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Immunity
Article

MicroRNA-155 Is a Negative Regulator of Activation-Induced Cytidine Deaminase

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DOI 10.1016/j.immuni.2008.03.015

SUMMARY

B lymphocytes perform somatic hypermutation and class-switch recombination (CSR) of the immunoglobulin locus to generate an antibody repertoire diverse in both affinity and function. These somatic diversification processes are catalyzed by activation-induced cytidine deaminase (AID), a potent DNA mutator whose expression and function are highly regulated. Here we show that AID was regulated posttranscriptionally by a lymphocyte-specific microRNA, miR-155. We found that miR-155 was upregulated in murine B lymphocytes undergoing CSR and that it targeted a conserved site in the 3′-untranslated region of the mRNA encoding AID. Disruption of this target site in vivo resulted in quantitative and temporal deregulation of AID expression, along with functional consequences for CSR and affinity maturation. Thus, miR-155, which has recently been shown to play important roles in regulating the germinal-center reaction, does so in part by directly downmodulating AID expression.

INTRODUCTION

Vertebrates are able to produce a vast repertoire of antibody molecules to combat infection. The number of antibody specificities available during a human lifetime is estimated to exceed 10^9, far greater than the coding capacity of the genome. Instead, the size of the antibody repertoire results from gene-diversification processes that occur in antibody-producing B lymphocytes.

During B cell development, antibody genes are assembled by DNA rearrangement [V(D)J recombination] to produce the primary repertoire of antibody specificities (about 10^5–10^6 different specificities). However, this repertoire is neither large enough nor specific enough to include high-affinity antibodies against the full range of antigens an animal might encounter. Thus, the generation of antibody diversity depends largely on processes that follow V(D)J recombination.

Two main processes drive the generation of the secondary antibody repertoire: somatic hypermutation [or SHM, where mutations are introduced in and around the productively rearranged V(D)J segment] and gene conversion (or GCV, where nonfunctional pseudogenes are used as templates for diversification). Both SHM and GCV are triggered by activation-induced cytidine deaminase (AID), an enzyme believed to function directly as a DNA mutator. In addition to these two processes, AID-mediated deamination initiates class switch recombination (CSR), a reaction that does not contribute to diversification of antibody specificity but replaces the default Ig constant region (Cμ) with a downstream constant region (Cγ, Cε or Cα) to alter the effector capacity of the antibody while retaining the specificity for antigen (Teng and Papavasiliou, 2007).

A potent mutator such as AID must be stringently regulated in the cell. Indeed, it has been proposed that AID is regulated transcriptionally (Dedeoglu et al., 2004; Gonda et al., 2003; Sayegh et al., 2003), by intracellular compartmentalization and trafficking (Ito et al., 2004; McBride et al., 2004), by posttranslational modification (Basu et al., 2005; Basu et al., 2007; McBride et al., 2006; Pasqualucci et al., 2006), and by interaction with specific cofactors (Chaudhuri et al., 2004; MacDuff et al., 2006). In addition to these more traditional modes of regulation, we show here that AID is also subject to posttranscriptional regulation by a specific microRNA, miR-155.

miR-155 belongs to a recently identified class of noncoding 21–23 nt RNAs that function as posttranscriptional modulators of gene expression. Encoded by diverse metazoan genomes, miRNAs target their cognate messenger RNAs for degradation or translational repression (Bartel, 2004; Bartel and Chen, 2004). Their functional influence is apparent in a host of biological processes, ranging from pancreatic insulin secretion (Poy et al., 2004) to B and T cell development (Zhou et al., 2007).

miR-155 is processed from the noncoding RNA transcribed from *Bic* (B cell integration cluster); this RNA serves as the primary miRNA precursor. *Bic* was originally identified as a common site for insertion of proviral DNA in lymphomas induced by the avian leukosis virus (Tam et al., 1997). A role for miR-155 in germinal-center development and function has recently been proposed on the basis of studies using in vivo loss-of-function and gain-of-function approaches to analyze the consequences of deletion or ectopic expression of miR-155, which revealed B cell autonomous defects in affinity maturation (Rodriguez et al., 2007; Thai et al., 2007). However, miR-155 has been shown to

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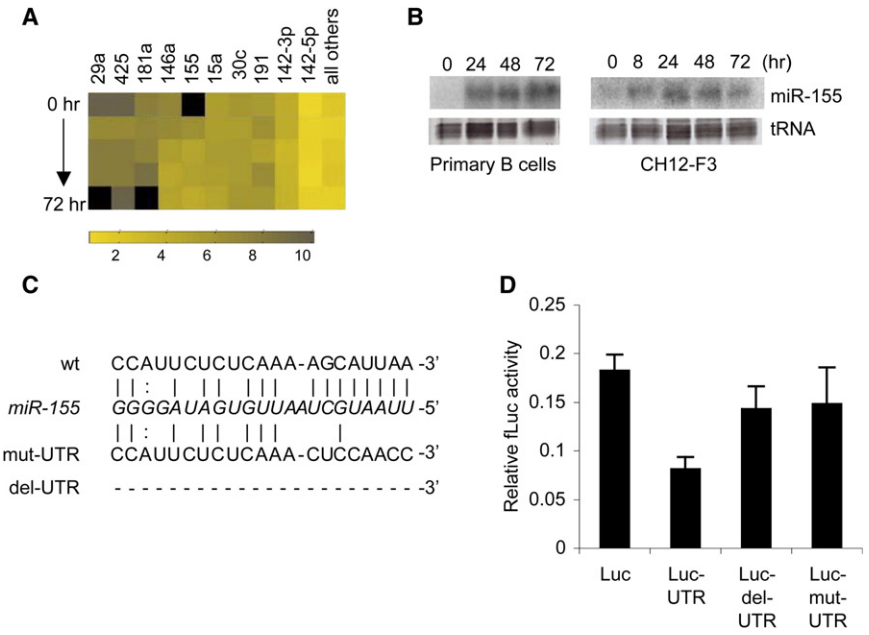

Figure 1. miR-155 Is Upregulated in B Cells Undergoing CSR and Targets the 3′ UTR of AID mRNA

(A) Relative cloning frequencies for ten of the most abundant miRNAs in murine splenic B lymphocytes stimulated with IL-4 and LPS (0, 8, 24, 48, and 72 hr). The color-coding scale is shown at bottom, and undetected miRNAs are indicated by black.

(B) RNA blots for miR-155 from total RNA isolated from murine splenic B cells (left) and the murine B cell line CH12-F3 (right), stimulated in vitro (0–72 hr) to undergo CSR. Ethidium-bromide staining of tRNA bands is shown as a loading control.

(C) Base pairing between murine miR-155 (italicized) and its target site in the wt AID 3′ UTR. Sequences of the mutated (mut-UTR) and deleted (del-UTR) target-site variants are shown underneath.

(D) CH12-F3 cells stimulated to undergo CSR were transfected with reporter constructs containing firefly luciferase alone (Luc) or fused to the wild-type AID 3′ UTR (UTR), del-UTR, or mut-UTR. Data represent mean values of eight independent experiments ± standard error of the mean (SEM). One-way ANOVA F statistic = 2.95 ($p = 0.05$).

target more than 60 different genes in B cells (Vigorito et al., 2007), and it is unclear which of those genes would be directly targeted by this microRNA to produce the observed phenotype. Furthermore, induction of miR-155 expression has been found in association with the activated phenotype of several types of immune cells, including T lymphocytes, dendritic cells, and macrophages (O'Connell et al., 2007; Rodriguez et al., 2007; Thai et al., 2007), suggesting synergistic action of miR-155 on numerous target genes in multiple cell types.

Here we present evidence that AID is a direct target for miR-155 regulation in vivo. Using a bacterial artificial chromosome (BAC) transgenic model of AID expression, we show that specific ablation of miR-155:AID interaction deregulates AID protein expression in germinal-center B cells and results in ectopic persistence of AID expression in B cells as they exit the germinal centers and enter the peripheral circulation. We also show that lack of AID regulation by miR-155 leads to defective affinity maturation. Hence, the central role of miR-155 in control of the germinal-center reaction (Thai et al., 2007) is at least in part due to its role in repression of AID expression.

RESULTS

miR-155 Is Upregulated in B Cells Undergoing Class-Switch Recombination

microRNAs (miRNAs) comprise a class of newly discovered small RNA species that regulate gene expression. To investigate the role these regulators might play in CSR, we cloned and sequenced miRNAs present in primary B cells before and after stimulation with LPS and IL-4, conditions that induce switching from IgM to IgG1. Of the 123 miRNAs that were cloned from these samples (data not shown), we identified one, miR-155, that was upregulated after stimulation (Figure 1A). Importantly, miR-155 was upregulated not only in primary B cells treated with LPS and IL-4 but also in CH12-F3 cells, a murine B cell line that switches from IgM to IgA after treatment with anti-CD40, IL-4, and TGF-β (Figure 1B). Thus, miR-155 is upregulated during CSR in a manner that is not isotype specific.

miR-155 Can Target the 3′ UTR of AID mRNA

We applied several miRNA target-prediction algorithms [PicTar (Krek et al., 2005), miRanda (Griffiths-Jones et al., 2006; John et al., 2004), and TargetScan (Grimson et al., 2007; Lewis et al., 2003)] to identify putative targets of miR-155 with relevance to either SHM or CSR. AID was consistently predicted as one such target. Depending on the algorithm used, a number of additional miRNA binding sites in the AID mRNA were identified (not shown); however, these miRNAs were not detected in switching B cells by cloning or by RNA blot. Furthermore, the stringent prediction criteria of the most recent version of the TargetScan algorithm (Grimson et al., 2007) identified miR-155 as the sole miRNA target site in the AID 3′ UTR. The 3′ UTRs of AID mRNA diverge substantially in sequence and length between various species (Figure S1 in the Supplemental Data available online). However, they coincide strikingly at an 8 nt motif corresponding to the predicted miR-155 target-site seed region. To test the possibility of posttranscriptional AID regulation by miR-155, we created reporter constructs containing the 3′ UTR of *Aicda* downstream of a firefly luciferase reporter gene (Luc-UTR), along with variants harboring deletion (Luc-del-UTR) or mutation (Luc-mut-UTR) of the miR-155 target site (Figure 1C). These constructs were transiently transfected into CH12-F3 cells stimulated to undergo CSR (and hence induced to express endogenous miR-155). We observed repression of luciferase activity by ~50% in Luc-UTR-transfected cells compared to cells transfected with a luciferase-only construct (Figure 1D). This repression was alleviated upon disruption of the miR-155 target site by deletion or mutation. These data indicate that the single target site in the 3′ UTR of the AID mRNA renders it susceptible to repression by physiological levels of miR-155 expressed during CSR.

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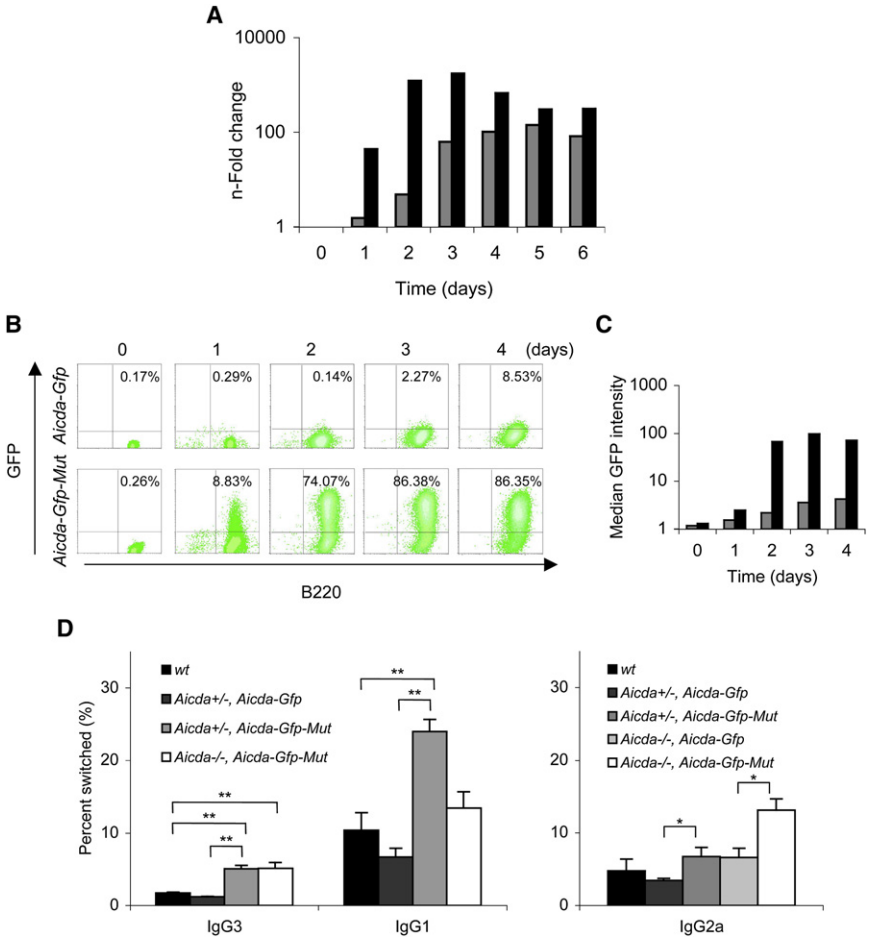

Figure 2. Mutation of the AID miR-155 Target Site Results in Deregulated AID-GFP Expression and Increased CSR Efficiency In Vitro

(A) AID-GFP mRNA expression was monitored by quantitative PCR in representative *Aicda-Gfp* control (gray) and *Aicda-Gfp-Mut* (black) mice. Data are normalized to Ku70 mRNA expression, and the scale is set to 1 for d0.

(B) AID-GFP protein expression was monitored by FACS up to 4 days after in vitro stimulation of splenic B lymphocytes with IL-4 and LPS. Percentages of GFP^+ $B220^+$ cells are indicated in the upper right quadrants. Cells from representative *Aicda-Gfp* control (Tg copy number approximately 5) and *Aicda-Gfp-Mut* (Tg copy number approximately 5) are shown.

(C) Median GFP fluorescence intensities, expressed in logarithmic units, are shown for the *Aicda-Gfp* (gray) and *Aicda-Gfp-Mut* (black) samples in (B).

(D) Splenic B lymphocytes from wild-type (n = 4); $Aicda^{+/-}$, *Aicda-Gfp* control (n = 5); $Aicda^{-/-}$, *Aicda-Gfp* control (n = 2); $Aicda^{+/-}$, *Aicda-Gfp-Mut* (n = 6), or $Aicda^{-/-}$, *Aicda-Gfp-Mut* (n = 3) mice were stimulated in vitro for 3 days with LPS (for inducing CSR to IgG3), LPS and IL-4 (for inducing CSR to IgG1), or LPS and IFNγ (for inducing CSR to IgG2a). Data represent mean values ± SEM. Note that $Aicda^{-/-}$, *Aicda-Gfp* control samples did not switch robustly to IgG1 or IgG3, although $IgG1^+$ and $IgG3^+$ populations were detected by FACS analysis (data not shown), but they did show quantifiable levels of CSR to IgG2a. t tests, $^*p < 0.05$, $^{**}p < 0.01$.

Mutation of the *Aicda* miR-155 Target Site Results in Deregulated AID Expression and Increased CSR in Splenic B Cells Stimulated In Vitro

To study the behavior of in vivo AID expression in response to miR-155, we took advantage of a recently generated transgenic *Aicda-Gfp* indicator mouse strain (Crouch et al., 2007). These mice carry between two and ten copies of a 75 kb BAC that contains the entire *Aicda* locus as well as its two adjacent gene loci (*Mfap5* and *Apobec1*). The *Aicda* locus in this BAC has been modified such that the gene encoding GFP is inserted downstream of the coding portion of the final *Aicda* exon (Figure S2A). The transgenic AID-GFP fusion protein expressed from this BAC was previously shown to replicate endogenous patterns of AID expression (Crouch et al., 2007). The AID-GFP gene product also retains catalytic activity; it can rescue CSR in $Aicda^{-/-}$ B lymphocytes in vitro (Figure S2B). Based on this published *Aicda-Gfp* BAC, we created a second transgenic construct in which the miR-155 target-site seed region was mutated to disrupt binding to miR-155 (*Aicda-Gfp-Mut*) (Figure S2A). We used this second construct to generate five independent founder lines carrying the *Aicda-Gfp-Mut* BAC transgene (founders carried varying copy numbers of the transgene, ranging from 5–20).

Splenic B lymphocytes from progeny of these transgenic founders were analyzed for AID-GFP expression in response to IL-4 and LPS stimulation in vitro. The dynamics of AID-GFP expression differed considerably between the *Aicda-Gfp* control and *Aicda-Gfp-Mut* mice. The controls exhibited a gradual increase of low-intensity AID-GFP fluorescence with time of stimulation [as also observed by (Crouch et al., 2007)]; this expression profile mirrored that of endogenous AID. In contrast, cells from *Aicda-Gfp-Mut* mice showed a rapid AID-GFP induction, peaking early around day 3, then reaching a plateau (Figures 2A and 2B). The median intensity of AID-GFP fluorescence was also higher in the *Aicda-Gfp-Mut* mice, suggesting more abundant AID-GFP protein than in controls (Figure 2C).

These disparities were not due to position effects resulting from differential integration of each BAC transgene because (1) large BACs such as this are not susceptible to overexpression due to random insertion in active expression sites (Gong et al., 2003; Hatten and Heintz, 2005) and (2) we have screened multiple independent *Aicda-Gfp-Mut* founder lines with identical results (Figure S3). In addition, these differences were not a result of AID-GFP overexpression due to copy number variation between the two BAC transgenics because we have screened animals with similar copy numbers and obtained identical results (Figure S3). Therefore, our results demonstrate that in vivo disruption of the AID miR-155 target perturbs the quantitative and temporal expression characteristics of AID.

To assess the functional consequences of disruption of the miR-155 target site, we bred the *Aicda-Gfp* control and

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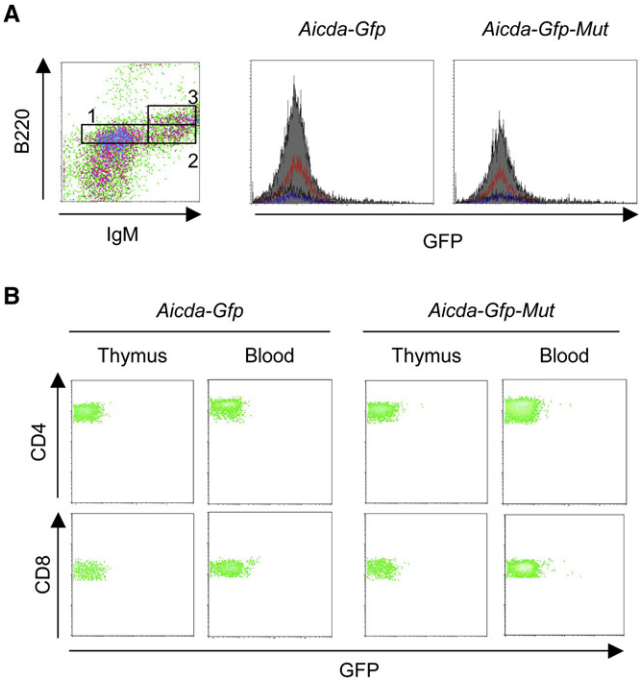

Figure 3. NP-Immunized *Aicda-Gfp-Mut* Mice Do Not Express AID-GFP in Developing B Lymphocytes or in T Lymphocytes

FACS analyses are shown for representative *Aicda-Gfp* and *Aicda-Gfp-Mut* mice.

(A) Developing B cells ($CD93^+$) from the bone marrow are subdivided into pro- and pre-B cells (box 1: IgM^-, $B220^{low}$), transitional B cells (box 2: IgM^+, $B220^{low}$), and recirculating B cells (box 3: IgM^+, $B220^{high}$). Histograms for GFP fluorescence show pro- and pre-B cells (red), transitional B cells (blue), and recirculating B cells (black).

(B) $CD8^+$ and $CD4^+$ T lymphocytes from thymus and peripheral blood are shown.

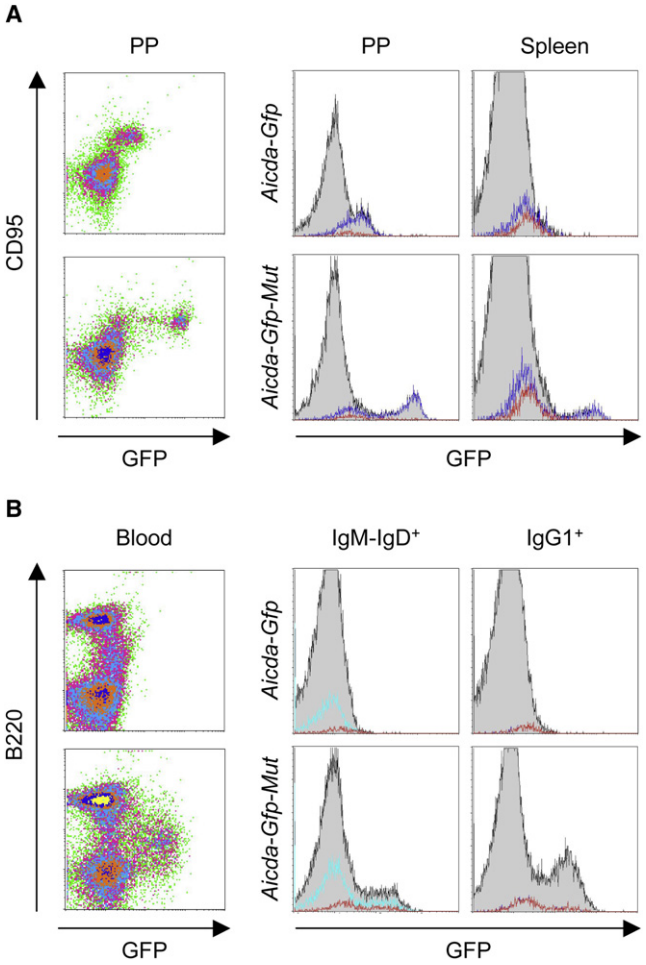

Figure 4. Mutation of the AID miR-155 Target Site Results in Deregulated In Vivo Expression of AID-GFP in NP-CGG Immunized Mice

(A) FACS analyses for AID-GFP expression in GCs from representative *Aicda-Gfp* and *Aicda-Gfp-Mut* mice. (Left) GC B cells from Peyer's Patches (gated on $B220^+$ B cells). (Right) Histograms show AID-GFP expression in B cell subsets from GCs of Peyer's patches (PP) and spleen. Shown in overlay are $CD95^+$ $B220^+$ GC B cells (blue) and the subset of GC B cells that recognize NP (red).

(B) FACS analyses for AID-GFP expression in peripheral blood from representative *Aicda-Gfp* and *Aicda-Gfp-Mut* mice. (Left) Total blood lymphocytes are shown. (Right) Histograms show AID-GFP expression in $IgM\text{-}IgD^+$, $B220^+$ (cyan) and $IgG1^+$, $B220^+$ (blue) B cell subsets. Red overlays indicate the respective subsets that recognize NP (note that the blue and red overlays in the $IgG1^+$ histogram overlap completely).

Aicda-Gfp-Mut mice onto an $Aicda^{-/-}$ background to extinguish the contribution of endogenous AID. We first compared in vitro CSR efficiency by stimulating splenic B lymphocytes from these mice by using LPS (to induce CSR to IgG3), both LPS and IL-4 (to induce CSR to IgG1), or both LPS and IFNγ (to induce CSR to IgG2a). $Aicda^{-/-}$ B cells perform negligible in vitro CSR (data not shown). We observed increased CSR in the *Aicda-Gfp-Mut* mice compared to *Aicda-Gfp* control or wild-type mice (Figure 2D). This was the case regardless of whether these BAC transgenics were deficient or heterozygous for *Aicda* in the endogenous locus (Figure 2D). These results indicate that miR-155 directly regulates AID expression in stimulated B lymphocytes.

Mutation of the miR-155 Target Site Leads to Local but Not Global Deregulation of AID In Vivo

To study in vivo effects of disrupting the AID miR-155 target site, we immunized the transgenic mice intraperitoneally with nitrophenol conjugated to chicken gamma globulin (NP-CGG). The NP hapten induces a well-characterized immune response (Cumano and Rajewsky, 1985; Furukawa et al., 1999; Taketani et al., 1995) in peripheral lymphoid germinal centers (GC), microenvironments where activated lymphocytes undergo Ig diversification. Eighteen to 21 days after immunization, we evaluated AID-GFP expression by FACS in various B lymphocyte subsets. Mutation of the miR-155 target site did not disrupt global transcriptional control of AID: Immature $CD93^+$ B lymphocytes in the bone marrow, along with thymic and peripheral $CD4^+$ or $CD8^+$ T lymphocytes, were devoid of AID-GFP (Figure 3). However, AID-GFP was detected in B cell populations associated with activation. GC B lymphocytes ($CD95^+$ $B220^+$) from spleen and intestinal Peyer's patches expressed AID-GFP in both the control and *Aicda-Gfp-Mut* mice. The latter, however, showed far more intense GFP fluorescence, indicating a similar overabundance of AID-GFP during the GC reaction, as observed during in vitro CSR (Figure 4A).

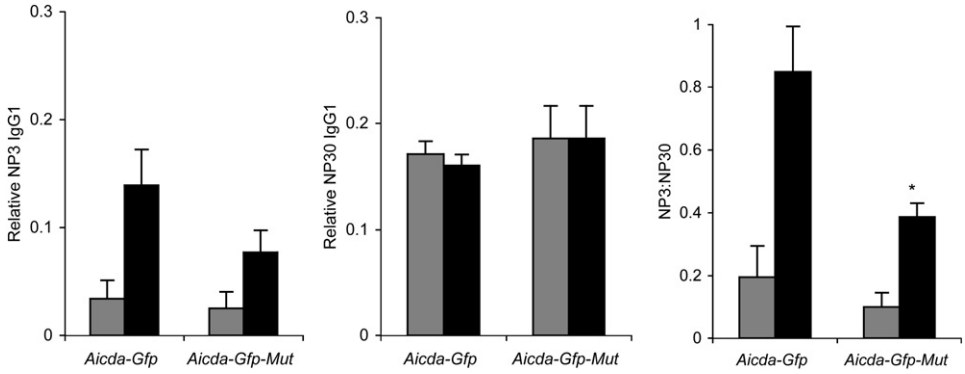

Figure 5. *Aicda-Gfp-Mut* Mice Show Loss of Affinity Maturation

Shown from left to right are average titers of NP3-binding IgG1, NP30-binding IgG1, and NP3:NP30 binding ratios as measured by ELISA on day 8 (gray) or day 19 or 21 (black) after immunization in *Aicda-Gfp* (n = 3) and *Aicda-Gfp-Mut* (n = 7) mice ± SEM. t test, *p = 0.0016.

After completing the GC reaction, activated B lymphocytes exit into the periphery and differentiate into plasma or memory cells. These post-GC B lymphocytes have been shown to cease expression of AID (Crouch et al., 2007). Accordingly, the *Aicda-Gfp* control mice did not express AID-GFP in B cells in the peripheral blood (Figure 4B). In contrast, a GFP^+ population of B cells was consistently detected in the blood of immunized *Aicda-Gfp-Mut* mice (Figure 4B). These appeared to be a heterogeneous population of B lymphocytes ($B220^{int-hi}$), which were mostly $IgG1^+$ but also included IgM^+ and IgD^+ nonswitched cells. This B lymphocyte population did not appear to arise ectopically as a consequence of AID-GFP overexpression; a similar $B220^{int}$ (though GFP^-) population was also present in the *Aicda-Gfp* controls. Thus, disruption of the AID miR-155 target site allows for improper persistence of AID-GFP expression beyond the GC compartment. These results confirm once again that the phenotypes we observed cannot be explained by variation in BAC copy number, which could be expected to increase protein expression but cannot lead to persistent expression in cells where the gene of interest is normally shut off. Thus, our data strongly support the idea that miR-155 controls AID expression levels and, specifically, plays a pivotal role in extinguishing AID expression in post-GC B cell populations.

Loss of miR-155 Regulation of AID Results in Impaired Affinity Maturation

To determine the functional impact of defective AID downregulation, we compared affinity maturation (increased antibody affinity achieved through SHM) of NP-binding IgG1 in sera collected at 8 days and 19 or 21 days after immunization. The ELISA capture substrate was NP conjugated to BSA in different ratios to detect high-affinity (NP_3-BSA) and total (NP_{30}-BSA) immunoglobulins (Ig) specific for NP. Affinity maturation was measured as the NP_3:NP_{30} binding ratio. As expected, we observed an increase in the proportion of high-affinity Ig from day 8 to day19 or 21 in control *Aicda-Gfp* mice. However, affinity maturation was significantly impaired in *Aicda-Gfp-Mut* animals (Figure 5).

To ascertain whether the observed affinity-maturation defect resulted from AID-GFP overexpression in GC's, we analyzed the mutation load at Ig loci in $CD95^+$ GC B lymphocytes. After sequencing the V_H186.2 gene segment that is selected for during the Ig response against NP (Cumano and Rajewsky, 1985) (to assess the mutation profile under selection) and the J_H4 intron (to assess the mutation profile in the absence of selection pressure), we found that the frequencies and overall patterns of mutation did not differ substantially between *Aicda-Gfp* and *Aicda-Gfp-Mut* mice (Table 1). Thus, we considered two possibilities: Either the repair mechanisms that process AID-mediated lesions are not overwhelmed by higher lesion loads, or excess AID was not specifically targeted to the Ig locus.

Aside from the Ig genes, a number of other genes, including the oncogene *Bcl6*, have been shown to hypermutate in the germinal centers (Pasqualucci et al., 2001; Shen et al., 1998). We reasoned that excess AID mutational activity, if not targeted to the Ig genes, could instead be targeted to these other non-Ig loci. To test this hypothesis, we cloned and sequenced *Bcl6* from GC B lymphocytes of NP-immunized *Aicda-Gfp* and *Aicda-Gfp-Mut* mice. We found that *Bcl6* from *Aicda-Gfp* GC B cells was mutated with a frequency of 0.17×10^{-3} per base [similar to reported *Bcl6* mutation frequency in murine GC B cells from Peyer's Patches (Muto et al., 2006) (Table 1)]. In contrast, *Bcl6* from *Aicda-Gfp-Mut* GC B lymphocytes mutated about three times as frequently (0.46×10^{-3} per base) (Table 1). This observation supports the hypothesis that excess AID activity within the GC is distributed to non-Ig targets for hypermutation and raises the possibility that loss of miR-155-mediated downregulation of AID could result in higher rates of AID-dependent translocation or lymphomagenesis.

Table 1. Mutation Frequencies × 10^{-3} per Base

Genotype[a]	V_H186.2	J_H4 Intron	*Bcl6*
Aicda-Gfp	31.8	6.8	0.17
	(394/12,400)[b]	(79/11,600)	(7/41,600)[c]
Aicda-Gfp-Mut	30.7	5.5	0.46
	(800/26,000)	(101/18,400)	(18/39,000)

Mutation frequencies (accumulated number of mutations in a given region divided by the total number of nucleotides sequenced from that region) are shown for V_H186.2, the J_H4 intron, and *Bcl6* from splenic GC B cells in NP-immunized *Aicda-Gfp* and *Aicda-Gfp-Mut* mice.

[a] Mutation data are pooled from two animals.

[b] Number of point mutations/total nucleotides sequenced.

[c] Chi square test, p = 0.0306.

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Table 2. Clonal Variability in Post-GC B Cells

Genotype[a]	Number of Unique Clones/Total	Intraclonal Mutation Frequency × 10^{-3}	Percent W33L or Y99G[d]
Aicda-Gfp	9/50[c]	0.55 (11/20,000)[b]	63
Aicda-Gfp-Mut	36/80[c]	0.87 (34/39,000)	18

Clonal variability of post-GC lymphocytes in *Aicda-Gfp* and *Aicda-Gfp-Mut* mice is shown in terms of clonal mutations in the J_H4 intron and amino acid substitutions associated with NP antibody affinity maturation.
[a] Mutation data represent unique clones pooled from four animals.
[b] Number of point mutations/total nucleotides sequenced.
[c] Fisher's exact test for the indicated data sets, p = 0.043.
[d] W33L and Y99G are substitutions associated with high affinity.

However, these data did not explain the loss of affinity maturation observed in the *Aicda-Gfp-Mut* mice. We then asked whether continued mutation outside of the GC (as a result of persistent AID-GFP expression in post-GC B cells) could account for this phenotype. To test this possibility, we cloned and sequenced the J_H4 intron from post-GC lymphocytes from peripheral blood of *Aicda-Gfp* and *Aicda-Gfp-Mut* mice. We found that B lymphocytes from the blood of *Aicda-Gfp* mice comprised a small number of highly related and affinity-matured clones, with some intraclonal heterogeneity (Table 2). This is not surprising because post-GC B cell clones in the blood have undergone stringent positive selection for those producing high-affinity Ig. In contrast, we found a higher number of unique post-GC B lymphocyte clones in the blood of *Aicda-Gfp-Mut* animals. Because these clones were so heterogeneous, we could not conclusively ascertain whether the mutational load per unique clone was higher in comparison to those from the blood of *Aicda-Gfp* mice (i.e., whether these cells were continually mutating their J_H4 intron). However, in agreement with the serum data, these clones did not appear to have acquired the amino acid substitutions associated with high-affinity NP antibodies [notably, the W33L substitution or the Y99G substitution (Furukawa et al., 1999)]. Thus, it did not appear that these clones emerged from the germinal center with high affinity, which would then be lost as a result of continuing mutation; rather, it appeared that persistent AID expression led to escape from the germinal center of cells bearing low-affinity NP antibodies.

DISCUSSION

Although hundreds of miRNAs are present in the mammalian genome, genetic studies addressing their physiological roles are at an early stage. In the present work, we address the function of miR-155 in the context of antibody diversification by identifying this particular miRNA as upregulated in B cells stimulated to undergo CSR, by bioinformatically identifying AID as a putative target, and by genetically mutating the target sequence in the 3′ UTR of the AID mRNA to allow the gene to escape miR-155 control. These experiments identify a target of a miRNA in vivo by manipulating not the expression of the miRNA itself but rather by genetically disrupting the association between a miRNA and its putative target gene.

Most genes contain multiple predicted target sites for several different miRNAs. Deletion of just one of these targeting miRNAs can result in protein upregulation in a dose-dependent manner, leading to the hypothesis that multiple microRNAs synergistically fine-tune the expression of a single target gene (Xiao et al., 2007). Surprisingly, the AID 3′ UTR is predicted to contain only one miRNA target site, binding miR-155. We have shown that mutation of the seed region of this site leads to dramatic protein overabundance and disruption of proper temporal protein downregulation. We do not exclude the possibility that the specific point mutations introduced at the miR-155 target site could result in additional miR-155-independent effects (for example, alteration of RNA secondary structure, mRNA stability, or disruption of a binding site for some unknown regulatory factor). However, the previously reported miR-155-deficient mouse also showed upregulated AID expression (though to a lesser degree than observed in our *Aicda-Gfp-Mut* mouse, to be discussed below) (Vigorito et al., 2007), and we believe that the phenotypes observed in the *Aicda-Gfp-Mut* mouse are explained by a model in which miR-155 directly regulates AID expression. In addition, AID expression is stringently regulated in B cells such that mice heterozygous for *Aicda* show clear signs of haploinsufficiency (data not shown). These observations together with our results suggest that, especially when protein amounts are already limiting, a single miRNA can contribute far more substantially to control of protein expression than previously appreciated.

Ablating miR-155 control of AID expression led to increased protein expression in switching cells, which undergo significantly higher levels of CSR. It also led to increased protein expression in germinal-center cells. However, increased AID expression did not lead to more SHM in the Ig locus, in accordance with previous work by the Honjo group (Muto et al., 2006), whose members have shown that transgenic AID overexpression does not lead to an increase in SHM. Our data presented here, and the data published by Muto et al., support the notion of the existence of a limiting factor that targets AID to the Ig locus so that, in its presence, excess AID cannot access the locus (though in its absence it can, possibly in a stochastic fashion).

Excess AID in germinal-center cells, however, appears to increase hypermutation in other loci, such as *Bcl6*, which are known secondary targets of the SHM apparatus. Excess AID is also likely to increase the rates of chromosomal translocations associated with errant hypermutation (Ramiro et al., 2006; Ramiro et al., 2004) (also, see Dorsett et al., 2008, in this issue). Therefore, lack of miR-155 control of AID expression might be causal for B cell lymphomagenesis. Indeed, Kluiver et al. have recently documented a lack of miR-155 expression in primary cases of B cell Burkitt's lymphomas, which constitutively express AID (Kluiver et al., 2006).

In addition to overabundance of AID, we find that lack of miR-155 control leads to persistence of AID expression in post-germinal-center B cells, thus effectively marking a unique subset of circulating B cells as recent emigrants from the germinal center. We also find that persistent AID expression is associated with specific defects in affinity maturation. It is possible that persistent AID expression supports ongoing mutation in the Ig locus well after cells exit the germinal center with an affinity-matured antigen receptor and thus effectively destroys the properly selected antigen-specific Ig repertoire. However, this scenario is not strongly supported by our data, emphasizing again that specific cofactors might be required to target AID to the Ig locus. Instead, we observe multiple low-affinity B cell clones in the blood

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of animals for which the AID gene is not subject to miR-155 control, suggesting a defect in positive selection of properly matured B cells. Although the mechanisms of positive selection and affinity maturation are not well understood, it is thought that B cells cycle between the dark zone of the germinal center, where mutation occurs, and the light zone, where their newly minted receptors are substrates for positive selection. Eventually, high-affinity B cells are thought to emerge after multiple rounds of recycling through the germinal center. Our data support a scenario where B cells overexpressing AID might not be allowed to "recycle" into a germinal center for proper affinity maturation. We could speculate on the existence of a mechanism for cellular sensing of AID expression that would form a feedback loop between proper AID extinction and GC cycling. However, this would be difficult to experimentally demonstrate because little is presently known about the cellular and molecular parameters governing the mechanism of proper selection and affinity maturation.

The previously described miR-155-deficient mouse models (Rodriguez et al., 2007; Thai et al., 2007) highlighted the immense contribution of miR-155-mediated regulation to various aspects of vertebrate immunity. The phenotypes therein—specifically, a modest increase in AID expression, decreased in vivo CSR to IgG1, and impaired affinity maturation—probably reflect the composite deregulation of at least 60 genes (Vigorito et al., 2007). In contrast to global ablation of miR-155 regulation, we describe here specific disruption of a single miR-155: target interaction. In our mouse model, we observed robust deregulation of AID-GFP expression, increased in vitro CSR, and impaired affinity maturation, effects that might not be apparent in the more complex context of total miR-155 deficiency.

As the central catalyst for antibody diversification processes, AID has been shown to be regulated at the point of transcription (Dedeoglu et al., 2004; Gonda et al., 2003; Sayegh et al., 2003), by nucleo-cytoplasmic trafficking (Ito et al., 2004; McBride et al., 2004), and by phosphorylation (Basu et al., 2005; Basu et al., 2007; McBride et al., 2006; Pasqualucci et al., 2006). Here we reveal an additional miRNA-mediated pathway of AID regulation, which controls AID expression in germinal-center B cells and ensures proper extinction of AID expression as cells undergo affinity maturation and exit the secondary lymphoid organs. Thus, miR-155 plays an important role in the molecular restraint of AID, an enzyme that confers great immunological benefit but whose mutagenic potential must limited by tight regulation.

EXPERIMENTAL PROCEDURES

B Lymphocyte Isolation and Cell Culture

$CD43^-$ naive splenic B lymphocytes were purified by magnetic separation (MACS, Miltenyi Biotec). Cells were maintained at 0.5×10^6–1×10^6 cells/ml in standard culture medium and were treated with 5 ng/ml IL-4 (Sigma) and 25 μg/ml LPS (Sigma) so that CSR would be induced to IgG1; with 25 μg/ml LPS so that CSR would be induced to IgG3; or with 25 μg/ml LPS and 0.5 μg/ml IFNγ (Sigma) so that CSR would be induced to IgG2a. CH12-F3 cells were maintained in standard culture medium and were treated with 5 ng/ml IL-4, 0.2 μg/ml anti-CD40 (eBioscience), and 0.1 ng/ml TGF-β (R&D Systems) so that CSR would be induced to IgA. Cell cultures were sampled at various time points for FACS analysis or preparation of RNA.

RNA Isolation and Small RNA Cloning

Total RNA was prepared with Trizol (Invitrogen), and 21–23 nt RNAs were isolated, cloned, sequenced, and cataloged as previously described (Landgraf et al., 2007).

RNA Blots

Total RNA (10–20 μg) was run on a 15% TBE-urea criterion gel (Bio-Rad) and transferred to a Hybond N+ nylon membrane (Amersham Biosciences) by semi-dry blotting. Membranes were UV-crosslinked and dried. A miR-155 probe (5′-ACCCCTATCACAATTAGCATTAA-3′) was prepared by T4 polynucleotide kinase labeling with γ^{32}P-ATP. Blots were hybridized in Denhardt's solution or QuickHyb (Stratagene) at 50°C and were washed twice with wash solution I (5% SDS, 5× SSC) and twice with wash solution II (1% SDS, 1× SSC).

Luciferase Assays

Reporter constructs were modified from the pRL-TK plasmid (Promega). The renilla luciferase gene was replaced by firefly luciferase (fLuc) for creation of the Luc construct. The 3′ UTR of the AID mRNA was cloned directly downstream of fLuc to create the Luc-UTR plasmid. This was then altered by Quikchange PCR (Stratagene) for creation of the Luc-UTR-Del and Luc-UTR Mut constructs. CH12-F3 cells were cotransfected with a reporter construct and pRL-TK by Amaxa nucleofection and were stimulated for CSR as above. Cells were lysed 48 hr after transfection, and the Dual Luciferase Reporter Assay System (Promega) was used for measuring firefly and renilla luciferase activities.

CSR Rescue by AID-GFP

Replication-deficient retroviruses were generated by transfection of 293T cells with either AID-GFP-pQCXIP or pQCXIP alone, along with pCL-Eco packaging plasmid. Viral stocks were used to transduce naive splenic B lymphocytes from *Aicda*$^{-/-}$ mice, which had been stimulated in culture with IL-4 and LPS.

Transgenic Mice

The *Aicda-Gfp-Mut* BAC was modified from the *Aicda-Gfp* BAC used for creating the previously described *Aicda-Gfp* reporter mouse (Crouch et al., 2007) (copy number ranging from 1–10). Mutation of the miR-155 target site was achieved by homologous recombination in bacteria as described previously (Misulovin et al., 2001). In brief, the AID miR-155 target site plus approximately 1 kb flanking sequence on either side (for homologous recombination) was amplified by PCR from BAC RP24-68I7 (GenBank AC158651) with primers 5′-GGCGCGCCGGTAAGTCTGCCTGTCTGTCTGCC-3′ and 5′-GCGGCCGC GCGTCATTTCCTTGCCACGG-3′. The PCR product was cloned into TOPO-pCR4 (Invitrogen). Point mutations in the miR-155 target site were introduced by Quikchange PCR (Stratagene). The sequence was again amplified with the same primers as above and cloned into shuttle vector pLD53.SC.AEB (which also contains the RecA and SacB genes). After propagation in PIR2 bacteria (Invitrogen), the construct was electroporated into bacteria carrying the previously described modified BAC RP24-68I7 containing the *Aicda-Gfp* locus (Crouch et al., 2007). Cointegrates were selected for in liquid culture in the presence of ampicillin (for the insert-containing shuttle vector) and chloramphenicol (for the BAC), during which RecA-mediated recombination occurred between the BAC and homologous sequences inserted into the shuttle vector. Cultures were plated on chloramphenicol, and the desired recombination event (introduction of the modified miR-155 target site into BAC and deletion of the shuttle vector sequence) was ensured by treatment of duplicate plates with UV illumination and sucrose so that the loss of the shuttle-vector-encoded RecA and SacB, respectively, would be ensured. The integrity of the newly generated *Aicda-Gfp-Mut* locus was confirmed by sequencing. Generation of the *Aicda-Gfp-Mut* transgenic founder mice (copy number ranging from 5–20) was performed by the Rockefeller University Transgenic Services Laboratory according to standard methods. Transgene copy numbers were estimated by comparison to copy-number standards in Southern analysis. Animals were housed and studied in accordance with institutional guidelines.

Quantitative PCR

cDNA was generated from DNase-I-treated RNA, and AID-GFP expression was monitored by quantitative PCR (5′-GACTTGCGAGATGCATTTCGTATG-3′ and 5′-GCTGAACTTGTGGCCGTTTAC-3′). cDNA samples were normalized by amplification of Ku70 (5′-TGCCCTTTACTGAGAAGGTGAC-3′ and 5′- TGCT GCAGGACTGGATTCTC-3′).

Immunization and ELISA

Mice were immunized with 100 μg alum-precipitated NP-CGG (Biosearch Technologies) by intraperitoneal injection. Serum was prepared from peripheral

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blood collected by retro-orbital bleed at various time points. Serum dilutions were incubated in NP_3-BSA or NP_{30}-BSA-coated wells of microtiter plates, and NP-specific Ig was detected by ELISA with reagents from Southern Biotechnologies Clonotyping System-HRP. Affinity maturation was calculated as the ratio of NP_3 binding (high-affinity anti-NP Ig) to NP_{30} binding (total anti-NP Ig).

FACS Analysis

Cell suspensions from bone marrow, spleen, Peyer's patches, thymus, and peripheral blood were prepared and stained for FACS according to standard procedures. The following reagents were used for staining cells for FACS analysis (all are from BD Biosciences except where indicated): CD93-PE (eBioscience), IgM-APC (Jackson Immunoresearch), CD95-PE-Cy7, B220-PerCP, B220-APC, IgG1-biotin, IgM-biotin, IgD-biotin (eBioscience), CD8-PerCP, CD4-APC, NP-PE (Biosearch Technologies), and Streptavidin-PerCP.

Mutational Analysis

Genomic DNA was prepared from sorted splenic germinal-center B cells and white blood cells from peripheral blood. The following were amplified by PCR with PfuTURBO polymerase (Stratagene): JH4 intron (5′-AGCCTGACATCTGAGGAC-3′ and 5′-TAGTGTGGAACATTCCTCAC-3′, annealing temperature 55°C for 35 cycles; followed by a nested reaction, 5′-CTGACATCTGAGGACTCTGC-3′ and 5′-GCTGTCACAGAGGTGGTCCTG-3′, annealing temperature 58°C for 35 cycles); VH186.2 (5′-TCTTTACAGTTACTGAGCACACAGGAC and 5′-GGGTCTAGAGGTGTCCCTAGTCCTTCATGACC, annealing temperature 50°C for 35 cycles; followed by a nested reaction, 5′-CAGTAGCAGGCTTGAGGTCTGGAC-3′ and 5′-GGGTCTAGAGGTGTCCCTAGTCCTTCATGACC-3′, annealing temperature 64°C for 35 cycles); *bcl-6* (5′-GGCCGGACACCAGGTGATTAT-3′ and 5′-AGGGAGGGAACTACCGCTGAG-3′, annealing temperature 68°C for 35 cycles).

PCR products were blunt-end cloned into pSC-B (Stratagene) and sequenced with a standard T3 primer.

SUPPLEMENTAL DATA

Three figures are available with this article online at http://www.immunity.com/cgi/content/full/28/5/■■■/DC1/.

ACKNOWLEDGMENTS

We thank S. Fugmann, D. Schatz, and S. Petersen-Mahrt for comments on the manuscript. This work was supported by grants from the Keck foundation, National Institutes of Health grant CA098495 (F.N.P.), National Institutes of Health National Research Service Award training grant GM066699 (G.T.), and the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health (R.C.).

Received: January 6, 2008
Revised: February 6, 2008
Accepted: March 4, 2008
Published online: May 1, 2008

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