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A new site directed transgenic Rheumatoid Factor mouse model demonstrates extrafollicular class switch and plasmablast formation

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Abstract

The AM14 rheumatoid factor (RF) transgenic (Tg) mouse has been valuable for studying how self-reactive B cells are regulated beyond central tolerance, because they remain ignorant in normal mice. AM14 B cell activation can be studied on autoimmune-prone strains or by inducing activation with IgG2a anti-chromatin antibodies. Despite the utility of conventional Ig-Tg mice, site-directed Ig Tg (sd-Tg) mice provide a more physiological model for B cell responses, allowing class switch and somatic hypermutation. We report here the creation of an AM14 sd-Tg mouse and describe its phenotype on both normal and autoimmune-prone backgrounds. AM14 sd-Tg B cells develop normally but remain unactivated on the BALB/c background, even after significant aging. In contrast, on the autoimmune prone strain MRL/lpr, AM14 sd-Tg B cells become activated and secrete large amounts of IgG RF antibody into the serum. Class-switched antibody forming cells were found in the spleen and bone marrow. IgG RF plasmablasts were also observed in extrafollicular clusters in the spleens of aged AM14 sd-Tg MRL/lpr mice. Class switch and antibody secretion were observed additionally in AM14 sd-Tg BALB/c B cells activated in vivo using IgG2a anti-chromatin antibodies. Development of IgG autoantibodies is a hallmark of severe autoimmunity, and is related to pathogenesis. Using the AM14 sd-Tg, we now show that switched autoantibody-forming cells develop robustly outside germinal centers, further confirming the extrafollicular expression of AID. This model will allow more physiological studies of B cell biology in the future, including memory responses marked by class switch.

Keywords

plasmablast; isotype switch; lupus; extrafollicular response

INTRODUCTION

Dysfunctional activation of autoreactive B cells in systemic autoimmunity results in pathogenic autoantibodies with restricted specificities. Although several mouse models exist to study specific autoreactive B cells, these represent different aspects of the continuum of B cell tolerance and activation. Thus, multiple models are required in order to get a complete picture of how autoreactive B cells are regulated in health and disease.

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The initial models of autoreactive B cells focused on antigens that were not targets of natural autoimmune responses, but instead had the advantage that the nominal antigens were under genetic control and could be manipulated. Anti-hen egg lysozyme (HEL) B cells were anergic in Tg mice expressing soluble HEL and deleted in mice expressing membrane HEL [1,2]. Similarly, B cells specific for MHC Class I were found to delete and receptor edit in the presence of self-antigen [3].

Although useful for studying regulation of self-reactive B cells, these models did not utilize self-targets found in systemic autoimmune disease. Perhaps for this reason, when crossed onto autoimmune-prone genetic backgrounds, these B cells did not show clear evidence of activation and loss of tolerance, and hence these models were not as useful for studying how B cells are dysregulated in disease [4,5].

Only certain types of autoantigen are recurring targets in systemic autoimmune disease, with DNA-related, RNA-related and self-IgG autoantigens comprising most of the autoreactive specificities [6]. Hence, there must be special features of these autoantigens and the B cells that recognize them. Newer models were therefore constructed that focused on such targets. Weigert and colleagues created mice with the Tg anti-DNA B cell receptor, 3H9, cloned from MRL/lpr mice [7]. These B cells were tolerant on non-autoimmune prone backgrounds; no anti-DNA antibodies were secreted. Anti-ssDNA Abs of low affinity, generated upon pairing with a V_H 8 L chain, were anergic. Higher affinity anti-dsDNA B cells were found to undergo receptor editing during development [8]. A parallel Tg model of anti-DNA B cells, D42 was made from NZB/W mice [9]. D42 B cells delete, receptor edit, or become anergic to avoid autoreactivity on non-autoimmune prone strains. In contrast to earlier studies of B cell regulation, when these anti-DNA B cells were studied on autoimmune prone strains, they produced anti-DNA antibodies at least under some circumstances. Similar results were obtained in mice Tg for anti-Sm [10,11] as well as in ArsA1 mice which react with ssDNA [12,13].

As useful as all these models have been, there were some limitations. First, the authentic autoantigens could not be controlled and thus it was hard to define the role of autoantigen in tolerance and activation, which was a major advantage of the anti-HEL and anti-MHCI models. Second, these B cells were typically undergoing developmental tolerance events, though with some escape from these mechanisms, making it difficult to determine the precursor-product relationships between tolerized B cells in normal environments and activated B cells in autoimmune-prone settings. Furthermore, it is well known that there are non-tolerant (so-called “ignorant”) B cells that are self-reactive but present in the normal repertoire [14–16]. The anti-DNA and anti-Sm models mentioned above did not necessarily represent such B cells.

The AM14 V_H Tg mouse, which has RF B cells with a moderate affinity for IgG2a^a, allows study of autoreactive B cells on non-autoimmune prone strains which are not anergized, edited, or deleted but instead are ignorant even in the presence of antigen [17,18]. Moreover, the autoantigen, IgG2a^a can be controlled genetically, by exogenous administration, or by cell transfer. Similar to anti-DNA B cells and anti-Sm B cells, RF B cells when studied on autoimmune prone backgrounds become activated and secrete autoantibodies [19–21]. Most interestingly, AM14 B cells on the MRL/lpr background spontaneously differentiate into extrafollicular plasmablasts and mutate at this extrafollicular site [19]. We recently found that this extrafollicular plasmablast response can be induced in either normal or autoimmune-prone mice by the introduction of IgG2a anti-chromatin Abs, which presumably form immune complexes in vivo with ubiquitous endogenous chromatin [22]. Surprisingly, this response was found not to require T cells, although it was entirely dependent on MyD88 signaling, downstream of both TLR7 and TLR9 [23].

A number of the B cell tolerance models have been converted into sd-Tg versions. In these mice, the VDJ (and/or VJ) is inserted into the correct germline locus using homologous recombination in ES cells. In contrast to conventional Ig Tg mice that were used in earlier studies, the V gene loci of these latter sd-Tg mice are under control of normal promoter elements and can undergo normal isotype switching and somatic hypermutation. They can also, under some circumstances, be substrates for receptor editing in which the original rearrangement is replaced. These mice have been mainly useful in studying development and receptor editing [9,24–27], as well as for monitoring isotype switch during disease [28–31]. Thus far, sd-Tg mice have not allowed us to investigate the activation of self-reactive B cells with a disease-associated specificity without the complications of receptor editing and other central and peripheral tolerance mechanisms.

Here we report the successful construction, validation and characterization of the AM14 sd-Tg mouse. B cell development on BALB/c was normal in the mice with no significant spontaneous activation of RF B cells. Thus, in the context of more normal development and IgM plus IgD expression on naïve cells, AM14 RF cells are clonally ignorant, as originally inferred from the conventional Tg mice. On MRL/lpr, as in the AM14 conventional Tg, there was spontaneous activation. However, an important difference was that we were able to observe early extrafollicular isotype switching as well as large amounts of isotype-switched serum RF Ab. This provides a significant clue to the origin of IgG autoantibodies typically associated with severe autoimmunity and pathogenesis. This also supported our earlier observation of mutation at this site as both processes require AID [32]; the presence of switch establishes the presence of functionally active AID. This model will prove useful for the study of both activation and the development of memory in autoreactive B cells, and is unique in that it is a sd-Tg model that does not appear to undergo overt negative regulation during development, making it suitable for the study of activation both *in vivo* and *in vitro* [33,34].

METHODS

Creation of sd-Tg Mice

The targeting construct was created from the original AM14 conventional Tg, except that a 1.2 kb region of homology to the germline D_H region was added at the 5' terminus and a thymidine kinase cassette was added at the 3' terminus (Figure 1A). The presence of upstream V_H and D_H gene segments in site-directed BCR transgenes renders them particularly susceptible to RAG-mediated V_H replacement during B cell development using an internal heptamer in the 3' coding region of the rearranged transgene [35] or the RSS segments of other downstream J segments. To improve stability of the transgene, we mutated the J_H4 heptamer from 5'-CACAATA (on the anti-sense strand) to 5'-TGCAATA and introduced a silent mutation to mutate the internal V_H heptamer from 5'-CACAATA to 5'-CTCAATA. Mutagenesis of RSS heptamers was performed with the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. ES cells were transfected and blastocysts injected by the Animal Genomics Service of the Yale Cancer Center, using their established protocols. PCR screening of transfected ES cell clones was performed with primers within the germline D_H region (5' ATC TAC ATA GCT AGA GAG CTA GAG G 3') and the neomycin resistance cassette (5' GCA TCG CAT TGT CTG AGT AGG TGT CA 3'). Southern blotting of EcoRI digests of genomic ES cell DNA was performed as described [17] with radiolabeled J_H4 probe. The 1.6 kb HindIII-EcoRI fragment from the targeting construct was labeled with ³²P-dCTP (Amersham), using the Random-Primed DNA Labeling Kit (Roche) according to the manufacturer's instructions. Chimeric pups derived from blastocyst injection of ES cells were first bred to Fas-intact MRL/Mp mice to confirm germline transmission of the sd-Tg. AM14 sd-Tg mice were then backcrossed for 8 generations to Fas-deficient MRL/Mp^{lpr/lpr} mice for analysis of

seroconversion. AM14 sd-Tg mice were also backcrossed at least 10 generations to the BALB/c strain. All mice were genotyped using PCR as previously described [17]. All mouse experimentation was approved by the Yale Institutional Animal Care and Use Committee.

Flow Cytometry

Splenocytes and peritoneal lavage were prepared as previously described [17]. Bone marrow was flushed from tibia and femur using RPMI with 2.5% Fetalplex™ (Gemini). RBC lysis was performed as above.

Antibodies were made on site as previously described [17] or purchased from vendors as indicated. The following staining reagents were used for these experiments: 4-44-biotin [17], PNA FITC (Vector), anti-CD95 PE (Jo2, Pharmingen), anti-CD19 Pacific Blue (1D3.2), anti-CD138 PE (281-2, Pharmingen), anti-CD44 Alexa 647 (IM7), anti-CD21/35 FITC (7G6, Pharmingen), AA4.1 PE (BD Biosciences), anti-CD23 Alexa 680 (B3B4), goat anti-mouse IgM PE Cy7 (Southern Biotech), anti-IgM Alexa 488 (RS3.1), goat anti-mouse IgG2a PE Cy7 (Southern Biotech), anti-CD23 FITC (B3B4, Pharmingen), anti-CD5 PE (53-7.5, Ebioscience), anti-IgM Alexa 647 (RS3.1), anti-CD11b APC Cy7 (M1/70, Biolegend), SA Alexa 488 (Molecular Probes), SA Alexa 647 (Molecular Probes), SA APC Alexa 750 (Invitrogen).

Samples were analyzed on a LSRII (BD). Data was analyzed with FlowJo software.

ELISpot Assay

ELISpot assay was performed and analyzed as previously described [18,36].

Immunofluorescence Histology

Sections were prepared, stained with antibodies and images were captured as previously described [22]. Abs used were described above as well as anti-CD4 Alexa 647 (GK1.5), anti-IgD Alexa 488 or 647 (AMS15), anti-CD19 Alexa 647 (1D3.2), anti-IgM^a Alexa 488 (RS3.1), anti-IgG2a^a 647 (20.8.3).

ELISA

ELISA Assay was performed and analyzed as previously described [17].

Ascites Preparation and Immunizations

PL2-3 hybridoma [37] was grown in RPMI with 10% fetal plex. 10 million cells were injected into *Rag*^{-/-} Balb/c mice i.p. Ascites fluid was collected approximately 1 week later. Lipid was separated from ascites by centrifugation at 4600 × g and removed. Ascites was sterile filtered using MILLEX-HV 33mm syringe filters with a hydrophilic PVDF membrane and 0.45 mm pores (Millipore). IgG2a concentration was determined by ELISA. Ascites was diluted with sterile PBS prior to injection. Mice were injected with diluted ascites containing 0.5 mg PL2-3 i.p. on day 0, 2, and 4, and sacrificed on day 6 for analysis. Immune complexes of TNP₁₁-KLH and Hy1.2 in alum were prepared and injected as previously described [38].

RESULTS

Generation of AM14 sd-Tg Mice

We created an AM14 sd-Tg mouse, in which the rearranged VDJ gene segments of the AM14 heavy chain were inserted into the J_H region germline immunoglobulin heavy chain (IgH) locus. To generate the mice, we transfected the AM14 sd-Tg targeting construct into

murine embryonic stem (ES) cells and screened them by PCR for homologous recombination of the transgene into the germline IgH locus (Figure 1A). Of 223 ES clones screened, three were PCR-positive for correct upstream integration. Genomic DNA from these three clones was digested with EcoRI and hybridized to a J_H4 probe by Southern blot. A 6.2 kb band corresponding to the native germline IgH locus was seen in all three clones, and a 9.4 kb band corresponding to the correct integration of the transgene was observed unambiguously in one clone (Figure 1B). ES cell clone 80 was then injected into blastocysts, which were transferred into pseudopregnant female mice. Breeding of chimeric offspring resulted in germline transmission of the AM14 sd-Tg transgene.

Development of AM14 sd-Tg B Cells

AM14 sd-Tg mice backcrossed at least 10 times to the BALB/c strain were examined by flow cytometry to assess B cell development. AM14 RF B cells were identified using the 4-44 idiotype antibody, which is specific only for those B cells which express the BCR heavy chain in combination with either one of two V_H 8 family light chains joined to J_H 4 or J_H 5 [17]. These light chains in combination with the AM14 heavy chain confer RF specificity [17,19,20]. Bone marrow was analyzed from BALB/c mice and AM14 sd-Tg BALB/c mice (Figure 2). As expected, due to the expression of a preformed VDJ and the requirement of light chain expression for 4-44 detection, there was not a significant population of 4-44⁺ pro-B cells or pre-B cells (AA4.1⁺ IgM⁻). Within the CD19⁺ 4-44⁻ population, pro- and pre-B cells were found in both AM14 sd-Tg BALB/c mice and BALB/c mice. A smaller proportion of the bone marrow B cells were pro- or pre-B cells in AM14 sd-Tg BALB/c mice compared with BALB/c mice, most likely due to premature expression of the preformed heavy chain. There was a commensurate increase in both immature (AA4.1⁺ IgM⁺) and mature (AA4.1⁻ IgM⁺) B cells among 4-44⁺ B cells in the knock-in compared to BALB/c. The 4-44⁺ CD19⁺ population in AM14 sd-Tg BALB/c mice contained an intermediate frequency of immature and mature B cells. Thus, although all stages of development are represented, development of AM14 sd-Tg B cells was accelerated in the bone marrow.

In the spleen, we examined T1 (AA4.1⁺ CD23⁻ CD21⁻ IgM⁺), T2 (AA4.1⁺ CD23⁺ CD21^{lo} IgM^{hi}), T3 (AA4.1⁺ CD23⁺ CD21^{lo} IgM^{int}), follicular (AA4.1⁻ CD23⁺ CD21^{lo} IgM⁺), marginal zone (AA4.1⁻ CD23⁻ CD21^{hi} IgM^{hi}), and B1 (AA4.1⁻ CD23⁻ CD21^{int} IgM^{int}) B cells. 4-44⁺ B cells were found in all analyzed populations with a significantly lower representation in the T1 population and the marginal zone population (Figure 3B). In the peritoneal cavity, the ratio of B1a to B1b cells was 1:2 in the knock-in mice, in both 4-44 positive and negative B cells. The ratio was reversed in BALB/c mice (Figure 4). The AM14 RF specificity or H chain sd-Tg does appear to skew development somewhat in favor of FO over MZ B cells in spleen and B1b over B1a cells in the peritoneum; effects of restricted specificity Tg and sd-Tg on selection or maintenance in B cell subsets or compartments are well-known [39–44]. Overall, detection of all expected populations throughout B cell development, with the specific alterations described above, provides background for further study in this model.

AM14 sd-Tg B Cells Are Not Activated in a Non-autoimmune Strain

AM14 sd-Tg B cells have potential self-reactivity since, for technical reasons, the mice were generated on the 129/Sv (IgH^a) background. It was important to determine whether AM14 sd-Tg B cells are spontaneously activated in a non-autoimmune prone environment. Young adult AM14 sd-Tg mice were found to lack detectable 4-44⁺ germinal center (GC) cells (PNA⁺ Fas⁺), 4-44⁺ plasmablasts (Syndecan⁺ CD44⁺), and 4-44⁺ IgG2a⁺ class switched cells (Figure 5). In contrast, GCs, plasmablasts and switched cells were found, albeit at very low levels, in the CD19⁺ 4-44⁻ population of both AM14 sd-Tg and BALB/c mice. Hence,

much like their conventional Tg counterparts [38], RF B cells in young AM14 sd-Tg mice have an “ignorant” phenotype in the spleen. Lack of activation on non-autoimmune strains provides an important baseline for the study of activation in autoimmune-prone genetic backgrounds or upon introduction of autoantigen.

AM14 conventional Tg mice on the BALB/c background accumulated 4-44⁺ GCs, but not antibody forming cells, after substantial aging [38]. We sought to determine whether AM14 sd-Tg B cells were regulated in a similar way. AM14 sd-Tg BALB/c mice, aged at least 30 weeks, were sacrificed and tested for antibody forming cells or germinal centers. 4-44⁺ antibody forming cells were found in spleens, lymph nodes, and Peyer’s patches of aged AM14 sd-Tg BALB/c mice (Figure 6A); however, compared with aged AM14 Tg MRL/lpr mice, which have upwards of 500 antibody forming cells per million splenocytes (see Figure 7), these levels, though detectable, were still very low.

The frequency of 4-44⁺ GC cells in the spleens of aged AM14 sd-Tg BALB/c mice was relatively low; however, it was significantly higher than young unimmunized controls (Figure 6B). Additionally, the frequency of 4-44⁺ GC phenotype cells in the mesenteric lymph nodes of aged mice was also significantly higher than young controls. In contrast, a vigorous 4-44⁺ GC response developed after immunizing young adult AM14 sd-Tg BALB/c mice with IgG2a^a anti-hapten-carrier conjugate immune complexes in alum, but not after immunizing with alum alone (Figure 6B). Thus, while spontaneous RF (4-44⁺) GCs were not abundant over time, they were clearly found in a subset of AM14 sd-Tg BALB/c mice, indicating that they breached a tolerance checkpoint as they aged.

Quantitative assessment of GCs by flow cytometry is informative; however, it does not evaluate lymphoid architecture, an important component of authentic GC identification. Immunofluorescence analysis of sections of spleens of alum-immunized young AM14 sd-Tg BALB/c mice revealed a lack of GCs (Figure 6C). In contrast, sections from mice immunized with hapten-based immune complexes as above showed robust 4-44⁺ GCs (Figure 6D,E). Approximately half of aged AM14 sd-Tg BALB/c mice did not develop spontaneous 4-44⁺ GCs that were identifiable by immunohistology (Figure 6F), even though there were small numbers of PNA⁺ CD95⁺ 4-44⁺ cells by flow cytometric analysis (Figure 6B). In one spleen and four Peyer’s patches, GCs were found that had scattered 4-44⁺ cells, but were not dominated by them (Figure 6F). In mesenteric lymph nodes a few GCs that were dominated by 4-44⁺ cells were found; however, these GCs were noticeably smaller compared with those elicited by immune complex immunization (Figure 6G, H, and data not shown). Thus our histological data supports the flow cytometry data in general; in aged AM14 sd-Tg BALB/c mice 4-44⁺ B cells can form germinal centers, but only in some mice and at relatively low levels. The small numbers of GC-phenotype cells identified by flow cytometry in animals that did not have GCs detectable by histology might be explained by the lower sensitivity of histology, which is limited by the number of sections that can be reasonably surveyed, or it could reflect a small number of cells that adopt a GC-phenotype without actually residing in bona fide GCs.

AM14 sd-Tg B Cells Are Activated in an Autoimmune Prone Strain

AM14 Tg mice on the MRL/lpr background have spontaneously activated 4-44⁺ B cells, a subset of which develops into plasmablasts [19,21]. To test whether 4-44⁺ sd-Tg B cells could be activated in a similar way, AM14 sd-Tg mice were crossed at least 8 times onto the MRL/lpr strain. These mice were aged and serum was tested for amounts of 4-44⁺ antibody of both IgM and switched isotypes (Figure 7A–C). 4-44⁺ IgM antibody was found in the serum, however, 4-44⁺ IgG2b was found at a much higher level as early as 11–13 weeks of age. 4-44⁺ IgA was also detected in a subset of the sera (Figure 7C).

Bone marrow and splenocytes were also tested directly *ex vivo* by ELISpot. Class switched 4-44⁺ antibody forming cells were found in both bone marrow and spleen with frequency increasing as mice aged (Figure 7D,E). Immunofluorescence analysis of splenic sections confirmed the presence of extrafollicular plasmablasts that expressed IgG2a (Figure 7F,G).

Thus, AM14 sd-Tg mice demonstrate age-dependent spontaneous extrafollicular activation of 4-44⁺ RF B cells on an autoimmune prone background. Most notably, there is robust class switching of these cells, a feature which was not apparent in prior models. The finding of plasmablasts that expressed isotype switched 4-44⁺ Ab confirms unequivocally that these cells have expressed AID; this is commensurate with our prior conclusions on conventional Tg mice that somatic hypermutation was taking place in similar cells.

AM14 sd-Tg B Cells Are Activated in a Non-autoimmune Strain with Anti-chromatin Antibodies

We previously found that extrafollicular activation of AM14 Tg B cells can be induced on both normal and autoimmune prone strains using PL2-3, an IgG2a^a anti-chromatin antibody [22]. We wanted to test whether this held true for AM14 sd-Tg B cells as it would be a useful model for studying dynamics of RF B cell activation over time. In particular, we were interested in the ability of this stimulus to elicit Ig class switch, which could not be determined previously. PL2-3 was delivered to AM14 sd-Tg BALB/c mice for 6 days. At this time point, plasmablasts had developed and a subset was class switched as detected by ELISpot assay (Figure 8A,B).

Histologically, no plasmablasts were seen in naïve AM14 sd-Tg BALB/c mice. In such naïve mice, most 4-44⁺ cells were within the follicle and IgD⁺ (Figure 8C,D). In PL2-3 treated mice, plasmablasts were identified as darkly staining cells with ample cytoplasm located at the T-B border as well as extrafollicularly at the marginal sinus bridging channels (Figure 8E–H, yellow arrows). Any GCs found in these mice were 4-44 negative (data not shown).

DISCUSSION

Studies of murine Tg B cells, including AM14 Tg B cells, have led to greater understanding of mechanisms controlling self-reactive B cells and of spontaneous activation in autoimmune prone environments. However, conventional Tg B cells have limitations. It is important to assess all basic functions of B cell biology including class switch and mutation, both of which are not possible or optimal in conventional Tg mice. In contrast, these processes can be studied with site directed B cell Tg mice. For example, it has long been known that autoantibodies in murine models of systemic autoimmunity as well as in human autoimmune diseases including SLE and RA are often isotype-switched, somatically mutated, and affinity matured. Hence, switch and physiological mutation is likely to be very important to the propagation of autoimmune disease, but is not currently well-understood in terms of both impact and origin. To address these questions, the AM14 sd-Tg mouse was created, and we have now studied AM14 sd-Tg B cells *in vivo* in normal settings, in spontaneous autoimmunity, and after induced activation using IgG2a anti-chromatin.

AM14 sd-Tg B cells develop normally and appear ignorant with minimal activation even at advanced ages. We did note that there were lower percentages of 4-44⁺ T1 and MZ B cells compared with the 4-44⁺ population (Figure 3). There were also fewer B1a B cells in the AM14 sd-Tg mouse compared to BALB/c (Figure 4). Aside from these few differences, B cell development was normal in AM14 sd-Tg mice. GCs and plasmablasts were absent in young AM14 sd-Tg BALB/c mice as detected by flow cytometry, ELISpot, and histology (Figures 5 and 6). As AM14 sd-Tg BALB/c mice became quite old, some plasmablasts and

GC were detectable (Figure 6). However, in contrast to the robust response observed upon induced GC activation, the plasmablasts and GCs observed in aged AM14 sd-tg BALB/c mice appeared only sporadically, and at low frequency.

Spontaneous isotype switch of AM14 B cells in MRL/lpr mice was confirmed by multiple means (Figure 7). This is important for understanding the origins of IgG autoantibodies, which are associated with pathogenesis. It also is definitive evidence that AID is turned on in these mice. GCs during autoimmunity, demonstrating that isotype-switched autoantibodies do not require GCs for development, in contrast to the contention that GCs are the exclusive or dominant source for such autoantibodies [45]. This finding is consistent with earlier work, showing that isotype switch can proceed during a normal extrafollicular response [46–48].

Moreover, we could induce the 4-44⁺ plasmablast response by administration of the anti-chromatin IgG2a^a antibody, PL2-3, to nonautoimmune prone AM14 sd-Tg BALB/c mice. Within one week of PL2-3 injection, 4-44⁺ IgM and IgG2a splenic ELISpots were detected. Plasmablasts were found outside the follicle, not in GCs (Figure 8). Thus, induced AM14 sd-Tg B cell activation recapitulates that observed in the spontaneous response, including class switch. This shows that AID is turned on after stimulation, in addition to spontaneously upon the onset of autoimmunity, and that this response proceeds quickly, and again in the absence of obvious GCs.

Accumulating evidence from a variety of systems suggests that the short-lived, extrafollicular, plasmablast pathway may be important in the generation of several types of autoantibodies. This evidence includes: 1) the histologic identification of the extrafollicular location of the RF response in non-Tg MRL/lpr mice [49,50] and of the anti-DNA response in 3H9 Tg MRL/lpr mice [51,52]; 2) the short-lived nature of autoantibody responses in mouse models and in humans with autoimmune disease, either as measured directly [21,53], or as inferred by the relatively rapid loss of serum titers of certain types of autoantibody after B cell depletion therapy [54–56]; 3) the T-independent nature of at least some types of autoantibody responses [23,28,57]; and 4) the strict TLR-dependence of these responses [23,57,58], which is more consistent with an extrafollicular than a GC origin [59,60]. Notably, BCR and TLR co-stimulation also leads to an exclusively extrafollicular plasmablast response in a different model antigen system [61].

Availability of a class switch-capable RF B-cell mouse model increases the scope of our ability to interrogate potential mechanisms involved in the initiation and propagation of autoimmune disease. With these new AM14 sd-Tg mice, we have here confirmed our previous findings from the conventional Tg AM14 mice. Continued investigation of this more physiologically relevant model will yield important and novel insights into the disease process. For example, by virtue of our ability to track AM14 class-switched B-cells, we will now be able to determine whether long term (*e.g.* memory B-cell or long-lived plasma cell) autoreactive B cell responses exist, and investigate their characteristics. These studies have begun, and should ultimately help us address the important question of whether memory cell development is related to the maintenance of autoimmunity.

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Figure 1.

Construction of AM14 sd-Tg mice. A) Targeting construct, germline IgH locus, and final integrated transgene are shown. Neo = neomycin resistance cassette; AM14 = AM14 rearranged AM14 VDJ₃; DQ52 = 3 germline D_H gene segment; J₁₋₃ and J₄ = germline J_H gene segments; E_μ = IgM enhancer; C_μ = IgM C region exons; TK = thymidine kinase cassette. Vertical lines indicate restriction sites: EcoRI (E), HindIII (H), SalI (S), and XhoI (X). Dashed lines indicate regions of homologous recombination with germline IgH locus. White arrows indicate site-directed mutagenesis of RSS heptamers. Black arrows indicate PCR primers to screen for correct upstream integration. Striped bar indicates J4 probe used in Southern blot. B) Southern blot of EcoRI digest of genomic DNA from 3 ES cell clones which were PCR positive for correct upstream integration. Germline IgH band is 6.2 kb, correct integration of transgene produces 9.4 kb band, indicated by asterisk in ES #80.

Figure 2.

Bone marrow B cell development is accelerated in AM14 sd-Tg BALB/c mice. AM14 sd-Tg BALB/c mice (n=4) and BALB/cJ mice from Jackson Laboratories (n=3) were sacrificed and bone marrow cells were stained as indicated. Representative plots are shown in (A). Cells were classified as mature (AA4.1⁻IgM⁺), immature (AA4.1⁺IgM⁺) or a combination of pro B cells and pre B cells (AA4.1⁺IgM⁻). (B) Quantitation for all mice is shown. Error bars are SEM.

Figure 3.

Splenic B cell development in AM14 sd-Tg BALB/c mice. Splenocytes were isolated from AM14 sd-Tg BALB/c mice (n=4) or Jackson BALB/cJ mice (n=3) and stained for flow cytometry as indicated. (A) Representative plots from one AM14 sd-Tg Balb/c mouse are. CD19⁺ cells were gated as 4-44⁺ or 4-44⁻ (middle left panel). Then both populations were gated on IgM⁺ cells (>97% were positive, not shown). These gated populations were then graphed (upper and lower left plots, indicated by arrows) with CD23 on the x- and AA4.1 on the y-axis. The cells in each of the four quadrants were then plotted (set of four plots on the right, labeled by quadrant within the plot) with respect to IgM (x) and CD21 (y) expression, to reveal relevant B cell subsets as follows: T1 (AA4.1⁺ CD23⁻ CD21⁻ IgM⁺), T2 (AA4.1⁺

CD23⁺ CD21^{lo} IgM^{hi}), T3 (AA4.1⁺ CD23⁺ CD21^{lo} IgM^{int}), follicular (FO: AA4.1⁻ CD23⁺ CD21^{lo} IgM⁺), marginal zone (MZ: AA4.1⁻ CD23⁻ CD21^{hi} IgM^{hi}), and B1 (AA4.1⁻ CD23⁻ CD21^{int} IgM^{int}). Populations of CD19⁺ cells from BALB/c mice were similar to the CD19⁺ 4-44⁻ population in the AM14 sd-TG BALB/c mice and are not shown. (B) Frequencies of cells as a percentage of IgM⁺ cells in populations as delineated in A from all mice are shown. Error bars are SEM. **p<0.01 by unpaired t-test compared with BALB/c.

Figure 4.

B1 B cell development in AM14 sd-Tg mice. AM14 sd-Tg BALB/c mice (n=3) or BALB/c mice (n=2) were sacrificed and peritoneal lavage was isolated ex vivo. Peritoneal cells were stained with the markers indicated and analyzed by flow cytometry. (A) Representative plots show the gating strategy for identifying B1a (CD19⁺ IgM^{hi} CD11b⁺ CD5⁺) or B1b cells (CD19⁺ IgM^{hi} CD11b⁺ CD5⁻). (B) The percent of B1 cells that was B1a or B1b is shown for the cell populations as indicated. Error bars are SEM.

Figure 5.

AM14 sd-Tg B cells are not activated on a normal strain background. Splenocytes from 7 wk old AM14 sd-Tg BALB/c mice (n=4) or Jackson BALB/cJ mice (n=3) were isolated and stained. GC B cells were identified as PNA⁺Fas⁺ for CD19⁺ 4-44⁺ or 4-44⁻ cells. Plasmablasts were identified as Syndecan⁺CD44⁺ for CD19⁺ 4-44⁺ or 4-44⁻ cells. Class switch was determined by IgG2a expression on CD19⁺ IgM⁻ 4-44⁺ or 4-44⁻ cells. Representative plots are shown. Percentages are of the CD19⁺ 4-44⁺ or 4-44⁻ total population. Cells not staining for IgG2a are a mixture of cells expressing other isotypes but most likely are predominantly IgM^{dull} non-switched cells.

Figure 6.

AM14 sd-Tg BALB/c mice do not develop uncontrolled activation over time. (A) Antibody forming cell frequency was determined in aged AM14 sd-Tg BALB/c mice or BALB/c controls using ELISpot assay. (B) AM14 sd-Tg BALB/c mice of the indicated ages were left untreated (-) or immunized with alum (A) or anti-TNP and TNP₁₁-KLH immune complexes in alum (ICA). At day 10 after immunization in these latter mice, or at the indicated age in non-immunized mice, splenocytes were isolated and stained for flow cytometry. After gating on $4-44^+$ cells, GC B cells were identified using PNA and CD95 (see Figure 5 for gating). * $p < 0.05$, ** $p < 0.005$ by Mann Whitney test compared with young controls. (C–G) Sections of spleen stained for GCs. $4-44^+$ GCs were not found in a 6 week old AM14 sd-Tg BALB/c mouse immunized with alum alone (C) but were found in a mouse immunized with immune complexes in alum (D,E). (F) $4-44^+$ GCs, as illustrated in panels G and H, were quantitated in sections from 30+ week old AM14 sd-Tg BALB/c mice. GCs were determined to be mainly comprised of $4-44^+$ cells (black symbols) or containing scattered $4-44^+$ cells (gray symbols). Although not abundant, some $4-44^+$ GCs were found in aged AM14 sd-Tg BALB/c mLN.

Figure 7.

AM14 sd-Tg MRL/lpr mice develop age-dependent spontaneous class-switched antibody and plasmablasts. (A–C) Serum from mice aged 8–23+ weeks was tested for 4-44⁺ IgM (A), IgG2b (B), or IgA (C) antibodies. (D–E) Cells were tested directly ex vivo for 4-44⁺ Ab secretion of the indicated isotypes in the bone marrow (D) or spleen (E). **p*<0.05, ***p*<0.005, ****p*<0.0005 by Mann Whitney test compared with youngest mice in cohort. (F–G) Splenic sections from an AM14 MRL/lpr mouse aged 25 weeks were stained as indicated in the panels. Clusters of class switched 4-44⁺ extrafollicular plasmablasts were observed, as indicated by the purple colored (red plus blue stained) cells in panel G that did not stain green for anti-IgM in panels F and G.

Figure 8.

PL2-3 activates AM14 sd-Tg BALB/c B cells to form plasmablasts. (A, B) Six days after initiation of treatment with PL2-3 (IgG2a anti-chromatin), splenocytes were harvested and analyzed for 4-44⁺ ELISpots directly ex vivo. Shown are IgM (A) or class switched spot frequencies (IgG2a and IgG2b compiled, B). C–H) Splenic sections from naive mice (C,D) or mice treated with PL2-3 (E–H) were stained as indicated in the panels. Plasmablasts are indicated by yellow arrows in PL2-3 treated mice as darkly stained red cells located at the T-B border (E, F) or the T zone-red pulp border (G, H), whereas most 4-44⁺ cells in naive mice are IgD⁺ and fall within the follicle.