Viral induction of AID is independent of the interferon and the Toll-like receptor signaling pathways but requires NF-κB

Polyxeni Gourzi, Tatyana Leonova, and F. Nina Papavasiliou

Laboratory of Lymphocyte Biology, The Rockefeller University, New York, NY 10021

Activation–induced cytidine deaminase (AID) is expressed in germinal centers of lymphoid organs during immunoglobulin diversification, in bone marrow B cells after infection with Abelson murine leukemia retrovirus (Ab–MLV), and in human B cells after infection by hepatitis C virus. To understand how viruses signal AID induction in the host we asked whether the AID response was abrogated in cells deficient in the interferon pathway or in signaling via the Toll–like receptors. Here we show that AID is not an interferon responsive gene and abrogation of Toll–like receptor signaling does not diminish the AID response. However, we found that NF–κB was required for expression of virally induced AID. Since NF–κB binds and activates the AID promoter, these results mechanistically link viral infection with AID transcription. Thus, induction of AID by viruses could be the result of several signaling pathways that culminate in NF–κB activation, underscoring the versatility of this host defense program.

CORRESPONDENCE
F. Nina Papavasiliou:
papavasiliou@rockefeller.edu

Activation-induced cytidine deaminase (AID) is a cytidine deaminase thought to function directly as a DNA mutator initiating somatic hypermutation and class switch recombination (CSR) of Ig genes. In addition to this role in producing antibody diversification within the vertebrate adaptive immune system, AID also mediates a form of host response against viral infection. Specifically, AID is induced in primary B cells in response to infection by a transforming retrovirus, Abelson murine leukemia retrovirus (Ab-MLV). As a result of AID induction, the proliferation of infected host cells is significantly restricted both in vitro and in vivo. Therefore, in addition to its roles in somatic hypermutation and CSR, AID is active in the innate antiviral response and serves to limit the proliferation of tumors of viral etiology.

A growing body of evidence indicates that AID functions directly as a DNA mutator to initiate Ig diversification. At the same time, inappropriate or ectopic expression of AID can lead to genomic instability (1, 2). It is therefore not surprising that AID needs to be tightly regulated at several levels. AID transcription is controlled by the synergistic action of several transcription factors, four of which have been identified: E47 (3), STAT6, NF-κB (4), and Pax5 (5). All of these are necessary, but none is sufficient to activate AID expression; thus, it is

likely that additional regulators of AID expression remain to be identified. AID activity is also controlled by posttranslational modifications (e.g., phosphorylation; references 6–9) and by subcellular localization (most AID is cytoplasmic although only the nuclear fraction appears to have DNA deamination activity; references 10–12).

AID induction is also part of the host response to infection by hepatitis C virus (13) and by herpes EBV, which maintains AID expression by latent membrane protein-1 signaling during the establishment of latency (14) until later stages of the EBV growth program down-regulate AID expression to unleash B cell proliferation (15). To understand how different viruses induce the AID host response, we asked whether AID expression during viral infection involved signaling by the two main antiviral pathways, namely the interferon pathway and the Toll-like receptor (TLR) pathway. Here we show that AID is not an interferon-responsive gene: treatment of B cells with type I or type II interferon does not lead to AID expression. Conversely, the AID response is not abrogated in IFN-αR–deficient or IFN-γ–deficient animals. In addition, even though TLR engagement leads to AID expression (this study and reference 16), the AID response is not abrogated in animals deficient in TLR signaling

(e.g., MyD88$^{-/-}$ or MyD88$^{-/-}$Trif$^{-/-}$ mice). Our results strongly suggest that neither of the two main antiviral pathways is required for the expression of virally induced AID.

We did find that NF-κB was required for the AID response to Ab-MLV infection of bone marrow B cells. The AID gene contains two NF-κB binding sites, both of which are occupied during CSR (4). NF-κB (p50) deficiency severely diminishes CSR both in vitro (this study) and in vivo (17, 18). Our data suggest a direct link between NF-κB activation and AID expression in infected B cells.

RESULTS AND DISCUSSION
AID up–regulation does not require intact interferon signaling

To determine the signaling cascade that induces AID expression in Ab-MLV–infected cells, we first asked whether it required the v-abl kinase, which is the single protein product made by the retrovirus and the cause of transformation. We therefore infected cells with the highly related Moloney murine leukemia virus (Mo-MLV), which does not encode the v-abl kinase. We found that Mo-MLV infection of bone marrow cells resulted in AID up-regulation, although the infected cells did not become transformed (Fig. 1 A).

Several other viruses induce or maintain AID expression upon infection (most prominently EBV [14, 19] and hepatitis C virus [13]), suggesting that these viruses activate a common signaling cascade culminating in AID expression in host cells. We first asked whether virally induced interferons signal AID expression but found that bone marrow B cells treated with

recombinant type I or type II interferon (IFN-αβ and IFN-γ) did not express AID (unpublished data). Therefore, AID is not an interferon–inducible gene. Conversely, we found that bone marrow B cells from IFN-γ–deficient mice not only expressed AID as a consequence of infection but expressed higher amounts of AID at earlier time points after infection than wild-type congenic controls (Fig. 1 B and reference 20). B cells from IFN-α receptor–deficient animals (IFN-αR$^{-/-}$) also expressed AID at wild-type levels upon infection with Ab-MLV. We conclude that viruses induce AID by a mechanism that does not depend on signaling through the interferon cascade.

AID is expressed in bone marrow B cells after ligation of endosomal TLRs

AID is expressed in B cells stimulated to class switch by treatment with LPS, a ligand for the cell surface TLR4. We therefore investigated whether viruses induce AID expression in the host is by signaling through TLRs.

Although TLR4 has been linked to recognition of several viruses (21), the TLRs most commonly associated with viral recognition reside in the endosomes and bind viral nucleic acid. We therefore tested whether ligation of endosomal TLRs would induce AID expression. We found that stimulation of bone marrow cells with CpG-containing single strand DNA (a ligand for TLR9), resulted in AID expression within 24 h (Fig. 2 A and reference 16). Transfection of bone marrow cells with synthetic double strand RNA (a ligand for TLR3) also induced AID expression, albeit with slower kinetics (Fig. 2 B). We conclude that, not only TRL4, but also two endosomal TLRs can lead to induction of AID expression in B cells.

Abrogation of TLR signaling does not affect virally induced AID expression

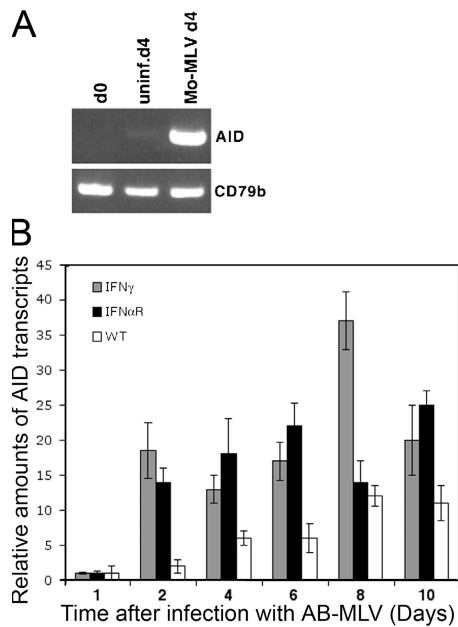Most TLRs signal through the adaptor molecule MyD88, with the exception of TLR3, which utilizes Trif (22). We

Figure 1. AID up–regulation does not require intact IFN signaling. (A) Mo-MLV infection of mouse bone marrow results in AID upregulation. CD79b expression is a loading control for the RT-PCR reaction. (B) IFN-γ and IFN-αR–deficient bone marrow cells induce AID expression upon viral infection.
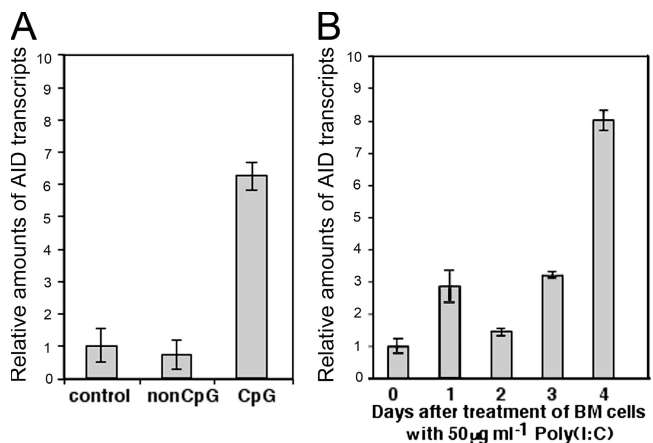

Figure 2. AID is up-regulated in bone marrow B cells after ligation of endosomal TLRs. (A) AID expression after transfection of BM cells with CpG for 24 h. (B) Time-course of AID expression after transfection of BM cells with double strand RNA.

therefore asked whether Ab-MLV could activate the AID response in B cells from mice deficient in one (MyD88$^{-/-}$) or both (MyD88$^{-/-}$Trif$^{-/-}$) of these adaptors. Surprisingly, we found that AID was still induced in B cells deficient in TLR signaling (Fig. 3 A). Furthermore, Ab-MLV could transform TLR signaling–deficient B cells with efficiencies indistinguishable from those seen in congenic wild-type controls (Fig. 3 B). Finally, MyD88-deficient mice were indistinguishable from congenic wild-type controls in their ability to up-regulate Rae-1 ligands (a hallmark of the AID host response to Ab-MLV infection; unpublished data).

In mice deficient in TLR signaling, B cells fail to properly respond to immunization either with T cell–dependent or T cell–independent antigens (23). However, these defects are attenuated when such B cells are activated in vitro: B cells from MyD88$^{-/-}$ mice can switch from IgM to IgG2a after stimulation with CD40 and IL-4 to levels similar to those seen in wild-type B cells (24 and unpublished data). To investigate the possibility that the AID response defect in MyD88$^{-/-}$ B cells is only evident in vivo, we monitored disease progression in mice engrafted with either infected wild-type B cells or infected MyD88-deficient B cells. We found that mice receiving infected wild-type bone marrow succumbed to Abelson disease with kinetics that were indistinguishable from those of mice receiving infected MyD88-deficient bone marrow (Fig. 3 C). We conclude that the AID response to viral infection does not depend on the TLR signaling pathway in vitro or in vivo. In contrast, AID-mediated adaptive immune responses appear to require an intact TLR signaling pathway, suggesting distinct pathways for AID expression in adaptive and innate immune responses (23, 24).

NF-κB is required for host expression of AID upon viral infection

Several known stimuli such as CD40 ligation, TLR ligation, cytokine stimulation, and now a component of viral infection induce AID expression in B cells. We have shown that the signaling pathways used by viral infection to induce AID expression in B cells are not the ones which induce Ig diversification (e.g., TLR4 ligation). Therefore, the pathways controlling AID transcription must converge in common elements further downstream.

NF-κB is required for the Ig diversification program (18) and can be activated by several overlapping though distinct signaling pathways, many of which are activated by viral infection (25). To determine whether virally induced AID expression requires NF-κB, we used Ab-MLV to infect bone marrow cells from mice deficient in the p50 subunit of the NF-κB heterodimer. We found that such NF- κB–deficient B cells were completely unable to up-regulate AID expression (Fig. 4 A). In addition, such cells were more susceptible to viral transformation (26 and unpublished data). Thus, the host response of NF-κB–deficient B cells to Ab-MLV infection and transformation closely parallels the one reported for AID-deficient B cells (20), suggesting a genetic link between the two.
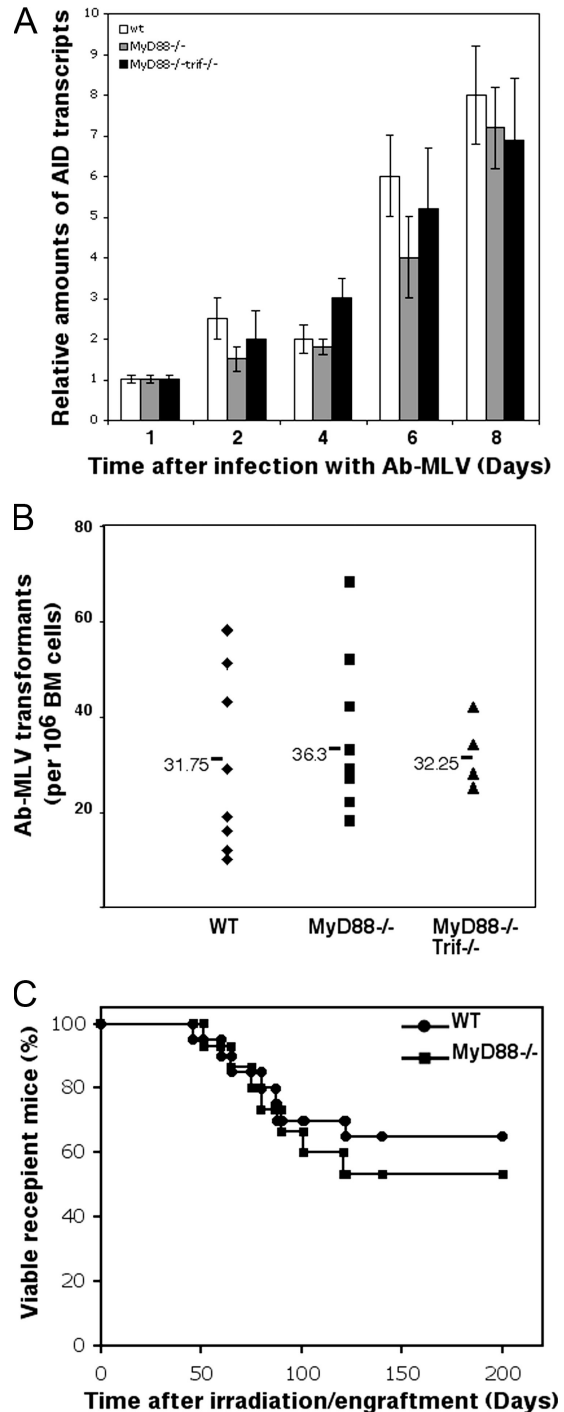

Figure 3. Abrogation of TLR signaling does not diminish the expression of virally induced AID. (A) Bone marrow cells from MyD88$^{-/-}$ and MyD88$^{-/-}$Trif$^{-/-}$ mice induce AID expression upon infection with Ab-MLV. (B) B cells deficient in TLR signaling are susceptible to transformation by Ab-MLV. (C) Mice deficient in MyD88 are as susceptible as their wild-type counterparts to death after Ab-MLV infection.

NF-κB is necessary for AID-mediated Ig diversification in vivo (17). To address the role of NF-κB in Ig diversification in vitro, we stimulated cells to undergo CSR. Using two

different stimulation protocols, we found that CSR was drastically diminished in NF-κB–deficient B cells compared with congenic controls (Fig. 4 B). NF-κB is thought to regulate recombination to specific Ig isotypes (18, 27) but has also been reported to directly modulate AID expression levels by binding and activating the AID promoter (4). Indeed, we found that NF-κB is necessary for optimal AID expression in splenic B cells stimulated to undergo CSR: NF-κB–deficient cells stimulated with CD40 and IL-4 or LPS and IL-4 showed a substantial reduction in AID expression compared with wild-type controls (approximately a 10-fold reduction; Fig. 4 C and unpublished data). Collectively, these data suggest that NF-κB directly modulates AID gene transcription both during CSR and in the context of viral infection.

NF–κB–p50 is recruited to the mouse AID promoter to activate AID expression during infection by Ab–MLV

The human AID promoter contains two NF-κB binding sites, and nuclear extracts from human cell lines that are stimulated to express AID can supershift oligonucleotides containing both sites. These in vitro experiments have suggested that AID induction during CSR can be at least in part attributed to NF-κB (4). In contrast, a scan of the mouse AID promoter revealed a single NF-κB binding site (gaGGGActtgtc; capital letters refer to the core binding

sequence), corresponding to positions -1447 to -1434 from the promoter. To directly confirm the link between viral AID induction and NF-κB activation, we asked whether this site was occupied in vivo by NF-κB–p50 after infection of B cells with Ab-MLV. Using chromatin immunoprecipitation we found that the site was not occupied by NF-κB before infection (Fig. 5, day 0). However, the site was clearly bound by NF-κB at day 6 after infection (Fig. 5 A), a time point which coincides with the initial peak of AID expression (Figs. 3 and 4 and reference 20). Thus, our experiments demonstrate that, in vivo, NF-κB is recruited to the AID promoter during infection, with kinetics that parallel those of AID induction.

To demonstrate that NF-κB was not only recruited to, but could also transactivate the AID promoter, we infected bone marrow cells with Ab-MLV together with retroviruses carrying the AID promoter upstream of a luciferase reporter gene. Bone marrow cells infected with both Ab-MLV (expressing GFP) and with reporter virus (expressing RFP) were sorted on the basis of dual color expression and were analyzed for luciferase activity during infection. We could detect above-background luciferase activity but only in bone marrow cells coinfected with Ab-MLV and a retroviral construct expressing luciferase under the control of the wild-type AID promoter (Fig. 5 B, wt κB Luc). In bone marrow cells
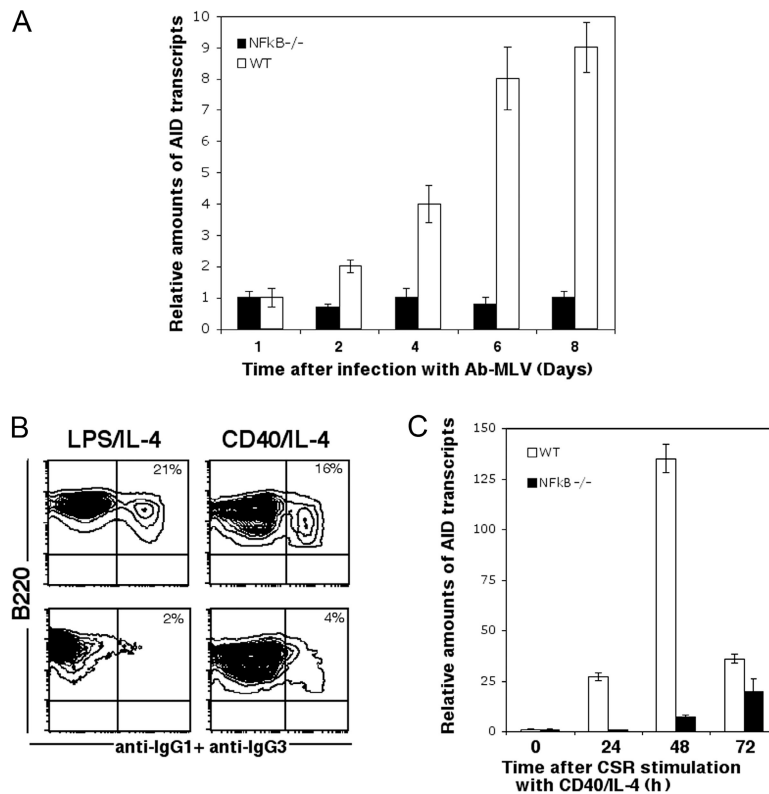
Figure 4. NF–κB is required for expression of virally induced AID. (A) Bone marrow cells from NF-κB (p50)$^{-/-}$ mice cannot induce AID expression upon infection with Ab-MLV. (B) NF-κB (p50)$^{-/-}$ splenic B cells are defective in CSR. Levels of CSR to IgG1 and IgG3 are shown 72 h after stimulation with LPS and IL-4 or CD40 and IL-4. (C) NF-κB is required for optimal AID expression in switching B cells.
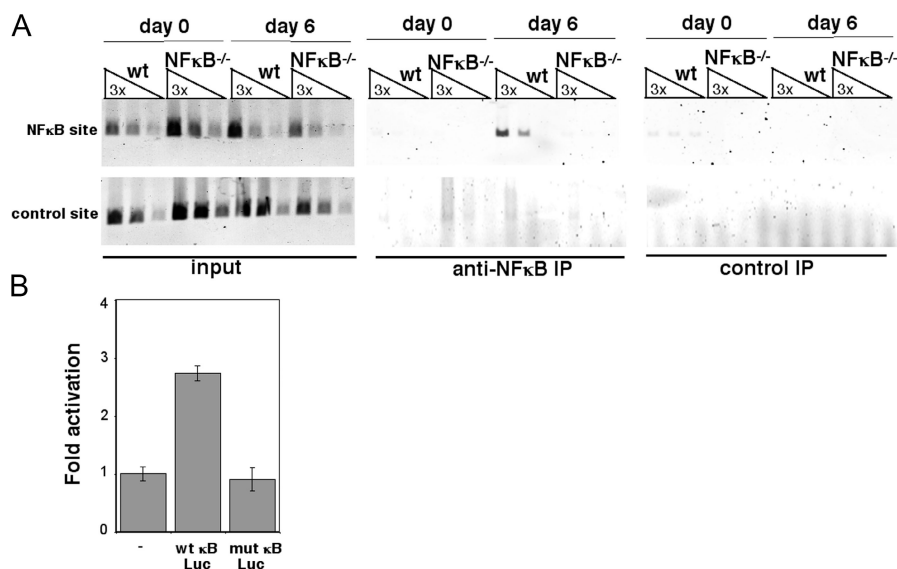
Figure 5. NF-κB–p50 is recruited to the mouse AID promoter to directly activate AID expression during infection by Ab-MLV. (A) Occupancy of the NF-κB binding site present in the AID promoter is assessed by ChIP before (day 0) or during (day 6) infection of wild-type or NF-κB–p50–deficient animals. Occupancy of an NF-κB binding site present in the nearby APOBEC1 promoter was evaluated as a negative control. Threefold titrations are shown for input, anti–NF-κB immuno- precipitation, and isotype control immunoprecipitation samples. (B) AID promoter activity in infected cells, in the presence or absence of the κB binding site. Bone marrow cells were coinfected with Ab-MLV and with a retroviral luciferase reporter construct containing either the wild-type κB site (wt κB Luc) or a mutated once κB site (mut κB Luc). Promoter activity for each construct was measured in relative light units per μg of protein lysate. Results are means ± SD ($n = 3$).

coinfected with Ab-MLV and a reporter construct in which the κB site was mutated (from gaGGGActtgtcc to gaAAAA- cttgtc), luciferase expression was at baseline levels (Fig. 5 B, mut κB Luc). Thus, during viral infection, NF-κB binds and directly activates the AID promoter.

We have found that AID expression in response to viral infection and transformation requires NF-κB. Many antiviral cascades depend on distinct signaling components ultimately linked by NF-κB activation (25). Our results suggest that viral induction of AID could use several signaling pathways that culminate in NF-κB activation, underscoring the versatility of this host defense program.

MATERIALS AND METHODS

Cells and mice. Bone marrow cells were flushed from the femurs and tibias of 5–8-wk-old mice and were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 20% heat-inactivated FCS and 50 μM 2-mercapto-ethanol (Sigma-Aldrich).

AID–deficient BALB/c Byj mice were obtained from M. Nussenzweig (The Rockefeller University). BALB/c Byj congenic controls and NF-κB– deficient (p50$^{-/-}$) C57BL/6 mice and IFN-γ–deficient C57BL/6 mice with appropriate congenic controls were obtained from The Jackson Laboratories. IFN-αR–deficient animals were a gift from David Levy (New York University Medical School, New York, NY), and MyD88-deficient mice and MyD88$^{-/-}$Trif$^{-/-}$ mice along with congenic controls were a gift from S. Akira (via M. Nussenzweig). All animal research has been approved by the Rockefeller University Institutional Animal Care and Use Committee and was in compliance with the relevant federal guidelines.

Virus production and infections. Replication-deficient Ab-MLV was generated by transfection of 293T cells with pMIG–Ab-MLV retroviral construct (a gift from Naomi Rosenberg, Tufts University School of Medicine, Boston, MA) and pCL packaging DNA (1:1 ratio) using Lipofectamine according to the manufacturer's protocol. Cells were cultured for 48 h, and the supernatant was filtered through a 45-μm filter and used as viral stock for infection of target cells.

Viral titers (infectious U/ml) were determined by counting the number of gfp$^+$ cells that arose after infection of 3T3 cells with different dilutions of viral stock (multiplied by the dilution factor). For Ab-MLV infections, 2×10^6 bone marrow cells were challenged for 2 h with a multiplicity of infection of 0.1.

Agarose transformation assay of bone marrow cells. 2×10^6 freshly prepared bone marrow cells from wild-type and congenic MyD88$^{-/-}$ and MyD88$^{-/-}$Trif$^{-/-}$-deficient mice were infected with equivalent titers of Ab-MLV for 4 h and seeded in 0.3% agar (United States Biological) containing RPMI–20% FCS–50 μM β-mercaptoethanol as described previously (28). Macroscopic colonies of transformed pre–B cells were counted between 9 and 12 d postinfection.

Tumorigenicity assay. Bone marrow cells were isolated from 5–6-wk-old wild-type and congenic MyD88$^{-/-}$ mice and were infected for 2 h with equivalent titers of replication-deficient Ab-MLV. After infection, the cells were washed once with PBS and were injected intravenously into lethally irradiated (800 rads) C57BL/6 8–10-wk-old female mice. Five recipients from each strain were injected with mock-infected bone marrow cells, and 20 recipients from each strain were injected with virus–infected bone marrow cells (1 million bone marrow cells/mouse). Mice were monitored daily and analyzed soon after death by gross pathology, histology, and FACS.

Flow cytometry analysis. For the quantification of gfp$^+$ B cells, we stained bone marrow cultures with anti–mouse B220 PE-conjugated antibody (BD Biosciences). 7-amino actinomycin D was used for dead cell exclusion. FACS analysis was done on a FACSCalibur (Becton Dickinson) using Cellquest software (Becton Dickinson).

Quantitative (real-time) PCR. Bone marrow cells from 3–5-wk-old IFN-γ$^{-/-}$, IFN-αR$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$Trif$^{-/-}$ mice were infected with Ab-MLV as described in earlier paragraphs. The cells were collected at different days postinfection and stained with anti–mouse B220 PE-conjugated antibody. Cell sorting was performed on a FACSVantage (Becton Dickinson).

Total RNA was extracted from gfp$^+$B220$^+$ samples using Trizol (Invitrogen), according to the manufacturer's instructions. RNA was treated with DNase I (Promega) at 1 U/μg. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) and random hexamers. Primers for AID amplification were as in reference 20. Results were normalized to GAPDH.

Reagents. Phosphorothioate-stabilized CpG oligos (TCCATGACGTTC-CTGATGCT) and phosphorothioate-stabilized non-CpG oligos (GCTTG-ATGACTCAGCCGGAA) were purchased from MWG Biotech. Interferon α-A/D was purchased from Sigma-Aldrich. Double stranded poly(I):poly(C) was purchased from GE Healthcare.

NF-κB chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitation (ChIP) assays were preformed using the ChIP kit from Upstate Biotech in conjunction with antibodies to NF-κB–p50 (also from Upstate Biotechnology) or with an isotype control antibody. The assay was done according to the manufacturer's instructions.

The following oligonucleotide pairs were used: 5–NF-κB, 5′-CCCCT-CCACTGCCAAGCACAGC and 3–NF-κB, 5′- GCCCCCATCCTCCT-TCTTCCTC to amplify a 354-bp region of the AID promoter surrounding the NF-κB binding site and 5-APO, 5′-GCCCAATGTGGGTGGTGC-CAC-3′ and 3-APO, 5′-CTCAGATTTGAGATCATTCTCTCCAAG to amplify a 313-bp region of the APOBEC1 promoter, which includes an APOBEC1-specific NF-κB binding site. PCR amplification reactions within the linear range were loaded onto agarose gels that were subsequently stained with SyBR green dye (Molecular Probes) and visualized using a Typhoon Fluor Imager.

NF-κB luciferase reporter assays. For NF-κB luciferase reporter assays, we constructed replication-deficient retroviral constructs carrying the firefly luciferase gene downstream the AID promoter region (nucleotides −1500 to −1) with the κB binding site intact (Fig. 5 B, wt κB Luc) or mutated (Fig. 5 B, mut κB Luc). These constructs were transfected into 293T cells to produce two varieties of murine stem cell virus–red virus, which were used to infect bone marrow cells in the presence or absence of Ab-MLV.

7 d after infection, cells were harvested and resuspended in PBS buffer with a proteinase inhibitor mixture (Sigma-Aldrich). Luciferase activity was determined by using the Promega luciferase assay kit on cell lysates (which were prepared as per the manufacturer's instructions). Luminescence was read on a Wallac 1450 Microbeta Trilux luminometer. Protein concentration in lysates was determined by Bradford protein assay (Bio-Rad Laboratories).

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