A Novel Mouse Model for the Hyper-IgM Syndrome: A Spontaneous Activation-Induced Cytidine Deaminase Mutation Leading to Complete Loss of Ig Class Switching and Reduced Somatic Hypermutation

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A Novel Mouse Model for the Hyper-IgM Syndrome: A Spontaneous Activation-Induced Cytidine Deaminase Mutation Leading to Complete Loss of Ig Class Switching and Reduced Somatic Hypermutation

Carin I. M. Dahlberg, Minghui He, Torkild Visnes, Magda Liz Torres, Elena M. Cortizas, Ramiro E. Verdu, Lisa S. Westerberg, Eva Severinson, and Lena Ström

We describe a spontaneously derived mouse line that completely failed to induce Ig class switching in vitro and in vivo. The mice inherited abolished IgG serum titers in a recessive manner caused by a spontaneous G→A transition mutation in codon 112 of the aicda gene, leading to an arginine to histidine replacement (AID<sup>R112H</sup>). Ig class switching was completely reconstituted by expressing wild-type AID. Mice homozygous for AID<sup>R112H</sup> had peripheral B cell hyperplasia and large germinal centers in the absence of Ag challenge. Immunization with SRBCs elicited an Ag-specific IgG1 response in wild-type mice, whereas AID<sup>R112H</sup> mice failed to produce IgG1 and had reduced somatic hypermutation. The phenotype recapitulates the human hyper-IgM (HIGM) syndrome that is caused by point mutations in the orthologous gene in humans, and the AID<sup>R112H</sup> mutation is frequently found in HIGM patients. The AID<sup>R112H</sup> mouse model for HIGM provides a powerful and more precise tool than conventional knockout strategies. The Journal of Immunology, 2014, 193: 4732–4738.

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In this study, we identified a spontaneously occurring arginine 112 histidine mutation in AID (AID<sup>R112H</sup>) and characterized its impact on the B cell Ig class switching in vitro and in the vivo immune response.

**Materials and Methods**

**Mice**

Mice were bred in the animal facility of the Department of Cell and Molecular Biology at Karolinska Institutet or the Department of Molecular Biosciences at Stockholm University or were bought from Charles River. All experiments were approved by the animal ethical committee of Stockholm Region North.

**In vitro cultures for class-switch analysis and retroviral transduction**

Spleen cells were enriched for total or small resting B cells, as described previously (7). Different stimuli were used to detect various Ig isotypes: 10 ng/ml LPS (Sigma-Aldrich) to measure switching to IgG2b and IgG3, 10 μg/ml anti-CD40 + 8–16 ng/ml IL-4 to measure IgG1 and IgE, and LPS + 5 ng/ml IL-5 + 0.5 ng/ml TGF-β to measure IgA (all cytokines were from PeproTech). For retroviral transduction, splenic B cells from wild-type or AID<sup>R112H</sup> mice were stimulated with LPS for 24 h and subsequently transduced with retroviral particles, as described (8). LPS or anti-CD40 + IL-4 were added, and the cells were cultured for three additional days. Cultured B cells were harvested, washed, and fixed, as described (7), and stained with biotinylated anti-mouse IgG1, IgG2b, IgG3, IgE, or IgA or isotype controls (all from BD Biosciences), followed by Streptavidin-FITC diluted in a balanced salt solution with 0.1% saponin. The percentages of cells expressing different Ig isotypes were determined using a FACSCalibur (BD Biosciences).

**RT-PCR**

At the indicated time points after the addition of stimuli, cells were harvested, and total RNA was prepared using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA was synthesized using AMV reverse transcriptase (Roche) at 42°C for 1 h, primed by Oligo dt (Roche). RT-PCR was run on a Corbett Rotor-Gene 6000 and analyzed with Rotor-Gene 6000 series software 1.7. The γ1 germline (GL) transcripts were quantified using SYBR Green (KAPA BIOSYSTEMS). The following primers were used: γ1 GL transcript, forward primer: 5′-GCCAGCTCTTCTTGGCCCT-3′ and reverse primer: 5′-CTGCAGGTCGAGGGCTG-3′ and Mb1, forward primer: 5′-CTCAGGTTAGAGGGCTG-3′ and reverse primer: 5′-CTTGGAGATCAGGAGG-3′. mRNA levels of Aicda, Ung, and β-actin were quantified with a TaqMan expression assay using Mm00507774_m1, Mm00449156_m1, and Mm00607939_s1, respectively (Life Technologies).

**Immunizations and ELISA for serum levels of Ig**

Wild-type and AID<sup>R112H</sup> mice were immunized by i.p. injection of 2.6-trinitrophenol (TNP)-conjugated SRBCs (TNP-SRBCs) diluted 1:100 in PBS. TNP conjugation to SRBCs was performed as described (9). Mice were euthanized 6 d after immunization, and spleens were imbedded in Tissue-Tek OCT compound (Sakura) for immunohistochemistry. To determine anti-TNP Ab titers in response to TNP-SRBC immunization, anti-TNP IgM and IgG1 Abs in sera were measured by standard ELISA. Total IgG and IgM were captured with purified anti-mouse IgG4 (Southern Biotech). Specific Abs against TNP were captured with SpecificAbs against TNP-agarose. The slides were incubated with primary Abs for 30 min at room temperature, washed with PBS, incubated at room temperature for 30 min with a secondary Ab, and washed again with PBS.

The following reagents were used: allopurinol (Sigma-Aldrich), streptavidin–Alexa Fluor 555, FITC–conjugated CD169 (MOMA-1; AbD Serotech), and biotinylated peanut agglutinin (Vector Laboratories). Images were acquired using a Leica DM IRBE confocal laser scanning microscope (Leica Microsystems) equipped with one argon and two HeNe lasers, using an HC PL APO lens at 10×/0.40 CS 90% glycerol (MP Biomedicals). Images were processed with Adobe Photoshop CS4 Version 11.0.2 (Adobe Systems).

**Western blot**

Cells were harvested, and protein extracts were prepared at indicated time points. A total of 100 μg total protein extracts was run on 12% SDS-PAGE and immunoblotted with monoclonal rat anti-mouse AID (Active Motif) or rabbit anti α-actin Abs (Sigma-Aldrich), followed by HRP-conjugated donkey anti-rat Ig (Jackson ImmunoResearch) or swine anti-rabbit Ig (Dako).

**Sequencing and SHM analysis**

Primary B cells were activated for 3 d with anti-CD40 + IL-4, and RNA was extracted and reverse transcribed. The aicda sequence was amplified using the following primers: AID F1, 5′-GCAAAGGCTCTTCTGCGCTA-3′ and AID R2, 5′-TAAACAAATGTGTGTGC-3′. The amplified DNA was purified and cloned into a TOPO TA cloning vector (Invitrogen). Three separate clones derived from HIGM mice were sequenced in both directions and compared with one clone of the wild-type control and to the published mRNA sequence (accession number NM_009645). For SHM analysis, V<sub>116</sub>2 transcripts were amplified from cDNA of GC-enriched B cells from mice immunized with 4-hydroxy-3-nitrophenylacetyl (NP)-keyhole limpet hemocyanin (KLH) using a nested PCR approach. The first amplification was performed with the following primers: V<sub>116</sub>2.5′-GTGCTATATCTGCTTCTCCG-3′ and Cm, 5′-AGGGGCTCTGCGAGGACGAGG-3′. The second set of primers was V<sub>116</sub>2.5′-GTGCTTACACTCCAGCGGCTA-3′ and Cm, 5′-AGGGGCTCTGCGAGGACGAGGAC-3′ for amplification of IgM transcripts. Resulting PCR products were cloned into TOPO TA (Invitrogen) and sequenced (Operon).

**Genotyping of aicda R112H mutation**

Exon 2 of the aicda gene was amplified from genomic DNA using Promega GoTaq polymerase supplemented with 1.6 mM MgCl<sub>2</sub> and the following primers: 5′-CTCTACCTCACCTGGAATGTG-3′ and 5′-TGATCCTCCATCTCCAGCTGTTA-3′, with 30 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. The resulting 259-bp PCR fragment was digested or not with 1 U BssHII at 37°C for 2 h and run on an agarose gel. PCR products from wild-type alleles were cut into 175- and 84-bp fragments, whereas the AID<sup>R112H</sup> band was resistant to digestion. The nibpl gene was genotyped as described (10).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed as previously described (11). In short, activated or nonactivated B cells were crosslinked with 1% formaldehyde for 20 min at room temperature, and the reaction was stopped by addition of glycine to 125 mM final concentration. Cells were washed twice with cold PBS, resuspended in RIPA buffer (150 mM NaCl, 1% [v/v] IGEPAL CA-630, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 50 mM Tris-HCl [pH 8], 5 mM EDTA, and protease and phosphatase inhibitors), and sonicated on a Bioruptor Next Gen (Diagenode). For immunoprecipitation, 0.5 μg (2 μg/μl) protein extract was cleared for 2 h with 30 μl Protein A/G-Sepharose slurry before Ab was added. Immunocomplexes were eluted, and cross-linking was reversed by adding to 200 mM NaCl, 1 mM EDTA, and 1 mM DTT and incubating overnight at 65°C in the presence of 5 μg proteinase K. DNA was purified using a QIAquick PCR purification kit and quantified with real-time PCR using SYBR Green.

**Statistics**

Statistical significance between groups was assessed by the two-tailed Student t test and ANOVA; a paired t test was used for the reconstitution experiments. The p values < 0.05 were considered significant.

**Results**

A mouse with complete absence of in vitro Ig class-switch response

During the course of a project analyzing mice heterozygous for a gene-trap mutation in nibpl (Nipped-B like) (10), we found that
B cells of one control mouse were incapable of activating Ig class switching in vitro. Purified peripheral B cells were stimulated with anti-CD40 + IL-4, LPS, LPS + anti–δ-dex, or LPS + IL-5 + TGF-β. After 3–5 d of culture, cells were harvested, stained for different Ig isotypes, and analyzed using flow cytometry. Although B cells from the nipbl<sup>+/-</sup> mouse mounted a seemingly normal isotype-switched response, the cells from the control mouse did not respond (Supplemental Table I). When the cultures were examined microscopically, cells appeared healthy and grew normally. To exclude the possibility that the animals or the samples were mistaken during the experimental procedure, genotyping was repeated on the cultured cells; the control cells were indeed wild-type with respect to the nipbl gene. This caught our interest, and we sought to understand the reason for the lack of Ig class switching.

**Identification of inheritance pattern of the HIGM trait**

The mouse with abolished Ig class switching in vitro was the offspring of a mating between a nipbl<sup>+/-</sup> male and two CD-1 wild-type females. This mating produced 18 pups, of which 6 were heterozygous for the nipbl transgene. We hypothesized that this breeding serendipitously gave rise to immunodeficient offspring. A consequence of this might be an inability to secrete IgG in the bloodstream. The 15 available nipbl<sup>+/-</sup> and nipbl<sup>+/+</sup> siblings of the mouse in which we had detected abolished in vitro Ig class switching were used for further analysis. We measured the IgM and IgG serum titers and found that 3 of 15 mice from two litters had IgG serum titers close to null but normal to slightly elevated IgM serum titers (Fig. 1A, F<sub>0</sub>CD1 generation). We called this phenotype HIGM, because it resembled the human hyper-IgM immunodeficiency. Breeding was initiated to monitor the inheritance of the HIGM phenotype. The two HIGM females were bred to the single HIGM male, and all of the resulting offspring had IgG serum titers close to the detection limit (Fig. 1A, F<sub>1</sub>CD1 generation), indicating that the HIGM trait is genetically inheritable. We crossed the same HIGM male to two wild-type C57BL/6 females and found that none of the 16 pups born had the HIGM

**FIGURE 1.** Inheritance of the HIGM phenotype. (A) ELISA analysis of total serum titers of IgM and IgG Abs in littermates of the founder HIGM mouse (F<sub>0</sub>CD1, n = 11) identified three mice as having the HIGM phenotype (indicated in red). Interbreeding of these mice resulted in 20 offspring (F<sub>1</sub>CD1) with very low IgG serum titers. (B) One HIGM male was bred with C57BL/6 females to start generating mice backcrossed to the C57BL/6 background [F<sub>1</sub>(B<sub>6</sub>/CD1) n = 16]. Further interbreeding generated F<sub>2</sub>(B<sub>6</sub>/CD1) mice (n = 54), of which a quarter displayed the HIGM phenotype. (C) Pedigree of mice carrying the HIGM trait, which was discovered in 4 of 18 littermates (F<sub>0</sub> generation). The originally discovered F0 mouse is crossed out, whereas breeding of the remaining male HIGM mouse with its female HIGM siblings resulted in 20 offspring all displaying the HIGM phenotype (F1). None of the 16 offspring from the HIGM male mated to C57/B6 females displayed the HIGM phenotype, whereas interbreeding the F1 offspring reproduced the HIGM phenotype in about one quarter of the offspring (F2).
phenotype (Fig. 1B, F1<sup>B6</sup>/CD<sub>1</sub> generation). In the F2<sup>B6</sup>/CD<sub>1</sub> generation, we got 54 pups that were analyzed for IgM and IgG serum titers (Fig. 1B). Roughly one fourth of these displayed very low IgG titers at 6 wk of age, suggesting that the observed HIGM phenotype displayed a recessive Mendelian inheritance pattern (Fig. 1C).

Mice from these subsequent generations were tested for the ability to perform Ig class switching in vitro. The class-switching defect identified in the original founder of the phenotype was then confirmed (Fig. 2A, 2B). This might be due to defective proliferation, but we found that the proliferative response was indistinguishable between HIGM and control B cells (Supplemental Fig. 1).

We then investigated whether the reason for the abolished Ig class switching was reduced expression of GL transcripts, which are necessary to create chromatin accessibility of S-regions in the Ig locus. After stimulation with anti-CD40 + IL-4, wild-type and HIGM B cells expressed equal levels of GL γ1 transcripts (Fig. 2C). <i>aicda</i> was an obvious candidate gene that, if mutated, could cause this HIGM phenotype. However, we found comparable quantities of AID in HIGM and wild-type B cells at both the mRNA and protein levels (Fig. 2C, 2D). In addition, we tested the expression of Ung mRNA and also found that to be equally expressed (Fig. 2C).

**Identification of the R112H mutation in aicda**

Of the genes causing the human HIGM syndrome, CD40L was not a likely candidate, because stimulation with anti-CD40 would then have resulted in normal Ig class switching in vitro. In an attempt to identify the mutation causing the HIGM phenotype, we sequenced cDNA for <i>aicda</i>, as a first candidate. Compared with the published sequence, we found one alteration at position 427 consisting of a G→A mutation, leading to the amino acid replacement arginine 112 to histidine (Fig. 3A). This mutation is localized in a region of the <i>aicda</i> gene between the cytidine deaminase domain and the APOBEC1-like domain. The corresponding residue in human <i>AICDA</i> is frequently mutated in HIGM patients harboring AID deficiencies (12). This region is localized just outside a short sequence that constitutes a recognition loop determining the substrate specificity toward deoxyctydines (Fig. 3B).

To confirm that the HIGM phenotype was due to the mutation in <i>aicda</i>, we reintroduced wild-type AID by means of retroviral transduction using a construct in which the <i>aicda</i> gene was flanked by GFP, separated by an internal ribosome entry site motif. LPS-activated B cells isolated from AID<sup>R112H</sup> or wild-type mice were transduced with retroviral particles containing the genes for AID and GFP or only GFP. Cells were activated with LPS or anti-CD40 + IL-4 for three additional days, and Ig class switching was measured among the GFP<sup>+</sup> cells. Ectopic expression of wildtype AID in AID<sup>R112H</sup> B cells fully rescued the Ig class-switching response (Fig. 3C); therefore, we concluded that the HIGM phenotype is due to the R112H mutation.

Because AID<sup>R112H</sup> was expressed at normal levels (Fig. 2D), we were interested in how the mutation influenced nuclear translocation and chromatin association. To address this, we performed chromatin immunoprecipitation in combination with real-time PCR using primers specific for the AID binding site in the Ig S<sub>m</sub> locus (11). DNA was isolated from wild-type and AID<sup>R112H</sup> B cells at 0 or 48 h.
after stimulation with anti-CD40 + IL-4. At 0 h, no binding could be detected in either population of cells (data not shown). At 48 h, maximum AID binding was detected in wild-type cells, whereas the AID<sup>R112H</sup> association was 50% of that.

**Naive AID<sup>R112H</sup> mice have increased peripheral B cell number and large GCs and fail to induce SHM**

HIGM patients with loss-of-function mutations in AID have highly proliferative IgM<sup>+</sup>IgD<sup>+</sup> B cells and enlarged GCs (13). Likewise, gene-targeted aicda<sup>−/−</sup> mice display expansion of B cell follicles and large GCs in the absence of Ag challenge (14). In the bone marrow, AID<sup>R112H</sup> mice and littermate controls showed similar B cell development, as characterized by the Hardy classification (C.I.M. Dahlberg and L.S. Westerberg, unpublished observations). When assessing peripheral B cell development and function, before and after immunization with TNP-SRBCs, AID<sup>R112H</sup> mice had an increased number of total B cells in the spleen compared with wild-type littermate mice (Fig. 4A). This increased number of B cells was evident in both the follicular and the marginal zone subsets of B cells (C.I.M. Dahlberg and L.S. Westerberg, unpublished observations). Wild-type mice showed few, if any, GCs before immunization but formed large GCs in response to TNP-SRBCs at day 6 (Fig. 4A, 4B). AID<sup>R112H</sup> mice already had enlarged GCs in the spleen before challenge, and they remained large after challenge with TNP-SRBCs (Fig. 4A, 4B). We next examined the anti-TNP Ab response in serum. Wild-type mice showed increased serum titers of TNP-specific IgG1 after immunization, whereas AID<sup>R112H</sup> mice, as expected from the in vitro defect in Ig class switching, failed to induce a TNP-specific IgG1 serum response (Fig. 4C). We did not detect any differences in total plasma cell number when comparing wild-type and AID<sup>R112H</sup> mice before and after TNP-SRBC immunization. When assessing IgG1-producing plasma cells, wild-type mice responded to TNP-SRBCs with an increased number of IgG1<sup>+</sup> plasma cells, whereas AID<sup>R112H</sup> failed to do so (Fig. 4D).

To investigate SHM, AID<sup>R112H</sup> mice were immunized with the T cell–dependent Ag NP-KLH. NP-KLH induces selection of up to 50% of NP-specific B cells that express the specific V<sub>H</sub> chain (V<sub>H1</sub>) V<sub>H186.2</sub> (15, 16). Upon immunization, wild-type mice had increased serum titers of NP-specific IgG1, whereas AID<sup>R112H</sup> mice lacked an IgG1 response (data not shown). To establish the rate of SHM and introduction of the reported high-affinity mutation in CDR1, W33L, in response to NP-KLH, we prepared cDNA from GC-enriched B cells and sequenced V<sub>H1</sub>/86.2 combined with the constant segment for IgM. Wild-type B cells induced SHM, as evident by many replacement mutations and presence of the high-affinity W33L mutation in 6 of 72 sequences (data not shown, Fig. 4E). In contrast, AID<sup>R112H</sup> B cells had an overall low rate of mutations and failed to induce the W33L mutation (0/86 sequences) (Fig. 4E).

**Discussion**

In this article, we describe an inherited trait in mice that is caused by a point mutation in aicda and results in a failure to switch Ig class. The arginine 112 histidine mutation renders AID catalytically inactive (17), thus explaining the lack of Ig class switching and SHM in AID<sup>R112H</sup> B cells. The AID<sup>R112H</sup> mutation has been observed in several unrelated HIGM patients, and it is a frequent cause of AID deficiency (12, 18).

It is interesting that the AID<sup>R112H</sup> mutation appeared with the nipbl<sup>+/+</sup> genotype. The most well-characterized function for NIPBL is DNA loading of the cohesin complex. Both NIPBL and

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**FIGURE 3.** The defect in class switching is caused by an AID arginine 112 histidine mutation. (A) Sequence of aicda cDNA, corresponding to amino acids 111–113. Mutant (upper panel); wild-type (lower panel). (B) Schematic illustration of the AID protein; the R112H mutation and important functional domains are indicated. (C) Primary splenic B cells were stimulated in vitro with LPS for 24 h, transduced with retroviral particles, and stimulated with either LPS or anti-CD40 + IL-4. After a total of 5 d, the cells were stained for expression of the indicated Ig isotypes and analyzed by flow cytometry. Average of three independent experiments is shown. *p < 0.05, **p < 0.01, ***p < 0.001, paired t test. Differences between AID-reconstituted wild-type and AID<sup>R112H</sup> mice were not significant. (R112H<sup>−/−</sup> mice have increased peripheral B cell number and large GCs and fail to induce SHM)
cohesin are important for chromosome segregation, gene regulation, and DSB repair (19–21). Thus, nipbl+/2 mice could benefit from lacking functional AID, because AID induces these types of DNA lesions.

The spontaneous AID<sup>R112H</sup> mouse model described in this article shares many similarities with aicda<sup>−/−</sup> mice described by Muramatsu et al. (14), as well as the human HIGM syndrome caused by mutations in AICDA that was identified by Revy et al. (13). They all have abolished in vitro Ig class switching, expansion of peripheral B cells, and large GCs. An important advantage of the AID<sup>R112H</sup> mouse is that AID is expressed normally, allowing for evaluation of its posttranslational regulation. Moreover, the AID<sup>R112H</sup> protein translocated to the nucleus and bound to the S<sup>m</sup> region, albeit with decreased efficiency. Possible reasons for this are deficient interactions with proteins important for targeting of AID to the S regions.
Although recent studies, to some extent, defined the requirements for GC selection of B cells and showed that GC B cells compete for help from T follicular helper cells (22), the exact cues that make B cells leave the GC remain unknown. It was suggested that mutant aicda<sup>−/−</sup> mice have larger than normal GCs because of decreased apoptosis (23). Why B cells without functional AID accumulate and/or fail to leave the GCs remains to be addressed.

A recent study examined the potential role of AID as an epigenetic eraser and transcriptional regulator (24). The investigators addressed the potential influence of the aicda<sup>−/−</sup> genotype but instead found that the identified alterations in the transcription profile were not related to the aicda<sup>−/−</sup> genotype. The AID<sup>R112H</sup> mouse strain described in this study could become a valuable tool in the study of AID function, as well as a model for the human HIGM syndrome.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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Value represent the percentage of total activated B cells

Supplemental Table I

A mouse with complete absence of in vitro Ig class switch response

Splenic B cells were stimulated in vitro with LPS +/- anti-δ-dex for switching to IgG2b and IgG3, with antiCD40 plus IL4 for switching to IgG1 and IgE and with LPS + IL-5 + TGFβ for switching to IgA. After 3-5 days the cells were stained for expression of indicated Ig isotype and analyzed by FACS.
Supplemental FIGURE 1. B cells from HIGM mice proliferate normally. Splenic B cells from a control or a HIGM mouse were labeled with CSFE, as detailed in Materials and Methods, cultured with anti-CD40 + IL-4 for 5 days and analyzed in a flow cytometer. Left: control B cells, right: B cells from a HIGM mouse.