reported by Suspène et al.[67] Human APOBEC3 cytidine deaminases target and edit single-stranded DNA, which can be of viral, mitochondrial, or nuclear origin. Retroviral genomes, such those of HIV, deficient in the *vif* gene, and hepatitis B virus, are particularly vulnerable.

The genomes of DNA viruses, such as herpesviruses, are also subject to editing. This is the case for herpes simplex virus type 1 (HSV-1), at least in tissue culture, where APOBEC3C (A3C) overexpression reduces viral titers and the particle/plaque forming unit (PFU) ratio by approximately 10-fold. A3A, A3G, and activation-induced cytidine deaminase (AICDA) can edit what is thought to be a small fraction of HSV genome in an experimental setting without seriously impacting viral titers. Hyper-editing was found to occur in HSV genomes recovered from four of eight uncultured buccal lesions, but the phenomenon was not restricted to HSV; hyper-mutated EBV genomes were readily recovered from four of five established cell lines, indicating that episomes are also vulnerable to editing [67]. These findings suggest that the widely expressed A3C cytidine deaminase can function as a restriction factor for some human herpesviruses.

Other studies reported sequence variation in BART miRNAs.[68] The significance of these mutations and their effect on miRNA processing, as well as the mechanism of mutation, whether it is mediated by A3C, members of other APOBEC families, or other mechanisms, have yet to be determined.

4. Regulation of EBV-encoded miRNA processing

4.1 Processing of miRNAs under normal versus cancerous conditions

The mechanism of miRNA biosynthesis involves sequential endonucleolytic cleavages mediated by two RNase III enzymes, Drosha and Dicer (Fig.1). Following transcription by RNA pol II, Drosha processes the primary miRNA transcript (pri-miRNA) into a 60-100 nt hairpin structure, termed the precursor miRNA (pre-miRNA), in the nucleus (Fig. 1). Following cleavage by Drosha, the pre-miRNA is transported out of the nucleus through an interaction with Exportin-5 and Ran-GTP. Then, the pre-miRNA undergoes further processing catalyzed by Dicer (Fig. 1). This cleavage event gives rise to an approximately 22 nt dsRNA product containing the mature miRNA guide strand and the miRNA* passenger strand (Fig. 1). Then, the mature miRNA guide strand is loaded onto the RISC, while the passenger strand is degraded (Fig. 1).

Although substantial progress has been made in understanding the basic mechanism of miRNA biogenesis, less is known about the mechanisms that regulate miRNA biogenesis and how these systems might be deregulated during oncogenesis. Several studies have reported that various regulatory mechanisms of miRNA biosynthesis are potentially involved in carcinogenesis.[69]

The tumor suppressor protein p53 was recently found to modulate miRNA processing through its association with p68 and Drosha.[70,71] Under conditions of DNA damage, several miRNAs, such as miR-143 and miR-16, are post-transcriptionally induced. This process requires p53, as p53-null HCT116 cells do not induce miRNAs in response to DNA damage.[72] Co-immunoprecipitation studies have indicated that p53 is present in a complex with both Drosha and p68, and the addition of p53 to *in vitro* pri-miRNA processing assays enhances the activity of Drosha. Interestingly, several p53 mutant-containing cells that are linked to oncogenesis have low post-transcriptional miRNA expression.[72]

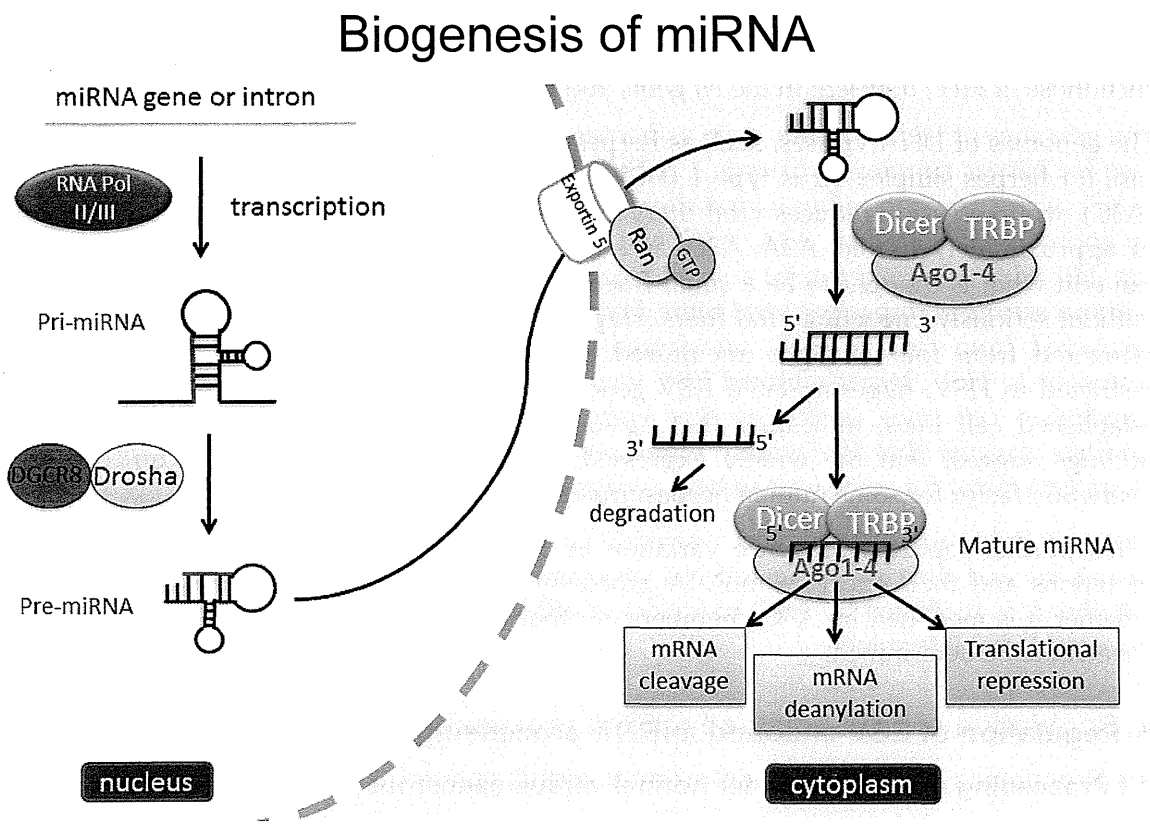

Fig. 1. Processing machinery of miRNA
miRNA genes are transcribed by RNA polymerase II or III into long primary (pri) miRNA transcripts, processed by the nuclear nuclease Drosha into ~60 bp hairpins termed precursor (pre) miRNAs, and further cleaved in the cytosol by the Dicer nuclease into mature miRNAs. Mature miRNAs are then incorporated into the multiprotein RNA-induced silencing complex (RISC), exerting post-transcriptional repression of target mRNAs, either by inducing mRNA cleavage, mRNA degradation or blocking mRNA translation.

4.2 Processing of EBV-encoded miRNAs

For EBV-encoded miRNAs, several regulatory processes have been reported[68]. Almost all of the EBV-encoded miRNAs originate from one of three sequence clusters. Two of the three clusters of miRNAs are made from the BARTs, a set of alternatively spliced transcripts that are highly abundant in NPC, but have not been shown to produce a detectable protein. Edwards et al. investigated the mechanism of BART-derived miRNA processing by comparing the processed miRNAs with the original BART transcript and residual transcripts after processing.[68] First, they showed that residual pieces of the intron sequence were detectable in the nucleus of cells that express the miRNAs. Characterization of these residual pieces indicated that the miRNAs were produced from one large initial transcript prior to splicing and that a specific spliced form of the transcript favored the production of miRNAs. Second, they found that miR-BART12 is not detected at all, even though the primary transcript is abundant. Third, pre-miR-BART5 could be detected in all cell lines and tumors tested, despite low or undetectable expression of the mature miR-BART5, indicating that the processing of pre-miR-BART5 was inhibited.

	function	target viral	Host target	
BLHF1-1	transformation	BFLF2	LILRB-5,E2F1,p53,CBFA2T2	BHRF1
BLHF1-2	transformation	BFLF2	PIK3R1	BHRF1
BLHF1-3	transformation	BFLF2	CXCL11,PRF1,TGIF,NSEP1	BHRF1
BART1-5p	Cancer development	LMP1	CXCL12	BART Cluster1
BART2-5p	viral replication	BALF5、LMP1	MIC B, Bim	
BART3		LMP1	IPO7, Bim	BART Cluster1
BART4		LMP1	Bim	BART Cluster1
BART5	Host cell survival	LMP1	PUMA, Bim	BART Cluster1
BART6	maintain viral latency	LMP1	Dicer, Bim	BART Cluster1
BART7		LMP1	Bim	BART Cluster2
BART8				BART Cluster2
BART9				BART Cluster2
BART10				BART Cluster2
BART11				BART Cluster2
BART12				BART Cluster2
BART13				BART Cluster2
BART14				BART Cluster2
BART15				BART Cluster1
BART16	Cancer development	LMP1	TOMM22	BART Cluster1
BART17	Cancer development	LMP1		BART Cluster1
BART18				BART Cluster2
BART19				BART Cluster2
BART20				BART Cluster2
BART21				BART Cluster2
BART22		LMP2		BART Cluster2

Table 1.

Amoroso et al. reported that the levels of the different BART miRNAs vary up to 50-fold within a given cell line.[73] However, this variation cannot be explained by differential miRNA turnover, as all EBV miRNAs appear to be remarkably stable, suggesting that miRNA maturation is a key step in regulating steady-state levels of EBV miRNAs. Future studies should further investigate the mechanism of miRNA transcript processing in EBV-infected cells, highlighting any differences between the three types of latent infections.

5. Secretory EBV-encoded miRNAs

5.1 Secretory miRNAs

Cellular and viral miRNAs control gene expression by repressing the translation of mRNAs into protein, a process that is tightly regulated in healthy cells, but is deregulated in cancerous and virus-infected cells. Curiously, miRNAs are not strictly intracellular, but are also secreted through the release of small vesicles called exosomes and, therefore, exist extracellularly in the peripheral blood and in cell culture media.[74] It has been suggested that exosome-associated miRNAs play a role in intercellular communication [74], although concrete evidence for this has been lacking. The dynamics of miRNA secretion via exosomes and the proposed transfer mechanisms remain poorly understood. In addition, it is unclear whether miRNAs are secreted in physiologically relevant amounts.

5.2 Existence of secretory EBV-encoded miRNAs

Pegtel et al. were the first to show that exosomes deliver viral miRNAs to non-infected cells.[75] They used EBV B95.8-immortalized LCLs and demonstrated that exosomes contained

BHRF1 miRNAs, which could target the *CXCL11/ITAC* gene in nearby uninfected cells. Furthermore, they showed that non-B cells in EBV-infected patients with elevated viral loads contained EBV miRNAs, demonstrating that exosomes apparently transfer miRNAs *in vivo* to uninfected cells. These findings were confirmed by two studies that demonstrated the release of exosomes from NPC cells. Gourzones et al. showed that EBV miR-BARTs present within exosomes can be detected in the serum of mice xenografted with human NPC cells and that the sera of NPC patients also contain BART miRNAs.[76]

6. Concluding remarks

EBV-related cancers are generally difficult to cure. Despite extensive studies based on well-known concepts and methods, the molecular basis by which EBV mediates tumorigenesis and eludes immunosurveillance remains unclear. Mouse models of EBV-mediated lymphoproliferative disease have recently revealed that EBV infection of B cells is necessary, but not sufficient, for tumorigenesis, as all peripheral mononuclear cells are needed to generate tumors in these mice.[77] Immune cells are also indispensable for EBV-mediated tumorigenesis . The relationship between these cells and EBV-infected cells with regard to tumorigenesis remains unclear. Moreover, the mechanism of drug resistance, which causes poor prognosis of EBV-related tumors, has not yet been elucidated. Therefore, it is important to study the tumor biology of EBV-related tumors from a fresh perspective, such as EBV-encoded miRNAs.

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Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

Toyotaka Kawamata, Jun Lu, Tadayuki Sato, Masafumi Tanaka, Hitoshi Nagaoka, Yasutoshi Agata, Takae Toyoshima, Kazuaki Yokoyama, Naoki Oyaizu, Naoya Nakamura, Kiyoshi Ando, Arinobu Tojo and Ai Kotani

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Brief report

Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

*Toyotaka Kawamata,[1] *Jun Lu,[2] *Tadayuki Sato,[3] Masafumi Tanaka,[4] Hitoshi Nagaoka,[5] Yasutoshi Agata,[6] Takae Toyoshima,[2] Kazuaki Yokoyama,[1] Naoki Oyaizu,[7] Naoya Nakamura,[8] Kiyoshi Ando,[9] †Arinobu Tojo,[1] and †Ai Kotani[2]

[1]Department of Hematology-Oncology/Molecular Therapy, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [2]Institute of Innovation Science and Technology, Tokai University, Kanagawa, Japan; [3]Center for Education and Support, Department of Medicine, Tokai University, Kanagawa, Japan; [4]Molecular Life Science, Department of Medicine, Tokai University, Kanagawa, Japan; [5]Molecular Pathology, Department of Medicine, Gifu University, Gifu, Japan; [6]Immunology, Department of Medicine, Kyoto University, Kyoto, Japan; [7]Department of Laboratory Medicine in Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [8]Pathology, School of Medicine, Tokai University, Kanagawa, Japan; and [9]Hematology and Oncology, School of Medicine, Tokai University, Kanagawa, Japan

Activation-induced cytidine deaminase (AID) is essential for class switch recombination and somatic hypermutation. Its deregulated expression acts as a genomic mutator that can contribute to the development of various malignancies. During treatment with imatinib mesylate (IM), patients with chronic myeloid leukemia often develop hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we provide evidence that class switch recombination on B-cell activation is apparently inhibited by IM through down-regulation of AID. Furthermore, expression of E2A, a key transcription factor for AID induction, was markedly suppressed by IM. These results elucidate not only the underlying mechanism of IM-induced hypogammaglobulinemia but also its potential efficacy as an AID suppressor. (*Blood*. 2012;119(13): 3123-3127)

Introduction

Activation-induced cytidine deaminase (AID) is essential for class switch recombination (CSR) and somatic hypermutation.[1] Deregulated expression of AID acts as a genomic mutator and can contribute to tumorigenesis through genomic recombination and aberrant somatic hypermutation.[2-4] E2A, which harbors 2 binding sites in the AID promoter, is the crucial transcription factor for induction of AID.[5] Imatinib mesylate (IM) has diverse immunomodulatory effects,[6,7] including reduction of T-cell proliferation and inhibition of T-cell effector functions.[8,9] Previously, we reported that serum titers of IgG and IgA, but not IgM, were significantly lower in chronic myeloid leukemia patients treated with IM versus those treated with IFN-α,[10] suggesting that IM impairs CSR. In the present study, we investigated the effects of IM on CSR both in vitro and in vivo. Here, we present evidence that IM inhibits CSR through down-regulation of AID expression in splenic B cells.

Methods

Mouse immunization

Eight-week-old mice were immunized as previously reported,[1] with or without 50 mg/kg imatinib mesylate. The experiments were approved by the Committee of Animal Care at the Institute of Medical Science, University of Tokyo.

Immunohistochemistry

Immunostaining for AID was performed on frozen sections following the manufacturer's instructions using an AID antibody (H-80; Santa Cruz Biotechnology).

Primer sequences, reagents, and more detailed methods are shown in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

CSR is induced in splenic B cells by stimulation with IL-4 and lipopolysaccharide (LPS).[11] After stimulation with IL-4 and LPS for 72 hours, IM decreased the proportion of IgG1-positive B cells dose-dependently. The proportion of B cells expressing surface IgG1 was approximately 16% without IM but was significantly reduced to approximately 3% with 10μM IM (Figure 1A). In the present culture system, only B cells can survive and proliferate,[1] suggesting that IM may act directly on B cells and inhibit their CSR.

Next, we examined expression of the germline transcript directed by the I promoter of IgG1 and AID, both of which are essential for CSR after B-cell stimulation.[12] Expression of AID was suppressed by IM dose-dependently (Figure 1B), whereas the IgG1 germline transcripts were not decreased by IM (Figure 1C). Likewise, IgA CSR in CH12F3-2A cells was impaired by IM in a dose-dependent manner (Figure 1D). These results

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*T.K., J.L., and T.S. contributed equally to this study.

†A.T. and A.K. contributed equally to this study as co–last authors.

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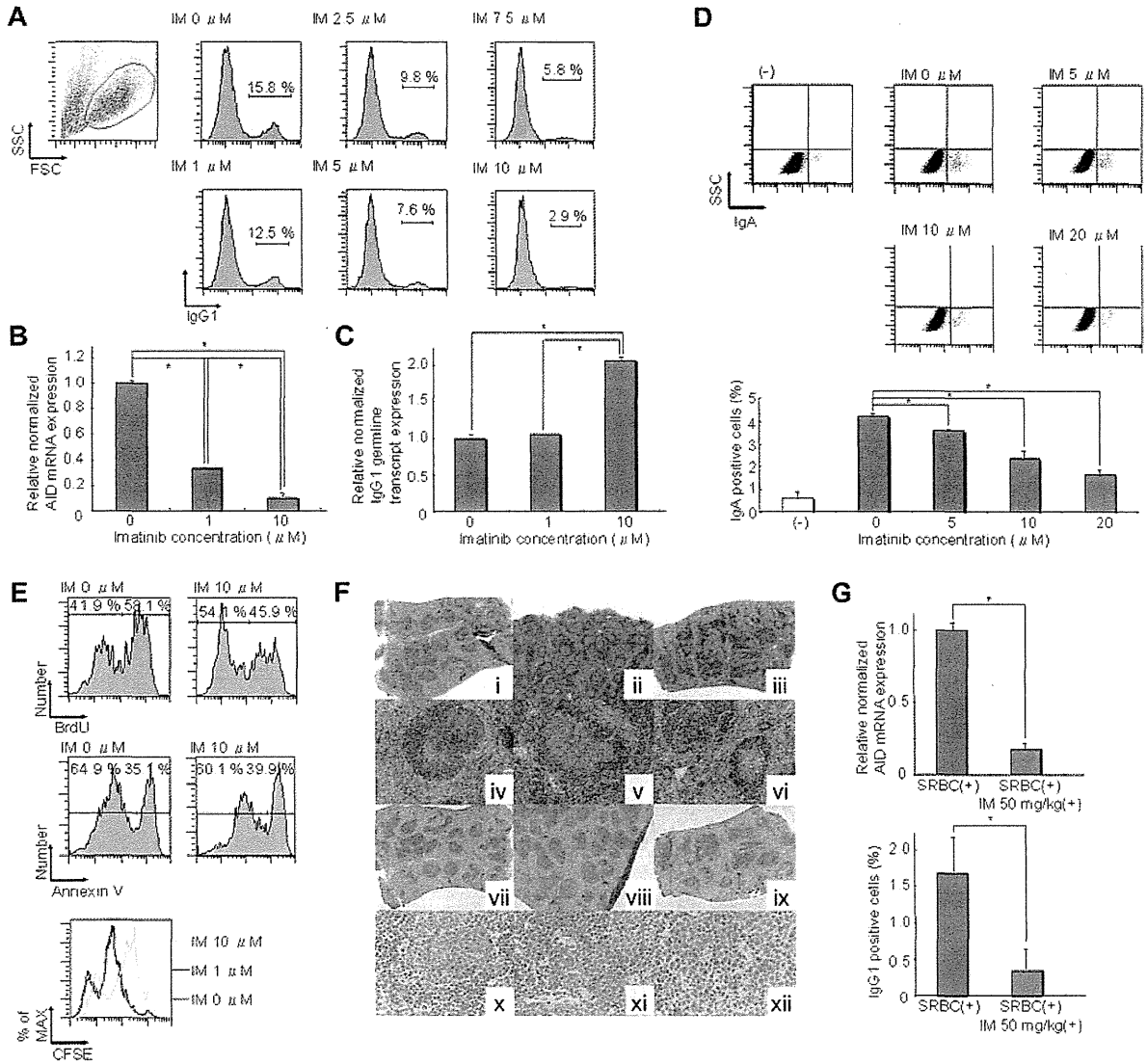

Figure 1. IM directly inhibits CSR in activated B cells through down-regulation of AID. (A) IgG1 expression levels in spleen cells cultured in conditioning medium containing 12.5 μg/mL LPS and 7.5 ng/mL IL-4 with 0, 1, 2.5, 5, 7.5, and 10μM IM for 72 hours were 15.8%, 12.5%, 9.8%, 7.6%, 5.8%, and 2.9% of untreated controls, respectively. Reduction of IgG1 expression was induced by IM dose-dependently. (B) Real-time RT-PCR in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours indicated that expression of AID was decreased by IM dose-dependently. Significant differences were found between 0 and 1μM or 10μM IM. $^*P < .05$. The y-axis represents AID mRNA levels relative to the no-IM control. The levels of AID mRNA at each IM concentration were calculated relative to the internal control (GAPDH); n = 6. (C) The level of the germline transcript of IgG1 in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours was not decreased in contrast to AID mRNA expression levels, which were decreased by IM in a dose-dependent manner. Significant differences were found between 0, 1, and 10μM IM. $^*P < .05$. The y-axis represents expression levels of the IgG1 germline transcript relative to the no-IM control in the same manner as that in panel B; n = 4. (D) IgA expression levels in CH12F3-2A cells cultured in conditioning medium containing 7.5 μg/mL IL-4, 0.3 ng/mL TGF-β1, and 40% CD40 ligand with 0, 5, 10, and 20μM IM for 72 hours were reduced in an IM dose-dependent manner. (E) Cell proliferation, division, and apoptosis in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were investigated using BrdU, annexin V, and CFSE assays. The BrdU incorporation rate of 10μM IM was 45.9%, whereas that of 0μM IM was 58.1%. Cell fluorescence of 10μM IM using the CFSE assay was shifted, but that of 1μM IM was not shifted to the right compared with that of 0μM IM. Annexin V analysis of 10μM IM was 39.9%, whereas that of 0μM IM was 35.1%. These results indicate that IM affects cell proliferation but not apoptosis. (F) Immunohistochemical analysis of spleens from mice that were administered SRBC with or without IM. Serial sections of spleens were prepared from nonimmunized (i,iv,vii,x), SRBC-immunized (ii,v,viii,xi), or SRBC-immunized + IM (50 mg/kg; iii,vi,ix,xii) animals. (i-vi) H&E staining. (vii-xii) Immunohistochemical analysis of AID. Low-power fields are shown in panels i to iii and vii to ix. High-power fields are shown in subpanels iv to vi and x to xii. Individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and were comparable with those from nonimmunized mice. AID expression, which was induced in germinal center-activated B cells, was barely detectable in spleens of IM-treated mice but was strongly positive in those of nontreated mice. Moreover, IM significantly suppressed AID expression, even in the residual germinal centers. (G) Real-time RT-PCR analysis of AID and FACS analysis of IgG1 expression of spleen cells harvested from SRBC-immunized mice with or without 50 mg/kg IM. The top panel shows relative normalized AID mRNA expression, and the bottom panel shows surface IgG1 expression of total splenocytes. A significant difference was found between SRBC (+) and SRBC (+) IM 50 mg/kg (+) for both AID and IgG1. $^*P < .05$. The y-axis represents the relative ratio of the relative expression level of AID mRNA (top panel) and the percentage of surface IgG1 expression of total splenocytes (bottom panel). Normalized values obtained for SRBC (+) IM 50 mg/kg (+) were derived from SRBC (+); n = 2.

showed that AID, but not the germline transcript, was responsible for inhibition of CSR by IM. BrdU, CFSE, and annexin V analysis revealed that IM affected proliferation but not apoptosis (Figure 1E). Importantly, 1μM of IM did not decrease proliferation but down-regulates AID (Figure 1B,E). In addition, 5-fluorouracil decreased proliferation but did not down-regulate AID (supplemental Figure 1), suggesting that proliferation is not necessarily coupled with expression of AID. Therefore, it is

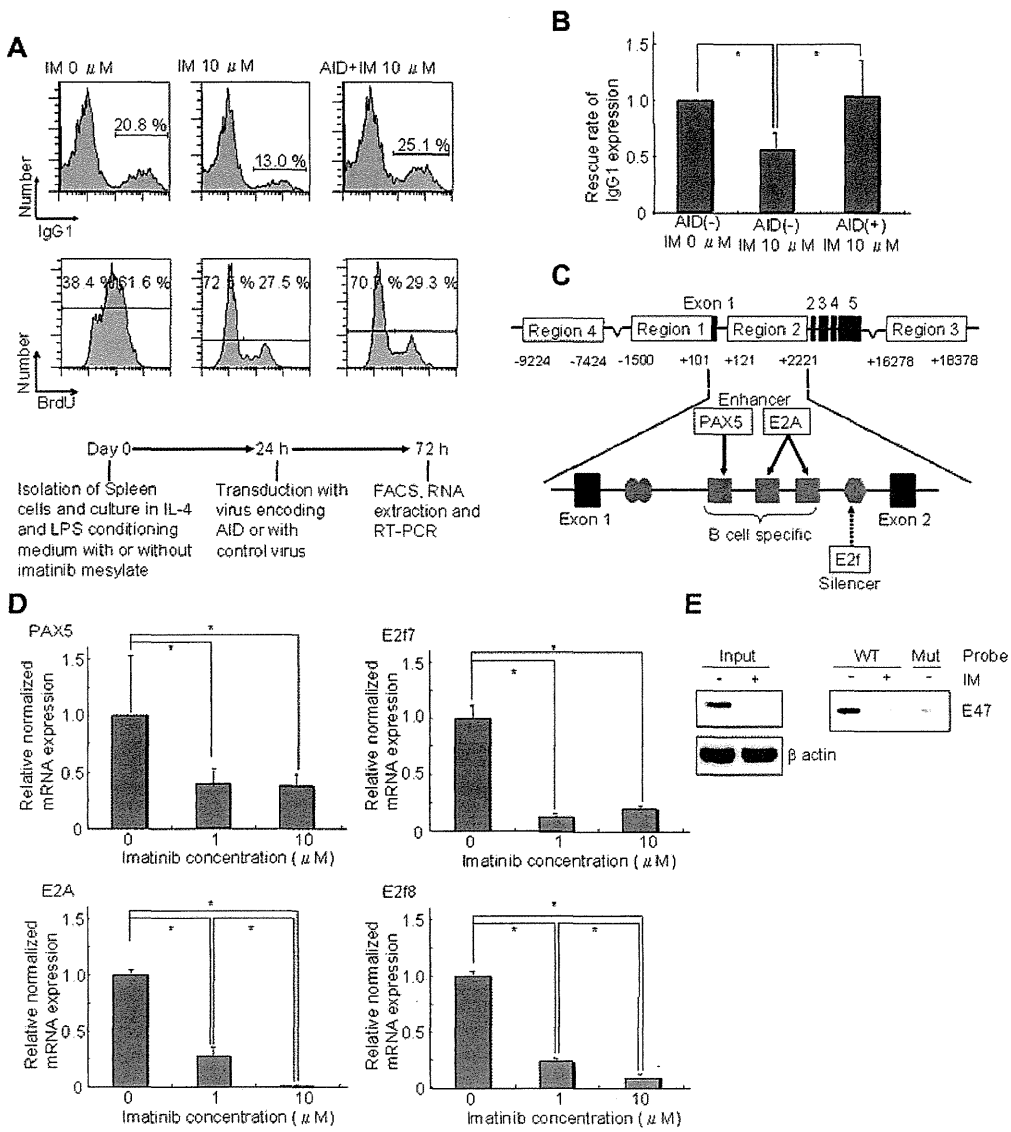

Figure 2. Down-regulation of AID mediated by E2A, Pax5, E2f7, and E2f8 is responsible for CSR impairment by IM. (A) Ectopic expression of AID completely rescued reduction of IgG1 expression caused by IM. Spleen cells were cultured in IL-4 and LPS conditioning medium with or without IM. After 24 hours of prestimulation culture, cells were transduced with retrovirus encoding AID-eGFP or retrovirus encoding eGFP only (control). After a further 48 hours, IgG1 expression was analyzed (bottom panel). A mononuclear cell fraction based on forward scatter/side scatter profiles was gated and sequentially subdivided into an eGFP-positive fraction. This eGFP-positive fraction was analyzed. Ectopic AID expression with 10μM IM increased IgG1 expression from 13.0% to 25.1% versus 20.8% without IM. The BrdU assay revealed that DNA synthesis decreased in the 10μM IM culturing condition. Although the BrdU assay was similar with or without ectopic expression of AID, IgG1 expression was completely rescued by ectopic expression of AID. (B) Average of the IgG1 expression rescue rate among 4 rescue experiments. IgG1 expression of AID(+) IM at 10μM was completely rescued by ectopic expression of AID. (C) Schema illustrating transcriptional binding sites in the *Aicda* gene promoter region, focusing particularly on region 2 in the first intron. PAX5 and E2A activate the *Aicda* promoter, whereas E2f7 and E2f8 have silencing effects. (D) The expression levels of 4 transcriptional factors (PAX5, E2A, E2f7, and E2f8) in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were determined by real-time RT-PCR. All were reduced by IM. E2A expression was most markedly reduced.*$P < .05$. The y-axis represents mRNA levels of the PAX5, E2A, E2f7, and E2f8 relative to the no-IM control. Levels of each transcriptional factor mRNA were calculated relative to the internal control (GAPDH); n = 2. (E) Protein expression and DNA-binding activity of E2A in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours. The E2A gene encodes 2 transcription factors: E12 and E47. Western blot analysis revealed that expression of E47 in splenocytes cultured in conditioning medium containing LPS and IL-4 was down-regulated by IM to a barely detectable level. DNA affinity precipitation analysis of the same cell extracts using biotinylated E-box probe and its mutant revealed that E-box binding activity of E47 in the extracts was similarly reduced by IM.

possible to differentiate the effect of IM on proliferation and AID expression.

To further confirm that CSR is impaired by IM through down-regulation of AID in vivo, immunohistochemical analysis was performed on splenic tissues from nonimmunized and sheep red blood cells–immunized C57BL/6 mice with or without IM treatment (Figure 1F). The individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and comparable with those from nonimmunized mice (Figure 1F). As expected from these findings, AID expression, which is induced in germinal center-activated B cells, was barely detectable in the spleens of IM-treated mice but was strongly positive in those of nontreated mice. In addition, IM significantly suppressed AID expression,

even in the residual germinal centers. Expression of AID was confirmed by real-time RT-PCR analysis. The results of IgG1 expression did not conflict with these results (Figure 1G). Compatible with the results obtained by in vitro stimulation of spleen cells, IM down-regulated expression of IgG1 as well as AID. Although enlargement of germinal center formation has been reported in AID knockout mice,[1] it is assumed that the immunomodulatory effects of IM on B cells, T cells, and dendritic cells[6,7] resulted in impairment of germinal center formation in our system.

Furthermore, we investigated whether ectopic expression of AID could rescue inhibition of CSR by IM. IgG1 expression in spleen cells decreased with IM treatment, whereas ectopic expression of AID completely rescued impairment of CSR under the condition that cell proliferation was suppressed by IM (Figure 2A-B). The results indicated that impairment of CSR by IM was at least in part the result of down-regulation of AID.

Finally, we examined the mechanism of down-regulation of AID by IM. Recently, Tran et al reported that *Aicda* regulation involved derepression by several layers of positive regulatory elements in addition to the 5′-promoter region.[5,13] Promoter region 2 in the first intron contains the functional binding elements for the ubiquitous silencers c-Myb and E2f and for the B cell–specific activators Pax5 and E2A (Figure 2C).[5,13] Surprisingly, all of these transcription factors were down-regulated by IM. Among them, expression of E2A was most markedly reduced (to 1 of 500) by IM (Figure 2D). We further found that levels of E2A protein as well as E-box binding activity were markedly reduced by IM (Figure 2E), suggesting that down-regulation of E2A by IM causes significant suppression of AID.

For the first time, our findings elucidate a mechanism of hypogammaglobulinemia caused by IM, which has been observed frequently in IM-treated chronic myeloid leukemia patients.[8,9] Its adverse effects as well as the immunomodulatory functions of each drug and their underlying mechanisms must be examined in more extensive studies.

AID was previously reported to be induced by BCR-ABL1 in $Ph1^+$ pre B-ALL cell lines and inhibited by IM through ID2 up-regulation. Interestingly, neither PAX5 nor E2A showed changes in expression.[14] In the present study using normal mature B cells, PAX5 and E2A levels were significantly decreased by IM, whereas ID2 was not increased (data not shown). PDGFR[15] and c-kit,[16] kinases that are also inhibited by IM, were not expressed in mature B cells. Together, these results could be induced by the off-target multikinase inhibitory effects of IM. The results of microarray analysis (supplemental Table 1; supplemental Figures 2-3) are consistent with this hypothesis. All microarray data are available for viewing on the Gene Expression Omnibus under accession number GSE35559.

Inappropriate expression of AID affects many diseases, such as malignancy and autoimmune diseases.[17,18] It probably also affects allergic disorders because AID is also essential to CSR from IgM to IgE, deregulation of which is an important causative factor of allergic disorders. The results of the present study suggest that IM, which has been used safely for several decades in clinical settings, can be used for various diseases involving AID. Indeed, dramatic resolution by IM has been reported in several cases of rheumatoid arthritis or asthma complicated with chronic myeloid leukemia.[19,20]

In conclusion, suppression of AID by IM is responsible for CSR impairment, leading to the frequent adverse effects of IM. IM may also be clinically useful as an AID suppressor.

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Authorship

Contribution: T.K., J.L., T.S., A.K., and M.T. designed, performed, and analyzed the experiments and wrote the manuscript; T.T., H.N., Y.A., K.Y., N.O., and N.N. contributed vital reagents; K.A. collected the clinical samples; and A.T. supervised the research.

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Correspondence: Arinobu Tojo, Department of Hematology-Oncology, Research Hospital, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: a-tojo@ims.u-tokyo.ac.jp; and Ai Kotani, Tokai University Institute of Innovative Science and Technology, Medical Science Division, Shimokasuya 143, Isehara City, Kanagawa Prefecture 259-1193, Japan; e-mail: ka102009@tsc.tokai-u.ac.jp.

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The AID Dilemma: Infection, or Cancer?

Tasuku Honjo,* Maki Kobayashi,* Nasim Begum,* Ai Kotani,† Somayeh Sabouri,* and Hitoshi Nagaoka‡

**Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Yoshida Sakyo-ku, Kyoto, Japan*

†*Medical Science Division, Tokai University Institute of Innovative Science and Technology, Shimokasuya, Isehara City, Kanagawa, Japan*

‡*Department of Molecular Pathobiochemistry, Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu, Japan*

Activation-induced cytidine deaminase (AID), which is both essential and sufficient for forming antibody memory, is also linked to tumorigenesis. AID is found in many B lymphomas, in myeloid leukemia, and in pathogen-induced tumors such as adult T cell leukemia. Although there is no solid evidence that AID causes human tumors, AID-transgenic and AID-deficient mouse models indicate that AID is both sufficient and required for tumorigenesis. Recently, AID's ability to cleave DNA has been shown to depend on topoisomerase 1 (Top1) and a histone H3K4 epigenetic mark. When the level of Top1 protein is decreased by AID activation, it induces irreversible cleavage in highly transcribed targets. This finding and others led to the idea that there is an evolutionary link between meiotic recombination and class switch recombination, which share H3K4 trimethyl, topoisomerase, the MRN complex, mismatch repair family proteins, and exonuclease 3. As Top1 has recently been shown to be involved in many transcription-associated genome instabilities, it is likely that AID took advantage of basic genome instability or diversification to evolve its mechanism for immune diversity. AID targets

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are therefore not highly specific to immunoglobulin genes and are relatively abundant, although they have strict requirements for transcription-induced H3K4 trimethyl modification and repetitive sequences prone to forming non-B structures. Inevitably, AID-dependent cleavage takes place in nonimmunoglobulin targets and eventually causes tumors. However, battles against infection are waged in the context of acute emergencies, while tumorigenesis is rather a chronic, long-term process. In the interest of survival, vertebrates must have evolved AID to prevent infection despite its long-term risk of causing tumorigenesis.

I. INTRODUCTION

The successful smallpox vaccine introduced by Jenner in 1789 paved the way for future vaccines against a wide variety of bacterial and viral infections. While some of medical science's critical contributions to human healthcare have come through microbiology and immunology, the reasons behind the efficacy of vaccines have long remained a mystery. The question is twofold: how does the immune system recognize specific antigens out of the huge variety of antigens the body is exposed to? And, how does the immune system recognize pathogens as the same antigens previously encountered in a vaccine? These two questions are central to modern immunology. Two contrasting hypotheses to answer the first question were extensively debated from the 1950s through the 1970s, one proposing that we have a large number of genes encoding antigen receptors, and the other suggesting that a limited number of genes are mutated to amplify the antigen receptor repertoire. The subsequent development of recombinant technology contributed to proof that the latter hypothesis, proposed by Burnet, is basically correct. However, the precise mechanism is more complex than originally anticipated.

Vertebrates have two types of antibody diversification mechanisms, each taking place at a different lymphocyte differentiation phase. VDJ recombination creates an enormous repertoire of both T- and B-cell receptors by assembling various combinations of V, D, and J segments into one exon (Bassing *et al.*, 2002; Fugmann *et al.*, 2000; Gellert, 2002). The mechanism is highly regulated, and proceeds in step with the T and B lymphocyte developmental program. RAG1 and RAG2, which mediate VDJ recombination, are biochemically well characterized and appear to have been introduced rather recently in evolution, most likely by a transposon-like element (Kapitonov and Jurka, 2005; Schatz, 2004). However, VDJ recombination does not explain how antigenic antibody memory is generated, since its process is completely independent from antigen stimulation.

A second layer of diversity, somatic hypermutation (SHM), is introduced in the V exon of B lymphocytes by antigen stimulation. Evidence for SHM has accumulated through a series of experiments by Milstein, Weigert, and Cohn, among others. The most striking observation by Weigert *et al.* (1970)

is that 7 out of 19 mouse Vλ amino acid sequences are highly homologous except for several amino acid substitutions, while the remaining 12 Vλ sequences are identical. Direct proof that SHM occurs through genetic modification was obtained through the work of Tonegawa, Weigert, and other researchers, who compared DNA sequences between the germline and rearranged immunoglobulin Vλ genes (Rajewsky, 1996).

Antigenic stimulation of mature B lymphocytes induces yet another genetic alteration, called class switch recombination (CSR), into the immunoglobulin heavy-chain locus. Class-switching phenomena were originally reported by Uhr, Nossal, and Cooper, whose combined observations clearly indicated that B cells that express IgM change their isotype to other classes after antigenic stimulation (Honjo *et al.*, 2002). In 1978, Kataoka and Honjo proposed class switching to be caused by DNA recombination with a looping-out deletion (Honjo and Kataoka, 1978). This genetic alteration was (Honjo and Kataoka, 1978) directly demonstrated by cloning class-switched immunoglobulin loci (Cory *et al.*, 1980; Dunnick *et al.*, 1980; Maki *et al.*, 1980; Rabbitts *et al.*, 1980; Yaoita and Honjo, 1980).

By the end of the 1990s, we had learned that antigen-specific antibody memory is represented by two genetic alterations in the immunoglobulin locus: SHM, which is a point mutation in the V region exon, and CSR, a recombination event that replaces the CH gene in the heavy-chain locus. Many researchers looked for the enzymes or proteins regulating these genetic alterations. The gene inducing SHM was considered to be a mutator gene, and the gene for CSR was expected to encode a recombinase. The two proteins were assumed to be different.

The year 2000 brought the surprising discovery that a single protein, activation-induced cytidine deaminase (AID), regulates both SHM and CSR (Muramatsu *et al.*, 2000; Revy *et al.*, 2000). AID was cloned by subtractive hybridization between stimulated and nonstimulated CH12F3-2A B lymphoma cells, which switch efficiently from IgM to IgA when stimulated (Muramatsu *et al.*, 1999; Nakamura *et al.*, 1996). AID was demonstrated to regulate both SHM and CSR, both in studies of AID-deficient mice and through the identification of AID mutations in hyper-IgM syndrome type II (HIGM II) patients (Revy *et al.*, 2000). Subsequently, using artificial constructs to measure SHM and CSR, it was demonstrated that AID induces SHM and CSR in nonlymphoid cells (Okazaki *et al.*, 2002; Yoshikawa *et al.*, 2002). It therefore became clear that AID is essential and sufficient to induce SHM and CSR.

AID overexpression was subsequently shown to cause tumors in mice, indicating that AID is indeed a mutator. Transgenic mice carrying AID cDNA under the chicken β actin promoter frequently develop T lymphoma and lung microadenoma, and less frequently, B lymphoma, muscle-derived tumors, and hepatoma (Okazaki *et al.*, 2003). Inversely, AID deficiency reduces the frequency of *c-myc/Ig* chromosomal translocations that lead to

the plasmacytoma formation associated with IL-6 overexpression (Ramiro *et al.*, 2004). More recently, several lines of evidence have indicated that viruses that can cause tumors also frequently induce AID. These include the Epstein–Barr virus (EBV) (Epeldegui *et al.*, 2007), HTLV-1 (Ishikawa *et al.*, 2011), and hepatitis virus type C (Endo *et al.*, 2007). In addition, *Helicobacter pylori* was shown to induce AID in gastric epithelial cells (Matsumoto *et al.*, 2007). Another interesting association between AID and the Philadelphia chromosome-encoded *Bcr-Abl* kinase is considered to link AID with tumorigenesis or tumor progression (Feldhahn *et al.*, 2007). Although AID's preferred target is the Ig locus, AID obviously attacks other genes as well, and there are many lines of evidence indicating that AID may be involved in tumorigenesis. Thus, although AID is essential for antibody memory generation as a core function of adaptive immunity, AID expression may simultaneously cause tumors. This dilemma regarding AID will be discussed from an evolutionary point of view.

II. TWO DISTINCT AID FUNCTIONS

AID function has been extensively studied using AID mutants. Strikingly, in HIGM II patients, AID mutation sites are found scattered across almost all of its 198 amino acid residues (Durandy *et al.*, 2006; Revy *et al.*, 2000). This finding clearly indicates that mutations of various AID regions affect CSR function. This could be due to the AID protein interacting with multiple proteins at its various regions, or to its structure being so unstable that point mutations in its various regions alter the structure enough to damage AID's function. Subsequent studies have revealed that both may be the case.

A. Phenotypes of AID Deficiency

AID mutations in HIGM II patients are scattered through almost all of AID's regions (Fig. 1 and Table I). Loss-of-function mutations are located not only within the catalytic region, which is well defined by conserved histidine (residue 50), tryptophan (residues 68 and 80), and cysteine (residue 87) residues, but also in the NLS (N-terminus), linker, apobec-like, and nuclear export signal (NES) (C-terminus) regions. In most cases, phenotypes result from defects in CSR alone or in both CSR and SHM, leading to severe immune deficiency. These findings strongly support the idea that AID is essential for these two genetic alterations required for antibody memory.

The phenotypes of AID knockout mice are almost identical to those of HIGM II patients, convincingly demonstrating that AID is essential for both CSR and SHM (Muramatsu *et al.*, 2000). AID-deficient mice showed

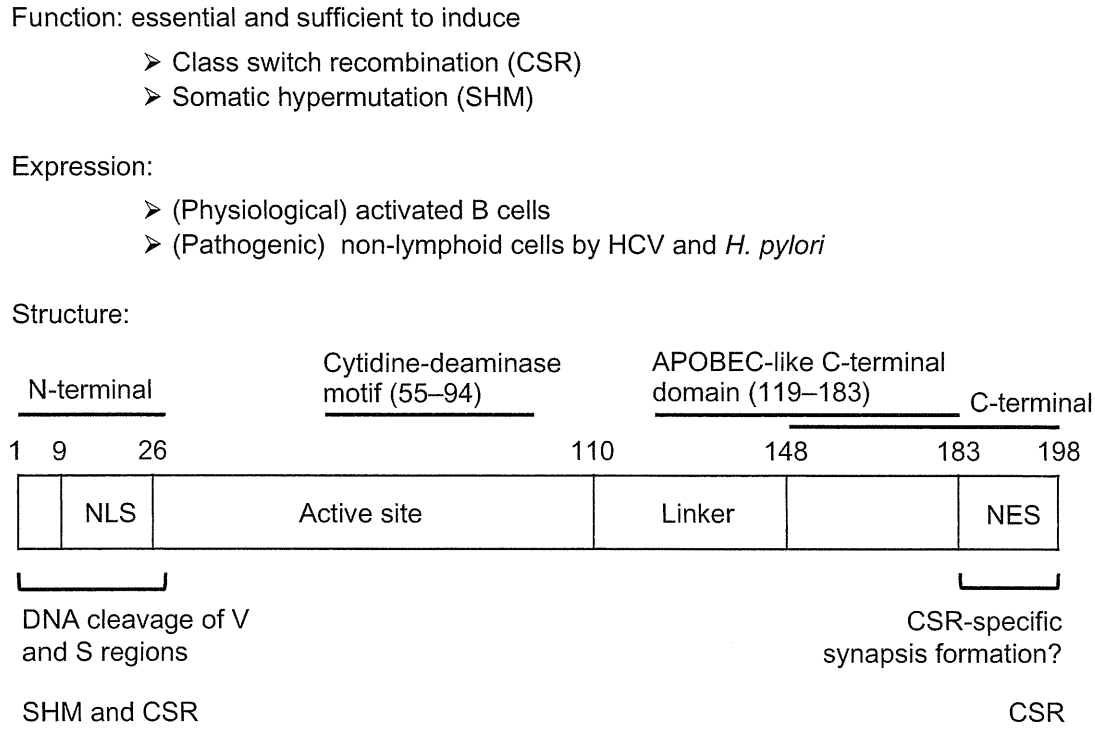

Fig. 1 AID structure and function. Note that the AID N-terminal region is required for DNA cleavage of the V and S regions, and the AID C-terminal region is required for CSR after DNA cleavage. Therefore, AID has two separate functions although it contains only one catalytic center in the middle of the protein.

generalized activation of immune cells that associates with enlarged lymphoid organs. Especially in the gut, the activation of immune cells caused hyperplasia of gut follicular structures such as Peyer's patches and isolated lymphoid follicles scattered throughout the length of the small intestine (Fagarasan *et al.*, 2002). The abnormal bacterial communities in gut mainly caused the activation of immune cells in $Acid^{-/-}$ mice (Fagarasan *et al.*, 2002). Subsequently, it was found that the gut flora in $Acid^{-/-}$ mice was composed mostly of uncultured anerobes, such as segmented filamentous bacteria, which are known to attach firmly to the gut epithelial cells and to cause activation of B and T cells in the gut (Suzuki *et al.*, 2004). Indeed, the treatment of AID-deficient animals with wide spectrum antibiotics resulted in the decrease of anerobic bacteria and simultaneous subsidence of activation of B cells and hyperplasia of germinal center (GC) in all lymphoid tissues. A mutant AID incapable to support diversification of IgAs by SHM (AID^{G23S}) showed very similar phenotype with $Acid^{-/-}$ mice (see later discussion) (Wei *et al.*, 2011). Together, these studies in $Acid^{-/-}$ mice revealed a previously underestimated role of IgAs in regulating the bacterial composition and diversity in the gut. They also showed that gut microbiota critically affects the whole body immune homeostasis and rekindled the study of immunology in the context of infection and immunity (Suzuki *et al.*, 2004).

B. Phenotypes of AID N-Terminal Mutations

AID N-terminal mutations in humans and mice affect both CSR and SHM. Doi *et al.* (2009) showed that in N-terminal-defective AID mouse mutants, DNA cleavage activity was deficient in both the V and S regions, indicating that the AID N-terminal region is indispensable for DNA cleavage. While SHM and CSR are deficient in most N-terminal mutants, they are augmented in others (Shinkura *et al.*, 2004; Shivarov *et al.*, 2008). In either case, CSR and SHM are both affected in the same direction. These results further imply that the cleavage mechanism may be common in the V and S regions. Whether the N-terminal mutation augments or decreases the AID activity, SHM is affected more strongly than CSR. These results are consistent with the observation that AID cleaves DNA at least five times more efficiently in the S region than in the V region (Doi *et al.*, 2009). One possible explanation for the high efficiency of S-region cleavage may be the abundance of repetitive sequences that are prone to form a non-B DNA structure (Dunnick *et al.*, 1993; Nikaido *et al.*, 1981). Thus, in several N-terminal region mutants, SHM is almost completely lost while the CSR activity is only slightly reduced (Shinkura *et al.*, 2004). One such mutant, G23S, is used to specifically abolish SHM and retain CSR *in vivo*, as we will discuss further. Taken together, these results show that AID introduces DNA cleavage in the V and S regions with different efficiencies, and that the target specificity between the V and S regions is not determined by AID itself, but probably by other mechanisms that control the status of the locus.

Studies of G23S knock-in animals show that without SHM, gut microbes expand aberrantly. AID deficiency and GC B cell hyperplasia are observed in the spleen as well as the gut, indicating that SHM is critical for maintaining gut microbial homeostasis (Wei *et al.*, 2011). Without preimmunization, G23S animals are sensitive to cholera toxin, whereas wild-type mice are completely resistant to cholera toxin at the same dose. This increased susceptibility of G23S mice is obvious even after cholera toxin immunization, indicating that in wild-type animals, the SHM that generates the gut immunoglobulin repertoire is spontaneously induced by gut microbes. This observation suggests that generation of a fully competent gut immunoglobulin repertoire depends not only on VDJ recombination, but also on diversification by SHM. How the gut microbes are sensed by the host immune cells is not yet clear. Bacteria might be taken up by M cells in Payer's patches, or by dendritic cells or macrophages situated beneath the epithelial layer. In any case, the results clearly indicate that gut microbes and the generation of the whole-body immune repertoire are mutually dependent. The lymphoid organ hyperplasia in HIGM II patients has been interpreted to be the result of recurrent infection (Quartier *et al.*, 2004). Interestingly, the lymph nodes