AWARD NUMBER: W81XWH-14-1-0305

TITLE:
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

REPORT DATE: October 2015

TYPE OF REPORT:
Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:
Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	15 Aug 2014 - 14 Aug 2015

4. TITLE AND SUBTITLE
B-Cell Activation and Tolerance Mediated by B-Cell Receptor, Toll-Like Receptor, and Survival Signal Crosstalk in SLE Pathogenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER W81XWH-14-1-0305

5c. PROGRAM ELEMENT NUMBER

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

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

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	28	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

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 known 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 this master transcription factor. Most ABCs display preferential class switch recombination (CSR) to the IgG2a/c isotype, are potent 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 signals, but can be blocked by IL4. These findings are summarized in **Figs. 1 and 2** and discussed below.

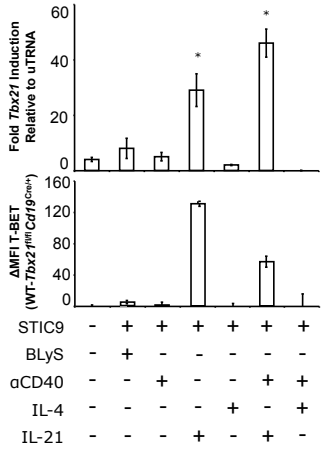


Fig. 1: IL21 & IL4 reciprocally regulate T-bet in STIC9 stimulated B cells. Splenic CD23+ B cells were cultured in the conditions shown for 72h, harvested and assessed for *Tbx21* message (upper) & T-bet protein (lower panel).

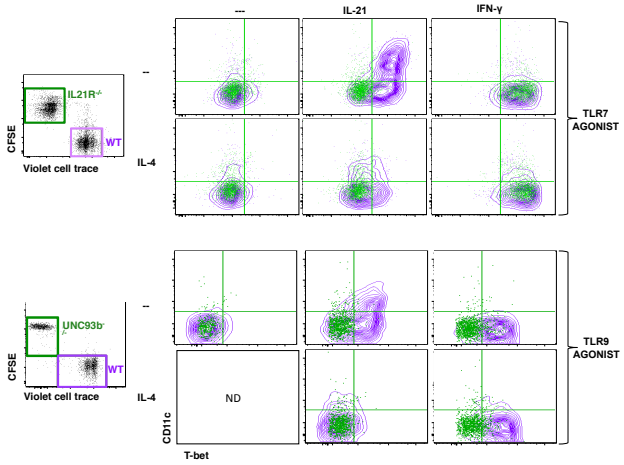


Fig. 2: IL21 and IL4 reciprocally regulate the T-bet+ CD11c+ fate in the context of TLR7 or TLR9 signals. Splenic CD23+ B cells from IL21 KO, UNC93b KO, or WT mice were loaded with CFSE (green) or VCT (violet) dyes respectively, co-cultured with the stimuli shown for 48h, and harvested and cytofluorimetrically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

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.

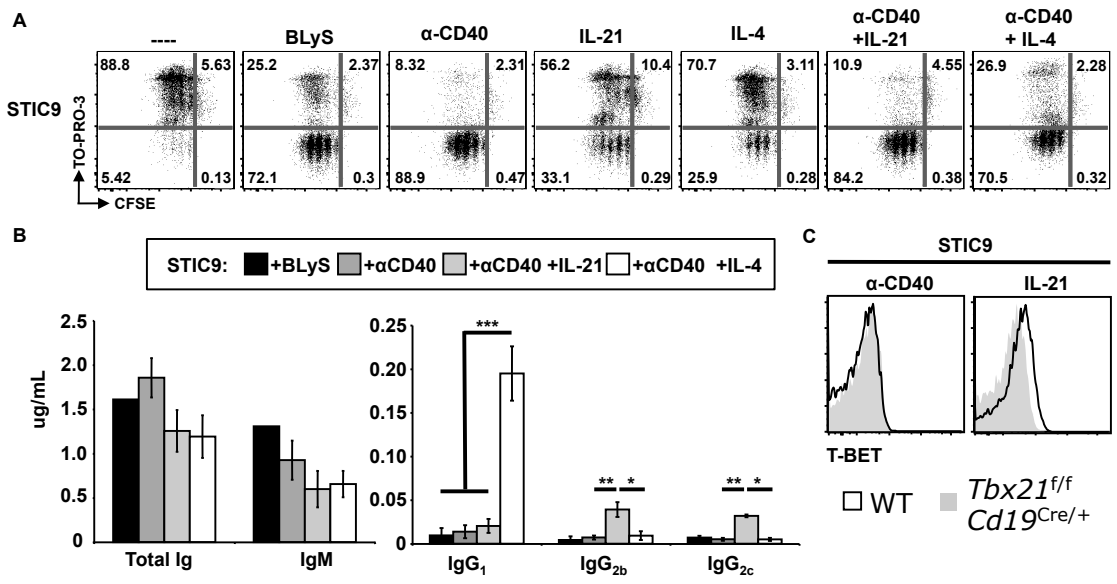


Fig. 3: IL21 and IL4 reciprocally regulate adoption of the ABC phenotype in STIC9 stimulated cells. A) FO B cells were cultured in the presence of the indicated additions, and monitored for division and survival at 72h. **B)** Supernates were assayed by ELISA for total Ig and Ig isotype following culture 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.

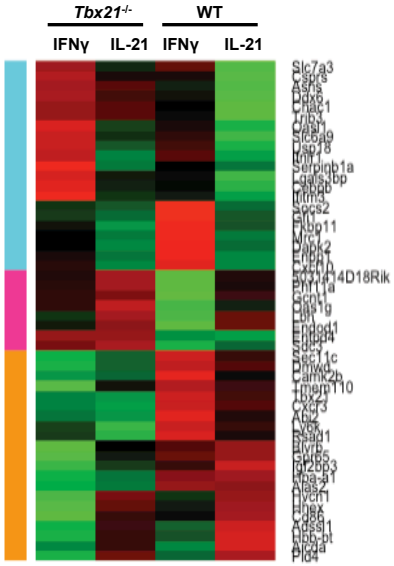


Fig 4: T-bet influences transcription patterns downstream of TLR9 plus IL21 or IFN-γ. FO B cells from WT or *Tbx21* KO mice were stimulated with CpG and either IL21 or IFN-γ for 20h, then RNA was prepared and analyzed using Illumina BeadChip mouse ref8-v2 array. This comparison assesses *within-gene* expression differences.

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 ABC 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.

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 recieves 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 plenmary 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.

1. Sharma S, Fitzgerald KA, **Cancro** MP, Marshak-Rothstein A. Nucleic Acid-Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation. J Immunol. 2015 Oct 15;195(8):3507-12. doi: 10.4049/jimmunol.1500964. Review. PubMed PMID: 26432899; PubMed Central PMCID: PMC4593056. (appended)

2. Rubtsova K, Rubtsov AV, **Cancro** MP, Marrack P. Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity. J Immunol. 2015 Sep 1;195(5):1933-7. doi: 10.4049/jimmunol.1501209. Review. PubMed PMID: 26297793; PubMed Central PMCID: PMC4548292. (appended)

3. Nündel K, Green NM, Shaffer AL, Moody KL, Busto P, Eilat D, Miyake K, Oropallo MA, **Cancro** MP, Marshak-Rothstein A. Cell-intrinsic expression of TLR9 in autoreactive B cells constrains BCR/TLR7-dependent responses. J Immunol. 2015 Mar 15;194(6):2504-12. doi: 10.4049/jimmunol.1402425. Epub 2015 Feb 13. PubMed PMID: 25681333; PubMed Central PMCID: PMC4382804. (appended)

4. Naradikian, MS, Hao, Y and **Cancro** MP. Age Associated B cells: Key mediators of both protective and autoreactive humoral responses. Immunol. Rev. *in press*.

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:

Name	**Michael P. Cancro**
Project Role	**P.I.**
Researcher Identifier	**N/A**
Nearest month worked	**2**
Contribution	**Principal Investigator; oversee all research**
Funding support	**This grant**

Name	**Jean L. Scholz**
Project Role	**Research Associate**
Researcher Identifier	**N/A**
Nearest month worked	**4**
Contribution	**Perform experiments and oversee logistics**
Funding support	**This grant**

Name	**Martin Naradikian**
Project Role	**Graduate student**
Researcher Identifier	**N/A**
Nearest month worked	**3**
Contribution	**Perform experiments related to ABCs**
Funding support	**This grant, NIH training grant.**

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

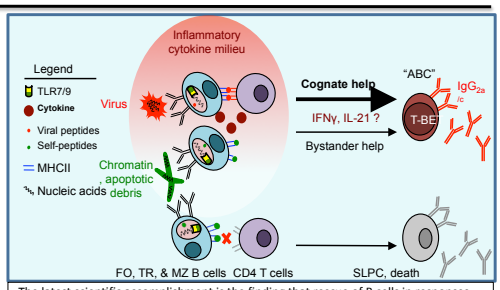
PI: Michael P. Cancro **Org:** University of Pennsylvania **Award Amount:** $1,200,000 (total direct costs)

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 differentiative 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 differentiative 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.



The latest scientific accomplishment is the finding that rescue of B cells in responses to antigens that contain TLR9 ligands can yield the Tbet driven 'ABC' phenotype associated with humoral autoimmunity.

Timeline and Cost

Activities CY	14	15	16
Aim 1: pathways in subsets			
Aim 2: differentiative outcomes			
Aim 3: mice with risk alleles			
Aim 3: SLE patient B cells			
Estimated Budget ($K)(TDC)	**$400**	**$400**	**$400**

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

Goals/Milestones

CY14 Goals – Project launch
☒ Begin Aim 1, 2, and 3 experiments with mice
☒ Begin Aim 3 work with B cells from SLE patients
CY15 Goals – Sustain research program
☐ Continue work on all aims
☒ Disseminate results via posters, talks, publications
CY16 Goal – Project completion
☐ Finalize work on all aims Disseminate results via posters, talks, publications

Comments/Challenges/Issues/Concerns

- None at this time

Budget Expenditure to Date
Projected Expenditure: $1,200,000 (total direct costs)
Actual Expenditure: $0

9. APPENDICES:

Appendix 1: Sharma S, Fitzgerald KA, Cancro MP, Marshak-Rothstein A. Nucleic Acid-Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation. J Immunol. 2015 Oct 15;195(8):3507-12.

Appendix 2: Rubtsova K, Rubtsov AV, Cancro MP, Marrack P. Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity. J Immunol. 2015 Sep 1;195(5):1933-7.

Appendix 3: Nündel K, Green NM, Shaffer AL, Moody KL, Busto P, Eilat D, Miyake K, Oropallo MA, Cancro MP, Marshak-Rothstein A. Cell-intrinsic expression of TLR9 in autoreactive B cells constrains BCR/TLR7-dependent responses. J Immunol. 2015 Mar 15;194(6):2504-12.

Brief Reviews

The Journal of Immunology

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

Received for publication April 28, 2015. Accepted for publication August 21, 2015.

This work was supported by Alliance for Lupus Research Grant 260146 (to S.S.), National Institutes of Health Grant AR050256 (to A.M.-R.), and Congressionally Directed Medical Research Programs Grant PR130769 (to M.P.C.).

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.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500964

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 splicesomes, 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 DNase Trex1, 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 Ifi204 (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 TLR 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 deoxynucleoside triphosphohydrolase SAMHD1, limit the exposure of NA sensors to ERE load (32). MyD88/TLR7-dependent B cell–dependent Ab responses are also required to curb the reactivation and emergence of infectious endogenous retroviruses (55). However, reactivated EREs share a complex relationship with host cells, because BCR-mediated induction of ERE transcription appears to provide a second signal to cytosolic NA sensors and, thereby, facilitate B cell activation by type II T-independent Ags (56).

Dysregulated catabolism of cellular components, such as lipids, proteins, and self-NAs resulting from lysosomal storage disorders, is another source of endogenous ligands (57). Self-

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 accelerated and more severe clinical disease (6, 14, 61–63). Exactly how TLR9 deficiency promotes disease remains unresolved, but, in general, *Tlr9*$^{-/-}$ mice appear to be hyperresponsive to TLR7 ligands. In vitro analyses of transduced cell lines and myeloid lineage cells suggest that endosomal sensors compete with one another for association with Unc93B1 (64) and imply that, in the absence of TLR9, more Unc93B1 is available for the RNA sensors, leading to their increased activity. TLR9 and TLR7 differentially traffic to endosomal compartments (64, 65) where they could potentially interact with distinct signaling complex components and trigger TLR-specific functions. However, remarkably little is known about distinguishing components of the TLR9 and TLR7 signaling cascades, how they interface with one another, or why a TLR7 signal would be more pathogenic than a TLR9-elicited response, especially in myeloid cells.

The distinct functional outcomes of TLR9 versus TLR7 activation have been rigorously explored in B cells. Mixed bone marrow chimera studies suggest that TLR9 deficiency in B lineage cells alone is sufficient to drive exacerbated autoimmunity (14). A variety of mechanisms may be involved. These include a unique requirement for TLR9 in the production of autoantibodies involved in the clearance of apoptotic debris (66) or the increased pathogenicity of autoantibodies specific for RNA or RNA-associated autoantigens. Alternatively, BCR/TLR7-stimulated B cells may have a greater capacity to activate autoreactive T cells (14) and/or to differentiate toward the plasma cell lineage (67). It is also possible that TLR9 preferentially contributes to the depletion of autoreactive B cells from the developing bone marrow repertoire (68).

The most direct comparisons between BCR/TLR9 and BCR/TLR7-dependent activation were carried out with B cells derived from autoreactive BCR-transgenic mice. Importantly, 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

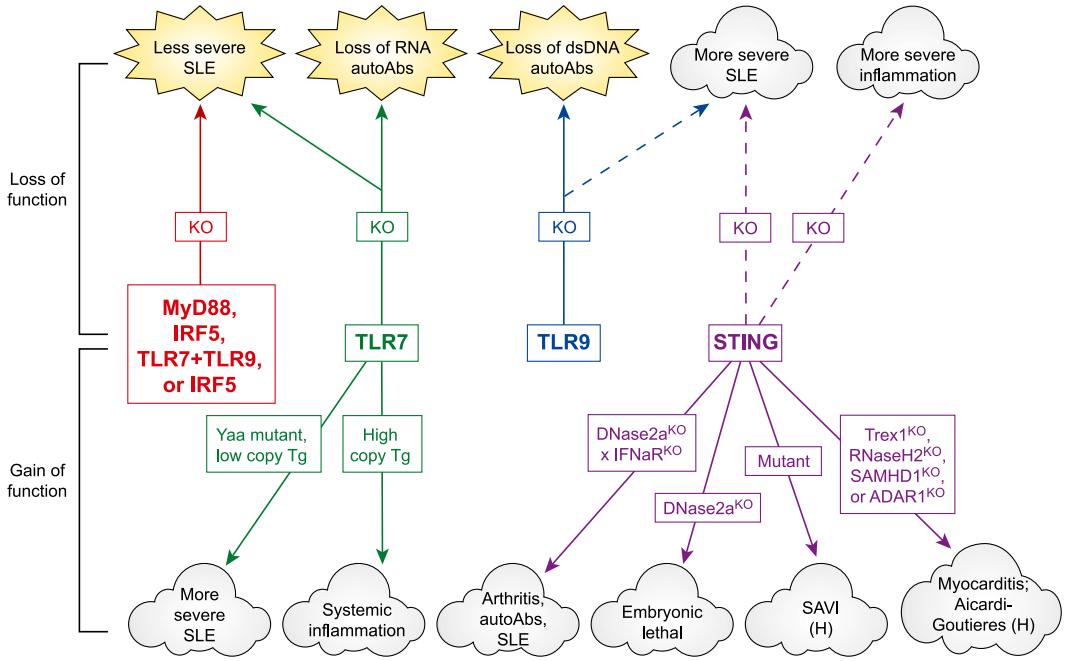


FIGURE 1. Endosomal and cytosolic sensors promote and negatively regulate systemic autoimmunity and inflammation. NA-sensing receptors detect endogenous ligands and promote autoimmunity and inflammation. Examples of the expected outcomes for loss-of-function or gain-of-function mutations that modulate 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.

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 NA ligands, an attractive possibility is that cells titer 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

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.

Acknowledgments

We thank Dr. M. Atianand for advice and helpful suggestions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
2. Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11: 373–384.
3. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
4. Lau, C. M., C. Broughton, A. S. Tabor, S. Akira, R. A. Flavell, M. J. Mamula, S. R. Christensen, M. J. Shlomchik, G. A. Viglianti, I. R. Rifkin, and A. Marshak-Rothstein. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202: 1171–1177.
5. Vollmer, J., S. Tluk, C. Schmitz, S. Hamm, M. Jurk, A. Forsbach, S. Akira, K. M. Kelly, W. H. Reeves, S. Bauer, and A. M. Krieg. 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J. Exp. Med.* 202: 1575–1585.
6. Christensen, S. R., J. Shupe, K. Nickerson, M. Kashgarian, R. A. Flavell, and M. J. Shlomchik. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417–428.
7. Nickerson, K. M., S. R. Christensen, J. Shupe, M. Kashgarian, D. Kim, K. Elkon, and M. J. Shlomchik. 2010. TLR9 regulates TLR7- and MyD88-dependent autoantibody production and disease in a murine model of lupus. *J. Immunol.* 184: 1840–1848.
8. Kono, D. H., M. K. Haraldsson, B. R. Lawson, K. M. Pollard, Y. T. Koh, X. Du, C. N. Arnold, R. Baccala, G. J. Silverman, B. A. Beutler, and A. N. Theofilopoulos. 2009. Endosomal TLR signaling is required for anti-nucleic acid and rheumatoid factor autoantibodies in lupus. *Proc. Natl. Acad. Sci. USA* 106: 12061–12066.
9. Pawaria, S., K. Moody, P. Busto, K. Nündel, C. H. Choi, T. Ghayur, and A. Marshak-Rothstein. 2015. Cutting Edge: DNase II deficiency prevents activation of autoreactive B cells by double-stranded DNA endogenous ligands. *J. Immunol.* 194: 1403–1407.
10. Yasuda, K., C. Richez, J. W. Maciaszek, N. Agrawal, S. Akira, A. Marshak-Rothstein, and I. R. Rifkin. 2007. Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J. Immunol.* 178: 6876–6885.
11. Savarese, E., C. Steinberg, R. D. Pawar, W. Reindl, S. Akira, H. J. Anders, and A. Krug. 2008. Requirement of Toll-like receptor 7 for pristane-induced production of autoantibodies and development of murine lupus nephritis. *Arthritis Rheum.* 58: 1107–1115.
12. Berland, R., L. Fernandez, E. Kari, J. H. Han, I. Lomakin, S. Akira, H. H. Wortis, J. F. Kearney, A. A. Ucci, and T. Imanishi-Kari. 2006. Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity* 25: 429–440.
13. Lee, P. Y., Y. Kumagai, Y. Li, O. Takeuchi, H. Yoshida, J. Weinstein, E. S. Kellner, D. Nacionales, T. Barker, K. Kelly-Scumpia, et al. 2008. TLR7-dependent and FcgammaR-independent production of type I interferon in experimental mouse lupus. *J. Exp. Med.* 205: 2995–3006.
14. Jackson, S. W., N. E. Scharping, N. S. Kolhatkar, S. Khim, M. A. Schwartz, Q. Z. Li, K. L. Hudkins, C. E. Alpers, D. Liggitt, and D. J. Rawlings. 2014. Opposing impact of B cell-intrinsic TLR7 and TLR9 signals on autoantibody repertoire and systemic inflammation. *J. Immunol.* 192: 4525–4532.
15. Pisitkun, P., J. A. Deane, M. J. Difilippantonio, T. Tarasenko, A. B. Satterthwaite, and S. Bolland. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 312: 1669–1672.
16. Subramanian, S., K. Tus, Q. Z. Li, A. Wang, X. H. Tian, J. Zhou, C. Liang, G. Bartov, L. D. McDaniel, X. J. Zhou, et al. 2006. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc. Natl. Acad. Sci. USA* 103: 9970–9975.
17. Hwang, S. H., H. Lee, M. Yamamoto, L. A. Jones, J. Dayalan, R. Hopkins, X. J. Zhou, F. Yarovinsky, J. E. Connolly, M. A. Curotto de Lafaille, et al. 2012. B cell TLR7 expression drives anti-RNA autoantibody production and exacerbates disease in systemic lupus erythematosus-prone mice. *J. Immunol.* 189: 5786–5796.
18. Deane, J. A., P. Pisitkun, R. S. Barrett, L. Feigenbaum, T. Town, J. M. Ward, R. A. Flavell, and S. Bolland. 2007. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity* 27: 801–810.
19. Sun, X., A. Wiedeman, N. Agrawal, T. H. Teal, L. Tanaka, K. L. Hudkins, C. E. Alpers, S. Bolland, M. B. Buechler, J. A. Hamerman, et al. 2013. Increased ribonuclease expression reduces inflammation and prolongs survival in TLR7 transgenic mice. *J. Immunol.* 190: 2536–2543.
20. Umiker, B. R., S. Andersson, L. Fernandez, P. Korgaokar, A. Larbi, M. Pilichowska, C. C. Weinkauf, H. H. Wortis, J. F. Kearney, and T. Imanishi-Kari. 2014. Dosage of X-linked Toll-like receptor 8 determines gender differences in the development of systemic lupus erythematosus. *Eur. J. Immunol.* 44: 1503–1516.
21. Guiducci, C., M. Gong, A. M. Cepika, Z. Xu, C. Tripodo, L. Bennett, C. Crain, P. Quartier, J. J. Cush, V. Pascual, et al. 2013. RNA recognition by human TLR8 can lead to autoimmune inflammation. *J. Exp. Med.* 210: 2903–2919.
22. Clancy, R. M., D. Alvarez, E. Komissarova, F. J. Barrat, J. Swartz, and J. P. Buyon. 2010. Ro60-associated single-stranded RNA links inflammation with fetal cardiac fibrosis via ligation of TLRs: a novel pathway to autoimmune-associated heart block. *J. Immunol.* 184: 2148–2155.
23. Graham, R. R., C. Kyogoku, S. Sigurdsson, I. A. Vlasova, L. R. Davies, E. C. Baechler, R. M. Plenge, T. Koeuth, W. A. Ortmann, G. Hom, et al. 2007. Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. *Proc. Natl. Acad. Sci. USA* 104: 6758–6763.
24. Napirei, M., A. Gültekin, T. Kloeckl, T. Möröy, J. Frostegård, and H. G. Mannherz. 2006. Systemic lupus-erythematosus: deoxyribonuclease 1 in necrotic chromatin disposal. *Int. J. Biochem. Cell Biol.* 38: 297–306.
25. Al-Mayouf, S. M., A. Sunker, R. Abdwani, S. A. Abrawi, F. Almurshedi, N. Alhashmi, A. Al Sonbul, W. Sewairi, A. Qari, E. Abdallah, et al. 2011. Loss-of-function variant in DNASE1L3 causes a familial form of systemic lupus erythematosus. *Nat. Genet.* 43: 1186–1188.
26. Crow, Y. J., B. E. Hayward, R. Parmar, P. Robins, A. Leitch, M. Ali, D. N. Black, H. van Bokhoven, H. G. Brunner, B. C. Hamel, et al. 2006. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutières syndrome at the AGS1 locus. *Nat. Genet.* 38: 917–920.
27. Rice, G. I., J. Bond, A. Asipu, R. L. Brunette, I. W. Manfield, I. M. Carr, J. C. Fuller, R. M. Jackson, T. Lamb, T. A. Briggs, et al. 2009. Mutations involved in Aicardi-Goutières syndrome implicate SAMHD1 as regulator of the innate immune response. *Nat. Genet.* 41: 829–832.
28. Rice, G. I., P. R. Kasher, G. M. Forte, N. M. Mannion, S. M. Greenwood, M. Szynkiewicz, J. E. Dickerson, S. S. Bhaskar, M. Zampini, T. A. Briggs, et al. 2012. Mutations in ADAR1 cause Aicardi-Goutières syndrome associated with a type I interferon signature. *Nat. Genet.* 44: 1243–1248.
29. Rice, G. I., M. A. Reijns, S. R. Coffin, G. M. Forte, B. H. Anderson, M. Szynkiewicz, H. Gornall, D. Gent, A. Leitch, M. P. Botella, et al. 2013. Synonymous mutations in RNASEH2A create cryptic splice sites impairing RNase H2 enzyme function in Aicardi-Goutières syndrome. *Hum. Mutat.* 34: 1066–1070.
30. Crow, Y. J., and J. Rehwinkel. 2009. Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. *Hum. Mol. Genet.* 18(R2): R130–R136.
31. Stetson, D. B., J. S. Ko, T. Heidmann, and R. Medzhitov. 2008. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134: 587–598.
32. Stetson, D. B. 2012. Endogenous retroelements and autoimmune disease. *Curr. Opin. Immunol.* 24: 692–697.
33. Gall, A., P. Treuting, K. B. Elkon, Y. M. Loo, M. Gale, Jr., G. N. Barber, and D. B. Stetson. 2012. Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. *Immunity* 36: 120–131.
34. Okabe, Y., K. Kawane, S. Akira, T. Taniguchi, and S. Nagata. 2005. Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. *J. Exp. Med.* 202: 1333–1339.
35. Yoshida, H., Y. Okabe, K. Kawane, H. Fukuyama, and S. Nagata. 2005. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat. Immunol.* 6: 49–56.
36. Kawane, K., M. Ohtani, K. Miwa, T. Kizawa, Y. Kanbara, Y. Yoshioka, H. Yoshikawa, and S. Nagata. 2006. Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* 443: 998–1002.
37. Kawane, K., H. Tanaka, Y. Kitahara, S. Shimaoka, and S. Nagata. 2010. Cytokine-dependent but acquired immunity-independent arthritis caused by DNA escaped from degradation. *Proc. Natl. Acad. Sci. USA* 107: 19432–19437.
38. Kimura-Kataoka, K., T. Yasuda, J. Fujihara, T. Toga, R. Ono, Y. Otsuka, M. Ueki, R. Iida, R. Sano, T. Nakajima, et al. 2012. Genetic and expression analysis of SNPs

in the human deoxyribonuclease II: SNPs in the promoter region reduce its in vivo activity through decreased promoter activity. *Electrophoresis* 33: 2852–2858.
39. Arvio, M. A., J. M. Rapola, and P. M. Pelkonen. 1998. Chronic arthritis in patients with aspartylglucosaminuria. *J. Rheumatol.* 25: 1131–1134.
40. Morishita, K., and R. E. Petty. 2011. Musculoskeletal manifestations of mucopolysaccharidoses. *Rheumatology (Oxford)* 50(Suppl. 5): v19–v25.
41. Li, X. D., J. Wu, D. Gao, H. Wang, L. Sun, and Z. J. Chen. 2013. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* 341: 1390–1394.
42. Unterholzner, L., S. E. Keating, M. Baran, K. A. Horan, S. B. Jensen, S. Sharma, C. M. Sirois, T. Jin, E. Latz, T. S. Xiao, et al. 2010. IFI16 is an innate immune sensor for intracellular DNA. *Nat. Immunol.* 11: 997–1004.
43. Xiao, T. S., and K. A. Fitzgerald. 2013. The cGAS-STING pathway for DNA sensing. *Mol. Cell* 51: 135–139.
44. Ahn, J., D. Gutman, S. Saijo, and G. N. Barber. 2012. STING manifests self DNA-dependent inflammatory disease. *Proc. Natl. Acad. Sci. USA* 109: 19386–19391.
45. Jeremiah, N., B. Neven, M. Gentili, I. Callebaut, S. Maschalidi, M. C. Stolzenberg, N. Goudin, M. L. Frémond, P. Nitschke, T. J. Molina, et al. 2014. Inherited STING-activating mutation underlies a familial inflammatory syndrome with lupus-like manifestations. *J. Clin. Invest.* 124: 5516–5520.
46. Liu, Y., A. A. Jesus, B. Marrero, D. Yang, S. E. Ramsey, G. A. Montealegre Sanchez, K. Tenbrock, H. Wittkowski, O. Y. Jones, H. S. Kuehn, et al. 2014. Activated STING in a vascular and pulmonary syndrome. *N. Engl. J. Med.* 371: 507–518.
47. Casciola-Rosen, L. A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179: 1317–1330.
48. Green, D. R., T. Ferguson, L. Zitvogel, and G. Kroemer. 2009. Immunogenic and tolerogenic cell death. *Nat. Rev. Immunol.* 9: 353–363.
49. Nagata, S. 2010. Apoptosis and autoimmune diseases. *Ann. N. Y. Acad. Sci.* 1209: 10–16.
50. Shlomchik, M. J. 2009. Activating systemic autoimmunity: B's, T's, and tolls. *Curr. Opin. Immunol.* 21: 626–633.
51. Lan, Y. Y., D. Londoño, R. Bouley, M. S. Rooney, and N. Hacohen. 2014. Dnase2a deficiency uncovers lysosomal clearance of damaged nuclear DNA via autophagy. *Cell Reports* 9: 180–192.
52. Rongvaux, A., R. Jackson, C. C. Harman, T. Li, A. P. West, M. R. de Zoete, Y. Wu, B. Yordy, S. A. Lakhani, C. Y. Kuan, et al. 2014. Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* 159: 1563–1577.
53. West, A. P., W. Khoury-Hanold, M. Staron, M. C. Tal, C. M. Pineda, S. M. Lang, M. Bestwick, B. A. Duguay, N. Raimundo, D. A. MacDuff, et al. 2015. Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* 520: 553–557.
54. Goodier, J. L., and H. H. Kazazian, Jr. 2008. Retrotransposons revisited: the restraint and rehabilitation of parasites. *Cell* 135: 23–35.
55. Young, G. R., U. Eksmond, R. Salcedo, L. Alexopoulou, J. P. Stoye, and G. Kassiotis. 2012. Resurrection of endogenous retroviruses in antibody-deficient mice. *Nature* 491: 774–778.
56. Zeng, M., Z. Hu, X. Shi, X. Li, X. Zhan, X. D. Li, J. Wang, J. H. Choi, K. W. Wang, T. Purrington, et al. 2014. MAVS, cGAS, and endogenous retroviruses in T-independent B cell responses. *Science* 346: 1486–1492.
57. Platt, F. M., B. Boland, and A. C. van der Spoel. 2012. The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction. *J. Cell Biol.* 199: 723–734.
58. Baum, R., S. Sharma, S. Carpenter, Q. Z. Li, P. Busto, K. A. Fitzgerald, A. Marshak-Rothstein, and E. M. Gravallese. 2015. Cutting edge: AIM2 and endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II-deficient mice. *J. Immunol.* 194: 873–877.
59. Arnoult, D., F. Soares, I. Tattoli, and S. E. Girardin. 2011. Mitochondria in innate immunity. *EMBO Rep.* 12: 901–910.
60. White, M. J., K. McArthur, D. Metcalf, R. M. Lane, J. C. Cambier, M. J. Herold, M. F. van Delft, S. Bedoui, G. Lessene, M. E. Ritchie, et al. 2014. Apoptotic caspases suppress mtDNA-induced STING-mediated type I IFN production. *Cell* 159: 1549–1562.
61. Lartigue, A., P. Courville, I. Auquit, A. François, C. Arnoult, F. Tron, D. Gilbert, and P. Musette. 2006. Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus. *J. Immunol.* 177: 1349–1354.
62. Santiago-Raber, M. L., I. Dunand-Sauthier, T. Wu, Q. Z. Li, S. Uematsu, S. Akira, W. Reith, C. Mohan, B. L. Kotzin, and S. Izui. 2010. Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice. *J. Autoimmun.* 34: 339–348.
63. Yu, P., U. Wellmann, S. Kunder, L. Quintanilla-Martinez, L. Jennen, N. Dear, K. Amann, S. Bauer, T. H. Winkler, and H. Wagner. 2006. Toll-like receptor 9-independent aggravation of glomerulonephritis in a novel model of SLE. *Int. Immunol.* 18: 1211–1219.
64. Lee, B. L., J. E. Moon, J. H. Shu, L. Yuan, Z. R. Newman, R. Schekman, and G. M. Barton. 2013. UNC93B1 mediates differential trafficking of endosomal TLRs. *eLife* 2: e00291.
65. Avalos, A. M., O. Kirak, J. M. Oelkers, M. C. Pils, Y. M. Kim, M. Ottinger, R. Jaenisch, H. L. Ploegh, and M. M. Brinkmann. 2013. Cell-specific TLR9 trafficking in primary APCs of transgenic TLR9-GFP mice. *J. Immunol.* 190: 695–702.
66. Stoehr, A. D., C. T. Schoen, M. M. Mertes, S. Eiglmeier, V. Holecska, A. K. Lorenz, T. Schommartz, A. L. Schoen, C. Hess, A. Winkler, et al. 2011. TLR9 in peritoneal B-1b cells is essential for production of protective self-reactive IgM to control Th17 cells and severe autoimmunity. *J. Immunol.* 187: 2953–2965.
67. Nündel, K., N. M. Green, A. L. Shaffer, K. L. Moody, P. Busto, D. Eilat, K. Miyake, M. A. Oropallo, M. P. Cancro, and A. Marshak-Rothstein. 2015. Cell-intrinsic expression of TLR9 in autoreactive B cells constrains BCR/TLR7-dependent responses. *J. Immunol.* 194: 2504–2512.
68. Isnardi, I., Y. S. Ng, I. Srdanovic, R. Motaghedi, S. Rudchenko, H. von Bernuth, S. Y. Zhang, A. Puel, E. Jouanguy, C. Picard, et al. 2008. IRAK-4- and MyD88-dependent pathways are essential for the removal of developing autoreactive B cells in humans. *Immunity* 29: 746–757.
69. Nickerson, K. M., S. R. Christensen, J. L. Cullen, W. Meng, E. T. Luning Prak, and M. J. Shlomchik. 2013. TLR9 promotes tolerance by restricting survival of anergic anti-DNA B cells, yet is also required for their activation. *J. Immunol.* 190: 1447–1456.
70. Sharma, S., A. M. Campbell, J. Chan, S. A. Schattgen, G. M. Orlowski, R. Nayar, A. H. Huyler, K. Nündel, C. Mohan, L. J. Berg, et al. 2015. Suppression of systemic autoimmunity by the innate immune adaptor STING. *Proc. Natl. Acad. Sci. USA* 112: E710–E717.
71. Dong, G., M. You, L. Ding, H. Fan, F. Liu, D. Ren, and Y. Hou. 2015. STING Negatively Regulates Double-Stranded DNA-Activated JAK1-STAT1 Signaling via SHP-1/2 in B Cells. *Mol. Cells* 38: 441–451.
72. Chen, H., H. Sun, F. You, W. Sun, X. Zhou, L. Chen, J. Yang, Y. Wang, H. Tang, Y. Guan, et al. 2011. Activation of STAT6 by STING is critical for antiviral innate immunity. *Cell* 147: 436–446.
73. Lawrence, T., and G. Natoli. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat. Rev. Immunol.* 11: 750–761.
74. Ravishankar, B., H. Liu, R. Shinde, P. Chandler, B. Baban, M. Tanaka, D. H. Munn, A. L. Mellor, M. C. Karlsson, and T. L. McGaha. 2012. Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase. *Proc. Natl. Acad. Sci. USA* 109: 3909–3914.
75. Jin, J., H. Hu, H. S. Li, J. Yu, Y. Xiao, G. C. Brittain, Q. Zou, X. Cheng, F. A. Mallette, S. S. Watowich, and S. C. Sun. 2014. Noncanonical NF-κB pathway controls the production of type I interferons in antiviral innate immunity. *Immunity* 40: 342–354.
76. Negishi, H., H. Yanai, A. Nakajima, R. Koshiba, K. Atarashi, A. Matsuda, K. Matsuki, S. Miki, T. Doi, A. Aderem, et al. 2012. Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. *Nat. Immunol.* 13: 659–666.
77. Parroche, P., F. N. Lauw, N. Goutagny, E. Latz, B. G. Monks, A. Visintin, K. A. Halmen, M. Lamphier, M. Olivier, D. C. Bartholomeu, et al. 2007. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proc. Natl. Acad. Sci. USA* 104: 1919–1924.
78. Wu, X., N. M. Gowda, S. Kumar, and D. C. Gowda. 2010. Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses. *J. Immunol.* 184: 4338–4348.
79. Sharma, S., R. B. DeOliveira, P. Kalantari, P. Parroche, N. Goutagny, Z. Jiang, J. Chan, D. C. Bartholomeu, F. Lauw, J. P. Hall, et al. 2011. Innate immune recognition of an AT-rich stem-loop DNA motif in the *Plasmodium falciparum* genome. *Immunity* 35: 194–207.
80. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.
81. Zucchini, N., G. Bessou, S. Traub, S. H. Robbins, S. Uematsu, S. Akira, L. Alexopoulou, and M. Dalod. 2008. Cutting edge: Overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. *J. Immunol.* 180: 5799–5803.
82. Schoggins, J. W., D. A. MacDuff, N. Imanaka, M. D. Gainey, B. Shrestha, J. L. Eitson, K. B. Mar, R. B. Richardson, A. V. Ratushny, V. Litvak, et al. 2014. *Pan*-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* 505: 691–695.

Brief Reviews

The Journal of Immunology

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 inflammaging (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. These pathologies may reflect causal relationships with overall changes in the immune system, the cumulative impact of environmental insults, or combinations of these factors. Alternatively, some of these features may instead initiate in young individuals and stem from normal immune activity, but their pathogenic actions may only become manifest once the underlying effectors reach a minimum threshold with advancing age.

At a cellular level, the output of primary lymphoid organs wanes, reflecting a shift toward myeloid lineage preference in hematopoietic stem cell specification (13, 14), reductions in key developmental gene expression (15–17), and altered microenvironmental and homeostatic feedback mechanisms (18, 19). Despite this dwindling lymphocyte production, total numbers of mature B and T cells remain relatively unchanged. Nevertheless, nearly all peripheral lymphoid pools exhibit altered dynamics, shifts in functional subset representation, and changes in clonal composition. Thus, the renewal rates of both T and B cell pools decline (15, 20, 21), in part explaining how overall numbers can be maintained in the absence of newly generated cells. Further, inordinate clonal expansions are observed in both T and B cell compartments. Although some of these age-associated changes may result from immune dysregulation, others may simply reflect the cumulative influence of antigenic experiences and normal homeostatic processes. In accord with this notion, the ratio of T cells displaying naive versus memory phenotype inverts with age. Similarly, a substantial shift in the composition of peripheral B cell pools accompanies advancing age, reflecting the gradual appearance of a novel B cell subset whose properties and origins are the focus of this review.

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 homeostatically 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 locale 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,

*Howard Hughes Medical Institute, National Jewish Health, Denver, CO 80206; †Department of Biomedical Science, National Jewish Health, Denver, CO 80206; ‡Department of Immunology and Microbiology, School of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO 80045; §Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; ¶Department of Medicine, School of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO 80045; and ‖Department of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO 80045

Received for publication May 27, 2015. Accepted for publication June 29, 2015.

This work was supported by U.S. Public Health Service Grants AI-18785 and T32 AI074491 (to K.R.) and R01AG-16841 and CDMRP PR130769 (to M.P.C.).

Address correspondence and reprint requests to Dr. Kira Rubtsova, National Jewish Health, 1400 Jackson Street, Denver, CO 80206. E-mail address: rubtsovak@njhealth.org

Abbreviations used in this article: ABC, age-associated B cell; FO, follicular; MZ, marginal zone; T_{FH}, T follicular helper, .

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1501209

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, Rubstov 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 Rubstov 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 mice. 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 simulation 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).

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

	Mice (Ref. 22)	Mice (Ref. 23)	Humans (Ref. 23)	Exhausted Human B Cells
CD19	N/A	High	+	+
B220	+	+	+	N/A
CD11c	+/−	+	+	+
CD11b	N/A	+	N/A	+
CD21	−	−	−	Low
CD23	−	−	−	+
Fas	N/A	+	+	N/A
CD138	N/A	Int	N/A	Low
CD5	−	Int	+	N/A
CD80/86	Low	High	High	High
MHC class II	Low	High	N/A	N/A
T-bet	N/A	+	+	N/A
Surface IgM	+	+/−	−	N/A
Surface IgD	Low	+/−	−	−

Int, intermediate; N/A, not available; −, negative; +, positive.

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 may 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 (T_{FH}) 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 T_{FH} cell program or directing pre-T_{FH} cells to alternative effector fates. In agreement with this idea, ABC presenters skew primed T cells to a Th17 fate in vitro (22).

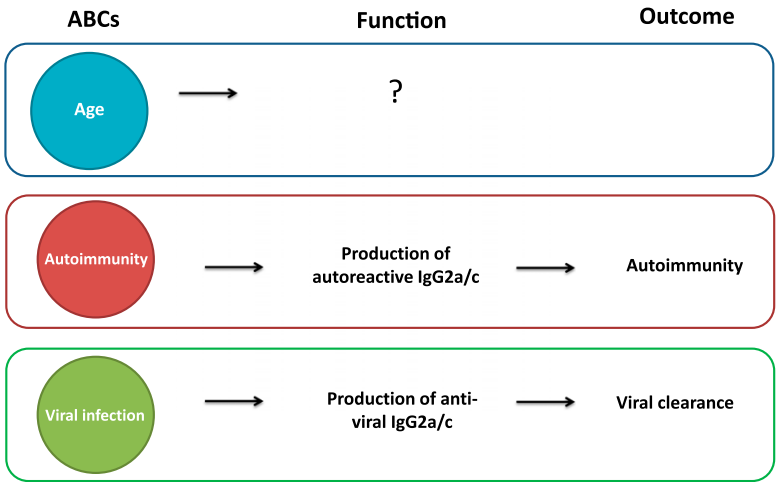


FIGURE 1. ABCs in age, autoimmunity, and infection. The function and outcome of the appearance of ABCs in aged animals are still unknown. In autoimmune animals, ABCs produce high titers of autoantibodies (mostly of IgG2a/c isotype) upon stimulation, which may be the cause of autoimmunity. During the infection, ABCs produce antiviral IgG (mostly IgG2a/c), which is required for efficient viral clearance.

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 cytotoxicity 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 milieus; 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).

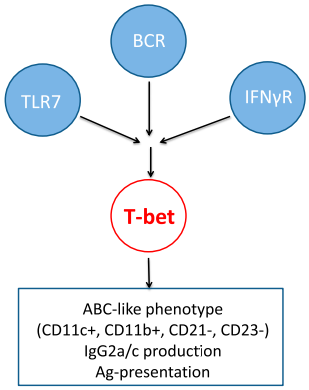


FIGURE 2. Model for T-bet induction in B cells and its role in B cell fate. Synergistic signaling via BCR, TLR7, and IFN-γR in B cells leads to the induction of high levels of T-bet expression, which, in turn, drives the expression of an ABC phenotype and class-switching to the production of IgG2a Abs.

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^{lpr}, NZB × WF1, $MER^{-/-}$, 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 $CD11c^{+}/CD21^{-}$ 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 $CD19^{high}/CD21^{low}$ (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 ribonuclear particles, 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-γR 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 $Mer^{-/-}$ 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 $MER^{-/-}$ 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 lyonized 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-γR. 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-differentiative 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

1. Ben-Yehuda, A., and M. E. Weksler. 1992. Immune senescence: mechanisms and clinical implications. *Cancer Invest.* 10: 525–531.
2. Pawelec, G. 2003. Immunosenescence and human longevity. *Biogerontology* 4: 167–170.
3. Miller, R. A. 1996. The aging immune system: primer and prospectus. *Science* 273: 70–74.
4. Doria, G., and D. Frasca. 1997. Genes, immunity, and senescence: looking for a link. *Immunol. Rev.* 160: 159–170.
5. Franceschi, C., M. Passeri, G. De Benedictis, and L. Motta. 1998. Immunosenescence. *Aging (Milano)* 10: 153–154.

6. Johnson, S. A., and J. C. Cambier. 2004. Ageing, autoimmunity and arthritis: senescence of the B cell compartment - implications for humoral immunity. *Arthritis Res. Ther.* 6: 131–139.
7. Franceschi, C., and M. Bonafè. 2003. Centenarians as a model for healthy aging. *Biochem. Soc. Trans.* 31: 457–461.
8. Franceschi, C., and J. Campisi. 2014. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J. Gerontol. A Biol. Sci. Med. Sci.* 69(Suppl. 1): S4–S9.
9. Dolcetti, R., and M. Boiocchi. 1996. Cellular and molecular bases of B-cell clonal expansions. *Clin. Exp. Rheumatol.* 14(Suppl. 14): S3–S13.
10. LeMaoult, J., S. Delassus, R. Dyall, J. Nikolić-Zugić, P. Kourilsky, and M. E. Weksler. 1997. Clonal expansions of B lymphocytes in old mice. *J. Immunol.* 159: 3866–3874.
11. LeMaoult, J., I. Messaoudi, J. S. Manavalan, H. Potvin, D. Nikolich-Zugich, R. Dyall, P. Szabo, M. E. Weksler, and J. Nikolich-Zugich. 2000. Age-related dysregulation in CD8 T cell homeostasis: kinetics of a diversity loss. *J. Immunol.* 165: 2367–2373.
12. Eaton-Bassiri, A. S., L. Mandik-Nayak, S. J. Seo, M. P. Madaio, M. P. Cancro, and J. Erikson. 2000. Alterations in splenic architecture and the localization of anti-double-stranded DNA B cells in aged mice. *Int. Immunol.* 12: 915–926.
13. Zediak, V. P., I. Maillard, and A. Bhandoola. 2007. Multiple prethymic defects underlie age-related loss of T progenitor competence. *Blood* 110: 1161–1167.
14. Miller, J. P., and D. Allman. 2003. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. *J. Immunol.* 171: 2326–2330.
15. Labrie, J. E., III, A. P. Sah, D. M. Allman, M. P. Cancro, and R. M. Gerstein. 2004. Bone marrow microenvironmental changes underlie reduced RAG-mediated recombination and B cell generation in aged mice. *J. Exp. Med.* 200: 411–423.
16. Sherwood, E. M., B. B. Blomberg, W. Xu, C. A. Warner, and R. L. Riley. 1998. Senescent BALB/c mice exhibit decreased expression of lambda5 surrogate light chains and reduced development within the pre-B cell compartment. *J. Immunol.* 161: 4472–4475.
17. Frasca, D., E. Van Der Put, R. L. Riley, and B. B. Blomberg. 2004. Age-related differences in the E2A-encoded transcription factor E47 in bone marrow-derived B cell precursors and in splenic B cells. *Exp. Gerontol.* 39: 481–489.
18. Stephan, R. P., D. A. Lill-Elghanian, and P. L. Witte. 1997. Development of B cells in aged mice: decline in the ability of pro-B cells to respond to IL-7 but not to other growth factors. *J. Immunol.* 158: 1598–1609.
19. Stephan, R. P., V. M. Sanders, and P. L. Witte. 1996. Stage-specific alterations in murine B lymphopoiesis with age. *Int. Immunol.* 8: 509–518.
20. Kline, G. H., T. A. Hayden, and N. R. Klinman. 1999. B cell maintenance in aged mice reflects both increased B cell longevity and decreased B cell generation. *J. Immunol.* 162: 3342–3349.
21. Tsukamoto, H., K. Clise-Dwyer, G. E. Huston, D. K. Duso, A. L. Buck, L. L. Johnson, L. Haynes, and S. L. Swain. 2009. Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. *Proc. Natl. Acad. Sci. USA* 106: 18333–18338.
22. Hao, Y., P. O'Neill, M. S. Naradikian, J. L. Scholz, and M. P. Cancro. 2011. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* 118: 1294–1304.
23. Rubtsov, A. V., K. Rubtsova, A. Fischer, R. T. Meehan, J. Z. Gillis, J. W. Kappler, and P. Marrack. 2011. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c^{+} B-cell population is important for the development of autoimmunity. *Blood* 118: 1305–1315.
24. Birjandi, S. Z., J. A. Ippolito, A. K. Ramadorai, and P. L. Witte. 2011. Alterations in marginal zone macrophages and marginal zone B cells in old mice. *J. Immunol.* 186: 3441–3451.
25. Rubtsova, K., A. V. Rubtsov, L. F. van Dyk, J. W. Kappler, and P. Marrack. 2013. T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. *Proc. Natl. Acad. Sci. USA* 110: E3216–E3224.
26. Ehrhardt, G. R., A. Hijikata, H. Kitamura, O. Ohara, J. Y. Wang, and M. D. Cooper. 2008. Discriminating gene expression profiles of memory B cell subpopulations. *J. Exp. Med.* 205: 1807–1817.
27. Ratliff, M., S. Alter, D. Frasca, B. B. Blomberg, and R. L. Riley. 2013. In senescence, age-associated B cells secrete TNFα and inhibit survival of B-cell precursors. *Aging Cell* 12: 303–311.
28. Gerth, A. J., L. Lin, and S. L. Peng. 2003. T-bet regulates T-independent IgG2a class switching. *Int. Immunol.* 15: 937–944.
29. Ehlers, M., H. Fukuyama, T. L. McGaha, A. Aderem, and J. V. Ravetch. 2006. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J. Exp. Med.* 203: 553–561.
30. Carrel, L., and H. F. Willard. 2005. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 434: 400–404.
31. Wang, N. S., L. J. McHeyzer-Williams, S. L. Okitsu, T. P. Burris, S. L. Reiner, and M. G. McHeyzer-Williams. 2012. Divergent transcriptional programming of class-specific B cell memory by T-bet and RORα. *Nat. Immunol.* 13: 604–611.
32. Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc. Natl. Acad. Sci. USA* 99: 5545–5550.
33. Rubtsov, A. V., K. Rubtsova, J. W. Kappler, J. Jacobelli, R. S. Friedman, and P. Marrack. 2015. CD11c-expressing B cells are located at the T cell/B cell border in spleen and are potent APCs. *J. Immunol.* 195: 71–79.
34. Keren, Z., S. Naor, S. Nussbaum, K. Golan, T. Itkin, Y. Sasaki, M. Schmidt-Supprian, T. Lapidot, and D. Melamed. 2011. B-cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging. *Blood* 117: 3104–3112.
35. Goenka, R., J. L. Scholz, M. S. Naradikian, and M. P. Cancro. 2014. Memory B cells form in aged mice despite impaired affinity maturation and germinal center kinetics. *Exp. Gerontol.* 54: 109–115.
36. Lefebvre, J. S., A. C. Maue, S. M. Eaton, P. A. Lanthier, M. Tighe, and L. Haynes. 2012. The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. *Aging Cell* 11: 732–740.
37. Yates, J. L., R. Racine, K. M. McBride, and G. M. Winslow. 2013. T cell-dependent IgM memory B cells generated during bacterial infection are required for IgG responses to antigen challenge. *J. Immunol.* 191: 1240–1249.
38. Kipps, T. J., P. Parham, J. Punt, and L. A. Herzenberg. 1985. Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. *J. Exp. Med.* 161: 1–17.
39. Coutelier, J. P., J. T. van der Logt, F. W. Heessen, A. Vink, and J. van Snick. 1988. Virally induced modulation of murine IgG antibody subclasses. *J. Exp. Med.* 168: 2373–2378.
40. Nimmerjahn, F., P. Bruhns, K. Horiuchi, and J. V. Ravetch. 2005. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. *Immunity* 23: 41–51.
41. Markine-Goriaynoff, D., and J. P. Coutelier. 2002. Increased efficacy of the immunoglobulin G2a subclass in antibody-mediated protection against lactate dehydrogenase-elevating virus-induced polioencephalomyelitis revealed with switch mutants. *J. Virol.* 76: 432–435.
42. Larson, J. D., J. M. Thurman, A. V. Rubtsov, D. Claypool, P. Marrack, L. F. van Dyk, R. M. Torres, and R. Pelanda. 2012. Murine gammaherpesvirus 68 infection protects lupus-prone mice from the development of autoimmunity. *Proc. Natl. Acad. Sci. USA* 109: E1092–E1100.
43. Rakhmanov, M., B. Keller, S. Gutenberger, C. Foerster, M. Hoenig, G. Driessen, M. van der Burg, J. J. van Dongen, E. Wiech, M. Visentini, et al. 2009. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc. Natl. Acad. Sci. USA* 106: 13451–13456.
44. Isnardi, I., Y. S. Ng, L. Menard, G. Meyers, D. Saadoun, I. Srdanovic, J. Samuels, J. Berman, J. H. Buckner, C. Cunningham-Rundles, and E. Meffre. 2010. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood* 115: 5026–5036.
45. Warnatz, K., C. Wehr, R. Dräger, S. Schmidt, H. Eibel, M. Schlesier, and H. H. Peter. 2002. Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. *Immunobiology* 206: 502–513.
46. Wehr, C., H. Eibel, M. Masilamani, H. Illges, M. Schlesier, H. H. Peter, and K. Warnatz. 2004. A new CD21low B cell population in the peripheral blood of patients with SLE. *Clin. Immunol.* 113: 161–171.
47. Moir, S., J. Ho, A. Malaspina, W. Wang, A. C. DiPoto, M. A. O'Shea, G. Roby, S. Kottilil, J. Arthos, M. A. Proschan, et al. 2008. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J. Exp. Med.* 205: 1797–1805.
48. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670–684.
49. Giltiay, N. V., C. P. Chappell, X. Sun, N. Kolhatkar, T. H. Teal, A. E. Wiedeman, J. Kim, L. Tanaka, M. B. Buechler, and J. A. Hamerman, et al. 2013. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. *J. Exp. Med.* 210: 2773–2789.
50. Allman, D., and S. Pillai. 2008. Peripheral B cell subsets. *Curr. Opin. Immunol.* 20: 149–157.
51. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
52. Jacob, C. O., P. H. van der Meide, and H. O. McDevitt. 1987. In vivo treatment of (NZB X NZW)F1 lupus-like nephritis with monoclonal antibody to gamma interferon. *J. Exp. Med.* 166: 798–803.
53. Balomenos, D., R. Rumold, and A. N. Theofilopoulos. 1998. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J. Clin. Invest.* 101: 364–371.
54. Ytterberg, S. R., and T. J. Schnitzer. 1982. Serum interferon levels in patients with systemic lupus erythematosus. *Arthritis Rheum.* 25: 401–406.
55. Christensen, S. R., J. Shupe, K. Nickerson, M. Kashgarian, R. A. Flavell, and M. J. Shlomchik. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417–428.

Cell-Intrinsic Expression of TLR9 in Autoreactive B Cells Constrains BCR/TLR7-Dependent Responses

Kerstin Nündel,*[,1] Nathaniel M. Green,*[,1] Arthur L. Shaffer,[†] Krishna L. Moody,* Patricia Busto,* Dan Eilat,[‡] Kensuke Miyake,[§] Michael A. Oropallo,[¶] Michael P. Cancro,[¶] and Ann Marshak-Rothstein*

Endosomal TLRs play an important role in systemic autoimmune diseases, such as systemic erythematosus 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 erythematosus lupus. ***The Journal of Immunology*****, 2015, 194: 2504–2512.**

Many of the autoantigens targeted during systemic autoimmune diseases act as autoadjuvants by associating with macromolecular complexes that stimulate innate immune receptors. In B cells, nucleic acid–associated autoantigens need to be bound by the BCR and transported to a TLR-associated compartment where TLR detection of DNA or RNA provides a second signal that promotes B cell activation. This paradigm, whereby BCR-delivered TLR agonists promote autoreactive B cell activation, initially emerged from in vitro studies (1) and has been supported by numerous in vivo observations. Thus, TLR7-deficient autoimmune-prone mice fail to make autoantibodies reactive with RNA-associated autoantigens, and TLR9-deficient autoimmune-prone mice fail to make autoantibodies reactive with dsDNA or chromatin (2). Moreover, autoimmune-prone mice lacking only TLR7 have markedly attenuated disease (2), whereas overexpression of TLR7 results in exacerbated clinical symptoms and accelerated mortality (3, 4). However, quite paradoxically, autoimmune-prone mice that lack functional TLR9 invariably develop more severe clinical disease and have shortened lifespans (5–9).

Remarkably little is known about the differential outcomes of TLR7 versus TLR9 engagement or how TLR9, but not TLR7, mitigates systemic autoimmunity. In mice, both TLR7 and TLR9 are expressed by B cells, dendritic cells (DCs), macrophages, and even neutrophils; therefore, any of these cell types could negatively regulate disease onset through a TLR9-dependent mechanism. However, the growing appreciation that B cells play a pivotal role in the etiology of systemic autoimmune diseases (10, 11) led us to monitor the direct effects of BCR/TLR7 and BCR/TLR9 coengagement on B cell differentiation. We used BALB/c mice expressing an IgG2a-specific site-directed transgene-encoded receptor, AM14, derived from an ∼6-mo-old Fas-deficient MRL/lpr mouse (12–14). These rheumatoid factor (RF) B cells bind IgG2a with sufficiently low affinity that they survive tolerance checkpoints and persist in BALB/c mice as resting naive follicular (FO) B cells, even in the presence of (monomeric) serum IgG2a (15). In fact, only IgG2a immune complexes (ICs) that incorporate endogenous nucleic acids capable of engaging either TLR7 or TLR9 can induce these RF B cells to proliferate in vitro (16). RF B cell responses to DNA-associated ICs are TLR9 dependent and inhibited by the addition of DNase I to the culture medium, whereas responses to RNA-associated ICs are TLR7 dependent and inhibited by the ad-

*Division of Rheumatology, Department of Medicine, University of Massachusetts School of Medicine, Worcester, MA 01605; [†]Lymphoid Malignancies Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; [‡]Hadassah University Hospital, Jerusalem 91322, Israel; [§]Department of Microbiology and Immunology, University of Tokyo, Tokyo 108-8639, Japan; and [¶]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA 19104

[1]K.N. and N.M.G. contributed equally to this work.

Received for publication September 23, 2014. Accepted for publication January 6, 2015.

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institutes of Health (NIH) Grant AR050256 (to A.M.-R.) and Department of the Army Grant PR120610 (to M.P.C.). M.A.O. was supported in part by NIH Training Grant T32 AI-055428, and A.L.S. was supported by the NIH intramural program.

The gene expression data presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE58756.

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Arthritis and Musculoskeletal and Skin Diseases or the National Institutes of Health.

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

The online version of this article contains supplemental material.

Abbreviations used in this article: AFC, Ab-forming cell; BLyS, B lymphocyte stimulator; DC, dendritic cell; FO, follicular; IC, immune complex; MZ, marginal zone; ODN, oligodeoxynucleotide; PC, plasma cell; qPCR, quantitative real-time PCR; RF, rheumatoid factor; SA, streptavidin; SLE, systemic lupus erythematosus; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402425

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 autoantibodies 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 $CD138^+$ plasmablasts. BCR/TLR7- and BCR/TLR9-activation pathways also have distinct functional outcomes in vivo, where again RF B cells activated through the BCR/TLR7 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 (s-oligodeoxynucleotide[ODN], kindly provided by Idera Pharmaceuticals), 0.1–1.0 μg/ml CL097 (InvivoGen), and 15 μg/ml goat anti-mouse IgM $F(ab')_2$ (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-β (300 U/ml; PBL). B cell proliferation was assessed by [^{3}H]thymidine incorporation at the times indicated or by fluorescent dye dilution at 72 h. B lymphocyte stimulator (BLyS), provided by Human Genome Sciences, was added to selected experimental groups maintained for 72 h at a final concentration of 50 ng/ml. The TLR9 inhibitory ODN (3′-CCT GGA TGG GAA CTT ACC GCT GCA-5′) was described previously (24).

Flow cytometry

B cell subsets were identified with CD22.2-FITC, CD138-PE, CD45.2-PE, CD45.1-allophycocyanin (BD Biosciences), CD45R/B220–eFluor 450, and CD44–eFluor 780 (eBioscience). RF B cells were detected with biotinylated-4G7 in combination with SA–PerCP–Cy5.5. IRF-4 was detected using an IRF-4 Ab (clone M-17; Santa Cruz Biotechnology) and anti-goat IgG Alexa Fluor 647 (Jackson ImmunoResearch). IRF4-PE and IRF8–PerCP–eFluor 710 (eBioscience) were used to costain IRF8 and IRF4. B cell proliferation was assessed by CFSE dilution (Life Technologies) (15) or VPD450 dilution (BD). Dead cells were distinguished with TO-PRO-3 (Life Technologies). To analyze TLR7 expression levels, unstimulated purified B cells or B cells stimulated for 24 h were fixed and permeabilized using the Foxp3 Fix/Perm Kit (eBioscience). TLR7 protein 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 LSR II with Diva 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, pax5, and prdm1 (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 6x80K 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 with biotinylated anti-clonotype 4G7 (for IgG1) or 4-44 (for IgG2a), as well as SA–alkaline phosphatase (BD Biosciences) (12). Spots were developed with BCIP/NBT substrate (Sigma-Aldrich) and counted using an ImmunoSpot reader (C.T.L.).

In vivo activation of AM14 B cells

B220-purified splenic B cells (15×10^6) 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 μM 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-dependent 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 [^{3}H]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 *Tlr9*$^{-/-}$ 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 (*Tlr9*$^{-/-}$ cells) recapitulated the BWR4 kinetics, whereas the TLR9-driven PL2-3 and PA4 responses peaked and declined at an earlier time point (Fig. 1C).

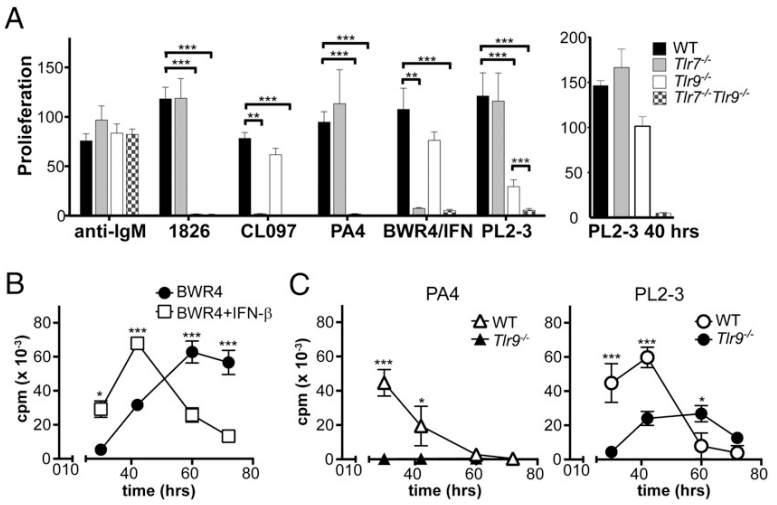


FIGURE 1. Monoclonal autoantibodies activate RF B cells through BCR/TLR9-dependent, BCR/TLR7-dependent, or both BCR/TLR7- and BCR/TLR9-dependent pathways. (**A**) Splenic B cells from RF WT, *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, and *Tlr7*$^{-/-}$*Tlr9*$^{-/-}$ mice were activated with the indicated ligands or monoclonal autoantibodies for 30 h (*left panel*) or 40 h (*right panel*) and proliferation was measured by [^{3}H]thymidine uptake. Data represent the mean ± SEM of three independent experiments. (**B**) RF WT B cells were stimulated with BWR4 in the presence (□) or absence (●) of IFN-β for the indicated times. (**C**) RF WT and RF *Tlr9*$^{-/-}$ B cells were stimulated with PA4 (*left panel*) or PL2-3 (*right panel*) for the indicated times. Data in (B) and (C) are representative of three independent experiments. Proliferation was assessed by [^{3}H]thymidine incorporation over the last 6 h of the experiment. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, unpaired Student *t* test.

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 CD22^{lo}, CD44^{hi}, 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.

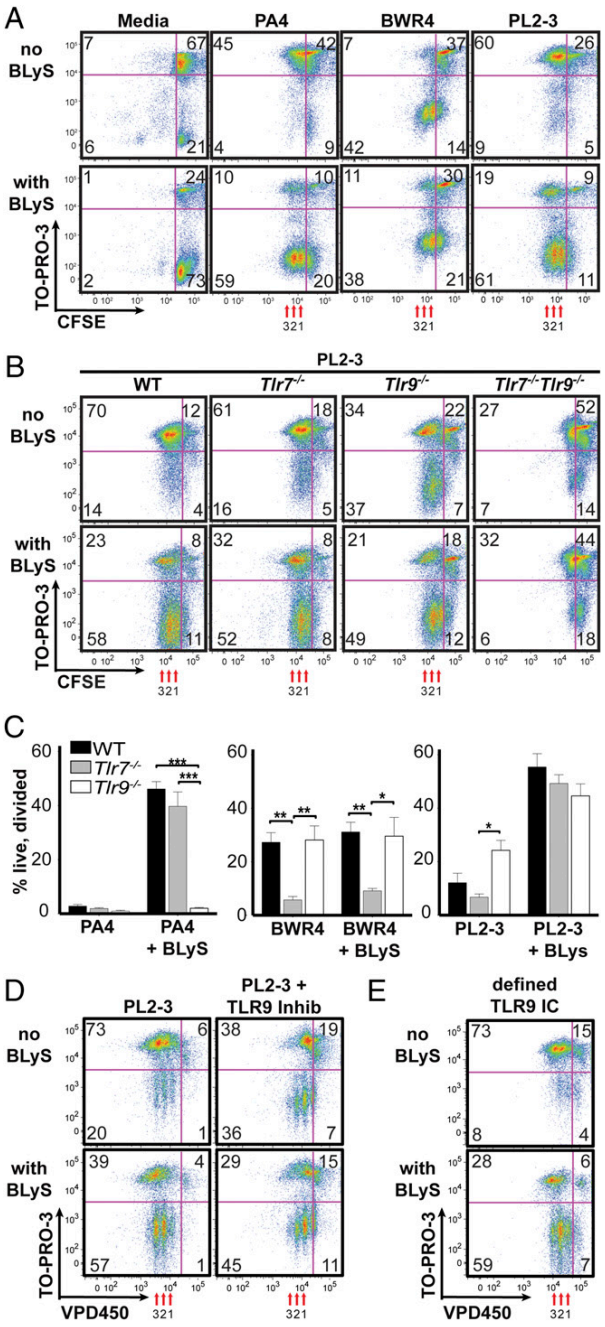


FIGURE 2. RNA-containing ICs induce BLyS-independent survival. (**A**) CFSE-labeled RF WT B cells were stimulated with the indicated ligands in the absence (*upper panels*) or presence (*lower panels*) of 50 ng/ml BLyS for 72 h. Proliferation was measured by CFSE dilution, and cell death was measured by uptake of TO-PRO-3. The quadrants depict the following: upper left, dead divided cells (TO-PRO-3$^+$ CFSE diluted); upper right, dead undivided cells (TOP-RO-3$^+$ CFSE undiluted); lower right, live undivided cells (TO-PRO-3$^-$ CFSE undiluted); lower left, live divided (TO-PRO-3$^-$ CFSE diluted). Division numbers are indicated by red arrows underneath the flow plots. Flow plots are representative of >10 experiments. (**B** and **C**) B cells from RF WT, *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, and *Tlr7*$^{-/-}$*Tlr9*$^{-/-}$ mice were stimulated as in (A). Representative flow plots of B cells stimulated with PL2-3 (B) or with PA4, BWR4, and PL2-3 in the presence or absence of BLyS (C). Data represent the mean percentage (± SEM) of the recovered cells that had divided and remained alive ($n = 9$). (**D**) RF WT B cells were stimulated with PL2-3 in the presence or absence of BLyS, with or without a TLR9 inhibitor for 72 h. (**E**) RF WT B cells were stimulated with IgG2a IC that incorporated a defined CG-rich DNA fragment for 72 h. Flow plots in (D) and (E) are representative of three independent experiments. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, 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). Coordinately, 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

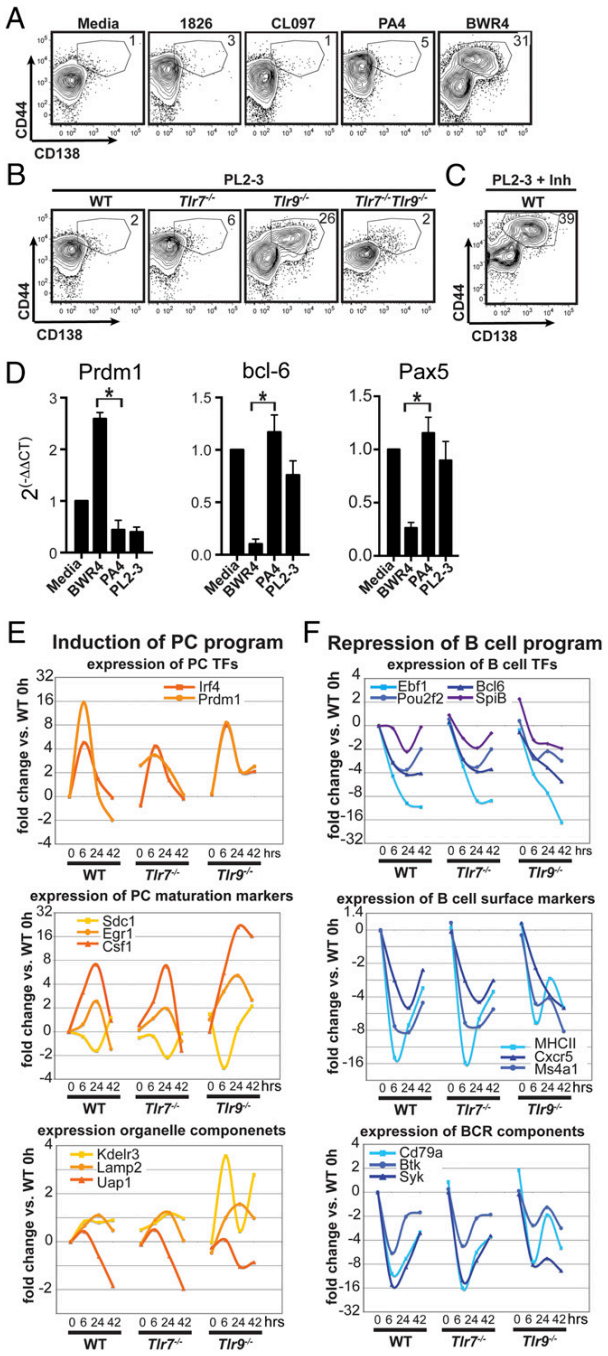


FIGURE 3. BCR/TLR7 coengagement promotes PC differentiation. (**A**) RF WT B cells were stimulated with the indicated ligands or monoclonal autoantibodies and stained for CD22, CD44, and CD138 to determine the frequency of $CD22^{lo}$ $CD44^{hi}$ $CD138^{+}$ plasmablasts (n = 4). (**B**) RF WT, $Tlr7^{-/-}$, $Tlr9^{-/-}$, and $Tlr7^{-/-}Tlr9^{-/-}$ B cells were activated with PL2-3 for 72 h and stained as in (A) (n = 4). (**C**) RF WT B cells were activated with PL2-3 in the presence of a TLR9-specific inhibitor for 72 h and stained as in (A) (n = 3). (**D**) Purified RF FcγR2b$^{-/-}$ B cells were activated with the indicated ICs for 96 h in the presence of 50 ng/ml BLyS. mRNA levels for bcl-6, Pax5, and prdm1 were determined by qPCR. Data were normalized to media control using the ΔΔCT method (n = 4). (**E** and **F**) Expression of B cell differentiation-associated genes in RF WT, $Tlr7^{-/-}$, and $Tlr9^{-/-}$ cells after stimulation with PL2-3. Gene expression changes are reported as ratios relative to RF WT 0 h (unstimulated), as measured by gene expression arrays. *$p \leq 0.05$, Student t test.

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 $Tlr9^{-/-}$ *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 $Tlr9^{-/-}$ B cells, described above, simply reflected amplified TLR7 expression, we compared TLR7 protein levels in unstimulated and stimulated WT and $Tlr9^{-/-}$ RF B cells by flow cytometry. In the unstimulated B cells, TLR7 levels were low but comparable between WT and $Tlr9^{-/-}$ cells and were slightly higher than $Tlr7^{-/-}$ 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 $Tlr9^{-/-}$ B cells. Stimulation with 1826 only led to increased expression of TLR7 in WT cells, because $Tlr9^{-/-}$ cells were not activated and $Tlr7^{-/-}$ cells did not express TLR7. Thus, it appears that, in both unstimulated and stimulated cells, WT and $Tlr9^{-/-}$ B cells express the same amount of TLR7. These expression levels are completely consistent with the overlapping dose-response curves of WT and $Tlr9^{-/-}$ 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, $Tlr9^{-/-}$, $Tlr7^{-/-}$, or $Tlr7^{-/-}Tlr9^{-/-}$ 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 $Tlr7^{-/-}Tlr9^{-/-}$ 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, $Tlr9^{-/-}$, and $Tlr7^{-/-}$ B cells all divided multiple times (Fig. 6A *lower panels*, 6B), although $Tlr7^{-/-}$ cells underwent fewer divisions than did $Tlr9^{-/-}$ 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 $Tlr7^{-/-}$ 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

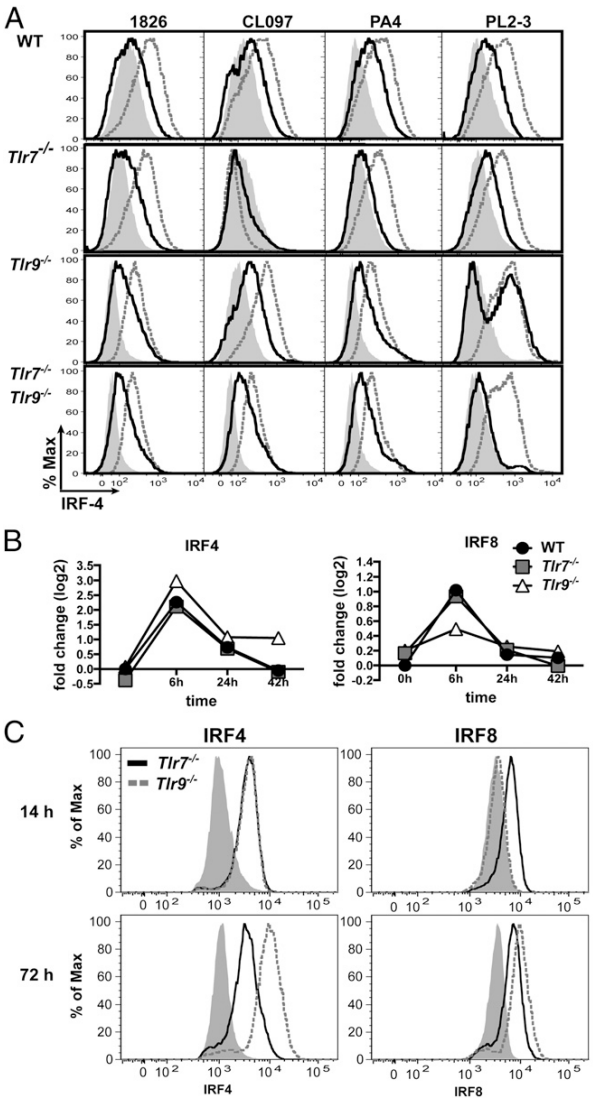


FIGURE 4. BCR/TLR7-dependent activation leads to the prolonged expression of IRF4, and BCR/TLR9-dependent activation leads to early expression of IRF8. (**A**) RF WT, *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, and *Tlr7*$^{-/-}$*Tlr9*$^{-/-}$ B cells were stimulated with the indicated ligands for either 24 h (dotted line) or 72 h (black line), permeabilized, and stained for IRF4. The filled graph represents the isotype control. Representative plots of three independent experiments are shown. (**B**) RF WT, *Tlr7*$^{-/-}$, or *Tlr9*$^{-/-}$ B cells were stimulated with PL2-3 for the indicated times, and gene expression for IRF4 (*left panel*) and IRF8 (*right panel*) was determined by microarray. (**C**) RF *Tlr7*$^{-/-}$ and *Tlr9*$^{-/-}$ B cells were stimulated with PL2-3 for 14 h (*top panel*) or 72 h (*bottom panel*) in the presence of 50 ng/ml BLyS. Expression of IRF8 (*right panels*) and IRF4 (*left panels*) was measured by flow cytometry (n = 2). The shaded histograms represent isotype controls.

known that PL2-3 IC activation of host DCs and neutrophils promotes the further production of BLyS (36, 37).

To assess the role of TLR7 and TLR9 in PL2-3–induced PC differentiation, CD45.1 BALB/c mice were injected with RF B cells and PL2-3 and then given additional injections of PL2-3 on days 3, 7, and 10. On day 13, the total number of RF B cells in the spleen was determined by flow cytometry, and the number of IgG1 and IgG2a AFCs was determined by a clonotype-specific ELISPOT assay. At this time point, we recovered comparable numbers of RF WT and *Tlr7*$^{-/-}$ B cells, approximately twice as 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 erythematosus 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-

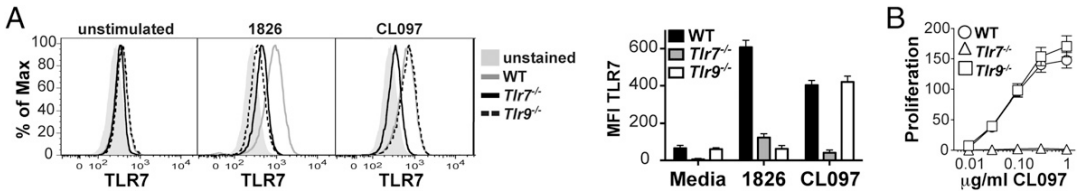


FIGURE 5. RF WT and *Tlr9*$^{-/-}$ B cells express comparable levels of TLR7. (**A**) Unactivated purified WT, *Tlr7*$^{-/-}$, and *Tlr9*$^{-/-}$ RF B cells or B cells activated with the indicated ligands for 24 h were permeabilized and stained for TLR7 expression. Unstained cells are also shown. Representative 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 [^{3}H]thymidine uptake. Data represent the mean ± SEM of eight independent experiments.

tended BLyS-independent response to PL2-3, an autoantibody that binds both DNA- and RNA-associated ligands. The addition of BLyS to the in vitro–activated TLR9-sufficient WT RF B cells prevented postproliferative cell death; however, these cells still did not show the same capacity to differentiate toward the PC lineage as did *Tlr9*$^{-/-}$ RF B cells. The capacity of TLR9 to constrain the survival of PL2-3–activated RF WT cells in vivo was less apparent, perhaps due to either basal levels of BLyS or to PL2-3 IC activation of plasmacytoid DCs (or other TLR-sufficient APCs that express FcγRs) and the ensuing production of pro-survival factors. This is a limitation of the use of PL2-3 ICs. In the context of a (nontransgenic) polyclonal repertoire, DNA-reactive (or other autoreactive) B cells would be exposed to DNA-associated autoantigens directly, prior to the production of ICs that could engage FcγR$^+$ cells. In fact, in a steady-state, MRL. *Fas*^{lpr} *Tlr9*$^{-/-}$ 3H9λ1$^+$ DNA-reactive B cells were shown to have a longer half-life in vivo than MRL.*Fas*^{lpr} *Tlr9*$^{+/+}$ 3H9λ1$^+$ DNA-reactive B cells (41), consistent with the premise that the life-span of naive B cells responding to DNA-associated autoantigens is curtailed through a TLR9-dependent mechanism. However, once RNA-associated ICs are present in the circulation and are able to elicit the production of survival factors, BCR/TLR9 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$^+$ (42–44). Most of these studies used a mixed population of FO and marginal zone (MZ) B cells. As we reported previously (15), BALB/c AM14 side-directed transgenic mice lack B1, MZ, and MZ precursor B cell compartments; therefore, our studies were carried out on a highly enriched population of naive FO B cells. These RF B cells express very low levels of TLR7 prior to activation and may not completely recapitulate the response of a polyclonal population. However, our data are consistent with the findings of Genestier et al. (45), who reported that TLR ligation predominantly induces MZ B cells and B1 B cells to differentiate into 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

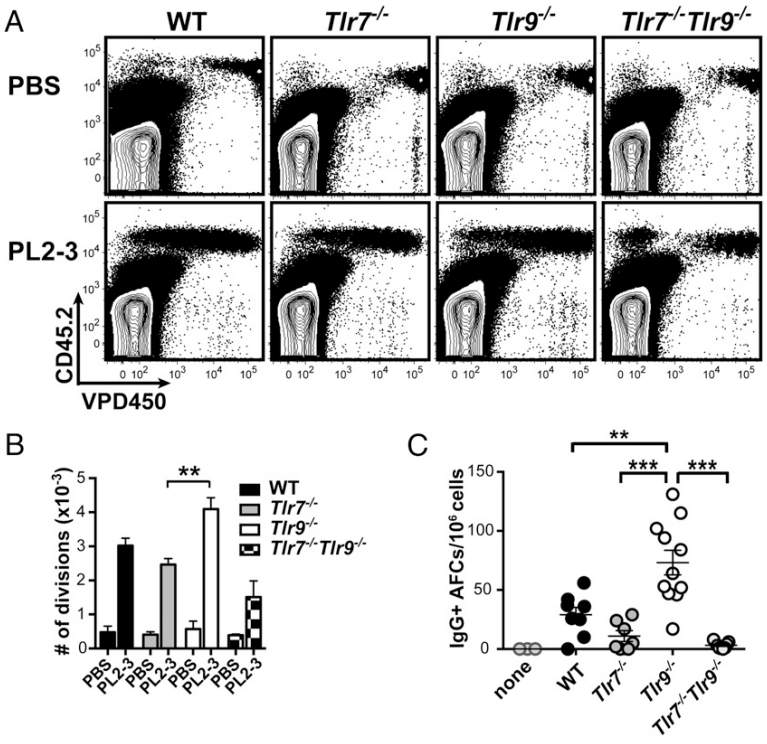


FIGURE 6. BCR/TLR7 activation promotes AFC differentiation in vivo. (**A**) BALB/c CD45.1 recipients were injected i.v. with 15 × 10^6 WT, *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, or *Tlr7*$^{-/-}$*Tlr9*$^{-/-}$ VPD450-labeled RF B cells and 50 μg PL2-3 on day 0; they were injected again with PL2-3 on day 3. 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). *$p \leq 0.05$, 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 IgG$^+$ AFCs was measured by ELISPOT. Data are compiled from four independent experiments. **$p \leq 0.005$, ***$p \leq 0.0005$, 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, *Irf8*$^{-/-}$ × 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, IRF8 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*^{lpr} 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^{lo} CD138^{+}. In contrast, in the current study, the number of IgG^{+} AFCs produced by WT B cells at day 13 was significantly lower than the number produced by *Tlr9*$^{-/-}$ B cells. The MRL/lpr study may be somewhat confounded by the accelerated disease in *Tlr9*$^{-/-}$ MRL/lpr 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% *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 for TLR7 and TLR9 in the formation of spontaneous germinal centers (48). Overall, the data point to a unique B cell–intrinsic role for TLR9 in the constraint of autoantibody production. Intriguingly, TLR8 was reported to negatively regulate murine SLE (40). These effects may reflect increased activity of TLR7, as a result of improved access to Unc93b (49) or, alternatively, distinct downstream components of the relevant TLR signaling cascades. These possibilities will be addressed in upcoming studies. It also will be important to determine whether TLR9 plays a similar role in the regulation of human autoimmunity, because a better understanding of the regulatory activity of the individual TLRs is likely to have implications for the optimal design of TLR-based therapeutics.

Acknowledgments

We thank Drs. Harinder Singh and Mark Shlomchik for helpful discussions. Furthermore, we thank Tara Robidoux and Purvi Mande for technical help.

Disclosures

The authors have no financial conflicts of interest.

References

1. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
2. Christensen, S. R., J. Shupe, K. Nickerson, M. Kashgarian, R. A. Flavell, and M. J. Shlomchik. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417–428.
3. Pisitkun, P., J. A. Deane, M. J. Difilippantonio, T. Tarasenko, A. B. Satterthwaite, and S. Bolland. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 312: 1669–1672.
4. Subramanian, S., K. Tus, Q. Z. Li, A. Wang, X. H. Tian, J. Zhou, C. Liang, G. Bartov, L. D. McDaniel, X. J. Zhou, et al. 2006. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc. Natl. Acad. Sci. USA* 103: 9970–9975.
5. Lartigue, A., P. Courville, I. Auquit, A. François, C. Arnoult, F. Tron, D. Gilbert, and P. Musette. 2006. Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus. *J. Immunol.* 177: 1349–1354.
6. Nickerson, K. M., S. R. Christensen, J. Shupe, M. Kashgarian, D. Kim, K. Elkon, and M. J. Shlomchik. 2010. TLR9 regulates TLR7- and MyD88-dependent autoantibody production and disease in a murine model of lupus. *J. Immunol.* 184: 1840–1848.
7. Yu, P., U. Wellmann, S. Kunder, L. Quintanilla-Martinez, L. Jennen, N. Dear, K. Amann, S. Bauer, T. H. Winkler, and H. Wagner. 2006. Toll-like receptor 9-independent aggravation of glomerulonephritis in a novel model of SLE. *Int. Immunol.* 18: 1211–1219.
8. Santiago-Raber, M. L., I. Dunand-Sauthier, T. Wu, Q. Z. Li, S. Uematsu, S. Akira, W. Reith, C. Mohan, B. L. Kotzin, and S. Izui. 2010. Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice. *J. Autoimmun.* 34: 339–348.
9. Jackson, S. W., N. E. Scharping, N. S. Kolhatkar, S. Khim, M. A. Schwartz, Q. Z. Li, K. L. Hudkins, C. E. Alpers, D. Liggitt, and D. J. Rawlings. 2014. Opposing impact of B cell-intrinsic TLR7 and TLR9 signals on autoantibody repertoire and systemic inflammation. *J. Immunol.* 192: 4525–4532.
10. Chan, O. T., L. G. Hannum, A. M. Haberman, M. P. Madaio, and M. J. Shlomchik. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J. Exp. Med.* 189: 1639–1648.
11. Teichmann, L. L., D. Schenten, R. Medzhitov, M. Kashgarian, and M. J. Shlomchik. 2013. Signals via the adaptor MyD88 in B cells and DCs make distinct and synergistic contributions to immune activation and tissue damage in lupus. *Immunity* 38: 528–540.
12. Shlomchik, M. J., D. Zharhary, T. Saunders, S. A. Camper, and M. G. Weigert. 1993. A rheumatoid factor transgenic mouse model of autoantibody regulation. *Int. Immunol.* 5: 1329–1341.
13. Sweet, R. A., S. R. Christensen, M. L. Harris, J. Shupe, J. L. Sutherland, and M. J. Shlomchik. 2010. A new site-directed transgenic rheumatoid factor mouse model demonstrates extrafollicular class switch and plasmablast formation. *Autoimmunity* 43: 607–618.
14. Wolfowicz, C. B., P. Sakorafas, T. L. Rothstein, and A. Marshak-Rothstein. 1988. Oligoclonality of rheumatoid factors arising spontaneously in lpr/lpr mice. *Clin. Immunol. Immunopathol.* 46: 382–395.
15. Nündel, K., P. Busto, M. Debatis, and A. Marshak-Rothstein. 2013. The role of Bruton's tyrosine kinase in the development and BCR/TLR-dependent activation of AM14 rheumatoid factor B cells. *J. Leukoc. Biol.* 94: 865–875.
16. Viglianti, G. A., C. M. Lau, T. M. Hanley, B. A. Miko, M. J. Shlomchik, and A. Marshak-Rothstein. 2003. Activation of autoreactive B cells by CpG dsDNA. *Immunity* 19: 837–847.
17. Lau, C. M., C. Broughton, A. S. Tabor, S. Akira, R. A. Flavell, M. J. Mamula, S. R. Christensen, M. J. Shlomchik, G. A. Viglianti, I. R. Rifkin, and A. Marshak-Rothstein. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202: 1171–1177.
18. Uccellini, M. B., L. Busconi, N. M. Green, P. Busto, S. R. Christensen, M. J. Shlomchik, A. Marshak-Rothstein, and G. A. Viglianti. 2008. Autoreactive B cells discriminate CpG-rich and CpG-poor DNA and this response is modulated by IFN-alpha. *J. Immunol.* 181: 5875–5884.
19. Green, N. M., A. Laws, K. Kiefer, L. Busconi, Y. M. Kim, M. M. Brinkmann, E. H. Trail, K. Yasuda, S. R. Christensen, M. J. Shlomchik, et al. 2009. Murine B cell response to TLR7 ligands depends on an IFN-beta feedback loop. *J. Immunol.* 183: 1569–1576.
20. Prak, E. L., and M. Weigert. 1995. Light chain replacement: a new model for antibody gene rearrangement. *J. Exp. Med.* 182: 541–548.

21. Monestier, M., and K. E. Novick. 1996. Specificities and genetic characteristics of nucleosome-reactive antibodies from autoimmune mice. *Mol. Immunol.* 33: 89–99.
22. Monestier, M., K. E. Novick, and M. J. Losman. 1994. D-penicillamine- and quinidine-induced antinuclear antibodies in A.SW (H-2s) mice: similarities with autoantibodies in spontaneous and heavy metal-induced autoimmunity. *Eur. J. Immunol.* 24: 723–730.
23. Eilat, D., and R. Fischel. 1991. Recurrent utilization of genetic elements in V regions of antinucleic acid antibodies from autoimmune mice. *J. Immunol.* 147: 361–368.
24. Lenert, P., K. Yasuda, L. Busconi, P. Nelson, C. Fleenor, R. S. Ratnabalasuriar, P. L. Nagy, R. F. Ashman, I. R. Rifkin, and A. Marshak-Rothstein. 2009. DNA-like class R inhibitory oligonucleotides (INH-ODNs) preferentially block autoantigen-induced B-cell and dendritic cell activation in vitro and autoantibody production in lupus-prone MRL-Fas(lpr/lpr) mice in vivo. *Arthritis Res. Ther.* 11: R79.
25. Kanno, A., C. Yamamoto, M. Onji, R. Fukui, S. Saitoh, Y. Motoi, T. Shibata, F. Matsumoto, T. Muta, and K. Miyake. 2013. Essential role for Toll-like receptor 7 (TLR7)-unique cysteines in an intramolecular disulfide bond, proteolytic cleavage and RNA sensing. *Int. Immunol.* 25: 413–422.
26. Herlands, R. A., S. R. Christensen, R. A. Sweet, U. Hershberg, and M. J. Shlomchik. 2008. T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29: 249–260.
27. Calame, K. L., K. I. Lin, and C. Tunyaplin. 2003. Regulatory mechanisms that determine the development and function of plasma cells. *Annu. Rev. Immunol.* 21: 205–230.
28. Angelin-Duclos, C., G. Cattoretti, K. I. Lin, and K. Calame. 2000. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J. Immunol.* 165: 5462–5471.
29. Sciammas, R., A. L. Shaffer, J. H. Schatz, H. Zhao, L. M. Staudt, and H. Singh. 2006. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* 25: 225–236.
30. Shaffer, A. L., K. I. Lin, T. C. Kuo, X. Yu, E. M. Hurt, A. Rosenwald, J. M. Giltnane, L. Yang, H. Zhao, K. Calame, and L. M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17: 51–62.
31. Tarte, K., F. Zhan, J. De Vos, B. Klein, and J. Shaughnessy, Jr. 2003. Gene expression profiling of plasma cells and plasmablasts: toward a better understanding of the late stages of B-cell differentiation. *Blood* 102: 592–600.
32. Shaffer, A. L., M. Shapiro-Shelef, N. N. Iwakoshi, A. H. Lee, S. B. Qian, H. Zhao, X. Yu, L. Yang, B. K. Tan, A. Rosenwald, et al. 2004. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 21: 81–93.
33. Ochiai, K., M. Maienschein-Cline, G. Simonetti, J. Chen, R. Rosenthal, R. Brink, A. S. Chong, U. Klein, A. R. Dinner, H. Singh, and R. Sciammas. 2013. Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* 38: 918–929.
34. Feng, J., H. Wang, D. M. Shin, M. Masiuk, C. F. Qi, and H. C. Morse, III. 2011. IFN regulatory factor 8 restricts the size of the marginal zone and follicular B cell pools. *J. Immunol.* 186: 1458–1466.
35. Fukui, R., S. Saitoh, A. Kanno, M. Onji, T. Shibata, A. Ito, M. Onji, M. Matsumoto, S. Akira, N. Yoshida, and K. Miyake. 2011. Unc93B1 restricts systemic lethal inflammation by orchestrating Toll-like receptor 7 and 9 trafficking. *Immunity* 35: 69–81.
36. Boulé, M. W., C. Broughton, F. Mackay, S. Akira, A. Marshak-Rothstein, and I. R. Rifkin. 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J. Exp. Med.* 199: 1631–1640.
37. Scapini, P., A. Carletto, B. Nardelli, F. Calzetti, V. Roschke, F. Merigo, N. Tamassia, S. Pieropan, D. Biasi, A. Sbarbati, et al. 2005. Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: implications for inflammatory diseases. *Blood* 105: 830–837.
38. Christensen, S. R., M. Kashgarian, L. Alexopoulou, R. A. Flavell, S. Akira, and M. J. Shlomchik. 2005. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J. Exp. Med.* 202: 321–331.
39. Nickerson, K. M., J. L. Cullen, M. Kashgarian, and M. J. Shlomchik. 2013. Exacerbated autoimmunity in the absence of TLR9 in MRL.Fas(lpr) mice depends on Ifnar1. *J. Immunol.* 190: 3889–3894.
40. Desnues, B., A. B. Macedo, A. Roussel-Queval, J. Bonnardel, S. Henri, O. Demaria, and L. Alexopoulou. 2014. TLR8 on dendritic cells and TLR9 on B cells restrain TLR7-mediated spontaneous autoimmunity in C57BL/6 mice. *Proc. Natl. Acad. Sci. USA* 111: 1497–1502.
41. Nickerson, K. M., S. R. Christensen, J. L. Cullen, W. Meng, E. T. Luning Prak, and M. J. Shlomchik. 2013. TLR9 promotes tolerance by restricting survival of anergic anti-DNA B cells, yet is also required for their activation. *J. Immunol.* 190: 1447–1456.
42. Rui, L., J. I. Healy, J. Blasioli, and C. C. Goodnow. 2006. ERK signaling is a molecular switch integrating opposing inputs from B cell receptor and T cell cytokines to control TLR4-driven plasma cell differentiation. *J. Immunol.* 177: 5337–5346.
43. Rui, L., C. G. Vinuesa, J. Blasioli, and C. C. Goodnow. 2003. Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat. Immunol.* 4: 594–600.
44. Boeglin, E., C. R. Smulski, S. Brun, S. Milosevic, P. Schneider, and S. Fournel. 2011. Toll-like receptor agonists synergize with CD40L to induce either proliferation or plasma cell differentiation of mouse B cells. *PLoS ONE* 6: e25542.
45. Genestier, L., M. Taillardet, P. Mondiere, H. Gheit, C. Bella, and T. Defrance. 2007. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J. Immunol.* 178: 7779–7786.
46. Pathak, S., S. Ma, V. Shukla, and R. Lu. 2013. A role for IRF8 in B cell anergy. *J. Immunol.* 191: 6222–6230.
47. Stoehr, A. D., C. T. Schoen, M. M. Mertes, S. Eiglmeier, V. Holecska, A. K. Lorenz, T. Schommartz, A. L. Schoen, C. Hess, A. Winkler, et al. 2011. TLR9 in peritoneal B-1b cells is essential for production of protective self-reactive IgM to control Th17 cells and severe autoimmunity. *J. Immunol.* 187: 2953–2965.
48. Soni, C., E. B. Wong, P. P. Domeier, T. N. Khan, T. Satoh, S. Akira, and Z. S. Rahman. 2014. B cell-intrinsic TLR7 signaling is essential for the development of spontaneous germinal centers. *J. Immunol.* 193: 4400–4414.
49. Fukui, R., S. Saitoh, F. Matsumoto, H. Kozuka-Hata, M. Oyama, K. Tabeta, B. Beutler, and K. Miyake. 2009. Unc93B1 biases Toll-like receptor responses to nucleic acid in dendritic cells toward DNA- but against RNA-sensing. *J. Exp. Med.* 206: 1339–1350.