

# Virus-induced Maturation and Activation of Autoreactive Memory B Cells

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## Abstract

We have examined B cell populations that participate in distinct phases of the immune response to the influenza virus A/PR/8/34 hemagglutinin (HA) for their susceptibility to negative selection in mice that express the HA as a neo-self-antigen (HA104 mice). We demonstrated previously that specificity for the neo-self-HA causes a population of immunoglobulin G antibody-secreting cells, which dominate the primary response to virus immunization in BALB/c mice, to be negatively selected in HA104 mice. We find here that in contrast to these primary response B cells, HA-specific memory response B cells developed equivalently in HA104 and nontransgenic (BALB/c) mice. Indeed, there was no indication that HA-specific B cells were negatively selected during memory formation in influenza virus-immunized HA104 mice, even though the neo-self-HA can be recognized by memory B cells. Furthermore, HA-specific autoantibodies were induced in the absence of virus immunization by mating HA104 mice with mice transgenic for a CD4<sup>+</sup> HA-specific T cell receptor. These findings indicate that specificity for a self-antigen does not prevent the maturation of autoreactive B cells in the germinal center pathway. Rather, the availability of CD4<sup>+</sup> T cell help may play a crucial role in regulating autoantibody responses to the HA in HA104 mice.

Key words: tolerance • autoantibodies • molecular mimicry • germinal center • CD4<sup>+</sup> T cells

## Introduction

After receptor engagement by antigen, B cells can undergo a variety of differentiative fates. In response to foreign antigens, mature B cells can become activated and differentiate into antibody-secreting cells (ASCs)<sup>1</sup> and/or memory B cells. These differentiated populations have distinct functional potentials for postactivation events such as antibody production and somatic hypermutation, and appear to have developed along different pathways as indicated by their discrete anatomical locations and their differential expression of variable region clonotypes (1–3). In some cases, the pathway along which responding B cells develop can be influenced by signals mediated via the B cell receptor (BCR [4]), yet there is also evidence that the preimmune repertoire contains separate populations of B cells that are predisposed to certain differentiative fates (5–11). The capacity for B cell populations to differentiate into either ASCs or

memory B cells may play an important role in providing effective humoral immunity to complex infectious agents such as viruses.

In addition to undergoing distinct differentiative fates in response to foreign antigens, B cells also appear to be heterogeneous in their responses to self-antigens. In recent years, studies using Ig transgenic (tg) mice have firmly established that although specificity of the BCR for a self-antigen can cause self-reactive B cells to be negatively selected from the primary B cell repertoire (12, 13), this is not absolute, as some B cells whose BCRs can react with a self-antigen can persist in the peripheral lymphoid organs of nonautoimmune mice (14–16). Moreover, although elimination of autoreactive B cells from the primary repertoire has been analyzed in considerable detail, the processes that govern the fate of autoreactive B cells during memory formation remain obscure. As it is well established that germinal center formation depends on cognate interactions between antigen-specific B cells and CD4<sup>+</sup> T cells, negative selection of autoreactive CD4<sup>+</sup> T cells could limit autoreactive memory B cell formation (17–19). However, self-reactive B cells activated by a cross-reactive antigen on a microbe would potentially have a source of linked CD4<sup>+</sup> T cell help (20, 21). Furthermore, B cells responding to foreign

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<sup>1</sup>Abbreviations used in this paper: AP, alkaline phosphatase; ASC, antibody-secreting cell; BCR, B cell receptor; ELISPOT, enzyme-linked immunospot; HA, hemagglutinin; HAU, hemagglutinating unit(s); HI, hemagglutination inhibition; HRP, horseradish peroxidase; PNA, peanut agglutinin; PR8, influenza virus A/PR/8/34; S1, site 1; tg, transgenic.

antigens, through somatic hypermutation, could acquire reactivity with self-antigens (22–24). Because of these possibilities, the germinal center has been proposed as a second window of BCR-mediated tolerance induction (19, 25). However, there is only indirect evidence that BCR specificity for self-antigens leads to negative selection of autoreactive B cells during memory formation (26–31). Indeed, that autoreactive B cells can undergo clonal expansion and somatic mutation in certain B cell-mediated autoimmune disorders strongly suggests that autoreactive B cells can mature through the germinal center pathway (23, 32, 33).

We have been examining how specificity for foreign and self-antigens affects B cell differentiation by using the influenza virus A/PR/8/34 (PR8) hemagglutinin (HA) as a well-characterized antigen (34, 35). We demonstrated previously that HA-specific B cells induced in response to PR8 virus immunization in BALB/c mice are composed of distinct populations that respond differently to antigenic stimulation by the HA. A major population of IgG-secreting ASCs dominates the primary response 5 d after immunization, and includes B cells that use a BCR clonotype (termed C12) that utilizes characteristic H chain variable (VH) and  $\kappa$  chain variable (Vk) gene segments (5, 36). Several lines of evidence indicate that C12 B cells predominantly undergo terminal differentiation into IgG ASCs in response to virus immunization and that they do not participate in germinal center reactions in BALB/c mice (37). However, other clonotypes (including a clonotype termed C4) have been identified that are typically isolated among IgM-secreting hybridomas 5 d after immunization, and as somatically mutated and IgG-secreting hybridomas after secondary challenge (38). Further evidence of the distinct phenotypic potentials exhibited by these two populations of HA-specific B cells emerged with the observation that C12 B cells are negatively selected in tg mice that express the HA as a neo-self-antigen (HA104 mice), whereas HA-specific IgM-secreting B cells (including C4 B cells) were readily isolated after primary immunization (5). The finding that C4 B cells evaded negative selection from the primary B cell repertoire in HA104 mice despite their specificity for the neo-self-HA provided an opportunity to examine the extent to which mechanisms exist to mediate their negative selection during memory formation. We show here that HA104 and non-tg(BALB/c) mice generate equivalent HA-specific B cell responses after secondary immunization, and that HA-specific C4 B cells can undergo somatic hypermutation and clonal expansion in HA104 mice. In addition, we examine the role that CD4<sup>+</sup> T cell help plays in the generation of autoantibody responses to the neo-self-HA.

## Materials and Methods

**Mice.** HA104 and site 1 (S1)-specific TCR transgene (TS1) mice were described previously (5, 39–41). Both lineages were backcrossed to BALB/c mice (Harlan) at least 7 and in most cases >10 generations before use in these experiments and were maintained in sterile microisolators at The Wistar Institute Animal Fac-

cility. In multiple experiments, non-tg littermates of HA104 mice generated equivalent ASC responses to BALB/c mice after virus immunization. Therefore, both non-tg and BALB/c mice were used in these experiments and are referred to as non-tg(BALB/c) mice.

**Viruses and Immunizations.** Influenza viruses PR8 (A/Puerto Rico/8/34 [H1N1]), T3 (a mutant PR8 virus containing an aspartic acid to glycine interchange at amino acid 227 [35, 42]), and J1 (a reassortant of PR8 containing the nonserologically cross-reactive H3 HA [H3N1]; references 43, 44) were grown in the allantoic cavity of 10-d-old fertilized chicken eggs, purified by sucrose gradient centrifugation, and titered by chicken RBC agglutination (45). Mice were primed for memory B cell responses by immunization with 1,000 hemagglutinating units (HAU) intraperitoneally purified T3 or PR8 virus. For primary and secondary responses, mice were killed 5 or 3 d, respectively, after immunization with 1,000 HAU T3 or PR8 virus intravenously in 0.2 ml PBS. Secondary immunizations were performed a minimum of 4 wk after primary immunization.

**Virus-specific ELISA.** ELISAs were done using purified T3, PR8, and J1 viruses as immunoadsorbents as described previously (5). In brief, polyvinyl 96-well plates were coated with 25  $\mu$ l diluted virus (1,000 HAU/ml in PBS plus azide). After incubation overnight at 4°C, plates were blocked with 1% BSA in PBS plus azide. Hybridoma culture supernatants or serum were added (diluted in 1% BSA in PBS plus azide) for 90 min. Bound antibody was detected by using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG and/or IgM (diluted 1:1,000 in PBS plus azide; Southern Biotechnology Associates, Inc.) and developed using *p*-nitrophenyl phosphate. Absorbances were read at 405 nm using a microplate reader.

**Hemagglutination Inhibition Assay.** The ability of antibodies to neutralize viral agglutination of chicken RBCs was measured as described previously (45). In brief, 4 HAU of PR8 virus were plated (25  $\mu$ l) in 96-well Multiscreen-HA plates (Millipore). Twofold dilutions of serum beginning with 1:100 were added (25  $\mu$ l) to the virus and incubated for 1 h. 50  $\mu$ l 1% RBCs was added and incubated for 30 min. Hemagglutination inhibition (HI) titers were determined as the highest serum dilution capable of inhibiting hemagglutination.

**Enzyme-linked Immunospots.** Virus-specific enzyme-linked immunospots (ELISPOTs) were done using purified T3, PR8, and J1 as described previously (5). In brief, 96-well Multiscreen-HA plates (Millipore) were coated with 50  $\mu$ l diluted virus (1,000 HAU/ml in PBS). After overnight incubation (4°C), plates were blocked with 0.2 ml supplemented IMDM plus 5% FCS (40). Splenocytes were plated in triplicate in 0.2 ml at  $1 \times 10^6$ ,  $2.5 \times 10^6$ ,  $6.25 \times 10^5$ , and  $1.25 \times 10^5$  cells/well for 4 h at 37°C. Bound antibody was detected with AP goat anti-mouse IgG and/or IgM (Southern Biotechnology Associates, Inc.). AP was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaHCO<sub>3</sub> plus 0.001 M MgCl<sub>2</sub>. ASC spots were counted using a dissecting scope.

**Immunohistochemistry.** Spleens were submerged in OCT (VWR Scientific), frozen with 2-methyl butane cooled with dry ice, sectioned, and fixed with acetone. Sections were stained as described (2). In brief, sections were blocked using PBS/2% BSA/0.1% Tween 20 and then stained with anti-IgD-biotin (Southern Biotechnology Associates, Inc.), peanut agglutinin (PNA)-horseradish peroxidase (HRP; EY Labs), and Extravidin-AP (Sigma-Aldrich) diluted in PBS/2% BSA/0.1% Tween 20. AP and HRP were developed using Fast-Blue BB base and 3-amino-9-ethyl carbazole (Sigma-Aldrich), respectively.

**Hybridoma Generation.** Hybridomas were generated from T3-immunized mice 3 d after secondary immunization by fusion with Sp2/0-Ag14 cells and subsequent selection with hypoxanthine-azaserine as described previously (5). 10 d after fusion, hybridoma supernatants were aspirated and replaced with IMDM plus 10% FCS. Supernatants were screened 2 d later by ELISA for reactivity against T3 virus. T3-reactive hybridomas were expanded and their supernatants were screened for reactivity with T3, PR8, and J1 viruses and for H chain isotype.

**Sequence Analysis of Antibody V Regions.** Sequence analysis of hybridoma Ig H and L chain mRNA V regions was performed as described previously (5). In brief, RNA was isolated from  $\sim 10^6$  hybridoma cells and reverse transcribed using a  $\kappa$  L chain and appropriate H chain C region-specific primer under standard conditions (46). PCR was carried out on the resulting cDNA using appropriate C chain and V region-specific primers: VH5'1 or VH5'2 to amplify H chain V regions and VkC4 to amplify L chains (5, 47). Amplified products were run on a 1% agarose gel and purified using GeneCleanII (Bio101). Sequences were obtained using four-color dye chemistries with the ABI 373S sequencer (PerkinElmer) at the Nucleic Acid Facility at The Wistar Institute.

**Flow Cytometry.** Flow cytometric analysis was performed on single cell suspensions made from pooled inguinal, brachial, axillary, and superficial cervical LNs on a FACScan™ flow cytometer (Becton Dickinson). 400,000–500,000 events were collected and analyzed using CELLQuest™ (Becton Dickinson). Antibodies used for staining were anti-CD4-FITC (GK1.5; BD Pharmingen), anti-CD69-PE (BD Pharmingen), and 6.5-biotin (41) detected by Streptavidin 670 (GIBCO BRL).

## Results

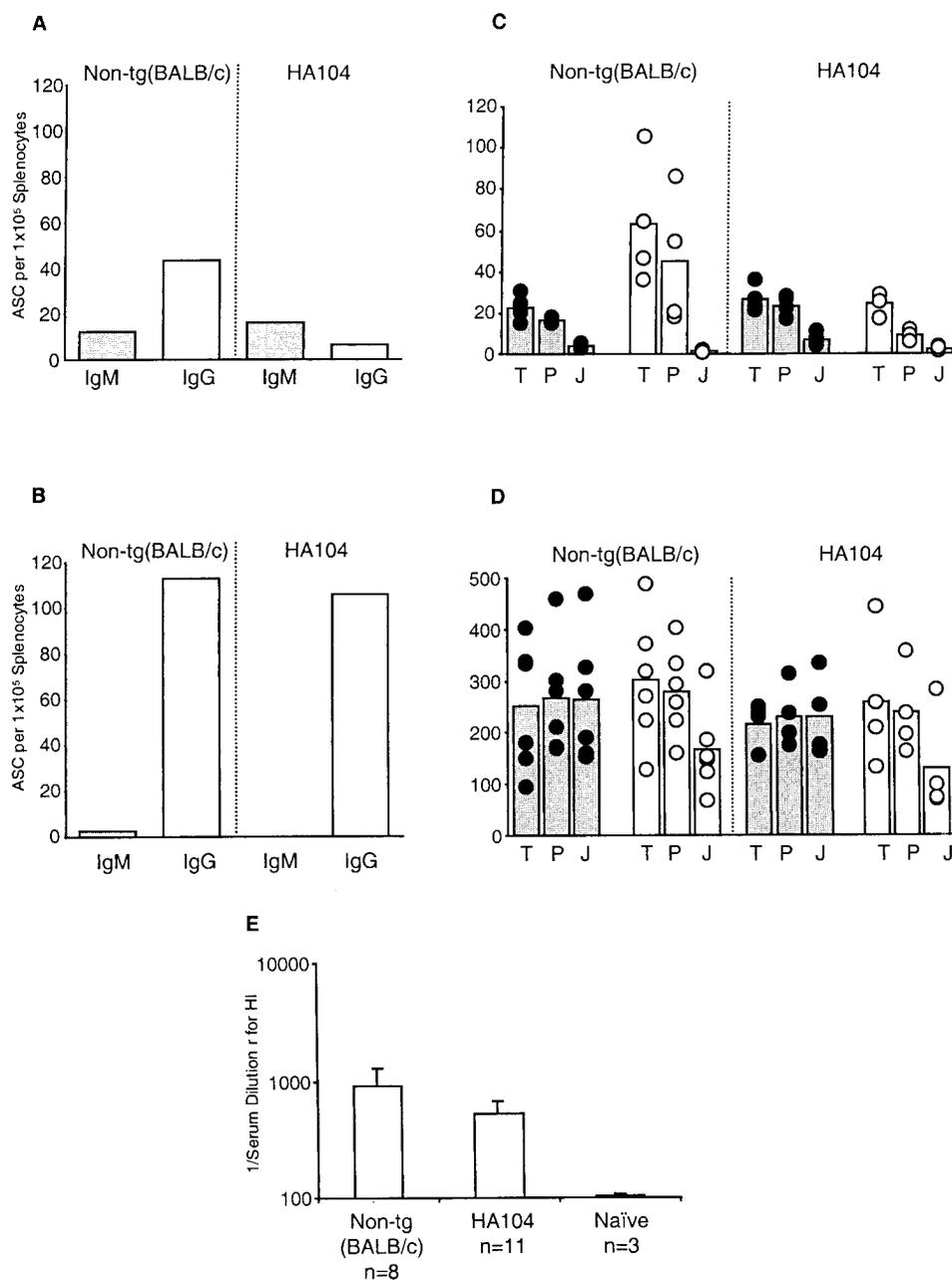
**HA104 and Non-tg(BALB/c) Mice Generate Equivalent HA-specific ASC Responses after Secondary Immunization.** HA104 mice express the influenza virus PR8 HA as a membrane-bound neo-self-antigen in a wide variety of tissues, including the bone marrow, spleen, and thymus (5, 39). To examine how the neo-self-HA affects the ability of HA104 mice to generate HA-specific B cell responses, HA104 and non-tg(BALB/c) mice were immunized with the virus T3 (a virus identical to PR8 except for a single amino acid difference in a B cell epitope of HA [35, 42]). Using whole virus as an immunogen ensured that T cell help would not be limiting for HA-specific B cells, as T cells directed to other proteins in the virus particle could provide a source of intermolecular cognate help for HA-specific B cells (20, 48). Additionally, using T3 rather than PR8 virus to challenge the mice permitted an analysis of the fine specificity of the anti-HA antibody response (see below). 5 d after primary, or 3 d after secondary immunization, splenocytes from immunized animals were analyzed for the frequency of ASCs by ELISPOT (Fig. 1). We initially determined the frequency of ASCs that could react with the HA by comparing the frequencies that could react with PR8 virus with those that could react with the reassortant virus J1. J1 is identical to PR8 except for the presence of a serologically non-cross-reactive HA, and quantitates the frequency of ASCs that are directed to non-HA viral components (i.e., other virus proteins and compo-

nents such as carbohydrates that derive from propagating the virus in hen eggs [43, 44]). The excess frequency of PR8-specific ASCs over J1-specific ASCs therefore indicates the number of HA-specific ASCs that were induced in response to virus immunization.

As described previously, splenocytes from non-tg(BALB/c) mice contained a sizable population of HA-specific IgG ASCs 5 d after primary immunization (5, 36; Fig. 1 A). The frequency of HA-specific IgG ASCs induced in HA104 mice was substantially lower than in non-tg(BALB/c) mice, recapitulating our previous demonstration that HA-specific primary response IgG ASCs are negatively selected in HA104 mice because of their specificity for the neo-self-HA (Fig. 1 A). As we also observed previously, HA-specific IgM ASCs were induced with similar frequencies in non-tg(BALB/c) and HA104 mice 5 d after primary T3 immunization (Fig. 1 A). When splenocytes from non-tg(BALB/c) and HA104 mice were examined 3 d after secondary immunization, equivalent frequencies of HA-specific IgG ASCs were detected (Fig. 1 B). The frequencies of HA-specific IgG ASCs induced in both strains of mice were roughly threefold higher than were induced in non-tg(BALB/c) mice after primary virus immunization, consistent with the activation of memory response B cells. Thus, in contrast to the primary response (Fig. 1 A), HA-specific IgG ASCs were as abundant after secondary immunization of HA104 mice as they were in non-tg(BALB/c) mice.

To examine the magnitude of the HA-specific memory B cell response at the level of serum antibody, we measured the ability of serum antibodies obtained after secondary virus immunization of HA104 and non-tg(BALB/c) mice to inhibit hemagglutination (HI assay). The ability of antibodies to neutralize virus-induced hemagglutination in vitro requires B cell recognition of conformation-dependent epitopes on the HA and correlates with the ability of antibodies to protect against viral infection (49, 50). As shown in Fig. 1 E, the HI titers of serum from HA104 and non-tg(BALB/c) mice after secondary virus immunization were equivalent. Together, the findings indicate that secondary virus immunization induces memory B cell responses in HA104 and non-tg(BALB/c) mice that are of equivalent magnitude and that in each case are directed towards conformation-dependent epitopes on the HA molecule.

**HA104 and Non-tg(BALB/c) Mice Do Not Differ in the Fine Specificity of Their Memory B Cell Response to T3 Virus.** As similar frequencies of HA-specific IgG ASCs could be activated from the memory B cell pool after a second exposure to virus in HA104 and non-tg(BALB/c) mice, this implied that HA-specific B cells are not negatively selected during memory B cell formation in HA104 mice. However, because B cell memory formation involves a series of poorly understood selection events that allow rare somatic mutants to preferentially expand and ultimately populate the memory pool (51, 52), we wanted to examine more closely whether HA-specific B cells are counterselected during the generation of the memory B cell pool. To this end, we examined the fine specificity of the response to T3 virus. The frequency of HA-specific B



**Figure 1.** Specificity of ASC formation in T3-immunized non-tg(BALB/c) and HA104 mice. Mice were analyzed 5 d after primary immunization ( $n = 4$  mice/group; A and C) or 3 d after secondary immunization ( $n = 6$  non-tg(BALB/c) and  $n = 4$  HA104 mice; B and D) for T3 (T)-, PR8 (P)-, and J1 (J)-specific ASCs by ELISPOT or HI titers (E). The magnitude of HA-specific component (A and B) was derived from subtracting the mean frequencies of ASCs measured on J1 virus from those measured on PR8 virus. Fine specificity (C and D) indicates absolute ASC values obtained using T3 virus (T), PR8 virus (P), and J1 virus (J). Filled circles and black bars, IgM ASCs; open circles and white bars, IgG ASCs. Symbols represent individual mice and bars indicate the mean frequencies in each set. (E) HI titers in naïve versus immunized non-tg(BALB/c) and HA104 mice 3 d after secondary immunization.

cells was related to those directed either to a non-self-epitope on the HA (the T3 mutation) or to other non-HA viral components (indicated by reactivity with J1). In this way, the frequency of B cells directed to T3 and J1, which provide a measure of those memory B cells specific for foreign (non-self) epitopes on the immunizing antigen, could be compared with the frequency with which anti-self-HA-specific B cells were generated in HA104 mice.

When the specificity of the primary response (Fig. 1 C) to T3 virus was examined in non-tg(BALB/c) mice, the majority of the ASCs were HA specific (i.e., did not react with J1 virus). A small excess of ASCs that could react with T3 but not with PR8 virus could also be detected, and corresponded to ASCs that recognize the mutant T3 epitope

on the HA. In HA104 mice, the magnitude and the specificity of the primary IgM ASC response closely paralleled that of non-tg(BALB/c) mice. However, as described above, the frequency of IgG ASCs that could react with PR8 virus was decreased roughly fivefold relative to non-tg(BALB/c) mice. Moreover, because HA-specific IgG ASCs were negatively selected in HA104 mice, the relative proportion of the total IgG ASCs that were specific for the non-self-T3 epitope more than doubled in HA104 mice compared with non-tg(BALB/c) mice (64 vs. 27%). Therefore, at the level of the primary response, negative selection of HA-specific B cells altered the fine specificity of the IgG ASC response, which became skewed toward reactivity with the T3 mutation.

After secondary immunization (Fig. 1 D), the frequencies of IgM and IgG ASCs that could react with T3 virus increased substantially relative to the primary response, and were of equivalent magnitudes in HA104 and non-tg(BALB/c) mice. The IgM ASCs from HA104 and non-tg(BALB/c) mice reacted equally well with T3, PR8, and J1 viruses, indicating that these secondary response IgM ASCs were almost exclusively specific for non-HA epitopes on the virus particle. Non-HA epitopes include other viral epitopes as well as components that derive from propagating influenza virus in hen eggs, and it is possible that these secondary response IgM ASCs are directed to egg-derived carbohydrate molecules. Strikingly, the frequencies of T3-, PR8-, and J1-specific IgG ASCs were also similar in HA104 and non-tg(BALB/c) mice, and in each case roughly half of the IgG ASCs could react with T3 and PR8 but not with J1 and were therefore HA specific. Thus, unlike the primary response in which the IgG ASC responses in HA104 mice were predominantly directed toward the non-self-T3 epitope, memory B cell responses in HA104 mice were not skewed toward either the T3 mutation or non-HA epitopes on the virus particle.

To examine the specificity of the antibody response to T3 virus at the level of individual B cells, B cell hybridomas were generated 3 d after secondary immunization of HA104 and non-tg(BALB/c) mice and analyzed for their ability to react with T3, PR8, and J1 viruses (Table I). Consistent with the ELISPOT data, the overwhelming majority of the IgM-secreting hybridomas isolated from either HA104 or non-tg(BALB/c) mice were specific for non-HA viral components. The IgG-secreting hybridomas also closely paralleled the ELISPOT analysis, in that both the absolute numbers of HA-specific hybridomas isolated from HA104 and non-tg(BALB/c) mice, and the relative numbers of HA-, T3-, and J1-specific hybridomas were similar. Thus, based on the specificity of the B cell response to T3 virus, HA-specific B cells do not appear to be negatively selected during memory B cell formation in HA104 mice. Indeed, anti-self-HA-specific B cells did not appear to be at any disadvantage relative to either T3-specific, or to non-HA antiviral B cells in their ability to enter and be reactivated from the memory B cell pool.

*HA-specific B Cells Undergo Clonal Expansion and Somatic Mutation in HA104 Mice.* To examine the extent to which HA-specific secondary response B cells in HA104 mice had undergone somatic mutation and clonal expansion, HA-specific hybridomas generated from HA104 mice after secondary T3 immunization were analyzed for the sequences of their Ig V regions. We focused this analysis toward identifying hybridomas that utilize a B cell clonotype (designated C4) that is characterized by the use of a Vk gene segment (VkC4) from the Vk8 gene family in conjunction with the Jk5 gene segment. B cells that use the C4 clonotype have been shown previously to participate in memory responses to the HA in non-tg(BALB/c) mice (38). Moreover, in our previous studies we showed that C4 B cells were frequently represented among IgM-secreting HA-specific B cell hybridomas that were isolated 5 d after

**Table I.** Specificity Analysis of Secondary Response Hybridomas

	Donor	Non-tg (BALB/c)		HA104	
		IgM	IgG	IgM	IgG
HA-specific*	1	0	7	2	17
	2	4	10	3	15
	3	0	17	2	10
	Total	<b>4</b>	<b>34</b>	<b>7</b>	<b>42</b>
Fine specificity					
T3 epitope‡	1	0	2	1	5
	2	0	1	2	0
	3	0	0	0	2
	Total	<b>0</b>	<b>3</b>	<b>3</b>	<b>7</b>
Viral, non-HA§	1	21	29	39	40
	2	18	8	39	12
	3	39	23	25	17
	Total	<b>78</b>	<b>60</b>	<b>103</b>	<b>69</b>

Specificity analysis of B cell hybridomas isolated after secondary T3 immunization of HA104 mice. Hybridomas from HA104 and non-tg(BALB/c) mice were generated 3 d after secondary immunization with T3 virus. Hybridoma supernatants were analyzed for reactivity with T3, PR8, and J1 viruses and for H chain isotype usage.

\*Number and H chain isotype of hybridomas that react with PR8 but not J1 virus (in most cases binding to J1 virus was undetectable and at least 100-fold lower than to PR8 virus).

‡Number and H chain isotype of hybridomas that react only with T3 (>10-fold higher binding to T3 virus than to PR8 virus; binding to PR8 and J1 was usually undetectable).

§Number and H chain isotype of hybridomas that react equally with T3, PR8, and J1.

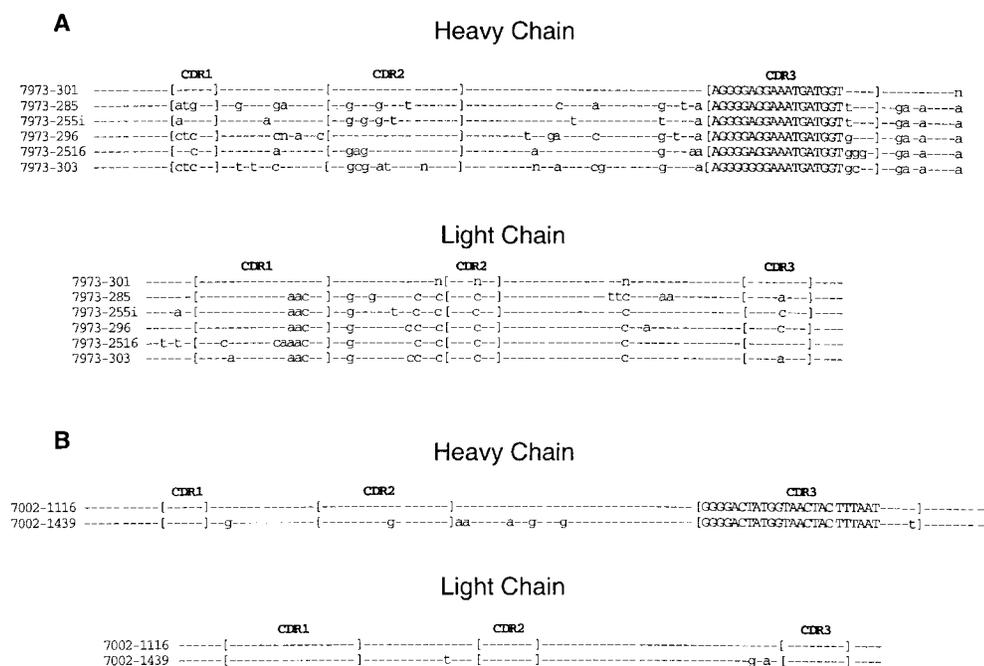
primary T3 immunization from HA104 mice (5). Accordingly, a panel of PR8 HA-specific hybridomas that had been isolated from HA104 mice were initially screened by reverse transcription PCR to determine whether they used L chain genes from the Vk8 gene family (53). 34 of 90 PR8 HA-specific hybridomas examined from HA104 mice were found to express L chains from the Vk8 gene family, and these hybridomas were selected for more detailed analysis of their H and L chain gene sequences. Of the 34 hybridomas that had initially been found to use a Vk8 gene family member, 19 were found upon sequence analysis to use the C4 clonotype (i.e., used the VkC4 gene segment joined to Jk5). Vk8<sup>+</sup> hybridomas were also analyzed for their H chain rearrangements. Hybridomas that utilize the C4 clonotype were found to use members of several different VH gene families, consistent with the previous characterization of C4 B cell hybridomas isolated from non-tg(BALB/c) mice (38). Among the C4<sup>+</sup> hybridomas isolated from one of the HA104 mice was a group of six (one IgM and five IgG3 secretors) that had identical CDR3 junctional sequences despite having other individual differences (Fig. 2 A). Their shared H chain junctional sequences indicate that these hybridomas had derived from the clonal

progeny of a single B cell, and therefore the individual differences between these hybridomas are almost certainly the result of somatic hypermutation that occurred during clonal expansion of these HA-specific B cells in vivo (24). A second group of clonally related HA-specific B cells was isolated from a different HA104 mouse (Fig. 2 B). In this case, a pair of IgG2a-secreting hybridomas was identified that use L chains encoded by a V $\kappa$ 8 gene segment other than V $\kappa$ C4 in conjunction with closely related H chain gene sequences. Again, these hybridomas displayed identical H chain junctional sequences and individual nucleotide differences that are likely the result of somatic hypermutation. As clonal expansion and somatic hypermutation occur predominantly in germinal centers (51, 54), these findings provide strong evidence that these HA-specific memory B cells had developed in the germinal centers of HA104 mice.

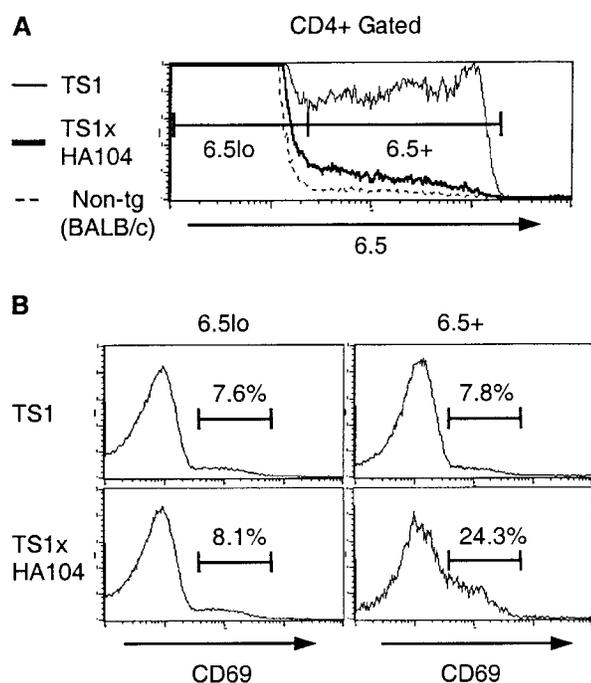
*The Neo-self-HA Can Stimulate HA-specific Autoantibody Production in the Absence of Virus Immunization.* As germinal center reactions and memory B cell formation appear to be strictly dependent on CD4<sup>+</sup> T cell help (17, 55, 56), it is likely that CD4<sup>+</sup> T cells directed toward non-HA viral proteins provided a source of intermolecular cognate help for HA-specific memory B cells in T3-immunized HA104 mice. To examine whether the provision of HA-specific CD4<sup>+</sup> T cells might allow autoantibody production in the absence of virus immunization, we examined whether HA-specific serum antibody could be detected in HA104 mice that had been mated with HA-specific TCR tg mice (TS1). TS1 mice express a tg TCR that is specific for the major I-Ed-restricted T cell determinant from the HA (designated S1 [57]). In TS1 mice, ~30% of the peripheral CD4<sup>+</sup> T cells express high levels of the clonotypic TCR as detected with the anticonotypic monoclonal antibody 6.5 (41). In TS1  $\times$  HA104 mice, the majority of the clonotypic T cells

are deleted during their development in the thymus (58). Nonetheless, a higher number of peripheral LN cells in TS1  $\times$  HA104 mice were detected by the 6.5 monoclonal antibody than were detected in non-tg(BALB/c) mice ( $1.1 \pm 0.6 \times 10^6$  vs.  $0.5 \pm 0.4 \times 10^6$ ), indicating that some HA-specific CD4<sup>+</sup> T cells evade deletion in TS1  $\times$  HA104 mice (Fig. 3 A). This conclusion is supported by recent studies in which we demonstrated that CD4<sup>+</sup> T cells that evade deletion in TS1  $\times$  HA104 mice could be activated by S1 peptide in vitro and by PR8 virus in vivo (58). To examine whether the T cells that evade deletion in TS1  $\times$  HA104 mice might have interacted with the S1 peptide in vivo, freshly isolated LN cells from non-tg(BALB/c), TS1, and TS1  $\times$  HA104 mice were stained with anti-CD4, 6.5, and anti-CD69 antibodies and analyzed by flow cytometry (Fig. 3 B). In the LN cells from TS1 mice, the percentage of 6.5<sup>+</sup> cells that were CD69<sup>+</sup> was similar to the percentage of total 6.5<sup>lo</sup> cells that were CD69<sup>+</sup> (7.6 vs. 7.8%). By contrast, the 6.5<sup>+</sup> cells in TS1  $\times$  HA104 mice contained higher percentages of CD69<sup>+</sup> cells than were found on 6.5<sup>lo</sup> cells (24.3 vs. 8.1%). As increased levels of CD69 are characteristic of antigen-experienced T cells (59, 60), the higher levels present on the autoreactive 6.5<sup>+</sup> T cells suggest that these T cells had interacted with the S1 peptide in vivo.

To examine whether T cells that evade deletion in TS1  $\times$  HA104 mice are capable of providing help for autoantibody responses to the HA in vivo, we examined unimmunized non-tg(BALB/c), HA104, TS1, and TS1  $\times$  HA104 mice for their levels of PR8 HA-specific serum antibody in an ELISA (Fig. 4). HA-specific serum antibody levels were below the limit of detection in non-tg(BALB/c), HA104, and TS1 mice. By contrast, TS1  $\times$  HA104 mice contained significant titers of anti-HA antibodies; indeed, the HA-

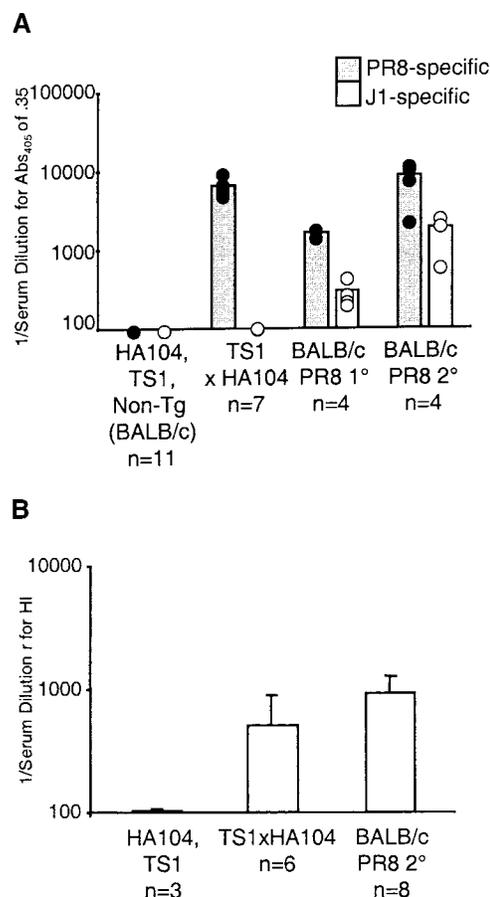


**Figure 2.** VH and V $\kappa$  sequence analysis identifies two sets of clonally related and somatically mutated HA-specific hybridomas from HA104 mice. VH and V $\kappa$  sequences of hybridomas derived from mouse 7973 (A) and 7002 (B) are arranged relative to an individual hybridoma from each set, and the relative locations of individual nucleotide differences are indicated by lowercase letters. The locations of CDRs are indicated by brackets (reference 70). The sequence for the H chain CDR3 nucleotide region is shown in uppercase letters. Hybridoma 7973-301 is an IgM secretor, and the remaining hybridomas from mouse 7973 are IgG3 secretors. Both 7002-1116 and 7002-1439 are IgG2a secretors. Sequence data are available from EMBL/GenBank/DBJ under accession nos. AF265682-AF265687.



**Figure 3.**  $6.5^+CD4^+$  LN cells that evade deletion in TS1  $\times$  HA104 show evidence of in vivo activation. LN cells from naive TS1, TS1  $\times$  HA104, and non-tg(BALB/c) mice were harvested and stained with anti-CD4, 6.5, and anti-CD69 for analysis by three-color flow cytometry. (A) Histogram of 6.5 staining on  $CD4^+$  LN cells from TS1, TS1  $\times$  HA104, and non-tg(BALB/c) mice. Gates marking  $6.5^{lo}$  and  $6.5^+$  cells are indicated. The average numbers (based on multiple mice) of  $6.5^+CD4^+$  LN cells ( $\times 10^{-6} \pm SD$ ) from the three lineages are as follows: TS1 mice ( $15.3 \pm 8.4$ ,  $n = 14$ ), TS1  $\times$  HA104 mice ( $1.1 \pm 0.6$ ,  $n = 11$ ), and non-tg(BALB/c) mice ( $0.5 \pm 0.4$ ,  $n = 7$ ). (B) Histograms of CD69 expression on  $6.5^{lo}$  and  $6.5^+$   $CD4^+$  LN cells from TS1 and TS1  $\times$  HA104 mice. The percentage of gated cells staining positive for CD69 is indicated. Scales for the CD69 histograms have been adjusted to account for differences in the number of  $6.5^{lo}$  and  $6.5^+$  cells. Data are representative of at least three experiments.

specific antibody titers of TS1  $\times$  HA104 mice exceeded those that were present in non-tg(BALB/c) mice 5 d after primary PR8 virus immunization, and were equivalent to the antiviral titers present after secondary virus immunization of non-tg(BALB/c) mice. In addition, whereas the sera from virus-immunized non-tg(BALB/c) mice contained antibodies that could react with both PR8 and J1 virus (and were therefore directed toward both the HA and to non-HA viral components), those from TS1  $\times$  HA104 mice displayed no detectable reactivity with J1 virus. The lack of reactivity with J1 virus in TS1  $\times$  HA104 mice is consistent with the activation of HA-specific B cells by the neo-self-HA (as opposed to viral particles). To determine whether the HA-specific antibodies elicited in naive TS1  $\times$  HA104 mice were directed against conformational epitopes on the HA molecule that are shared between the neo-self-HA and the virally expressed HA, sera from TS1  $\times$  HA104 mice were examined for their ability to inhibit viral hemagglutination. As indicated in Fig. 4 B, serum from unimmunized TS1  $\times$  HA104 mice exhibited HI titers that were comparable to those of virus-immunized

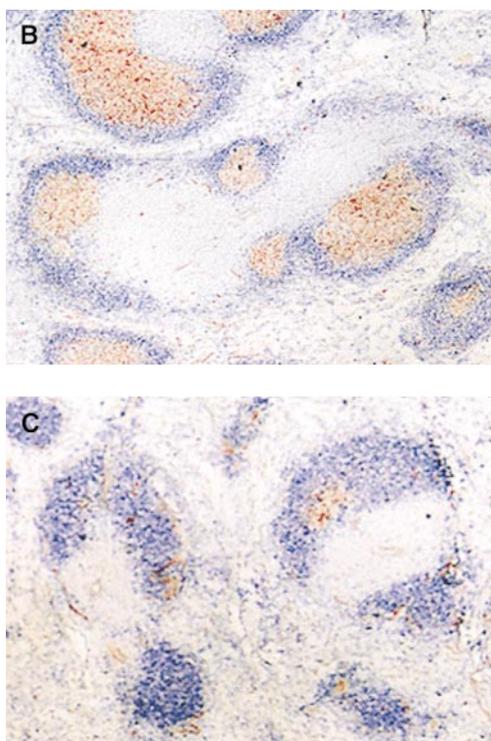
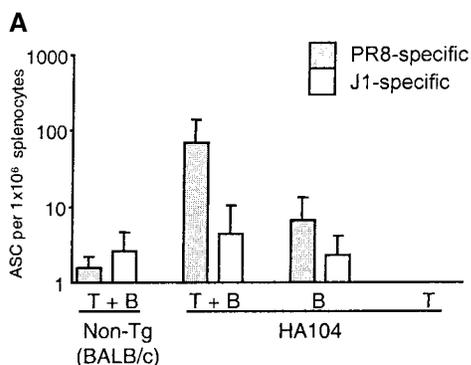


**Figure 4.** TS1  $\times$  HA104 mice contain high titers of HA-specific serum antibody. Serum from unimmunized HA104, TS1, and TS1  $\times$  HA104 mice, as well as serum from non-tg(BALB/c) mice collected either 5 d after primary ( $1^\circ$ ) or 3 d after secondary immunization with PR8 virus ( $2^\circ$ ), was assayed for reactivity with PR8 and J1 viruses in an ELISA (A) or for their ability to inhibit hemagglutination (B). (A) Data are expressed as the reciprocal of the dilution necessary to yield an absorbance at 405 nm ( $Abs_{405}$ ) of 0.35. Filled circles, PR8-specific titers; open circles, J1-specific titers from individual mice; bars, mean titers for each set of mice. (B) Data are expressed as the reciprocal of the dilution necessary to inhibit hemagglutination (HI).

non-tg(BALB/c) mice (Fig. 4 E). As signals mediated via the BCR are necessary for B cell differentiation into antigen-specific ASCs (61), the presence of high titers of conformation-dependent PR8 HA-specific serum antibody in TS1  $\times$  HA104 mice provides strong evidence that the neo-self-HA is recognized by autoreactive HA-specific B cells in HA104 mice. Moreover, because HA-specific antibody was detected in TS1  $\times$  HA104 but not in HA104 mice, the generation of these HA-specific antibodies appeared to depend on HA-specific  $CD4^+$  T cell help that was provided by the rare  $6.5^+$  T cells that evaded deletion by the HA.

*The Neo-self-PR8 HA in HA104 Mice Can Be Recognized by HA-specific Memory B Cells.* Although the preceding studies indicated that the HA can be recognized as B cell autoantigen in HA104 mice, we wanted to examine directly whether the HA can be recognized by memory B

cells. Accordingly, non-tg(BALB/c) mice were immunized with PR8 virus, and after 6 wk their splenocytes were depleted of T cells and then transferred into naive HA104 or non-tg(BALB/c) mice with or without the addition of LN



**Figure 5.** HA-specific memory B cell activation by transgene-encoded HA in HA104 mice. (A) The frequency of ASCs in splenocytes was determined by ELISPOT in either non-tg(BALB/c) or HA104 mice 5 d after receiving T cell-depleted splenocytes (termed B cells [B]) from non-tg(BALB/c) mice primed 6 wk earlier with PR8 virus either alone (a source of virus-primed B cells) or in conjunction with LN cells from TS1 mice (termed T cells plus B cells [T + B]). Additionally, one group of mice received only LN cells from TS1 mice (T cells [T]). Bars indicate the mean frequency of ASCs that react with PR8 virus (gray bars) and J1 virus (white),  $\pm$  SEM ( $n = 3-4$  mice per group). (B and C) Immunohistochemical analysis of spleen sections of HA104 (B) or non-tg(BALB/c) (C) recipients of T cell-depleted splenocytes from non-tg(BALB/c) mice and TS1 LN cells. Spleen sections were stained with anti-IgD-biotin, avidin-AP, and PNA-HRP. HRP and AP were developed using the substrates 3-amino-9-ethyl carbazole (red) and Fast-Blue BB base (blue), respectively. Staining is representative of at least three separate experiments. Original magnifications:  $\times 100$ .

cells from TS1 mice as a source of HA-specific CD4<sup>+</sup> T cells (Fig. 5). 5 d after transfer, the spleens of recipient mice were assayed for the presence of ASCs by ELISPOT and by immunohistochemistry for histologic signs of B cell activation (e.g., germinal center formation). In non-tg(BALB/c) mice that received both primed B cells and TS1 T cells, there was little or no B cell activation as evidenced either by ASC formation or by histologic examination (Fig. 5). The few ASCs that were detected by ELISPOT in non-tg(BALB/c) mice were primarily specific for non-HA viral components, and presumably include ASCs reacting to residual antigen that was transferred with the splenocytes. In HA104 mice that received primed B cells but no TS1 T cells, the frequency of ASCs directed to nonviral components was the same as in non-tg(BALB/c) mice, but the frequency of HA-specific ASCs (i.e., those that could react with PR8 but not with J1) now exceeded by roughly two-fold those directed to non-HA viral components. When both primed B cells and TS1 T cells were transferred into HA104 mice, HA-specific ASCs were roughly 10-fold more abundant than those directed to non-HA viral components. The spleens of HA104 mice that received both primed B cells and TS1 T cells also contained abundant, well-organized germinal centers that displayed characteristic staining with PNA. HA104 mice that received T cells alone did not mount a detectable HA-specific ASC response (Fig. 5), and the spleens of these recipients did not show evidence of germinal center formation (data not shown), indicating that the HA-specific B cell response was due to the activation of transferred memory B cells. Therefore, the neo-self-HA is expressed in HA104 mice in a form and location recognized by HA-specific memory B cells, and in the presence of HA-specific T cell help, induces both germinal center and HA-specific ASC formation.

## Discussion

We have examined tg mice that express the influenza virus HA as a membrane-bound neo-self-antigen (HA104 mice) for their ability to generate HA-specific B cell responses after primary and secondary virus immunization. We found that a population of IgG-secreting ASCs that dominates the day 5 primary response to HA in virus-immunized BALB/c mice is negatively selected from the primary B cell repertoire in HA104 mice. By contrast, a population of HA-specific B cells that evades negative selection from the primary B cell repertoire gave rise to memory B cell responses in virus-immunized HA104 mice that were of equal magnitude to those induced in non-tg(BALB/c) mice. Inasmuch as the HA-specific autoreactive B cells were somatically mutated and clonally expanded, these studies provide evidence that specificity for a self-antigen does not prevent the maturation of autoreactive B cells in the germinal center pathway. Indeed, if provided with CD4<sup>+</sup> T cell help, autoreactive HA-specific B cells appeared to be selected into and reactivated from the memory B cell pool as efficiently as B cells directed to non-self-epitopes on an immunizing influenza virus.

Although processes governing the negative selection of autoreactive B cells from the primary B cell repertoire have been extensively studied, how and if autoreactive B cells that evade negative selection from the primary B cell repertoire (or that acquire autoreactivity via somatic mutation) are regulated during memory formation has received little analysis. Because we found previously that separate populations of HA-specific B cells differed in their susceptibility to negative selection from the primary B cell repertoire in HA104 mice, we were able to evaluate whether a second window of tolerance induction might lead to the elimination of HA-specific B cells during memory B cell formation. We found that HA104 and non-tg(BALB/c) mice generated HA-specific memory B cell responses that were of equivalent magnitude. Moreover, the relative frequencies of memory B cells directed to the HA versus those directed either to a mutated epitope on the HA (the T3 mutation) or to non-HA epitopes on the virus (e.g., the nucleoprotein) were equivalent in HA104 and non-tg mice. Previous studies of antibody responses to species variants of self-proteins such as cytochrome c, hemoglobin, and MHC class II have demonstrated that negative selection of autoreactive B cells can focus the antibody responses toward epitopes that differ from the self-protein (62–64). However, in these previous studies, the extent to which focusing of the response to non-self-epitopes reflected negative selection of autoreactive B cells from the primary repertoire versus during formation of the memory B cell pool was not established. We found that whereas negative selection of HA-specific B cells skewed the T3-induced primary response IgG ASCs toward specificity for the non-self-T3 mutation, this was not true for the memory response in HA104 mice. Thus, even though memory B cells are produced through stochastic processes that require the generation, selection, and expansion of mutated B cells (11, 51, 52, 54), we did not find any evidence that HA-specific B cells in HA104 mice were at a disadvantage relative to B cells directed to epitopes other than HA on the same immunizing virus particle.

Why are HA-specific B cells not negatively selected during memory formation in HA104 mice? Antigen sequestration has been proposed as a reason that autoreactive B cells can avoid regulation by (or be “ignorant” of) self-antigens (65). However, three lines of evidence suggest that the HA is accessible to and can be recognized by HA-specific B cells in HA104 mice. First, the neo-self-HA is responsible for the negative selection of a major subset of HA-specific B cells from the primary B cell repertoire. Second, the neo-self-HA was able to activate HA-specific memory B cells from non-tg(BALB/c) mice when adoptively transferred with HA-specific T cells into HA104 mice, and this activation led to ASC formation and germinal center development in the spleens of recipient mice. Third, the neo-self-HA was able to stimulate autoantibody production in HA104 mice that had been mated with mice transgenic for an HA-specific CD4<sup>+</sup> TCR. Moreover, the autoantibodies elicited by the neo-self-HA were able to recognize conformation-dependent epitopes on the viral HA molecule.

Based on these lines of evidence, neither antigen inaccessibility nor incorrect folding of the neo-self-HA appears to be likely explanation for the ability of autoreactive HA-specific memory response B cells to develop in HA104 mice. There is also evidence that the presence of autoantibodies may mask self-antigens and thus limit the accessibility of the antigens to self-reactive B cells (18). In the case of HA-specific antibodies elicited by acute viral immunization, masking of HA by these antibodies may indeed allow for autoreactive B cells that emerge after immunization to escape tolerance induction mechanisms. However, that we were able to measure autoantibody production in naive TS1 × HA104 mice suggests that masking of autoantigens by virus-induced antibodies is not necessary for the initiation of autoantibody production to HA.

An alternative explanation for the HA-specific memory B cell responses that could be induced in HA104 mice is that B cells are not subjected to negative selection during memory formation based on the specificity of their BCR for a self-antigen. Instead, the availability of CD4<sup>+</sup> T cell help may play a crucial role in regulating memory autoantibody responses. Germinal center reactions depend on cognate interactions between antigen-specific B cells and CD4<sup>+</sup> T cells (17), and it has long been recognized that negative selection of autoreactive CD4<sup>+</sup> T cells could play an important role in regulating autoantibody responses (18, 66, 67). Yet, because of the possibility that a foreign antigen (such as a virus) could contain B cell epitopes that cross-react with self-antigens and provide autoreactive B cells with help directed to non-self-proteins (20, 21), several studies have examined whether B cell intrinsic tolerance mechanisms exist to prevent the development of autoreactive B cells in germinal centers. Evidence in support of such mechanisms is mostly indirect, and has been derived from examining the effects of administering high concentrations of soluble antigens to germinal center B cells and/or examining how genes that affect lymphocyte survival influence germinal center reactions (25, 27–31). We demonstrated here that clonally expanded and somatically mutated B cells using a clonotype (C4) that is typical of PR8 HA-specific memory B cell responses of influenza virus-immunized mice BALB/c mice could be isolated from virus-immunized HA104 mice. Because clonal expansion and somatic hypermutation occur predominantly in germinal centers (51, 54), this provides direct evidence that autoreactive HA-specific B cells had developed in the germinal center pathway. In addition, two observations suggest that HA-specific antibodies also underwent affinity maturation in HA104 mice. First, HA104 and non-tg(BALB/c) mice contained similar frequencies of HA-specific ASCs. Second, sera from HA104 and non-tg(BALB/c) mice contained similar titers of hemagglutination-inhibiting antibodies. Unless ASCs from HA104 mice secreted more antibody per ASC than did those in non-tg(BALB/c) mice (for which there is no precedence), equivalent serum titers of HI antibodies suggest that the overall affinity for the HA of serum from HA104 mice is comparable to that of non-tg BALB/c mice. Therefore, to the extent that the

levels of hemagglutination-inhibiting antibodies that are present in the sera of non-tg(BALB/c) mice after secondary immunization reflect the outcome of somatic mutation and affinity maturation (38), that HA104 and non-tg(BALB/c) mice contain both similar frequencies of HA-specific ASCs and comparable levels of hemagglutination-inhibiting serum antibodies suggests that HA-specific antibodies also undergo affinity maturation in HA104 mice. In this regard, it is noteworthy that a low affinity for self-antigens has been shown previously to allow B cells to evade negative selection from the primary repertoire (68). As HA104 and non-tg(BALB/c) mice differ substantially in the frequency of HA-specific IgG ASCs that are activated after primary immunization, it is difficult to assess the relative affinity of their primary serum responses. However, if the HA-specific B cells that evade negative selection from the primary B cell repertoire in HA104 mice are of relatively low affinity for the HA, the studies here suggest that affinity maturation allows the secondary response to achieve an affinity comparable to those in non-tg(BALB/c) mice.

Finally, it is significant to note that the ability of HA-specific C4 B cells to develop in the germinal center pathway in HA104 mice was dependent on virus immunization. Whereas HA-specific C4 B cell hybridomas isolated 3 d after secondary immunization in HA104 mice were somatically mutated and had undergone H chain class switching, those isolated 5 d after primary immunization of HA104 mice expressed unmutated IgM (5). This requirement for virus immunization most likely reflects a need for cognate T cell help to permit activation of these autoreactive B cells. We have demonstrated previously that HA-specific CD4<sup>+</sup> T cells are negatively selected in HA104 mice (69), and the idea that negative selection of HA-specific CD4<sup>+</sup> T cells plays a role in regulating autoantibody production in HA104 mice was also inferred from studies here using TS1 × HA104 mice. Even though HA-specific T cells undergo negative selection during their development in TS1 × HA104 mice (58), the rare autoreactive CD4<sup>+</sup> T cells that evade deletion by the neo-self-HA are sufficient to induce autoantibody production in TS1 × HA104 mice. It will be interesting in future experiments to determine whether the HA-specific autoantibodies in TS1 × HA104 mice derive from B cells that would have otherwise undergone deletion from the primary repertoire or from HA-specific B cells that were induced to form memory by the neo-self-HA in the presence of persistent T cell help. Regardless of the source of autoantibodies in TS1 × HA104 mice, it is clear that HA as a neo-self-antigen is capable of being recognized by HA-specific B cells *in vivo*. Moreover, the findings presented here provide evidence that provision of CD4<sup>+</sup> T cell help can play a crucial role in the activation of autoreactive B cells.

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