethal systemic inflammation (35). These data point to a critical balance between Unc93b1 and its capacity to bind TLR7 and TLR9 in the regulation of TLR-dependent responses. To determine whether the unique functional activity of Tlr9<sup>−/−</sup> B cells, described above, simply reflected amplified TLR7 expression, we compared TLR7 protein levels in unstimulated and stimulated WT and Tlr9<sup>−/−</sup> RF B cells by flow cytometry. In the unstimulated B cells, TLR7 levels were low but comparable between WT and Tlr9<sup>−/−</sup> cells and were slightly higher than Tlr7<sup>−/−</sup> or unstained cells (Fig. 5A). Stimulation for 24 h with CL097 led to significantly increased, but again comparable, levels of TLR7 expression in WT and Tlr9<sup>−/−</sup> B cells. Stimulation with 1826 only led to increased expression of TLR7 in WT cells, because Tlr9<sup>−/−</sup> cells were not activated and Tlr7<sup>−/−</sup> cells did not express TLR7. Thus, it appears that, in both unstimulated and stimulated cells, WT and Tlr9<sup>−/−</sup> B cells express the same amount of TLR7. These expression levels are completely consistent with the overlapping dose-response curves of WT and Tlr9<sup>−/−</sup> RF B cells in response to increasing concentrations of CL097 (Fig. 5B).

These values reflected relative mRNA levels, as determined by qPCR (data not shown).

**RNA-associated ICs induce AFCs in vivo**

It was important to determine whether the preferential survival and plasmablast differentiation of BCR/TLR7-activated B cells, which was apparent in vitro, extended to in vivo responses. To ensure that activation conditions were as comparable as possible, we again stimulated TLR-sufficient and -deficient RF B cells with PL2-3. WT, Tlr7<sup>−/−</sup>, Tlr9<sup>−/−</sup>, or Tlr7<sup>−/−</sup>/Tlr9<sup>−/−</sup> RF B cells were labeled with VPD450 and injected i.v., together with 50 μg of PL2-3 or PBS into BALB/c recipients. To accurately track the injected cells, CD45.2 RF B cells were injected into CD45.1 hosts. The mice were given a second injection of PL2-3 or PBS on day 3, and spleens were harvested on day 6. All genotypes engrafted comparably, because similar numbers of RF cells were recovered from the PBS-injected control groups (Fig. 6A, upper panels). RF Tlr7<sup>−/−</sup>/Tlr9<sup>−/−</sup> B cells showed a minimal response, reiterating the critical role for BCR/TLR coengagement in the response to PL2-3 (Fig. 6A, lower panels, 6B). However, in contrast to the survival pattern observed in vitro whereby BCR/TLR9 activation induced postproliferative cell death, PL2-3-stimulated WT, Tlr9<sup>−/−</sup>, Tlr7<sup>−/−</sup>, or Tlr7<sup>−/−</sup>/Tlr9<sup>−/−</sup> RF B cells were labeled with VPD450 and injected i.v., together with 50 μg of PL2-3 or PBS into BALB/c recipients. To accurately track the injected cells, CD45.2 RF B cells were injected into CD45.1 hosts. The mice were given a second injection of PL2-3 or PBS on day 3, and spleens were harvested on day 6. All genotypes engrafted comparably, because similar numbers of RF cells were recovered from the PBS-injected control groups (Fig. 6A, upper panels). RF Tlr7<sup>−/−</sup>/Tlr9<sup>−/−</sup> B cells showed a minimal response, reiterating the critical role for BCR/TLR coengagement in the response to PL2-3 (Fig. 6A, lower panels, 6B). However, in contrast to the survival pattern observed in vitro whereby BCR/TLR9 activation induced postproliferative cell death, PL2-3-stimulated WT, Tlr9<sup>−/−</sup>, and Tlr7<sup>−/−</sup> B cells all divided multiple times (Fig. 6A lower panels, 6B), although Tlr7<sup>−/−</sup> cells underwent fewer divisions than did Tlr9<sup>−/−</sup> cells. There also was a trend toward fewer divisions in the WT group. The inability of BCR/TLR9 engagement to more effectively limit cell expansion of the transferred WT and Tlr7<sup>−/−</sup> cells most likely reflects rescue through steady-state or induced B cell survival factors. It is difficult to determine whether constitutive levels of BLyS are sufficient to maintain the survival of these cells because it is

![Figure 3](http://www.jimmunol.org/)
many RF Tlr9−/− B cells, and very few RF Tlr7−/− Tlr9−/− B cells (data not shown). These data are consistent with the day-6 results and further support the premise that TLR9 expression limits expansion of autoreactive B cells. Importantly, Tlr9−/− B cell–injected mice had almost 10-fold more IgG AFCs than did Tlr7−/− B cell–injected mice, consistent with the propensity of the in vitro–activated Tlr9−/− cells to acquire PC markers (Fig. 6C). The mice injected with WT cells also had more AFCs (2.5 fold) than did Tlr7−/−–injected mice, as might be predicted by greater division. These data demonstrate that BCR/TLR7 B cell activation, in the absence of BCR/TLR9, preferentially induces autoreactive B cells to differentiate into isotype-switched AFCs compared with BCR/TLR9-activated cells, as well as promoting improved survival.

Discussion

The analysis of TLR9-deficient murine models of systemic rheumatoid lupus (SLE) has given paradoxical results. Although these mice fail to make autoantibodies reactive with dsDNA, as determined by the immunofluorescent staining of mitotic plates in anti-nuclear Ab assays, they invariably develop more severe SLE that is associated with a decreased lifespan (2, 6, 38, 39). B cells were shown to play a critical role in SLE, both through the production of autoantibodies that form pathogenic ICs and through their capacity to activate autoreactive B cells. A previous study found that Tlr9−/− B cells obtained from 2-mo-old autoimmune-prone Nba2 Yaa mice expressed higher levels of TLR7 mRNA and responded better to the TLR7 ligand imiquimod than did B cells obtained from age-matched TLR-sufficient Nba2 Yaa mice (8). However, these Tlr9−/− Nba2 Yaa mice develop a hyperaccelerated autoimmune disease, and survival is already compromised by 3 mo of age. Therefore, it is difficult to determine whether the enhanced TLR7 response of Tlr9−/− Nba2 Yaa B cells reported in this study was due to the loss of TLR9 expression per se or to the fact that these B cells had already been activated in vivo by the autoimmune disease process, because we now document upregulation of TLR7 protein levels in response to TLR activation. In a separate report, purified B cells obtained from Tlr9−/− and WT nonautoimmune-prone C57BL/6 mice were compared and shown to produce comparable amounts of cytokine in response to the TLR7 ligand R848 (40). We now show that purified B cells from nonautoimmune-prone mice responded comparably to increasing concentrations of a TLR7 ligand and that both unstimulated and stimulated WT and Tlr9−/− B cells expressed comparable levels of TLR7, as detected by flow cytometry with a TLR7-specific Ab (Fig. 5). Therefore, TLR9 deficiency in B cells does not seem to impact the TLR7 signaling threshold. Nevertheless, as shown in the current study, BCR/TLR7 activation and BCR/TLR9 activation can lead to distinct functional outcomes, especially with regard to autoantibody production.

We found that isolated in vitro–activated BCR/TLR7-activated Tlr9−/− B cells are more likely to differentiate toward the PC lineage than are BCR/TLR9-activated Tlr7−/− B cells and that the BCR/TLR7-activated Tlr9−/− B cells preferentially give rise to IgG autoantibody–producing cells in vivo. Moreover, BCR/TLR9 activation can at least partially block the BCR/TLR7–driven response. Consistent with the studies of nontransgenic B cells (M.A. Oropallo, V.J. Sindhava, K. Moody, L. Zhou, N. Green, K. Nündel, W. Stohl, A.M. Schmidt, C.A. Lowell, C. Lamagna, T. Kambayashi, A. Marshak-Rothstein, and M.P. Cancro, submitted for publication), BCR/TLR9 coengagement in vitro induced postproliferative cell death, even in cells coactivated by BCR and TLR7. Only Tlr9−/− RF B cells could sustain an ex-
Panel activated with the indicated ligands for 24 h were permeabilized and stained for TLR7 expression. Unstained cells are also shown. Representative plots of three experiments are shown (left panel). Data from the three experiments are summarized as MFI of the TLR7-staining intensities over background (right panel). (B) RF WT, Tlr7+/−, and Tlr9+/− splenic B cells were activated with the indicated concentrations of the synthetic TLR7 ligand CL097 for 30 h, and proliferation was measured by [3H]thymidine uptake. Data represent the mean ± SEM of eight independent experiments.

coengagement of DNA- or chromatin-reactive cells may no longer lead to cell death but, rather, activation.

It was somewhat surprising to find that the small molecule ligands 1826 and CL097 did not induce CD138 expression, because other investigators found that TLR ligands can drive B cells to become CD138+ plasmablasts and AFCs. BCR/TLR9 engagement of nontransgenic polyclonal B cells also preferentially induces MZ B cells to differentiate into AFCs (M. A. Oropallo et al., submitted for publication).

The association between BCR/TLR9 activation and early up-regulation of IRF8 is consistent with the phenotype of Irf8+/− mice. B cell–conditional Irf8+/− mice have twice the number of

FIGURE 5. RF WT and Tlr9+/− B cells express comparable levels of TLR7. (A) Unactivated purified WT, Tlr7+/−, and Tlr9+/− RF B cells or B cells activated with the indicated ligands for 24 h were permeabilized and stained for TLR7 expression. Unstained cells are also shown. Representative plots of three experiments are shown (left panel). Data from the three experiments are summarized as MFI of the TLR7-staining intensities over background (right panel). (B) RF WT, Tlr7+/−, and Tlr9+/− splenic B cells were activated with the indicated concentrations of the synthetic TLR7 ligand CL097 for 30 h, and proliferation was measured by [3H]thymidine uptake. Data represent the mean ± SEM of eight independent experiments.

FIGURE 6. BCR/TLR7 activation promotes AFC differentiation in vivo. (A) BALB/c CD45.1 recipients were injected i.v. with 15 × 10⁶ WT, Tlr7+/−, Tlr9+/−, or Tlr7+/− Tlr9+/− VPD450-labeled RF B cells and 50 µg PL2-3 on day 0; they were injected again with PL2-3 on day 3. Spleens were harvested on day 6. B cell engraftment was ascertained by CD45.2 staining, and proliferation was measured by dilution of VPD450. Representative plots of three independent experiments are shown. (B) The mean (±SEM) number of cell divisions based on the data in (A) was calculated for each mouse (n = 3 mice/group). *p ≤ 0.05. Student t test. (C) BALB/c CD45.1 recipients were injected with AM14 B cells, as above, but were injected with PL2-3 on days 0, 3, 7, and 10. Additional BALB/c CD45.1 mice were injected only with PL2-3 and not RF B cells (none). Spleens were harvested on day 13; the number of clonotype-positive IgG7 AFCs was measured by ELISPOT. Data are compiled from four independent experiments. **p ≤ 0.005, ***p ≤ 0.0005, one-way ANOVA including the Tukey multiple-comparison test.
mature B cells, as well as greater numbers of MZ and B1 cells (34). In addition, they spontaneously produce anti-dsDNA autoantibodies by 3 mo of age (46). Also, in contrast to MD4 × sHEL mice, IgR8+/− × MD4 × sHEL B cells differentiate to a more mature phenotype and spontaneously produce anti-HEL Abs (46). Together, the data point to a major role for IRF8 in the maintenance of B cell tolerance; therefore, IFR8 expression by PL2-3-activated B cells may account, in part, for the negative regulatory role of TLR9.

An interesting comparison can be made between our in vivo experiments and a previous report that involved the day−7 in vivo PL2-3 response of autoimmune-prone MRL.Fas−/− AM14 B cells (26). Although this study found that TLR-sufficient mice appeared to have more AFCs than did Tlr7−/− or Tlr9−/− mice, as detected by the number of ELISPOT+ cells, Tlr9−/− mice had a higher percentage of plasmablasts than did Tlr7−/− or WT mice, as determined by the phenotype CD22−/−CD138+. In contrast, in the current study, the number of IgG3 AFCs produced by WT B cells at day 13 was significantly lower than the number produced by Tlr9−/− B cells. The MRL/pr study may be somewhat confounded by the accelerated disease in Tlr7−/− MRL/pr mice, as well as subsequent changes in total spleen cell number and cell subset distribution. Nevertheless, the data suggest that the CD138+ cells are not full-fledged AFCs but rather are a distinct subset that is preferentially generated by BCR/TLR7 engagement, but only in the absence of BCR/TLR9 engagement. Additional studies will be necessary to further elucidate the direct impact of TLR7 and TLR9 on the long-term survival of RF B cells, as well as their capacity to move into specific short-lived and long-lived PC compartments.

Several laboratories have produced 80% μMT (or JhD+20%) + 20% Tlr9−/− mixed chimeras, in which TLR9 deficiency is predominantly limited to the B cell lineage (9, 41, 47). These B cell Tlr9−/− mice invariably develop more severe clinical features, including higher autoantibody titers, more extensive isotype switching of the autoantibody-producing cells, and increased activation of potentially autoreactive T cells. However, the potential contribution of other TLR9-expressing cell types cannot be completely ruled out because a significant proportion (20%) of the myeloid lineage also could be TLR9 deficient. Nevertheless, the 80% μMT + 20% Tlr9−/− chimeric mice invariably developed greater numbers of autoantibody-producing PCs, as well as effector/memory T cells, more extensive ectopic follicles, and more severe renal disease (9), consistent with the notion that Tlr9−/− B cells are more likely to differentiate into autoantibody-producing plasmablasts and more effectively activate autoreactive T cells. Exacerbated disease in these chimeras also could be attributed to the absence of TLR9-expressing cells that make protective Abs required for the clearance of apoptotic debris (47) or to cytokines produced by residual TLR9−/− myeloid cells. The current study clearly shows that BCR/TLR9 and BCR/TLR7 coengagement lead to distinct functional phenotypes. Our findings are strengthened by a recent publication that documents opposing roles specific to a unique B cell-intrinsic role of TLR9 (22). These studies support a critical role for TLR9 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice. J. Autoimmun. 34: 339–348.

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