# A Role for Activation-Induced Cytidine Deaminase in the Host Response against a Transforming Retrovirus

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## Summary

**Activation-induced cytidine deaminase (AID) is specifically expressed in the germinal centers of lymphoid organs, where it initiates targeted hypermutation of variable regions of immunoglobulin genes in response to stimulation by antigen. Ectopic expression of AID, however, mediates generalized hypermutation in eukaryotes and prokaryotes. Here, we present evidence that AID is induced outside the germinal center in response to infection by the Abelson murine leukemia virus. The genotoxic activity of virally induced AID resulted in checkpoint kinase-1 (chk1) phosphorylation and ultimately restricted the proliferation of the infected cell. At the same time, it induced NKG2D ligand upregulation, which alerts the immune system to the presence of virally transformed cells. Hence, in addition to its known function in immunoglobulin diversification, AID is active in innate defense against a transforming retrovirus.**

## Introduction

The mouse genome encodes three functional cytidine deaminases with distinct functions in vivo. Apolipoprotein B editing catalytic polypeptide-1 (APOBEC1) has long been recognized as an mRNA-editing enzyme, producing a truncated version of Apolipoprotein B that forms the chylomicron (Anant et al., 2003). Activation-induced cytidine deaminase (AID) was the second member of this family to be characterized. AID is an arbiter of antigen receptor gene diversity, as it mediates both the introduction of point mutations into the variable regions of immunoglobulin (Ig) genes and the deletional recombination between constant regions resulting in Ig isotype switching (Chaudhuri and Alt, 2004). Murine Apolipoprotein B editing complex-3 (APOBEC3) is only weakly homologous to the human APOBEC3 family, members of which have specific activity against retroviruses like HIV (Goff, 2003).

Upon infection of a cell with a retrovirus, human APOBEC3 deaminases are packaged in the virion and carried to the next target cell. The encapsidated enzymes deaminate the viral minus strand cDNA (Goff, 2003), but the mechanism by which these deaminases restrict viral infectivity does not require their catalytic activity (Chiu et al., 2005; Newman et al., 2005); hence, how these deaminases function in antiviral immunity is not fully understood. Therefore, although human APOBEC3s have

a profoundly deleterious effect on HIV, the molecular details of how these deaminases function in the context of antiviral immunity remain unknown. Murine APOBEC3s also can be incorporated into the virions of simple viruses (e.g., the murine leukemia virus MLV) and can deaminate the MLV genome, but their effect on viral infectivity is weak (Goff, 2003).

Despite their distinct functions in vivo, all three mouse cytidine deaminases display similar activities in vitro: they all deaminate single-stranded nucleic acid (DNA or RNA) (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003). In addition, all three molecules mutagenize DNA when expressed ectopically in *E. coli* (Harris et al., 2002). Although only APOBEC3 deaminases have as their primary function in vivo the reduction of viral infectivity, there is no a priori reason why AID or APOBEC1 could not possess similar antiviral activity.

Abelson murine leukemia virus (Ab-MLV) is a highly oncogenic transforming retrovirus that causes primary pre-B cell transformation in vitro and pre-B cell leukemia in vivo. In both settings, the activity of the single protein product of the virus, the v-Abl protein tyrosine kinase, is required. However, despite the presence of this strongly transforming oncogene, Ab-MLV-induced lymphomas are usually clonal or oligoclonal, suggesting that other events in addition to expression of v-Abl are required for tumor formation and therefore that Ab-MLV-induced transformation is a multistep process (Rosenberg, 1994).

Here, we show that infection of mouse primary B cells by Ab-MLV induces AID expression, but in contrast to APOBEC3s, AID is not packaged in the virion and it does not deaminate the retroviral genome. Instead, we show that AID induction within infected cells leads to checkpoint kinase-1 (chk1) phosphorylation, delays cell-cycle progression, and restricts the proliferation of the infected cell. At the same time, the genotoxic action of AID leads to upregulation of the NKG2D Rae-1 ligand on the surface of infected cells, which increases their susceptibility to killing by NK cells. The presence of Rae-1 on the surface of a developing Abelson tumor enhances the survival of the infected animal, further suggesting that AID is central in the ability of the host to limit the proliferation of infected cells, resulting in their eventual elimination from the system.

## Results

### AID Deficiency Increases the Susceptibility to Viral Transformation

To test whether AID might play a role in antiviral immunity, we infected bone marrow cells from wild-type (Balb/cByJ) and Balb/cByJ AID-deficient (*Aicda*$^{-/-}$) mice (Ramiro et al., 2004) with replication-deficient Abelson murine leukemia virus (Ab-MLV) in vitro. Even though bone marrow consists of a complex mixture of cells, Ab-MLV naturally transforms with highest specificity a subset of developing B cells (specifically proto-pre-B cells), and the efficiency of transformation
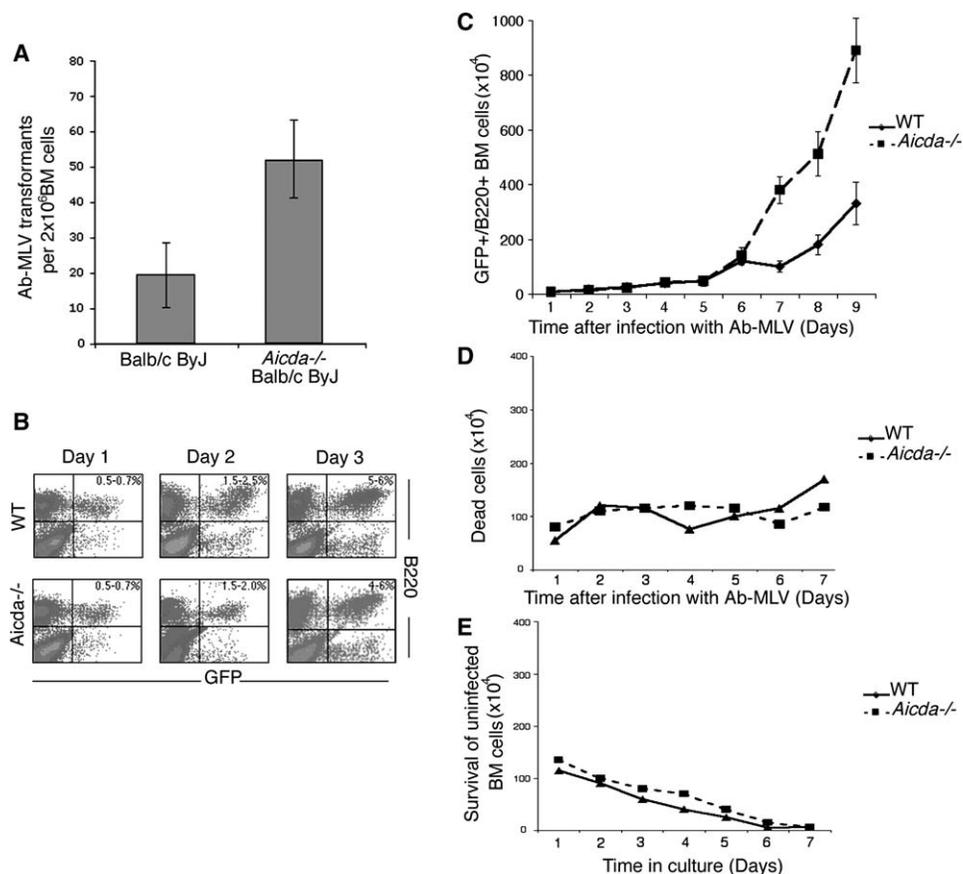
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Figure 1. Response of Wild-Type and $Aicda^{-/-}$ Cells to Ab-MLV Infection

(A) AID-deficient B cells produce higher numbers of transformants upon infection with Ab-MLV virus. $2 \times 10^6$ bone marrrow cells were infected with replication-deficient Ab-MLV and cultured in soft agar. Foci of transformed cells were counted 10 days postinfection. Uninfected primary cells produced no foci (moi = multiplicity of infection).

(B) Ab-MLV infects wt and $Aicda^{-/-}$ B cells with equivalent efficiencies. $2 \times 10^6$ bone marrrow cells were infected with replication-deficient Ab-MLV-GFP (moi 0.1), and the proportions of infected (GFP$^+$) cells in each culture were analyzed by FACS via antibodies to B220+ (a pan-B cell marker) and the internal green fluorescence produced by infected cells. The numbers in the upper right corner indicate the range of percentages of GFP$^+$ B cells recovered at the indicated time points in 10 independent experiments.

(C) Wild-type cells transformed by Ab-MLV are restricted in their proliferation compared to AID-deficient counterparts. $2 \times 10^6$ bone marrow cells were infected with replication-deficient Ab-MLV-GFP (moi 0.1), and the absolute numbers of live infected cells were counted on the days indicated. Error bars indicate standard deviation from the mean (5 experiments).

(D) The number of dead cells was compared in cultures of wt or $Aicda^{-/-}$ infected cells.

(E) Uninfected, primary B cells of either wild-type or $Aicda^{-/-}$ genotype were tested for survival in culture in the absence of infection.

can be precisely quantified by a semisolid agar assay and by counting the resulting foci (which are usually macroscopically visible 9 to 12 days postinfection) (Rosenberg and Baltimore, 1976). We found that AID-deficient B cells produce on average three times more transformed foci compared to wild-type mice of the same genetic background, and the number of foci is proportional to the multiplicity of infection (Figure 1A and Figure S1A in the Supplemental Data available with this article online).

An increase in susceptibility to transformation could be a consequence of perturbations in lymphoid subpopulations in AID-deficient mice, and hence of more virus-susceptible targets. To determine the numbers of Ab-MLV targets in AID-deficient and wt bone marrow, we labeled bone marrow cells with antibodies against B220 (a general B cell marker) and analyzed the kinetics of appearance of GFP$^+$ Ab-MLV transformed cells. We found that 0.5%–0.7% of bone marrow cells were GFP

positive the first day, and all of them were B cells (Figure 1B, day 1, and Figure S1B). This range was the same for both strains (10 independent experiments). Therefore, the subpopulation of B cells infected by Ab-MLV is equivalent in wt and AID-deficient strains.

However, although infection of $Aicda^{-/-}$ and wt bone marrows began with similar numbers of GFP-positive cells, the transformed cells did not proliferate equally. Expansion of both sets of cultures was identical until days 5–6 after infection; however, after day 7, $Aicda^{-/-}$ transformed cells proliferated markedly faster than their wt counterparts (Figure 1C), and this proliferation differential was also evidenced in the number of transformed foci at day 10 (Figure 1A) as well as in their size (with $Aicda^{-/-}$ foci being on average larger than wt foci; data not shown). The difference in proliferation cannot be explained by increased cell death in the infected wt cultures, because equivalent numbers of trypan blue-positive dead cells were counted in the
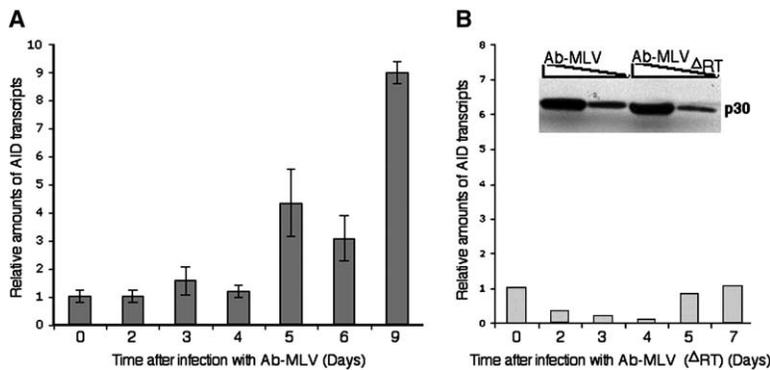
Figure 2. Infection with Ab-MLV Induces AID Expression, and AID Induction Requires that the Viral Genome Is Reverse Transcribed

(A) Kinetics of AID upregulation in Ab-MLV-infected (GFP^{+}) cells as analyzed by quantitative real-time PCR and normalized against uninfected controls that did not express AID (moi = 0.1). Error bars represent the standard deviation from the mean (5 independent experiments).

(B) Kinetics of AID upregulation in cultures of cells infected with reverse transcriptase mutant (ΔRT) Ab-MLV. Insert: immunoblot confirming equal detection of p30-gag from viral supernatants of Ab-MLV and Ab-MLV ΔRT, after centrifugation on sucrose cushions (20 μL and 5 μL).

wt and $Aicda^{-/-}$ culture (Figure 1D). Additionally, uninfected $Aicda^{-/-}$ and wt cells had identical survival curves in culture (Figure 1E). Therefore, AID-sufficient wt cells appear genetically more resistant to viral transformation.

### Bone Marrow, Wild-Type Cells Induce AID Expression upon Viral Infection

AID is thought to be expressed solely in germinal centers, which are regions of lymphoid organs where SHM and class switch recombination take place (Muramatsu et al., 1999). To determine whether AID is also expressed in primary bone marrow cells in response to viral infection, we sorted GFP^{+} B cells after infection, isolated their mRNA, and analyzed it by quantitative PCR. We found that AID mRNA was induced 5 days after infection and that expression continued to increase subsequently (Figure 2A). We did not observe substantial AID expression in uninfected bone marrow cells (Figure 2A, day 0; Muto et al., 2000).

The expression of infection-induced AID was about 10% of AID mRNA at the peak of the class switch recombination reaction (data not shown). When we infected bone marrow cells with a reverse-transcriptase mutant of the virus (ΔRT), it failed to upregulate AID (Figure 2B), even though the number of viral particles of the RT mutant virus was identical to that of the wt (Figure 2B, insert). Therefore, we conclude that AID induction in infected cells requires reverse transcription of the viral genome.

The increase in AID mRNA after day 5 (Figure 2A) precedes the increased proliferation of the $Aicda^{-/-}$ cells at days 6–7 (Figure 1C). That this proliferative burst is not observed in wild-type cells suggests that AID may play a role in reducing the proliferative potential of wt transformed cells.

AID normally targets the immunoglobulin locus, but when expressed in nongerminal center cells, it can initiate mutation of a number of highly transcribed genes (Martin et al., 2002). To determine whether the AID expression we observed was physiologically functional, we cloned and sequenced the $J_H4$ intron of the immunoglobulin locus from wt and $Aicda^{-/-}$ transformed cells 10 days postinfection. We found that the $J_H4$ locus was mutated in 10 out of 34 of the wt clones (16 mutations in 23,800 nts, mutation frequency $0.68 \times 10^{-3}$), but only one clone was mutated in 38 sequences from $Aicda^{-/-}$ cells (1 mutation in 26,600, mutation frequency $0.09 \times 10^{-3}$) (Table 1 and Figure S2). Within the B cell compartment, Ab-MLV transforms exclusively pre-B cells, and thus it is unlikely that the $J_H4$ mutations we detected were due to contaminating recirculating B cells. We also cloned and sequenced the λ5 gene, which is highly expressed in pre-B cells, with similar results (Table 1 and Figure S2). We conclude that the virally induced AID expression that we observe results in the production of enzymatically active protein.

### AID Protects Wild-Type Mice from Acute Death Resulting from Ab-MLV Infection and Transformation

To determine whether the altered transformation profile of $Aicda^{-/-}$ cells in culture reflects a potential cell-autonomous effect on disease progression in the infected $Aicda^{-/-}$ animal, we engrafted equivalent numbers of Ab-MLV-infected bone marrow cells from either wt Balc/cByJ or congenic $Aicda^{-/-}$ mice into Balc/cByJ recipients. Because infected cells of both genotypes are

Table 1. Mutation Frequencies of the $J_H4$ and λ5 Loci in Wild-Type or $AID^{-/-}$ Cells Infected with Ab-MLV

| | Clones Mutated | | Mutation Frequency $\times 10^{-3}$ | | % dG·dC | |
|---|---|---|---|---|---|---|
| Locus | Wild-Type | AID$^{-/-}$ | Wild-Type | AID$^{-/-}$ | Wild-Type | AID$^{-/-}$ |
| $J_H4$ | 10/34[a] | 1/38[a] | 0.68 | 0.09 | 75 | -[b] |
| λ5 | 6/24[c] | 0/18[c] | 1.19 | 0 | 100 | - |

Statistical significance was evaluated by the $\chi^2$ test for the indicated sets of data.
[a] $p < 0.01$
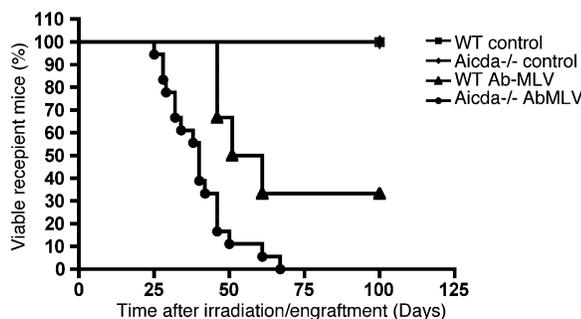[b] All mutations were from dA·dT.
[c] $p < 0.025$

Figure 3. AID Protects Wild-Type Mice from Acute Death after Ab-MLV Infection

The survival of lethally irradiated Balb/cByJ mice engrafted with Ab-MLV-infected wild-type (20 recipients; shown in triangles) or Ab-MLV-infected AID-deficient BM cells (20 recipients; shown in circles) is presented as a Kaplan-Meyer plot. After engraftment, irradiated recipients were monitored daily and analyzed shortly after death.

injected into animals with a wt complement of all antibody isotypes, this experiment uncouples a direct role of AID in the response to viral infection from an indirect role in generating neutralizing isotypes.

Sick mice presented with impaired gait, paraplegia, and in many cases a detectable subcutaneous tumor mass, all pathologies symptomatic of Ab-MLV-induced disease (Abelson and Rabstein, 1970). We confirmed the diagnosis of Abelson disease by performing gross pathological examination, histochemical staining, and FACS analysis (not shown). Only mice receiving infected bone marrow became ill (Figure 3). Mice receiving infected wt bone marrow died beginning 48 days postinfection, and 70% were dead after 65 days; 30% remained disease free (Figure 3).

In contrast, in mice receiving infected $Aicda^{-/-}$ bone marrow, disease progressed far more rapidly: mice began dying 24 days after infection, and more than 90% were dead by 48 days. In other words, almost all mice receiving infected $Aicda^{-/-}$ bone marrow died before any of their wt counterparts did (Figure 3). Indeed, a statistical comparison of the two survival curves revealed that the difference between them is highly significant (log-rank test, p = 4.8 × 10$^{-5}$). A third of the mice transplanted with infected $Aicda^{-/-}$ bone marrow did not present with classical features of Abelson disease. Rather, they died suddenly of thoracic hemorrhage due to a highly aggressive and invasive pre-B cell tumor. These in vivo data strongly suggest that AID functions in a cell-autonomous fashion in the host response to infection caused by transforming viruses by restricting the acute phase of the disease. Importantly, this function of AID is independent of its role in antibody diversification.

### Functional AID in Infected Wild-Type Cells Leads to NKG2D Ligand Expression and to chk-1 Phosphorylation

Because AID is expressed well after the virus is integrated in the host genome, it is unlikely that it directly deaminates the viral genome during the early stages of infection, prior to provirus formation. Indeed, we did not find any mutations when we amplified and sequenced parts of the viral genome at early time points after infection (the GFP and gag genes; data not shown).

Thus, unlike the human APOBEC3 deaminases, mouse AID does not specifically target the virus, and as a result all transformed cells are GFP$^+$.

To investigate whether virally induced AID could damage the genome of infected cells, we assayed cell-surface expression of ligands, which have recently been identified as markers of genotoxic stress (Gasser et al., 2005). We monitored the expression of the Rae-1 and H60, which are NKG2D ligands on the surface of Balb/cByJ and AID-deficient Balb/cByJ B cells at several time points after infection. The Rae-1 ligand, which is upregulated in response to DNA damage (Gasser et al., 2005), appears on the surface of Ab-MLV-infected Balb/cByJ cells by 6 days after infection (Figure 4A, top, black trace). In contrast, surface expression of Rae-1 ligands were significantly reduced in Ab-MLV-infected $Aicda^{-/-}$ cells (Figure 4A, gray histogram). Furthermore, expression of the NKG2D ligand H60, which is constitutively present on the surface of Balb/cByJ pre-B cells (Figure 4A; Malarkannan et al., 1998) but has not been linked to the DNA damage response, is not further induced in Ab-MLV-infected Balb/cByJ cells (Figure 4A, bottom, black trace). Therefore, coincident with AID upregulation, virus-infected wt cells upregulate the Rae family of NKG2D ligands whereas congenic $Aicda^{-/-}$ infected cells do not.

The inability of AID-deficient cells to upregulate Rae1 ligands after infection by Ab-MLV is not due to a general deficiency in the response of such cells to DNA damage: both Balb/cByJ and $Aicda^{-/-}$ cells can upregulate Rae-1 ligands after exposure to ionizing radiation (Figure 4B). The magnitude of the NKG2D response to ionizing radiation is similar to that seen in wt infected cells (day 7 after infection and beyond, Figure 4A).

Finally, Rae ligand upregulation makes infected Balb/cByJ cells sensitive to killing by NK cells (Figure 4C). This finding could explain why Ab-MLV-infected $Aicda^{-/-}$ cells kill wt mice rapidly (Figure 3): essentially, AID$^{-/-}$ cells, which do not upregulate Rae ligands (Figure 4A, top, gray histogram), lack the surface molecule that marks their wt counterparts for clearance by NK cells.

To further confirm that the observed increase in Rae-1 ligand expression was due to AID activity and not to other cellular processes initiated by viral infection and transformation, we examined splenic B cells for Rae-1 expression during class switch recombination. Splenic B cells can be induced to isotype switch after stimulation with LPS and IL-4. Although the new isotype is not detectable on the cell surface until about 72 hr postinduction (Figure 5A), switch recombination is completed at the molecular level by 48 hr after induction, coincident with AID expression and DNA break formation (Figure 5C; Schrader et al., 2005). We found that Rae-1 ligands were dynamically expressed on the surface of wt B cells between 48 and 60 hr postinduction. Rae-1 surface expression was dependent on the presence of AID (Figure 5B) and was reduced to background amounts by 80 hr after induction. Thus, Rae-1 ligand expression appears to be closely linked to the presence of functional AID.

NKG2D ligand upregulation has been linked to activation of the ataxia telangiectasia mutated (ATM) damage checkpoint (Gasser et al., 2005), which eventually inhibits entry of damaged cells into mitosis by activation of the effector kinase Chk1. This activation is achieved
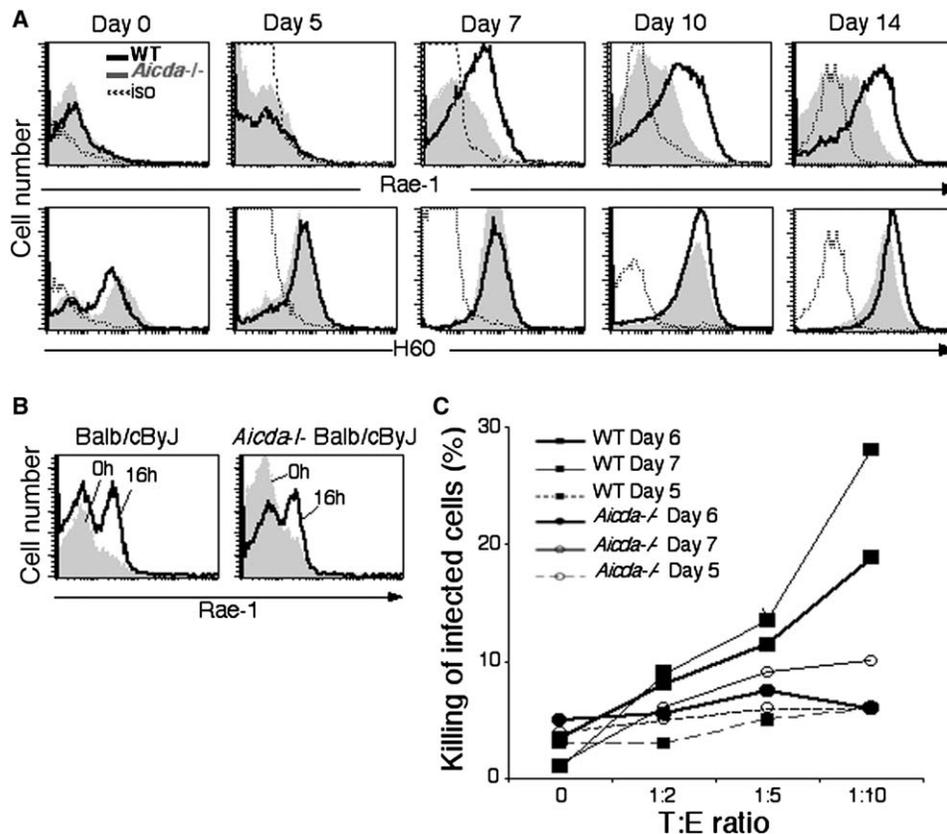
Figure 4. Virally Induced AID Mediates NKG2D Ligand Expression

(A) Expression of the NKG2D ligands Rae1 (top) and H60 (bottom) in wild-type (black traced histogram) and $Aicda^{-/-}$ (gray shaded histogram) infected cells as a function of time. The dashed line indicates staining with an isotype control antibody (iso). Note that the same isotype control is used for Rae as well as H60 staining.

(B) Rae1 expression in wt and $Aicda^{-/-}$ cells after exposure to ionizing radiation (4 Gy).

(C) Wild-type cells that express Rae ligand as a consequence of the host response to Ab-MLV infection can be eliminated by NK cells. Infected, GFP+ wt and $Aicda^{-/-}$ cells were incubated with MACS purified, splenic NK cells at days 5, 6, and 7 postinfection. Infected cells were incubated for 1 hr 30 min with increasing amounts NK cells and then stained with propidium iodide (which stains live cells rendered permeable by NK cells). Double positive (GFP+PI+) cells have been successfully attacked by NK cells. The experiment was done in duplicate.

when ATM directly phosphorylates chk1 on Ser345 (Zhao and Piwnica-Worms, 2001) and NKG2D ligand expression closely follows chk1 phosphorylation (Gasser et al., 2005). To determine whether chk1 phosphorylation correlated with Rae ligand expression in Ab-MLV-infected cells, we monitored the amounts of Ser345-phosphorylated chk1 by immunoprecipitation followed by immunoblotting. By using an antibody specific to the phosphorylated form of chk1, we were able to detect low levels of chk1 phosphorylation in wt infected cells as early as 6 days after infection (Figure 6). In contrast, $Aicda^{-/-}$ infected cells did not accumulate substantial amounts of phosphorylated chk1 (Figure 6). This finding could directly explain the delay in proliferation between wt and $Aicda^{-/-}$ cells (Figure 1C) after infection. We conclude that virally induced AID leads to genotoxic stress in wt cells, which leads to a decrease in the proliferating potential of the infected cell population by a mechanism that employs chk1 phosphorylation.

## Discussion

The targeted deamination of cytidine residues in nucleic acids has emerged as a central mechanism in the host

cell arsenal against retroviruses (Sheehy et al., 2002). At the same time, targeted deamination of antibody genes has been recognized as crucial for the generation of antibody diversity, specificity, and even memory (Muramatsu et al., 2000).

AID is the cytidine deaminase that is essential for the initiation of Ig diversification in the secondary lymphoid organs and was therefore thought to be expressed only within germinal centers where somatic hypermutation and class switch recombination take place. We report here that infection of primary cells by a transforming virus, Ab-MLV, induced AID expression in nongerminal center B cells. In contrast to the APOBEC3s, AID was not constitutively present in B cells, but was expressed by the cell after viral infection. AID was not packaged in the virion (not shown) and did not reduce viral infectivity. Rather, it restricted the proliferation of cells infected by the Abelson retrovirus.

Virus-induced AID is catalytically active: we have shown that it can mutate transcribed genes. Previous work from a number of laboratories has shown that ectopically expressed AID can deaminate many transcribed genes in addition to those encoding Ig (Petersen-Mahrt et al., 2002; Poltoratsky et al., 2004; Sale
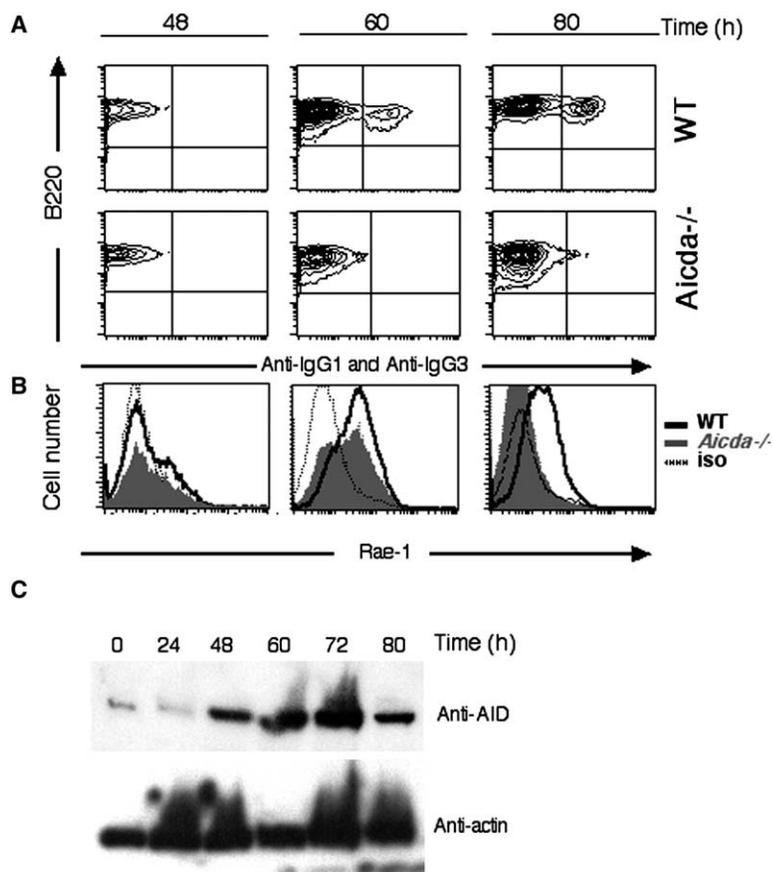
Figure 5. Rae-1 Is Expressed on the Surface of Switching B Cells

(A) Time course of surface expression of IgG1 and IgG3 after treatment of wt and *Aicda*$^{-/-}$ splenic B cells (Balb/cByJ strain) with LPS and IL-4.

(B) Time course of Rae-1 receptor surface expression after treatment of wt and AID$^{-/-}$ splenic B cells (Balb/cByJ strain) with LPS and IL-4. iso, isotype control.

(C) Levels of AID protein in wt cells stimulated with LPS and IL-4 for the indicated times (whole-cell extract is run on 4%–20% SDS-PAGE, blotted onto PVDF membranes, and probed with the indicated antibodies).

and Neuberger, 1998; Wang et al., 2004; Yoshikawa et al., 2002). We therefore hypothesized that virally induced AID would retard the proliferation of primary transformed cells by causing generalized DNA damage.

Recent work has linked DNA damage in primary cells to upregulation of ligands of the NKG2D receptor. We therefore asked whether the activity of virally induced AID leads to NKG2D ligand upregulation. Indeed, we found that wild-type cells upregulate the Rae-1 NKG2D ligand with kinetics that correlate with the kinetics of AID induction. In contrast, congenic *Aicda*$^{-/-}$ primary transformants do not upregulate Rae-1. A similar correlation between the kinetics of AID expression and Rae-1 ligand upregulation can be seen in B cells stimulated to undergo class switch recombination in vitro. Our data



Figure 6. Virally Induced AID Mediates chk1 Kinase Phosphorylation

Equal amounts of whole-cell extracts from infected wt and *Aicda*$^{-/-}$ cells were probed for the presence of chk1 and phospho-chk1 as indicated.

suggest that NKG2D ligand upregulation is not a result of viral infection or primary transformation but rather a direct result of AID activity in the transformed cells.

Furthermore, NKG2D ligand upregulation in response to genotoxic stress is directly dependent on the effector kinase chk1 that is phosphorylated by the ATM kinase upon DNA damage (Gasser et al., 2005). We find that Ab-MLV-infected wt cells but not *Aicda*$^{-/-}$ cells accumulate phospho-chk1 with kinetics similar to those of AID induction and Rae-1 surface expression. Therefore, our data mechanistically link AID activity to chk1 phosphorylation. As ATM has been shown to be the kinase responsible for chk1 phosphorylation, we speculate that AID activity in infected cells activates the ATM damage-response pathway, which would explain the reduced proliferation of Ab-MLV-transformed wild-type cells.

Finally, we have compared the progression of Abelson disease in wt mice carrying infected bone marrow cells from either wt or *Aicda*$^{-/-}$ congenic animals. We found that all wt mice carrying AID null tumors died of pre-B cell lymphomas very early, whereas wt mice carrying wild-type tumors lived significantly longer. These in vivo data provide a genetic demonstration that AID expressed in a tumor of viral etiology is important in restricting disease.

In this study, we have used the finding that NKG2D ligands can be upregulated in cells in response to DNA damage as a tool to probe whether AID expression in response to infection can lead to the activation of the DNA damage response. Our observation that AID activity can
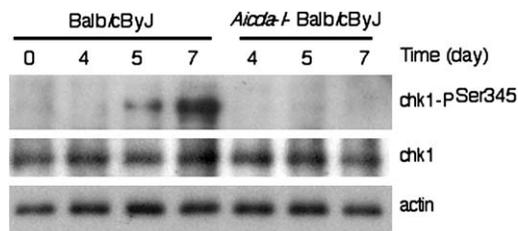
be correlated with NKG2D ligand expression on the surface of wt cells as well as the resulting differential susceptibility to NK cell-mediated clearance in vitro could partially explain why Ab-MLV-infected $Aicda^{-/-}$ cells kill wt mice rapidly. However, conclusive proof for the involvement of NK cells in clearance of wild-type Ab-MLV-infected cells from the mouse would require NK cell-depletion experiments for the duration of the in vivo studies. Clearly, more work needs to be done both in vivo and in vitro to conclusively address the role of these receptors in the NK cell-mediated clearance of virally infected cells.

It is important to note that the limitation of disease seen in the wild-type cannot be due exclusively to Rae-1 upregulation and NK cell killing. Indeed, we have shown that it is the AID-dependent activation of the DNA damage response that limits cell proliferation in vitro in a cell-autonomous manner. Therefore, tumor development in the mouse could be limited independently of NK cell killing. It is quite likely that limitation of disease requires both the AID-mediated proliferation arrest as well as the clearance of infected cells by NK cells.

AID-mediated restriction of proliferation of virally induced tumors may not be limited to Ab-MLV. EBV infection can induce AID expression early after viral entry (our preliminary data). Others have shown that HCV infection of the human Burkitt's lymphoma line Ramos, which hypermutates constitutively in culture (Sale and Neuberger, 1998), raises levels of AID, resulting in genome-wide mutation and a loss of proliferative potential (Machida et al., 2004). These viruses are quite different from each other in capsid composition, making it unlikely that they induce AID expression by binding a common cell-surface receptor. Furthermore, common signaling cascades initiated by virally induced interferons do not upregulate AID expression (our preliminary data). It is possible that AID is induced by some element of the process of transformation. In that regard, it will be important to determine whether viruses causing nontransforming yet persistent infections upregulate the AID response in the host.

We describe a novel role for AID in the host response to infection with transforming viruses. Unlike the APO-BEC3 deaminases that target the virus to reduce infectivity, AID restricts infected cell proliferation in vitro and the acute (hyperproliferative) phase of the disease in vivo. Hence, the APOBEC3 deaminases and AID have complementary roles.

## Experimental Procedures

### Cells and Mice
293T cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Sigma).

Bone marrow cells were flushed from the femurs and tibias of 5- to 8-week-old mice and were grown in RPMI 1640 medium (Sigma) supplemented with 20% heat-inactivated FCS and 50 $\mu$M 2-mercaptoethanol (Sigma).

Balb/cByj mice were obtained from Jackson Laboratories, and congenic AID-deficient mice were obtained from Dr. M. Nussenzweig (Rockefeller University) (Ramiro et al., 2004). All animal work was approved by the Rockefeller Institutional Animal Care and Use Committee.

### Retroviral Expression Vectors
The pMIG-vAbl-MLV-120 retrovirus expressing v-Abl tyrosine kinase was a gift from Dr. Naomi Rosenberg (Tufts University School

of Medicine, Boston, MA). The pCNL construct for the production of Abelson virus with a mutant reverse transcriptase (YMDD motif) was a gift from Alice Telesnitsky (University of Michigan) via S. Goff (Columbia University).

### Virus Production and Infections
Replication-deficient Ab-MLV was generated by transfection of 293T cells with pMIG-Ab-MLV retroviral construct and pCL packaging DNA (1:1 ratio) by the PerFectin Transfection Reagent (Gene Therapy Systems) according to the manufacturer's protocol. Cells were cultured for 48 hr, and the supernatant was filtered through a 45 $\mu$m filter and used as viral stock for infection of target cells.

Viral titers (infectious units/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, $2 \times 10^6$ bone marrow cells were challenged for 2 hr with a multiplicity of infection (moi) of 0.1. For the experiments with RT mutant virus, titers were determined by Western blots of viral gag protein (see below).

### Agarose Transformation Assay of Bone Marrow Cells
$2 \times 10^6$ freshly prepared bone marrow cells from BALB/cByJ and AID-deficient mice were infected with equivalent titers of Ab-MLV for 4 hr and seeded in 0.3% agar (usb) containing RPMI-20%FCS-50 $\mu$M-$\beta$-mercaptoethanol as described previously (Rosenberg and Baltimore, 1976). Macroscopic colonies of transformed pre-B cells were counted between 9 and 12 days postinfection (data shown are for day 10 foci).

### Tumorigenicity Assay
Bone marrow cells were isolated from 5- to 6-week-old BALB/cByJ and AID$^{-/-}$ congenic mice and were infected for 2 hr with equivalent titers of replication-deficient Ab-MLV. After infection, the cells were washed with 1$\times$ PBS and injected intravenously into lethally irradiated (800 rads) BALB/cByJ 8- to 10-week-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.

### NK Susceptibility Assay
NK cells were prepared by depletion of splenocytes of all other cell types via MACS beads (NK isolation kit, Miltenyi Biotech). NK cells were then incubated with GFP$^+$ infected B cells at the indicated effector-to-target ratios for 1.5 hr at 37ºC. After incubation, cells were washed with PBS-5%BSA buffer and directly stained with propidium iodide (PI), which stains cells rendered permeable by NK treatment (a more extensive protocol is provided with the NKTest kit, Orpegen Pharma). Percentages of double positive (GFP$^+$PI$^+$) intact cells at different effector-target ratios were then plotted.

### Flow Cytometry Analysis
For the quantification of GFP$^+$ B cells, bone marrow cultures were stained with mouse B220 PE-conjugated antibody (BD Pharmigen). 7AAD was used for dead cell exclusion.

NKG2D ligand upregulation was monitored by means of primary antibodies against Rae1 (pan-Rae, R&D Systems clone 186107, rat IgG2a), H60 (R&D Systems, clone 205326, rat IgG2a), and isotype controls (rat IgG2a, R&D Systems) in combination with a PE-labeled anti-rat IgG (R&D Systems).

Class switch recombination was monitored with antibodies against IgG1 and IgG3 in conjunction with anti-B220 (BD Pharmingen).

### Immunoblots
For detection of viral proteins, viral supernatants were centrifuged on a sucrose cushion. Virions were collected, resuspended in 1$\times$ Laemmli buffer (with 100 mM DTT), boiled for 10 min and then immediately loaded onto a Tris-glycine 4%–20% acrylamide gradient gel (BioRad). The gel was transferred onto PVDF membrane and incubated with antibodies against gag (Cell Signaling Technologies).

Chk1 expression was assessed by Western blotting infected cell lysates with antibodies against total chk1(via a chk1 antibody from

Cell Signaling Technologies), phospho-chk1 (Ser345 specific [Cell Signaling Technologies]), or β-actin.

## Quantitative Real-Time PCR

Bone marrow cells from 5- to 8-week-old BALB/cByJ mice were infected with Ab-MLV as described above. The cells were collected at different days postinfection and stained with mouse B220 PE conjugated antibody. Cell sorting was performed on a FACSVantageTM (Becton Dickenson).

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

## Sequencing Analysis

DNA was prepared from infected or uninfected bone marrow cells, and gene fragments were amplified with Pfu DNA polymerase (Stratagene). Amplification products were ligated into the TOPO cloning vector (Invitrogen), and individual clones were sequenced by the M13F primer. For the amplification of JH4, the sense primer was 5′-gtatccgatgcatagggacaaag and the antisense was 5′-cctctccagt ttcggctgaatcc; for λ5 the sense primer was 5′-gcccttagaacactggc cttg and the antisense was 5′-gcagtgtagatcaagggcg.

## Supplemental Data

The two supplemental figures can be found with this article online at http://www.immunity.com/cgi/content/full/24/6/779/DC1/.

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