Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants

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Memory B cells (MBCs) and long-lived plasma cells (LLPCs) persist after clearance of infection, yet the specific and nonredundant role MBCs play in subsequent protection is unclear. After resolution of West Nile virus infection in mice, we demonstrate that LLPCs were specific for a single dominant neutralizing epitope, such that immune serum poorly inhibited a variant virus that encoded a mutation at this critical epitope. In contrast, a large fraction of MBC produced antibody that recognized both wild-type (WT) and mutant viral epitopes. Accordingly, antibody produced by the polyclonal pool of MBC neutralized WT and variant viruses equivalently. Remarkably, we also identified MBC clones that recognized the mutant epitope better than the WT protein, despite never having been exposed to the variant virus. The ability of MBCs to respond to variant viruses in vivo was confirmed by experiments in which MBCs were adoptively transferred or depleted before secondary challenge. Our data demonstrate that class-switched MBC can respond to variants of the original pathogen that escape neutralization of antibody produced by LLPC without a requirement for accumulating additional somatic mutations.

Long-lived plasma cells (LLPCs) constitutively secrete antibody to neutralize antigen immediately upon reinfection, whereas memory B cells (MBCs) produce antibody only upon restimulation by specific antigen (Manz et al., 1997; Slifka et al., 1998). Although preexisting serum antibody titers correlate with vaccine efficacy, the importance of the MBC response in conferring protection to reinfection has remained controversial (Zinkernagel and Hengartner, 2006). As both LLPC and MBC have been retained through mammalian evolution, these cell types must have distinct functions that remain to be fully delineated.

Hapten-based studies have reported that the affinities of LLPC B cell receptors (BCRs) are higher than those of MBC (Smith et al., 1997). Studies using fixed BCR demonstrated that cells with high-affinity antigen receptors were recruited preferentially to the LLPC pool (Phan et al., 2006). In contrast, MBC can be formed even when BCR affinities are low (Dal Porto et al., 2002). Because of the reduced stringency of selection, the repertoire of the MBC pool may be more diverse and reactive to pathogen variants than a more oligoclonal LLPC population. Experiments with influenza and HIV have supported these predictions, although several of these studies suggest that MBCs achieve broader reactivity primarily through somatic hypermutation and affinity maturation that occurs after secondary encounter with heterologous viruses (Wrammert et al., 2008, 2011; Galli et al., 2009; Scheid et al., 2009).

One caveat to these conclusions is the recent finding that isotype-switched MBCs, which dominate the initial antibody recall response, have a limited ability to form new germinal centers and thus may be incapable of undergoing further affinity maturation (Dogan et al., 2009; Pape et al., 2011). Based on these studies, we...
hypothesized that MBC, but not LLPC, would recognize efficiently variant antigens before secondary encounter with heterologous viruses. To test this, we used a mouse model of West Nile virus (WNV) infection with a WT and variant virus that differ only by a single amino acid in a dominantly neutralizing epitope. Our results suggest that LLPCs function to prevent secondary infection with homologous viruses, whereas MBCs generated during the primary response can recognize pathogen variants that escape neutralization of preexisting serum antibody produced by LLPCs.

RESULTS AND DISCUSSION

In mice, both B cells and antibody are required for survival after WNV infection (Diamond et al., 2003). Strongly neutralizing mouse monoclonal and polyclonal antibodies bind to the lateral ridge (LR) epitope on domain III (DIII; DIII-LR, residues K307, T330, and T332) of the viral E glycoprotein (Beasley and Barrett, 2002; Oliphant et al., 2005, 2007). Virtually all antibodies specific for this epitope can be identified by comparing binding to WT and loss-of-function proteins with mutations at two of the critical contact residues (K307E and T330I). We measured a significant reduction (7.4-fold, P < 0.01) in serum antibody recognition of DIII-K307E/T330I in comparison with WT DIII at days 30–100 after infection in WT C57BL/6 mice (Fig. 1 A). Serum antibody titers against this epitope were sustained as late as 500 d after infection in WT C57BL/6 mice (Fig. 1 A). Thus, after WNV infection in C57BL/6 mice, a significant fraction of LLPC expressed BCRs that are restricted to a single immunodominant epitope.

To measure the frequency of WNV-specific LLPCs, we interrogated bone marrow cells from infected mice for their ability to secrete IgG that recognized WT and variant DIII. We detected DIII-specific LLPCs at 100 d after infection by ELISPOT (Fig. 1 B) and, similar to the ELISA data with serum, a substantial (9.3-fold, P < 0.01) reduction in binding to the DIII-LR epitope was observed. As an independent measure of LLPC specificity, we analyzed bone marrow cells for intracellular binding to a bivalent fusion protein composed of WNV DIII or DIII-K307E/T330I and human γ1 Fc (DIII-Fc, DIII-K307E/T330I Fc). We identified a small population of plasma cells that recognized DIII that was not present in mice infected with an unrelated virus (Fig. 1, C and D). Importantly, there were notably fewer LLPCs that recognized DIII-K307E/T330I compared with DIII (P < 0.005). Thus, after WNV infection in C57BL/6 mice, a significant fraction of LLPC expressed BCRs that are restricted to a single immunodominant epitope.

Given the epitope specificity of the LLPC response, we hypothesized that the antibody derived from these cells would strongly neutralize homologous WNV but might be impaired at neutralizing a variant (WNV-K307E) with a single mutation introduced at the dominant epitope. Convalescent serum from either WNV-infected or vaccinated mice was tested in neutralization assays with the homologous WNV-WT or variant WNV-K307E. With serum collected 60 d after WNV-WT infection, we observed a reduction (8.5-fold, P < 0.05) in the EC50 value for WNV-K307E compared with WNV-WT (Fig. 2, A and B). Similar results were observed with serum from mice 60 d after immunization with a formalin-inactivated vaccine, with less efficient neutralization of the variant WNV-K307E virus (Fig. 2, A and B).

To confirm these results, we performed protection experiments by transferring immune serum from vaccinated mice immediately before challenge with WNV-WT or WNV-K307E. Transfer of 1 µl of immune serum, but not a mutant K307E/T330I epitope.

Figure 1. LLPCs recognize the K307/T330, but not a mutant K307E/T330I epitope. (A) Mice were infected with WNV, and serum was collected at the indicated time points after infection. Levels of DIII or DIII-K307E/T330I-specific IgG were measured by ELISA. Endpoint titers are expressed as the reciprocal serum dilution that was 3 SD above background. The data reflect 6–10 mice per time point from four experiments. (B) Bone marrow cells were collected at the indicated time points after WNV infection and DIII or DIII-K307E/T330I-specific plasma cells were enumerated by ELISPOT assay. The data reflect six to nine mice per time point from four experiments. (C) Bone marrow cells were collected from mice infected with WNV or Chikungunya virus (CHIKV) and antigen-specific plasma cells were enumerated by intracellular staining. Data are shown as percentage of total B220+CD138+ cells. The percentage of cells staining as DIII or DIII-K307E/T330I-specific LLPC in CHIKV-infected mice was considered background and subtracted (0.1%). The data reflect five mice from three experiments. Horizontal bars in A–C indicate the mean. (D) Representative flow cytometry plots showing DIII or DIII-K307E/T330I-specific plasma cells from mice at 60 d after infection with WNV or CHIKV. Statistical significance (**, P < 0.01; *, P < 0.05) was determined by an unpaired, two-tailed Student’s t test.
saline, into naive 5-wk-old mice protected from WNV-WT challenge (100 vs. 5% survival, P < 0.001; Fig. 2 C). In comparison, the same immune serum did not improve survival significantly after challenge with the variant virus WNV-K307E (56 vs. 35% survival, P > 0.05). WNV-K307E was mildly attenuated in mice, as reported previously (Zhang et al., 2009). In mice receiving immune serum, viremia was reduced at 36 h (10-fold, P < 0.001) and 72 h (11-fold, P < 0.05) after infection with WNV-WT (Fig. 2 D). However, the same serum failed to reduce viremia after WNV-K307E infection (P > 0.2). Thus, LLPC-derived antibody was inefficient at controlling infection with a variant WNV that was altered at a single amino acid within the dominant neutralizing epitope.

To evaluate the frequency of MBCs specific to the neutralizing epitope, we used two approaches: (1) limiting dilution analysis (LDA) of supernatant from cultures of stimulated MBCs and (2) direct enumeration of antigen-specific MBCs by flow cytometry. At different time points after WNV-WT infection, CD19+ B cells from the spleen were diluted serially, antibody secretion was induced (Amanna and Slifka, 2006), and supernatants were tested for reactivity to DIII or DIII-K307E/T330I. At day 30 after infection, there were, on average, 26 DIII-specific MBCs per 10^6 B cells (Fig. 3 A). This frequency increased to 57 at day 60 but waned to 7 DIII-specific MBCs per 10^6 B cells by day 300. Next, we compared the relative frequencies of DIII and DIII-K307E/T330I-specific MBCs. In contrast to antibody produced by LLPCs, we did not measure a significant decrease in binding to the K307E/T330I DIII mutant relative to the WT DIII protein (P > 0.1).

As an independent measure of MBC frequency and specificity, we determined the relative number of virus-specific MBC by flow cytometry. Immune splenocytes were stained for MBC markers (CD19, CCR6, and CD80; Bhattacharya et al., 2007) and DIII-FcHu or DIII-K307E/T330I-FcHu. We defined a population of isotype-switched CCR6+CD80+ MBCs specific to DIII in WNV immune mice, but not in mice infected with an irrelevant virus (Fig. 3, B and C; and Fig. S1 A). The antigen specificity was confirmed by sorting DIII+ MBCs, activating them in vitro, and testing the supernatant by ELISA (Fig. S1 B). DIII+-sorted MBCs recognized both DIII and DIII-K307E/T330I, whereas DIII− MBC from WNV-immune mice failed to produce antibody that recognized DIII. All DIII-specific MBCs were within the CCR6+CD80+ gate, as adoptive transfer of isotype-switched CD80−CCR6− cells from WNV-immune mice or CD80+CCR6+ cells from naive mice failed to produce anti-DIII antibody upon challenge with WNV-WT (Fig. S1 C). The kinetics of antigen-specific MBC accumulation and maintenance was consistent in both the LDA and flow cytometric experiments (Fig. 3, A and B). In contrast to the LDA, we observed a small decrease by flow cytometry in the frequency of MBCs that recognized DIII-K307E/T330I at days 30 and 60 after infection. Nonetheless, the LDA and flow cytometry experiments both suggest that compared with the LLPC pool, MBCs contain a much larger frequency of B cell clones that recognize the mutant DIII-K307E/T330I. We calculated the frequency of cells that recognize DIII-K307E/T330I relative to the WT DIII protein (P > 0.1).

Figure 2. LLPCs weakly neutralize a variant WNV. (A) Mice were infected with WNV or WNV-K307E (left) or immunized with an inactivated WNV vaccine (right), and sera were collected at day 60. Neutralizing antibody titers were measured by plaque reduction assay. Means and SD are shown of six experiments. (B) EC50 titers were calculated from neutralization curves of WNV-WT or WNV-K307E incubated with serum from WNV-infected or vaccinated mice. Means and SD are shown from three or six experiments. The dashed line represents the limit of detection of assay. (C) Mice were injected with 1 µl WNV immune serum or PBS before infection with WNV or WNV-K307E or PBS. Data reflect 20–26 mice per group in three experiments. Survival differences were determined by the log-rank test. NS, not significant. (D) Mice were injected with 1 µl of immune or PBS immediately before infection with WNV (left) or WNV-K307E (right) mice. Viral RNA in serum was measured by qRT-PCR. Means and SD are shown of two experiments with five mice per group. Statistical significance (***, P < 0.001; **, P < 0.01; *, P < 0.05) was determined by an unpaired, two-tailed Student’s t test.
were more poised, compared with LLPCs, to recognize variant DIII carrying mutations that cause functional escape from neutralization.

Given the broader antigen specificity of the MBC population, we asked whether a subset of MBCs encoded antibody that preferentially recognized the variant epitope that was not present during primary infection. Remarkably, wells that contained single B cell clones as isolated by LDA revealed MBC specificities that recognized the mutant DIII-K307E/T330I but bound WT DIII so poorly that it was below the limit of assay detection (Fig. 4 A). To corroborate these findings, we isolated single DIII-specific or DIII-K307E/T330I-specific MBCs by cell sorting and expanded them in culture on feeder cells expressing BAFF and CD40L. Although DIII-K307E/T330I-specific MBC clones were isolated (Fig. 4 B), others produced antibody that recognized DIII and DIII-K307E/T330I equivalently (Fig. 4 C). Moreover, one clone (2F2) bound the variant DIII-K307E/T330I protein better than the WT protein. To confirm this observation, the heavy and light chain genes of 2F2 were cloned and expressed ectopically.

Figure 3. MBCs recognize mutant DIII-K307E/T330I epitopes. (A) Mice were infected with WNV, splenocytes were collected at the indicated time points, and CD19+ B cells were isolated by positive selection. The frequency of DIII- or DIII-K307E/T330I-specific IgG+ MBC was assessed by LDA. Virus-specific IgG in supernatants from LDA cultures were measured by ELISA and frequencies of virus-specific MBC determined by linear regression analysis. The limit of detection was ∼2 MBCs per 10^6 B cells (dashed line), and data reflect 5–14 mice per group from four experiments. P-values were determined using an unpaired, two-tailed Student’s t test. (B) The frequency of splenic DIII- or DIII-K307E/T330I-specific MBC was assessed by flow cytometry. The percentage of cells staining as DIII- or DIII-K307E/T330I+ MBC in CHIKV-infected mice was considered background and subtracted (6.1 and 5.4 MBCs per 10^6 CD19+ cells). Data reflect 7–11 mice per group from five experiments and are displayed as frequency of antigen-specific MBCs per 10^6 CD19+ cells. P-values were determined using an unpaired, two-tailed Student’s t test. (C) Flow cytometry plots were from B and show MBC recognizing DIII or DIII-K307E/T330I from WNV- or CHIKV-infected mice. (D) Ratio of frequency of DIII-K307E/T330I- to DIII-specific MBC or LLPC in individual mice as measured by flow cytometry, LDA, and ELISPOT. MBC frequencies were shown in A and B, and LLPC frequencies were from Fig 1 (B and C). P-values were determined by the Mann-Whitney test (***, P < 0.001; **, P < 0.01; *, P < 0.05). Horizontal bars indicate the mean.

Figure 4. MBC clones can recognize variant epitope better than WT DIII. (A) Percentage of MBC clones that bind DIII, both DIII and DIII-K307E/T330I, or DIII-K307E/T330I by ELISA. Positive-scoring wells of the lowest dilution from LDA in A were assigned specificities based on binding to DIII and DIII-K307E/T330I. Data represent ∼0.8 clones per mouse from 8–14 mice (low frequency of MBC at day 300 precluded analysis). [B and C] Individual WNV-specific MBCs were sorted and cultured. Supernatant was tested for binding to DIII or DIII-K307E/T330I by ELISA to identify DIII-LR-specific (B) or DIII-cross-reactive (C) MBC. Data represent four independent experiments. (D) Clone 2F2 was expressed ectopically in 293T cells and supernatant was tested for binding to DIII or DIII-K307E/T330I. Representative results are shown, and statistical significance was determined using a paired, two-tailed Student’s t test using data from four independent experiments performed in duplicate (*, P < 0.01).
Notably, 293T cells transfected with 2F2 heavy and light chain genes produced antibody that recognized DIII-K307E/T330I better than WT DIII (Fig. 4 D). These results suggest that antigen specificities can be generated within the MBC compartment through mutation in the primary germinal center reaction, which preferentially recognizes variant epitopes.

To define the diversity within the MBC compartment, we analyzed the immunoglobulin gene sequences of individual WNV-specific MBCs. The analysis of nine cross-reactive clones revealed vast sequence variation within the complementarity-determining regions (CDRs), with no apparent consensus for gene family or amino acid usage (Tables S1 and S2). Among 11 DIII-LR–specific clones, we noted some conservation of the light chain Vk19-25 gene segment. For comparison, sequencing data from the previously generated DIII-LR–specific monoclonal antibodies E16, E24, and E34 was included (Oliphant et al., 2005). Still, heavy chain usage was variable, even at contact residues between E16 and K307/T330 previously identified by crystallographic analysis (Nybakken et al., 2005). Although a similar analysis on LLPCs was attempted, the low frequency (~1%) of antigen-specific cells, low levels of cell surface BCR, and nonproliferative nature of these cells precluded isolation of individual WNV-DIII–specific LLPC clones. Still, the selection of MBCs with divergent immunoglobulin gene sequences is consistent with the broad reactivity of this population.

Because MBCs were better able to recognize DIII-K307E/T330I than LLPCs were, we hypothesized that antibody derived from these cells would more efficiently neutralize WNV-K307E. To test this, we tested the ability of antibody-containing supernatant from polyclonally stimulated MBCs to neutralize WT or variant WNV. Although the total antibody concentration in MBC culture supernatant was lower than in immune serum (~500-fold less, not depicted), resulting in less inhibition, antibody produced by MBC neutralized WNV and WNV-K307E equivalently (P > 0.9; Fig. 5 A). This contrasts with the relatively poor neutralizing activity of LLPC-derived antibody against WNV-K307E (Fig. 2 A).

We used two approaches to assess the ability of MBCs to respond to heterologous infection. We adoptively transferred CD19−CCR6−CD80−IgM−IgD−Lin− MBCs from vaccinated C57BL/6J-Igha mice into antibody allotype-mismatched Ighb C57BL/6 mice and challenged recipients with either WNV-WT or WNV-K307E. Using IgG2a−specific secondary antibodies, we detected MBC-derived antibody by ELISA. Transferred MBCs preferentially produced DIII-LR–specific antibodies when mice were challenged with WNV-WT (Fig. 5 B, left), suggesting that homotypic MBCs recognizing the DIII-LR epitope outcompete the cross-reactive MBCs in a secondary response, perhaps as a result of higher affinity for antigen. In contrast, MBCs produced antibodies that recognized both DIII and DIII-K307E/T330I equivalently when recipient mice were challenged with WNV-K307E (Fig. 5 B, right).

To evaluate the ability of MBCs to recognize naturally occurring viral variants without relying on adoptive transfer experiments, we used a CD20 antibody that depletes MBCs but does not affect LLPCs (Uchida et al., 2004; Ahuja et al., 2008; DiLillo et al., 2008). In the absence of MBCs, mice would be predicted to respond more poorly to viral variants. To test this, mice were immunized with DIII of a related flavivirus, Japanese encephalitis virus (JEV). Although JEV and WNV share ~75% amino acid sequence identity (Sumiyoshi et al., 1987), the cross-reactive DIII–specific antibody generated after immunization with JEV DIII is not directed at the DIII-LR epitope and does not affect LLPCs (Uchida et al., 2004; DiLillo et al., 2008). 60 d after immunization with JEV-DIII, mice were administered CD20 or isotype control mAb. After 2 wk, the depletion of CD19+ B cells was confirmed by analysis of peripheral blood.

Figure 5. MBCs can respond to and neutralize variant virus. (A) Neutralization curves of WNV-WT or WNV-K307E with supernatant from stimulated MBCs. Data reflect four independent experiments performed in duplicate. Error bars indicate SD. (B) Igha MBCs (CD19+IgM−IgD−Lin−CCR6−CD80+) from WNV-vaccinated mice were sorted and transferred into allotopic Ighb recipients and challenged with WNV-WT or WNV-K307E 1 d later. Specificity of the MBC-derived antibody at different time points was determined by ELISA using an IgG2a−specific detection antibody. Data reflects eight mice per group and is the average of five experiments. (C) Mice were immunized with JEV-DIII and treated with B cell–depleting (CD20 mAb) or an isotype control antibody. Serum was tested before WNV infection (left) and at days 3 and 8 (right) after infection for IgG binding to WNV-DIII by ELISA. The data reflect a total of five to nine mice per group from three experiments. P-values were determined using an unpaired, two-tailed Student’s t test (*, P < 0.01). NS, indicates not significant.

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Mice were rested for 13 wk to allow restoration of naive CD19+ B cells before challenge with WNV. Antibody response to an irrelevant hapten was normal in these mice, demonstrating that the naive B cell pool had fully recovered, whereas in vitro MBC cultures revealed the continued absence of JEV-DIII–specific MBC at this time point (unpublished data).

To evaluate the contribution of MBCs to heterologous challenge, JEV-DIII–immunized control or CD20 mAb-treated mice were infected with WNV. Serum was tested before WNV infection (Fig. 5 C, left), at day 3, and at day 8 (Fig. 5 C, right) after infection for binding to WNV-DIII. Importantly, before and at day 3 after infection, both control and CD20 mAb–treated mice had equivalent levels of anti–WNV-DIII IgG antibody that was derived exclusively from the initial JEV-DIII immunization (Fig. 5 C, P > 0.6). Thus, normal antibody titers derived from LLPCs were present in the CD20 mAb–treated mice. However, CD20 mAb–treated mice exhibited a reduced anti–WNV-DIII IgG response at day 8 in comparison with control mice (P < 0.001). Thus, MBC generated by JEV–DIII immunization produced anti–WNV-DIII antibody after heterologous challenge despite the presence of nonneutralizing antibody derived from LLPCs.

Our data support a model in which broadly reactive MBCs are generated upon primary pathogen exposure, expanded without a requirement for further somatic hypermutation after secondary challenge, and preferentially neutralize variant viruses better than LLPC–derived serum antibody. It also remains possible that IgM+ memory cells undergo additional somatic hypermutation as they differentiate into IgG+ plasmablasts. Consistent with this, antibody-secreting cells were reported to have increased numbers of somatic mutations relative to resting IgG+ MBC (Wrammert et al., 2008). More comprehensive comparisons of these cell types from the same individual are required to fully address this issue.

Analysis of selected HIV-infected individuals with low levels of viremia revealed MBC specificities with a diverse repertoire (Scheid et al., 2009). Indeed, our immunoglobulin gene sequencing results of DIII–specific MBCs with WNV confirm this. Thus, although the frequency of any given individual MBC that produces highly potent neutralizing antibody may be low, the breadth of the repertoire allows for recognition of divergent epitopes and viruses, resulting in a capacity for broad neutralization. Our results support this concept, as the supernatant from stimulated MBCs neutralized both WT and variant WNV. Nonetheless, only a limited number of MBC clones that produce potently neutralizing antibodies and recognize a diverse array of HIV strains have been characterized (Walker et al., 2009; Corti et al., 2010; Wu et al., 2010). The ability of MBCs to target variant epitopes was revealed in our studies with MBC clones that preferentially recognized the mutant compared with the WT epitope in DIII. By LDA, we calculated that ∼10% of our DIII–specific MBC response recognized variant protein significantly better than WT protein. Our observation of MBCs preferentially recognizing a protein variant is analogous to that observed with the anti–HIV gp120 antibody HJ16, which recognizes a mutant (D368R) gp120 more strongly than the WT protein (Corti et al., 2010). This particular antibody is potently neutralizing across different clades, suggesting that antibodies with variant specificities may be functionally relevant in the context of the breadth of the response.

**MATERIALS AND METHODS**

**Viruses and quantification of viral burden.** The WNV strain (3000.0259) was isolated in New York in 2000 and passaged once in C6/36 Aedes albopictus cells. Mice were inoculated subcutaneously in the footpad with 10^7 PFU of WNV diluted in Hanks balanced salt solution and 1% heat-methylated fetal bovine serum. The WNV-K307E strain was isolated as an in vivo escape mutant of the neutralizing humanized mAb E16 from brains of RAG1−/− mice (Zhang et al., 2009) and was passaged once in C6/36 cells. CHIKV (La Reunion 2006-OPY1) was generated from an infectious clone (gift of S. Higgs, University of Texas Medical Branch, Galveston, TX) and passaged once in C6/36 cells. For viral RNA quantification, RNA was purified from serum samples using a Qia-Amp RNA recovery kit (QIAGEN) and copies were determined by TaqMan real-time reverse transcription PCR, as previously described (Diamond et al., 2003).

**Immunizations and immunodepletion.** The West Nile Innovator vaccine was obtained commercially (Valley Vet Supply). Mice were administered two doses (100 µl each) of vaccine by intraperitoneal injection at days 0 and 1. DIII (residues 300–406) of JEV was expressed in E. coli using a pET21 vector and purified by oxidative refolding as previously described (Oliphant et al., 2005). Mice were immunized with 25 µg JEV-DIII and 20 µg CpG (Integrated DNA Technologies) in alum subcutaneously. For depletion of B cells, mice were administered 50 µg of an antibody against CD20 (MB20-11; Uchida et al., 2004) by retroorbital injection. Depletion was confirmed by flow cytometric analysis of CD19+Ter119− cells from peripheral blood 2 wk after antibody treatment, and B cell reconstitution was confirmed at least 13 wk later.

**Mice.** C57BL/6 (WT and RAG1−/−) and C57BL/6-Igh-1- Thy1. Gpi1−/− mice were purchased from The Jackson Laboratory. All mice were bred in the animal facilities of the Washington University School of Medicine under pathogen-free conditions, and experiments were performed in compliance with the Washington University Animal Studies guidelines. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (Assurance Number: A3381-01).

**Flow cytometry and cell sorting.** Single cell suspensions were generated from spleen or bone marrow. Lymphocytes were isolated by differential centrifugation (850 g, 10 min at room temperature) through a ficoll gradient composed of Histopaque-1119 and Histopaque-1077 (Sigma-Aldrich). Cells were resuspended in PBS containing 2% FBS (HyClone) and incubated on ice with the directly conjugated antibodies. MAb were purified from the following hybridomas (Bio-X-Cell): 2C11 (anti-CD3), GK1.5 (anti-CD4), 53–6.7 (anti-CD8), 8C5 (anti-Gr-1), TER119 (anti-Ter119), and 1D3 (anti-CD19). Antibodies were conjugated to Pacific Blue succinimidyl ester (Invitrogen) according to the manufacturer’s instructions. The following antibodies were purchased from ebioscience: anti-B220 (6B2) conjugated to PE–Cy7; anti–CD19 (ID3) conjugated to Alexa Fluor 700; anti–CD80 (16–10A1) conjugated to PE; and anti-igD (11–26) and anti–GL7 (GL-7) conjugated to biotin. The following antibodies were purchased from Bio-X-Cell: 2C11 (anti-CD3), GK1.5 (anti-CD4), and anti–B220 (6B2) conjugated to Pacific Blue succinimidyl ester (Invitrogen) according to the manufacturer’s instructions. The following antibodies were purchased from ebioscience: anti-B220 (6B2) conjugated to PE–Cy7; anti–CD19 (ID3) conjugated to Alexa Fluor 700; anti–CD80 (16–10A1) conjugated to PE; and anti–IgD (11–26) and anti–GL7 (GL-7) conjugated to biotin. The following antibodies were purchased from BD: anti–IgM (11/41) conjugated to PE–Cy5.5; anti–CCR6 (140706) conjugated to biotin. The following antibodies were purchased from Bio-X-Cell: 2C11 (anti-CD3), GK1.5 (anti-CD4), and anti–B220 (6B2) conjugated to Pacific Blue succinimidyl ester (Invitrogen) according to the manufacturer’s instructions.

For staining of antigen-specific MBCs, cells were incubated with 0.8 µg of a chimeric fusion protein (DIII-Fc) of WNV-DIII (or a mutant DIII–K307E/T330I) linked to a human Fc–γR heavy chain with a point mutation in the N-linked glycosylation site that abolishes Fc–γR and C1q binding (Tao and Morrison, 1989). This protein was generated as follows: WNV DIII–Fc-
amino acids. Bone marrow cells were depleted of erythrocytes by differential
neutralization assay. Plaque reduction neutralization assays on BHK21-15
serological analysis. WNV-specific antibody titers in serum were calculated
ELISPOT assay. Mixed celluelore filter plates (Millipore) were coated initially
Neutralization assay. Plaque reduction neutralization assays on BHK21-15
elements were stained for surface expression of CD8, CD19, and B220 and CD138. Cells were washed, fixed with 4% paraformaldehyde for 10 min, and permeabilized with sonoun buffer (Hanks balanced salt solution containing 10 mM Hepes and 0.1% wt/vol sonoun) for 10 min. Intracellular DIII-specific antibody was detected using the same method as described for staining of antigen-specific MBCs.
Serological analysis. WNV-specific antibody titers in serum were calculated as previously described (Oliphant et al., 2007). In brief, 96-well plates (Nalge Nunc) were coated with recombinant protein (5 µg/ml DIII or DIII-K307/T330I overnight at 4°C. After blocking for 1 h with PBS supplemented with 2% BSA and 0.05% Tween 20, wells were incubated with recombinant WNV DIII protein in PBS was added overnight at 4°C. Plates were washed twice with PBS with 0.05% Tween 20 and incubated with 1 µg/ml biotinylated anti-IgG (Sigma–Aldrich) for 1 h. After washing, 2 µg/ml streptavidin-conjugated horseradish peroxidase (Invitrogen) was added to each well and plates were incubated for 1 h. Plates were developed using tetramethylbenzidine substrate (Dako) and quenched with H2SO4. The optical density was read at 450 nm and endpoint dilutions were calculated as 3 SD above the background (BSA-coated wells).
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