# 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.

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Abbreviations used: BCR, B cell receptor; CDR, complementarity-determining region; CHIKV, Chikungunya virus; DIII, domain III; JEV, Japanese encephalitis virus; LDA, limiting dilution analysis; LLPC, longlived plasma cell; LR, lateral ridge; MBC, memory B cell; WNV, West Nile virus. 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

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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, a time at which short-lived plasma cell-derived antibody titers wane, suggesting that the LLPC population was skewed toward recognizing the DIII-LR 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  $\gamma$ 1 Fc (DIII-Fc<sub>Hu</sub>, DIII-K307E/T330I Fc<sub>Hu</sub>). 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  $\mu$ l of immune serum, but not



# 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<sup>-</sup>CD138<sup>+</sup> 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 < 0.001; \*\*, P < 0.01; \*, P < 0.05) was determined by an unpaired, two-tailed Student's *t* test.

Brief Definitive Report



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<sup>+</sup> 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 DIIIspecific MBCs per 10<sup>6</sup> B cells (Fig. 3 A). This frequency increased to 57 at day 60 but waned to 7 DIII-specific MBCs per 10<sup>6</sup> 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

#### 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 plague 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  $\mu I$  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 gRT-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.

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-Fc<sub>Hu</sub> or DIII-K307E/T330I-Fc<sub>Hu</sub>. We defined

a population of isotype-switched CCR6<sup>+</sup>CD80<sup>+</sup> 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<sup>+</sup> 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 WNVimmune mice failed to produce antibody that recognized DIII. All DIII-specific MBCs were within the CCR6<sup>+</sup>CD80<sup>+</sup> gate, as adoptive transfer of isotype-switched CD80<sup>-</sup>CCR6<sup>-</sup> cells from WNV-immune mice or CD80<sup>+</sup>CCR6<sup>+</sup> cells from naive mice failed to produce anti-DIII antibody upon challenge with WNV-WT (Fig. S1 C). The kinetics of antigenspecific 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 DIII between MBCs and LLPCs. At each time point, MBCs, whether enumerated by LDA or flow cytometry, were enriched in antibodies that recognized DIII-K307E/T330I compared with LLPCs (Fig. 3 D). Therefore, cells in the MBC compartment



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.





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 complementaritydetermining 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  $(\sim 1\%)$  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 assess 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-Igh<sup>a</sup> mice into antibody allotype-mismatched Igh<sup>b</sup> C57BL/6 mice and challenged recipients with either WNV-WT or WNV-K307E. Using IgG2a<sup>a</sup>-specific secondary antibodies, we detected MBC-derived antibody by ELISA. Transferred MBCs preferentially produced DIII-LRspecific 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



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 reflects four independent experiments performed in duplicate. Error bars indicate SD. (B) IgH<sup>a</sup> MBCs (CD19<sup>+</sup>IgM<sup>-</sup> IgD<sup>-</sup>lin<sup>-</sup>CCR6<sup>+</sup>CD80<sup>+</sup>) from WNV-vaccinated mice were sorted and transferred into allotypic IgH<sup>b</sup> 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<sup>a</sup>-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.

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  $\sim$ 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 efficiently neutralize WNV (unpublished data). 60 d after immunization with JEV-DIII, mice were administered CD20 or isotype control mAb. After 2 wk, the depletion of CD19<sup>+</sup> B cells was confirmed by analysis of peripheral blood.

Mice were rested for 13 wk to allow restoration of naive CD19<sup>+</sup> 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 mAbtreated 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<sup>+</sup> memory cells undergo additional somatic hypermutation as they differentiate into IgG<sup>+</sup> plasmablasts. Consistent with this, antibody-secreting cells were reported to have increased numbers of somatic mutations relative to resting IgG<sup>+</sup> 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  $\sim 10\%$  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<sup>2</sup> PFU of WNV diluted in Hanks balanced salt solution and 1% heat inactivated 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  $\mu$ l each) of vaccine by intraperitoneal injection at days 0 and 1. DIII (residues 301–406) 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  $\mu$ g JEV–DIII and 20  $\mu$ g CpG (Integrated DNA Technologies) in alum subcutaneously. For depletion of B cells, mice were administered 50  $\mu$ g of an antibody against CD20 (MB20-11; Uchida et al., 2004) by retroorbital injection. Depletion was confirmed by flow cytometric analysis of CD19<sup>+</sup>Ter119<sup>-</sup> 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/6J-Igh-1<sup>a</sup> Thy1<sup>a</sup> Gpi1<sup>a</sup> 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 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. MAbs 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 BD: anti-IgM (11/41) conjugated to PE-Cy5.5; anti-CCR6 (140706) conjugated to Alexa Fluor 647; anti-CD138 (281-2) conjugated to biotin; and anti-CD138 (281-2) conjugated to PE. Streptavidin conjugated to Qdot-605 was purchased from Invitrogen. Cells were stained at 4°C for 30 min.

For staining of antigen-specific MBCs, cells were incubated with 0.8  $\mu g$  of a chimeric fusion protein (DIII-Fc\_Hu) of WNV-DIII (or a mutant DIII-K307E/T330I) linked to a human Fc- $\gamma 1$  heavy chain with a point mutation in the N-linked glycosylation site that abolishes Fc- $\gamma R$  and C1q binding (Tao and Morrison, 1989). This protein was generated as follows: WNV DIII-Fc

(DIII residues 586-705) proteins were expressed in CHO-S cells (Life Technologies). The respective N297Q and K307E/T330I mutations were introduced by site directed mutagenesis using the QuikChange method (Agilent Technologies) in the mammalian expression vector pCIneo (Promega) before transfection. The mutated DIII-Fc proteins were then purified from conditioned medium by sequential protein A affinity and size exclusion chromatography. After binding of DIII-Fc<sub>Hu</sub>, cells were washed and incubated with an Alexa Fluor 488-conjugated goat anti-human IgG antibody (Invitrogen). For intracellular staining of antigen-specific LLPCs, bone marrow cells were depleted of CD4, CD3, CD19, GR-1, and Ter119 using anti-rat IgG magnetic beads (Dynabeads; Invitrogen). Remaining cells 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 saponin buffer (Hanks balanced salt solution containing 10 mM Hepes and 0.1% wt/vol saponin) for 10 min. Intracellular DIII-specific antibody was detected using the same method as described for staining of antigen-specific MBCs.

For adoptive transfer experiments, non–B cell lineage cells and IgM<sup>+</sup>, IgD<sup>+</sup>, CD138<sup>+</sup>, and GL-7<sup>+</sup> cells were depleted with magnetic beads (Dynabeads). CD19<sup>+</sup>CCR6<sup>+</sup>CD80<sup>+</sup> class-switched IgM<sup>-</sup>IgD<sup>-</sup> cells were sorted into media (DME supplemented with 10% FBS, 10 mM Hepes, 10 mM nonessential amino acids, 10 mM sodium pyruvate, and penicillin and streptomycin) using a FACSAria II (BD). Cells were transferred adoptively by retroorbital injection at a fixed 3:1 donor to recipient ratio resulting in 30,000–140,000 total memory cells per recipient.

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  $\mu$ g/ml DIII or DIII-K307E/T330I) overnight at 4°C. After blocking for 1 h with PBS supplemented with 2% BSA and 0.05% Tween 20 at 37°C, diluted serum samples were added to wells and incubated for 1 h at room temperature. Plates were washed with PBS with 0.05% Tween 20 and incubated with 1  $\mu$ g/ml biotinylated anti-IgG (Sigma-Aldrich) for 1 h. After washing, 2  $\mu$ g/ml streptavidin-conjugated horse-radish peroxidase (Invitrogen) was added to each well and plates were incubated for 1 h. Plates were developed using tetramethylbenzidine substrate (Dako) and quenched with H<sub>2</sub>SO<sub>4</sub>. The optical density was read at 450 nm and endpoint dilutions were calculated as 3 SD above the background (BSA-coated wells).

ELISPOT assay. Mixed cellulose filter plates (Millipore) were coated initially with a capture antibody (100 µl WNV E111 Fab fragment [Sukupolvi-Petty et al., 2007] at 40 µg/ml in PBS for 2 h at 37°C), washed twice with 200 µl PBS, and then 40 µg/ml of recombinant WNV DIII protein in PBS was added overnight at 4°C. Plates were washed twice with PBS with 0.1% Tween-20, twice with PBS, and blocked for 1 h at room temperature with DME (Sigma-Aldrich) supplemented with 10% FBS, penicillin and streptomycin, 10 mM Hepes, 50  $\mu$ M  $\beta$ -mercaptoethanol, and 10 mM nonessential amino acids. Bone marrow cells were depleted of erythrocytes by differential centrifugation as described in Flow cytometry and cell sorting, resuspended at  $2 \times 10^7$  cells/ml, and serially diluted into wells. After 5 h at 37°C, plates were washed three times with PBS and three times with PBS + 0.1%Tween-20 (PBST). Wells were incubated with 1  $\mu$ g/ml biotinylated anti-IgG (Sigma-Aldrich) diluted in PBST with 1% FBS and incubated overnight at 4°C. After washing with PBST, wells were incubated with streptavidin conjugated to 1.25 µg/ml HRP diluted in PBST with 1% FBS. Plates were washed a final time with PBST and PBS. Spots were developed using a chromogen substrate (AEC [3-amino-9 ethyl-carbazole]; Sigma-Aldrich), and the reaction was quenched by thoroughly rinsing wells with water. Spots were enumerated using a stereomicroscope.

**Neutralization assay.** Plaque reduction neutralization assays on BHK21-15 cells were performed after mixing serial dilutions of serum with a fixed amount (10<sup>2</sup> PFU) of WNV as previously described (Diamond et al., 2003). For MBC neutralization assays, MBCs were cultured as described in the next section, and pooled supernatant was concentrated using a centrifugal filter device (Millipore) before mixing with virus. The concentration of IgG was determined by

quantitative ELISA to normalize data between experiments. Neutralization data were fit to a variable slope to calculate the EC50 values by linear regression.

MBC LDA cultures. WNV-specific MBC frequencies were determined by measuring the reactivity of supernatants by LDA to recombinant protein by ELISA and was adapted from published protocols (Amanna and Slifka, 2006). Single cell splenocyte suspensions were generated from WNV-infected mice. Erythrocytes were lysed (ACK lysing buffer; Invitrogen) and CD19<sup>+</sup> B cells isolated by positive selection using magnetic beads (Miltenyi Biotec). B cells were resuspended in LDA medium (RPMI, 10% FBS, 1× penicillinstreptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes, and 50  $\mu$ M  $\beta$ -mercaptoethanol). B cells were cultured in twofold serial dilutions starting at 50,000-120,000 cells per well in 96-well flat-bottom plates in the presence of Staphylococcus aureus Cowan strain (SAC) lysate (diluted 1:10,000; Sigma-Aldrich), 1 µg/ml pokeweed mitogen lectin (Sigma-Aldrich), 10 µg/ml lipopolysaccharide (from E. coli 0.111:B4; Sigma-Aldrich), 1 µg/ml phosphorothioated murine CpG (5'-TCCAT-GACGTTCCTGATGCT-3'; Integrated DNA Technologies), 16 ng/ml mIL-2, 10 ng/ml mIL-6, 17 ng/ml mIL-10 (PeproTech), and 5,000 cells per well of irradiated syngeneic splenocytes. Plates were sealed with an adhesive lid to prevent evaporation and incubated at 37°C and 5% humidified CO2 for 6 d. To calculate the frequency of WNV-specific MBC that produced IgG, half-area 96-well plates (Corning-Costar) were coated with 5 µg/ml WNV E protein overnight at 4°C. Plates were washed and blocked with PBS-T for 1 h at 37°C. Supernatants from LDA plates were added to the ELISA plates (15 µl per well), and detection of WNV-specific antibody was conducted as described in ELISPOT assay. Positive wells were defined as wells that scored twofold over the mean OD of negative control wells (wells containing irradiated splenocytes and supplements alone). Supernatant from cultured naive splenocytes did not score positive, and only LDA that contained at least two positive wells at the highest dilution were analyzed.

Single-cell MBC cultures and reexpression of immunoglobulin genes. NIH 3T3 cells (American Type Culture Collection) were retrovirally transduced with a murine stem cell retrovirus (Bhattacharya et al., 2002) expressing CD40L-IRES-GFP or BAFF-IRES-human CD4. Antigen-specific MBCs were sorted using a FACSAria II into individual wells of a flatbottom 96-well plate containing LDA medium supplemented as described in the previous section. Each well was seeded with 5,000 mitomycin Ctreated BAFF+CD40L+ NIH 3T3 cells. After 6 d, supernatant was harvested and tested for reactivity to DIII or DIII-K307E/T330I by ELISA and total RNA was extracted with Trizol (Invitrogen). cDNA was generated, and immunoglobulin heavy and light chain genes were amplified by PCR and sequenced as previously described (Tiller et al., 2009). Sequences were analyzed for immunoglobulin genes using IgBlast. Primers for specific Vh, Vĸ, Jh, and J $\kappa$  segments were used to amplify variable regions for both heavy and light chain genes (Tiller et al., 2009), and these segments were cloned inframe into pEF1\alpha-myc-His expression vectors (Invitrogen) downstream of a consensus immunoglobulin leader sequence and upstream of either the mouse Cy1 or C $\kappa$  constant region. Six individual clones of both heavy and light chain genes were resequenced, and individual heavy and light chain expression constructs containing the consensus sequence without point mutations were cotransfected into 293T cells by calcium phosphate transfection. Culture supernatant was harvested 3 d after transfection for ELISA assays.

**Online supplemental material.** Supplemental material shows the gating and sorting strategy of splenic MBC ex vivo (Fig. S1, A and B) and adoptive transfer studies in  $RAG1^{-/-}$  mice (Fig. S1C). Gene family usage (Table S1) and immunoglobulin CDR sequences (Table S2) from WNV-specific MBC clones also are described. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110740/DC1.

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S. Johnson is an employee of MacroGenics, which has licensed monoclonal antibodies against WNV from Washington University for possible commercial use. M. Diamond is a paid consultant for MacroGenics. The authors have no other competing financial interests.

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### SUPPLEMENTAL MATERIAL.

### Purtha et al., http://www.jem.org/cgi/content/full/jem.20110740/DC1



**Figure S1.** Specificity of DIII<sup>+</sup> MBC as analyzed by flow cytometry. (A) Gating strategy of splenic MBCs. (B) MBCs from WNV-vaccinated or naive mice were sorted and cultured for 6 d. Two populations within the CD19<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup>CD80<sup>+</sup>CCR6<sup>+</sup> gate were sorted from WNV-vaccinated mice: DIII<sup>+</sup> or DIII<sup>-</sup>, DIII-K307E/T330I<sup>-</sup> (MBC). Specificity of secreted antibody from MBC culture supernatant was determined by ELISA. Data are representative of five mice from two independent experiments. Error bars indicate SD. (C) Isotype-switched IgM<sup>-</sup>IgD<sup>-</sup> MBC subsets (B220<sup>+</sup>CCR6<sup>+</sup>CD80<sup>+</sup> or B220<sup>+</sup>CCR6<sup>-</sup>CD80<sup>-</sup>) from vaccinated or naive mice were transferred into *RAG1<sup>-/-</sup>* recipient mice. MBC-derived antibody was determined by ELISA using an IgG-specific detection antibody at different time points after infection. The data reflect four to five mice per group from three independent experiments. Horizontal bars indicate the mean. The dashed line indicates the limit of the detection assay.

Clone	Epitope	Vh	Dh	Jh	Vк	Jĸ
E16	LR	J558.17	Dsp2.2	Jh2	Vĸ19-25	Jĸ5
E24	LR	Vha1	Dst4.3	Jh2	Vк19-25	Jк1
E34	LR	Vha1	Dsp2.7	Jh2	Vк19-25	Jĸ5
8A2	LR	V186.2	Dsp2.2	Jh3	Vк19-17	Jĸ2
9A10	LR	V165.1	Dsp2.13	Jh2	Vк19-25	Jĸ5
9B2	LR	V165.1	Dfl16.1j	Jh3	Vк19-25	Jк1
9C4	LR	J588.39.129	Dsp2.12	Jh4	Vк19-25	Jк1
9F7	LR	Mvarg2	Dfl16.1	Jh1	Vк19-25	Jĸ5
9E12	LR	V165.1	Dsp2.2	Jh2	Vк19-25	Jĸ5
1B2	Cross-reactive	VhSM7.1.44	Dfl116.2	Jh2	Vк23-48	Jĸ2
1G11	Cross-reactive	Vh7183.7.10	Dsp2.9	Jh2	Vк23-39	Jĸ5
2D8	Cross-reactive	Mvarg2	Dfl116.1	Jh1	Vĸ21-5	Jĸ2
2F2	Cross-reactive	J558.39.129	Dsp2.12	Jh4	Vкbd-2	Jк1
4C1	Cross-reactive	J558.74.176	Dsp2.8	Jh3	Vкat4	Jĸ5
8C5	Cross-reactive	Vh7183.21b	Dsp2.9	Jh3	Vк8-27	Jĸ2
8D3	Cross-reactive	J558.67.166	Dst4.3	Jh3	Vкat4	Jĸ4
8G3	Cross-reactive	J558.5A	Dsp2.13	Jh3	Vк19-17	Jĸ1
10B2	Cross-reactive	J558.39.129	Dsp2.11	Jh4	Vк21-2	Jĸ1

Table S1. DIII-specific MBCs use a diverse repertoire of heavy and light chain variable genes

Heavy and light chain gene regions were amplified by RT-PCR from cultures initiated from single WNV-specific MBC clones. Immunoglobulin gene families from heavy and light chains are listed for both DIII Ir-specific and DIII cross-reactive MBC clones.

Table S2.	DIII-specific	MBCs use a	wide variety of	CDRs to	recognize antigen
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Clone	IgH CDR1	IgH CDR2	IgH CDR3	lgk CDR1	lgk CDR2	lgk CDR3
E16	DY WIE	DILCGTGRT <i>R</i> YNEKLKA	SASYGDYADY	KASQDVSTAVA	WASTRHT	QQHYTT <u>P</u> LT
E24	SYDIN	WIYPGDGRIKYNEKFKG	<u>GG</u> SSG <u>T</u> YFDY	KASQDVSTAVA	WASTRHT	QQHYSNP <u>P</u> T
E34	SYDIN	WIFPGDGRIKYNEQIKD	<u>A</u> SYYGS <u>I</u> FDY	KASQDVSTAVA	WASTRHT	QQHYTTPLT
8A2	NYWIH	RIDPNSGRTKYNERFMS	<u>DG</u> YDYD <u>LFI</u> Y	KASQDVTTAVA	SATYRYT	QQHFST <u>P</u> YT
9A10	SYWIT	HIFPDSGRTHYNETFKS	<u>EGGN</u> GDY <u>V</u> DY	KASQDVSTAVA	WASTRHT	QHHYNI <u>PP</u> T
9B2	NYWIT	DIYP <b>Y</b> SG <b>RC</b> NYNEKFKS	<u>ORG</u> D <u>GDI</u> FD	KASQDVSTAVA	WASTRHT	QQHYSIP <u>R</u> T
9C4	DYSIH	VINPNYGTISYNPKFKG	YSY <u>YG</u> YYSMDY	KASQDVSTAVA	WASTRHT	QQHYSIP <u>R</u> T
9F7	STDIN	WIFPGDGRTRYNEKFKG	<u>SV</u> YDGS <u>S</u> FDV	KASQDVSTAVA	WTSTRHT	QQHYTTPLT
9E12	SYWIT	NTYPGSGSNNYNEKFKR	<u>EG</u> LR <u>GI</u> YFDY	KASQDVSTAVA	WASTRHI	QQHYRNP <u>P</u> T
1B2	DYYMH	RIDPEDGDTEYAPKFQG	YYG <u>R</u> DY	RASQSIGTSIH	YASESIS	QQSNSW <u>P</u> YT
1G11	SYGMS	NISGGYTYIYYPDSVKG	MMVTYFDF	RARDSIGDYLH	FASQSIS	QNGHSFPLT
2D8	SYDLN	WVYPKDGNTNYNERFKG	<u>SVG</u> YGSSHWYFDV	RASESVDSYGNSFMH	RASNLES	QQSNED <u>P</u> YT
2F2	DYSIH	VINPNYGTISYNPKFKG	YSY <u>YG</u> YY <b>S</b> MDY	KSSQSLLDSDGKTYLN	LVSKLDS	COGTHFPOT
4C1	DYTLH	WFHPGSNTLKYNEKFKD	<u>PQI</u> NY <u>P</u> FAY	SASSSVDYLY	DTSNLAS	QQWSSY <u>PF</u> T
8C5	SYAMS	TISDGGSYTYYPDNVKG	<u>VG</u> DGY <u>VL</u> AWFAY	KSSQSVLYSSKQKNYLA	WASTRES	HQYLS <u>S</u> YT
8D3	NYWMH	MIHPNSGSINYNEKFKS	S <u>GTDG</u> FPY	SASSSVNYMY	DTSNLAS	QQWSSSPFT
8G3	DYNIH	YNVPNTGITSYSQKFKG	<u>KD</u> L <u>HLRGFP</u> Y	KGSQDVSTAVA	SASYRYT	QQHYSTP <u>P</u> T
10B2	DYIIN	VINPNYGTVSYNQKFKG	YR <u>WV</u> LDYVMDY	RASETVDNYGISFMN	AASNQGS	QQTKEV <u>P</u> WT

Bold = somatic mutations, underline = N or P nucleotide additions, italics = contact residues with DIII (Nybakken et al., 2005). Immunoglobulin CDR sequences from heavy and light chains are listed for both DIII Ir-specific and DIII cross-reactive MBC clones. For comparison, sequencing data from previously generated DIII Ir-specific monoclonal antibodies E16, E24, and E34 is included (Oliphant et al., 2005).