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The Role of Activation-Induced Cytidine Deaminase in Antibody Diversification and B Cell Malignancies

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THE ROLE OF ACTIVATION-INDUCED CYTIDINE DEAMINASE IN ANTIBODY DIVERSIFICATION AND B CELL MALIGNANCIES

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Activation-induced cytidine deaminase (AID) is indispensable for somatic hypermutation (SHM) and class switch recombination (CSR) in B cells. SHM alters the affinity of an antibody for its corresponding antigen by introducing point mutations within the variable region of the immunoglobulin (Ig) gene heavy chain locus. CSR replaces the Igμ constant region with one of the downstream constant region, thereby changing the physical properties of the antibody.

To study the molecular mechanism of AID in these two antibody diversification events, I cloned, expressed and purified the recombinant AID protein from *E. coli* and showed that it can deaminate cytidines within single-stranded DNA molecules *in vitro*. In addition, I showed that AID does not have a preference for mutational hotspot sequences within a short DNA oligo *in vitro*. By studying truncated recombinant AID protein and AID point mutants I confirmed that AID activity is due to its typical cytidine deaminase active site. Hence, the role of AID in SHM and CSR is beneficial to an organism as it gives rise to antibodies of high affinity and avidity crucial in clearance of the antigen by humoral immune response.

Aberrant AID activity, however, can lead to c-myc/IgH chromosomal translocations and ultimately to B cell lymphomas and plasmocytomas in mice. Such translocations can also be detected in B cells stimulated to switch *in vitro.* I showed that switching B cells also upregulate Rae-1, a ligand for the NKG2D activating receptor found on natural killer (NK) cells, which renders them sensitive to NK attack. Furthermore, I demonstrated that IgH/c-myc translocations can be detected *in vivo* in germinal center (GC) B cells of immunized mice, and that these cells also express Rae-1. However, these calls are found in same frequencies in NK^+ and NK^- mice, implying that NK cells may be actively excluded from the GC. In addition, my data suggest a role for NK cells in clearance of B cells bearing IgH/c-myc translocations outside the protective environment of the germinal center.

"ONO CEMO BITI U STA VERUJEMO,

ONOLIKO BITI KOLIKO VERUJEMO"

TIN UJEVIC

MOJOJ PORODICI I TETKI BRANKI S LJUBAVLJU

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CHAPTER 1. INTRODUCTION

1.1 Immune System

The immune system of mammals relies on a combination of innate and adaptive mechanisms to defend the host against a wide range of pathogens. In traditional classifications, innate immunity utilizes germline-encoded pathogen recognition receptors to identify and respond to general non-self threats, while adaptive immunity employs combinatorial and mutational strategies to generate an exceedingly diverse set of antigen receptor specificities. But while the immune system can be activated to respond to foreign invaders (also termed “non-self”), it must also remain inert on encounter with self antigens (1, 2). An important exception to this rule is recognition of some tumor cells as non-self, or perhaps modified-self, so that the immune system can protect against cancer (3).

All cells of the immune system originate in the bone marrow from a haematopoietic stem cell, which ultimately gives rise to very diverse progeny which can be divided into two functional categories (4, 5). One group of cells, the cells of the innate immune system, is able to recognize the common features of pathogens to respond immediately (6). In contrast, the cells of the adaptive immune system are able to mount a specific response only days after encountering the antigen. During the time required for adaptive immunity to develop, the innate response plays a crucial role in controlling an infection.

1.2 Innate Immunity

The innate immune system provides a fast and general response to infection. Found in almost all multicellular organisms, this system was first described a century ago and has only recently become a focus of research (7). It recognizes highly conserved pathogen-associated molecular patterns (PAMPs) by germ-line encoded Toll receptors (6). These receptors were first described in Drosophila, and mammalian homologues were soon identified (8). Together, they constitute a family of ten Toll-like receptor (TLR) proteins, each recognizing a different microbial pattern (9). Phagocytic cells, such as macrophages and neutrophils play an important role in innate immunity. They carry almost all TLR receptors on their surface, which gives them the ability to recognize common surface molecules on microorganisms, further allowing them to engulf and digest them. Upon encountering bacteria, macrophages are also able to secrete cytokines and chemokines, thus initiating inflammation of the infected tissue. TLRs are also expressed on mast cells, which are also capable of phagocytosing microorganisms as well as initiating a potent inflammatory response. In addition to phagocytic cells, other granulocytes, eosinophils and basophils, play a crucial role in parasitic infections and allergy responses respectively.

Another important cell type that mediates innate immunity is the NK cell. It is of lymphoid origin, but unlike B and T cells, it lacks an antigen receptor. NK cells provide the first line of defense against viruses and tumors; they will be discussed in more detail later in the introduction.

1.3 Adaptive Immunity

In addition to the innate immune system, jawed vertebrates have developed an anticipatory, or adaptive, immune system. Adaptive immunity rests on the ability of the main effector cells, B and T lymphocytes, to diversify their surface receptors and generate more than 10^{11} different antigen recognition receptors. Unlike the cells of the innate immune system, which recognize general features of pathogens, B and T cells recognize specific antigenic features of pathogens in the context of major histocompatibility (MHC) molecules, after processing by antigen presenting cells.

Within the effector cell compartment there is a distinct delineation of functions, with T cells are responsible for cell-mediated immunity and with B cells providing humoral immunity. Generally, with the exception of superantigens, T cells are unable to recognize a pathogen unless it is on the surface of antigen-presenting cells such as dendritic cells (10). Therefore for T cells to effectively recognize pathogens, these have to be encountered and ingested by dendritic cells, which during that process mature into professional antigen-presenting cells and in addition to presenting antigen acquire the ability to secrete specific sets of cytokines (11, 12). Once they encounter their cognate antigen, T cells become activated: they undergo clonal expansion and differentiate either into cytotoxic T cells or helper T cells. While cytotoxic T cells kill infected cells, helper T cells can further activate cells such as macrophages and B cells, or help them maintain their activated status, again through the secretion of particular sets of cytokines.

On the other hand, B cells, upon encountering soluble antigen, further diversify their receptor genes by somatic hypermutation to create antibodies of higher affinity for the particular antigen. They also change the physical properties of the antibody by switching the isotype (class switch recombination). Successful cells are clonally selected to differentiate into plasma cells that secrete large amounts of antibodies to combat infection. B cells that were not able to successfully differentiate their receptor are negatively selected and deleted from the repertoire.

1.3.1 B cells

B cells originate from a common lymphoid progenitor in the bone marrow (4). The stages of B cell development are defined by a stepwise series of gene rearrangements and expression of immunoglobulin (Ig) and other characteristic proteins on the surface (Figure 1) (13-17). Early pro-B cell begins to rearrange its heavy chain Ig gene by VDJ rearrangement. After D_H to J_H joining, pre-B cell moves to the late pro-B stage undergoing V_H to previously rearranged DJ_H joining. Successful VDJ_H rearrangement leads to the expression of m heavy chain on the surface together with a surrogate light chain, forming the pre-B-cell receptor. This defines the next stage of development, the large pre-B cell. Signaling by the pre-B-cell receptor is contingent upon a productive rearrangement of the heavy chain, and the large pre-B cell divides, giving rise to a small pre-B cell in which light chain rearranges and is expressed on the surface

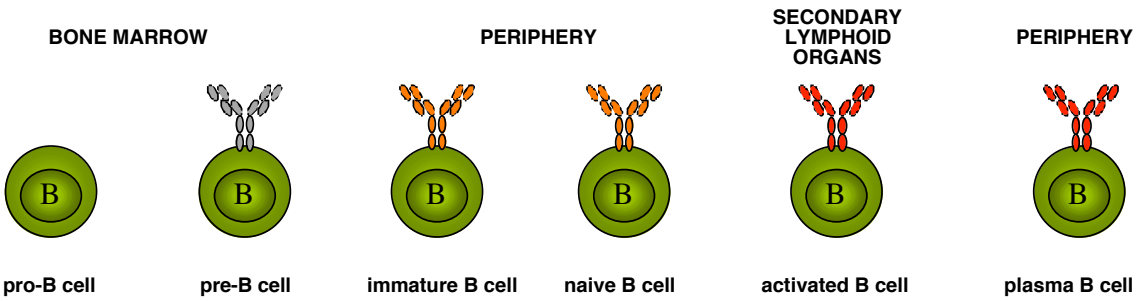

Figure 1. Stages of B cell development. B cells originate in the bone marrow, and start to diversify their Ig heavy chain by VDJ recombination at the pro-B stage. Once successfully rearranged, the heavy chain is expressed on the surface, with surrogate light chain, forming a pre-B receptor (grey) and defining a pre-B cell. Pre-B cells rearrange their light chain, and the B cell receptor is formed (orange) and expressed on the surface of immature B cell. Immature B cells are only allowed to exit into periphery if they are not autoreactive. There, they circulate as naïve B cells, and they become activated once they encounter the antigen. Activated B cells travel to the secondary lymphoid organs where they further diversify their Ig genes by SHM and CSR. Successful ones are positively selected, and they exit to the periphery as plasma cells where they produce and secrete antibody molecules (red) to combat infection.

replacing the surrogate chain to form a true B cell receptor; this defines the IgM^+ immature B cell. Before immature B cell emerges from the bone marrow, it undergoes negative selection for self-tolerance, i.e. auto-reactive cells are eliminated (18). B cells that leave the bone marrow and survive in the periphery further differentiate into mature B cells, expressing both IgM and IgD. These cells, also called naïve B cells, travel through peripheral lymphoid tissues, where they become activated if they encounter their cognate antigen.

1.3.2 Antibodies

The main function of B cells in defense of the organism is their ability to evolve, produce and secrete large amounts of antibodies specific for a particular antigen. Antibodies, also referred to as immunoglobulins, are a secreted form of the B cell receptor, composed of a heavy (H) and a light (L) chain, each with two distinct regions, called variable (V) and constant (C) (19, 20). The variable region recognizes and binds the antigen, while the constant region governs the way bound antigens are cleared from the body.

1.4 Diversification

1.4.1 VDJ Recombination

As mentioned before, lymphocytes are capable of recognizing a large number of antigens, due to the series of genetic rearrangements that take place within the Ig, or B cell receptor (BCR) gene. Antibody diversification begins

before exposure to antigen during B cell development in the bone marrow with the rearrangement of the variable (V), diversity (D) and joining (J) segments of the immunoglobulin gene (Figure 2). VDJ recombination joins a single V, D, and J segment into a single variable region through superbly well coordinated set of reactions starting with cleaving DNA within specific, well-conserved recombination signal sequences (RSS). This process is dependent on recombination-activating genes 1 and 2 (RAG1 and RAG2) which together form RAG endonuclease (21). Finally, DNA segments are re-assembled by common repair mechanism.

Each coding segment is flanked by an RSS, and the RAG proteins introduce double stranded DNA breaks between the coding sequence and RSS. In fact, in test-tube experiments, RAGs are sufficient to introduce DNA breaks in the synthetic DNA oligonucleotide with appropriate RSS (22). These RSS consist of highly conserved heptamer and nonamer sequences, separated by either a 12-mer or a 23-mer. A 12/23 rule states that if one RSS contains as 12-mer, the second RSS has to contain a 23-mer for efficient recognition and cleavage by RAG.

After cleavage, the double-stranded DNA breaks have to be repaired. Imperfect joining of the segments in the non-homologous end joining reaction (NHEJ), with nucleotides added or subtracted, adds to junctional diversity. The whole process has the potential to create as many as 10^{11} unique variable regions from the germline immunoglobulin genes.

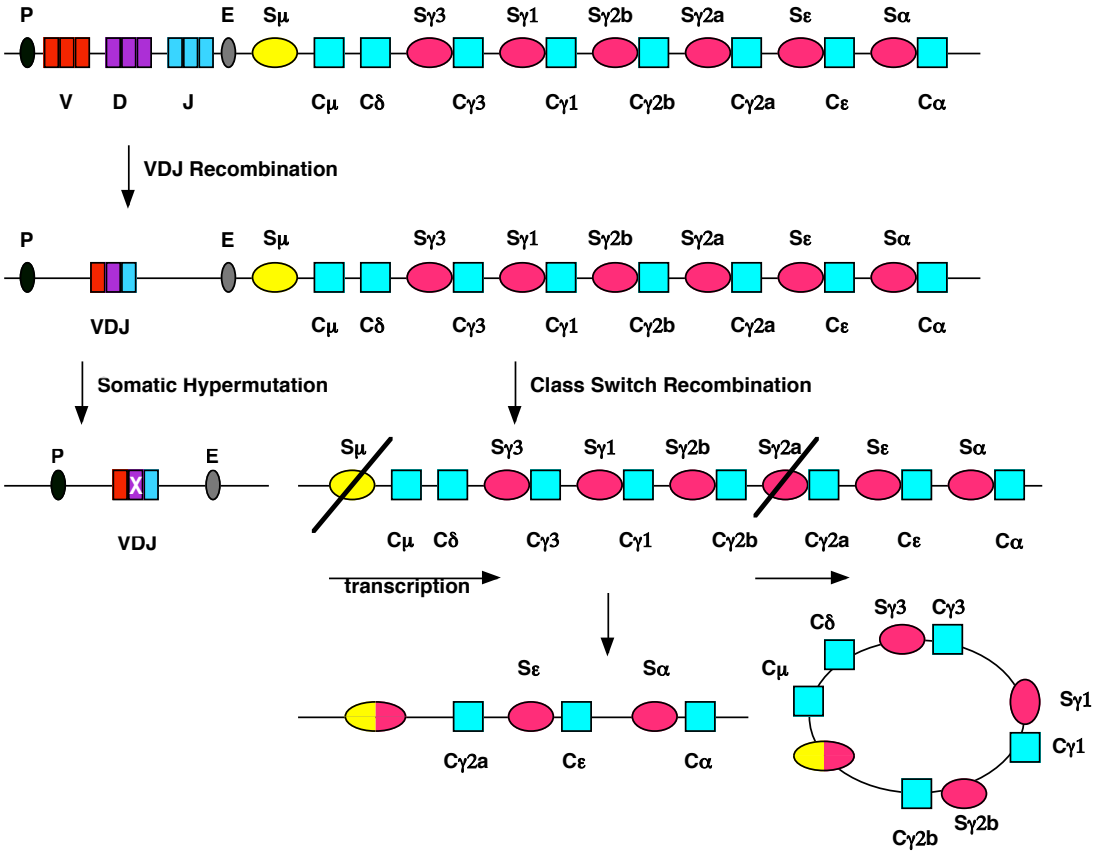

Figure 2. Antibody diversification events. V (red), D (purple) and J (blue) segments are recombined in VDJ reaction to yield a single variable region. Somatic hypermutation introduces point mutations (X) with the newly formed variable region. Class switch recombination proceeds via double stranded DNA breaks in the switch regions (Sμ – yellow, Sγ, Sε, and Sα – fuscia). Sμ is joined with a downstream S region, while the intervening DNA is looped out. Constant regions are shown in turquoise.

1.4.2 Somatic Hypermutation and Class Switch Recombination

Mature B lymphocytes that have successfully rearranged their Ig genes by VDJ recombination migrate through the periphery and become activated once they encounter an antigen. In secondary lymphoid organs, activated B cells proliferate and form organized compartments called germinal centers (GC) (23) where further diversification of Ig genes takes place: somatic hypermutation and class switch recombination.

1.4.2.1 Somatic Hypermutation

Somatic hypermutation (SHM) introduces point mutations and small deletions within the immunoglobulin V region of both heavy and light chains (24, 25), thereby changing the affinity of the antibody for antigen (Figure 2). The rate of somatic hypermutation is about million times higher than the background level of spontaneous somatic mutation.

Transcription is required for somatic hypermutation (26). It has been shown that mutations can be found within Cκ, a gene that normally does not mutate, when Ig promoter is duplicated upstream of Cκ (27). In addition, mutation rates are directly proportional to the transcription rates throughout the locus (28). For example, when a mutant gfp gene, carrying a revertible stop codon, is cloned under tetracycline-inducible promoter and transfected into B cells, the levels of reversion mutations are directly correlated with the levels of transcription (28).

Additionally, the mutation initiation site is determined by the distance from the promoter (27, 29). SHM has been shown to peak about 200 base pairs after the promoter, and frequency decreases exponentially with distance from promoter (30). The Ig enhancers have been shown to be important and perhaps necessary for SHM, as their presence facilitates mutation but their absence does not completely abolish it (31-35). Mutations are clustered within the complimentarity determining regions, which are shown to form the binding site in the three-dimensional structure of the antibody (36). Although it is not sequence specific, mutation shows preference for certain mutational “hotspot” motifs (RGYW/WRCY, where R=A/G, Y=C/T, and W=A/T; or better yet DGYW/WRCH, where D=A/G/T, and H=T/C/A), which are at least twice as likely to be mutated than other sequences (37, 38). However, this sequence is not responsible for targeting of mutation since not every RGYW motif is targeted (39).

It is important to note that Ig genes are not the only genes that can be mutated, and I will further expand on this issue in the discussion (see Chapter 6).

1.4.2.2 Class Switch Recombination

The constant region of the Ig heavy chain locus consists of several C genes, which are directly preceded by switch regions (S) (Figure 2). Class switch recombination replaces the default Ig constant region, Igμ, with a downstream constant region, Cγ, Cε, or Cα (Figure 2). This results in changing the antibody protein isotype from IgM to IgG, IgE or IgA respectively, while retaining the

antibody specificity for antigen while altering the physical properties of the antibody. This recombination takes place between two switch regions, while the intervening DNA is looped out and eventually removed (40, 41).

Switch regions are different in nucleotide sequence, but they are all highly repetitive. In mice, Sμ region primarily contains pentameric units that are repeated throughout (42), while repeats within Sγ contain 49 base pairs, repeats in Sε contain 40 base pairs (43), and finally repeats in Sα repeats are the largest, consisting of 80mers (44). This is not unique to mice, as switch regions of other species, including humans, show similar sequence patterns (45). In addition to being highly repetitive, switch regions also contain palindromic sequences, which allow for formation of stem-loops when DNA is denatured. Switch regions are necessary for CSR, as their deletion results in complete abolishment of the reaction (46, 47). However, it appears that the features of switch regions (such as repetitive and palindromic sequences) are more important than the sequences themselves. For example, when Sα was replaced by Sε in the CH12F3-2 model cell line that specifically switches to IgA, the efficiency of this reaction was retained (48). Also, changing the orientation of Sα region did not unduly influence switching efficiency.

Initially, breaks are made within switch regions directly preceding the constant regions participating in recombination (49, 50). Although these can be detected as blunt ends by ligation-mediated PCR (51), it is unclear whether they are clean double stranded breaks as opposed to nicked cleavage breaks, or

even staggered breaks made blunt by post-cleavage processing. Nevertheless, double stranded DNAs breaks are CSR-specific.

These newly formed breaks have to be repaired in order to preserve genomic stability. It is thought that the intervening DNA sequence is looped out, and the breaks themselves are repaired by a non-classical NHEJ pathway (52-54). Proteins required for NHEJ (DNA-PKcs, Ku-70 and Ku80) are also required for CSR (53, 55, 56). However, 53BP1, a protein induced upon genotoxic stress (specifically DSBs), is also required for CSR, as its deficiency is shown to produce significantly lower levels of switch junctions (54, 57). There is no sequence consensus at the newly formed joints, and no homology between the parental sequences. The newly repaired DNA will give rise to the new transcript, reading the desired constant region.

Transcription is necessary for CSR, and it appears to play a role in selection and targeting of a particular S region, as different cytokines initiate transcription of different S regions (58, 59). The levels of transcription directly correlate with the levels of recombination (60), as it has been previously mentioned for SHM.

1.4.3 Activation-Induced Cytidine Deaminase (AID)

Although seemingly very different, both SHM and CSR have been shown to depend on a presence of a single enzyme: activation-induced cytidine deaminase (AID) (61). AID was first discovered as CSR specific factor by

subtraction hybridization of cDNA libraries of switch-induced and uninduced CH12F3-2 cells (62). Furthermore, overexpression of AID in the same cell line enhanced levels of CSR, regardless of other stimulants, such as cytokines.

Mice and humans lacking AID undergo neither somatic hypermutation nor class switch recombination. AID-deficient mice do not mutate V regions of their Ig genes, even upon immunization with a common immunogen such as NP-ova (61). At the same time, IgG, IgE, and IgA titers are not present in blood of AID-deficient animals, with or without prior immunization (with the exception of young animals that carry maternal IgG). The only isotype that can be detected is IgM. In addition, B splenocytes isolated from AID-deficient mice fail to switch their isotypes when stimulated by LPS and IL-4 *ex vivo*. Furthermore, both mice and humans deficient in AID show signs of Hyper IgM Syndrome: their spleens are enlarged and blood IgM titers are high, but they do not produce any other antibody isotype (63). Mutations are not found within V regions of Ig genes in B cells from AID-deficient patients.

AID is a relatively small protein (198 amino acids, ~ 25 kDa), encoded by a large gene (2500 bp) on human chromosome 12p13 (62, 64). A small part of the gene encodes the active protein (594 bp), while most of the mRNA consists of a long 3' untranslated region. AID has been identified in all higher vertebrates, starting from cartilaginous or bony fish, consistent with the observation that they hypermutate (65-67).

AID is almost exclusively expressed in germinal center B cells (62): precisely the type of cells undergoing SHM and CSR *in vivo*. When overexpressed, AID can mutate non-germinal center B cells, such as hybridomas, suggesting that AID does not require any centroblast specific factors for SHM (68). Furthermore, overexpression of AID in non-B cells, such as NIH-3T3 fibroblasts, leads to mutations in artificial substrates (69). The levels of mutation can be correlated to levels of transcription, and the distribution of mutations is very similar to SHM in B cells, suggesting that AID is the only B cell specific factor required for mutation. Most strikingly, overexpression of AID mutated the RpoB gene in *Escherichia coli* (70). At the same time, AID transgenic mice show increased accumulation of mutations in T cells (T cell receptor gene and MYC oncogene), as well as increased incidence of T cell lymphomas (71). Together, these data indicate that AID alone is sufficient to cause mutation in highly transcribed genes, although under physiologic circumstances it is restricted to the Ig locus in B cells.

Cytidine deaminases are characterized by a bipartite active site (72), and AID is no exception. Centrally located, the first part of the active site ($\underline{H}^{56}V\underline{E}^{58}L$) is involved in proton shuffling, followed by the zinc coordinating domain ($\underline{C}^{87}YD\underline{C}^{90}$): both necessary for cytidine deamination (72).

AID is homologous to APOBEC-1, a catalytic subunit of the RNA-editing complex of apolipoprotein-B mRNA (62). APOBEC-1 deaminates cytosine at position 6666 of this mRNA, converting it to uracyl and thus giving rise to a new

mRNA, which is then translated into a shorter protein that forms chilomicrons (73, 74). APOBEC-1 is also found on human chromosome 12p13 (64), close to AID. Because of their homology, as well as close genetic mapping, it was initially proposed that AID acts on one or more mRNA molecules, thereby giving rise to novel proteins that would function in SHM and CSR, but no RNA targets have been identified (61, 75-78).

On the other hand, it has been shown that uracil DNA glycosylase (UDG) is also involved in both somatic hypermutation and class switch recombination. This enzyme is responsible for recognition and elimination of uracil when it is found within DNA. When UDG is blocked by a specific inhibitor, the SHM mutation spectrum is skewed and CSR is severely reduced, suggesting that AID may be converting cytidines within DNA directly to uridine (79, 80). A large part of my thesis work is devoted to understanding the biochemistry of AID, and my work on this will be discussed in later chapters.

1.5 B Cell Lymphomas

Like any other cell type in human body, B cells can become malignant. Lymphomas are cancers of the lymphatic system; B cell lymphomas are the most common, representing about 85% of all lymphomas, with the remainder being T cell lymphomas and NK cell lymphomas.

Chromosomal abnormalities were long postulated to be a cause of cell transformation and malignancy, but it was only in 1960 that a marker

chromosome, the Philadelphia chromosome, was strongly associated with chronic myelogenous leukemia (81). This abnormality was later characterized as reciprocal chromosomal translocation between chromosomes 9 and 23 (82). B cell lymphomas are often marked by chromosomal translocations (83-85), involving the IgH locus and an oncogene locus, such as IgH/cyclinD1 (mantle cell lymphoma (86)), IgH/bcl2 (follicular lymphoma (87)), and IgH/c-myc (Burkitt's lymphoma (88)). This genetic rearrangement results in deregulation of the oncogene, leading to the transformation of the affected cell.

One of the best-characterized chromosomal translocations is IgH/c-myc translocation, a hallmark of Burkitt's lymphoma in humans, plasmocytomas in mice and immunocytomas in rats (89-92). During this rearrangement, no genetic material is lost or gained, so that the translocation is balanced and reciprocal. C-myc, which is usually tightly regulated within a cell cycle, is thus brought to close proximity and transcriptional regulation of a strong IgH promoter, resulting in its deregulation and ultimately cellular transformation.

Analysis of IgH/c-myc junctions showed that most of these translocations involve IgH switch regions, suggesting they arise from an anomalous resolution of DNA breaks during the class switch reaction (88, 90, 93-98). In fact, it has been shown that AID is required for IgH/c-myc translocations *in vivo* (99). In this study, IL-6 transgenic mice were treated with pristein oil to induce translocations, and translocations were only detected in $AID^{+/-}$ mice, while they were absent in $AID^{-/-}$ mice. Furthermore, divergent pathways seems to lead either to class switch

recombination or IgH/c-myc translocation (100): although both events require AID and proceed through double stranded DNA breaks, it is the resolution of breaks that is different in the two processes *in vitro*. Hence, deficiency in tumor suppressors such as ATM, p19 or p53 increases the observed frequency of AID-induced translocations: the frequency of the translocations in p53-deficient B cells stimulated to switch *ex vivo* is about 30 percent. However, $p53^{-/-}$ mice do not succumb to B cell lymphomas *in vivo*, indicating that there must be additional mechanisms of suppression.

1.6 NK Cells in Innate Tumor Immunity

Natural killer cells bridge the adaptive and innate immunity in fighting pathogens and tumors. As one of the major effector cells of innate immunity, they have an ability to recognize and eliminate some virus-infected cells and tumor cells (101-103). NK cells were first recognized in 1970s by their spontaneous cytotoxic activity towards tumors (104). Although of the same origin and B and T cells, they are different in that they lack typical surface antigen receptors.

Instead, NK cells carry two types of germline encoded receptors, which are either inhibitory or activating (107). The killer inhibitory receptor (KIR) engages with MHC class I molecule on the target cell, and attenuates the activation of NK cells through the activating receptors (108, 109). The discovery of inhibitory receptors lead to the “missing-self” hypothesis: NK cells constantly survey their surrounding for MHC-I expression, which inhibits their activation.

Due to the constant engagement of inhibitory receptors with their ligands in a healthy organism, and thus constant NK cells inactivation, it has been very difficult to study activating receptors and their ligands, of which there are many. An activating receptor of major interest, as it can be upregulated upon genotoxic stress, is NKG2D, a type II dimer linked by disulfide bonds and containing lectin-like domains(110, 111). The mouse homologue of NKG2D was readily identified and only recently shown to bind H60 and Rae-1 as well as their human orthologs MICA and MICB (112, 113). The Rae-1 family of molecules is comprised of α, β, γ and δ isoforms, and it is known to be induced in embryonic carcinoma cells upon exposure to retinoic acid (114) and genotoxic stress (114, 115).

Transfection of Rae-1 into otherwise unsusceptible targets renders them sensitive to NK killing (112). This interaction appears to be specific, as it can be blocked by polyclonal serum made against soluble NKG2D. In particular, Rae-1 is expressed on some tumor cells, and the level of its expression correlates with the ability of NK cells to eliminate these tumors (116).

During my thesis work I have hypothesized that DNA damage incurred by B cells during CSR-dependent translocations leads to Rae-1 expression on their surface and eventual clearance by NK cells. Data pertaining to this hypothesis will be discussed in later chapters.

CHAPTER 2. MATERIALS AND METHODS

2.1 Mice

All mice were purchased from Jackson Labs, with the exception of $AID^{-/-}$ mice, which were a kind gift from Dr. Tasuku Honjo and were obtained from Dr. Michel Nussenzweig. The following strains were used in the study: C57BL/6J (000664), Balb/cByJ (001026), B6.MRL-Fas^{Lpr}/J (000482), B6.129S2-$Trp53^{tm1Tyj}$/J (002101).

2.2 Purification of strepAID and AID-his

Human AID constructs, wild type and catalytic site mutant (E58Q/C87,90A) were cloned into the pET-3d vector (Novagen) and transformed into Rosetta bacterial strain (Novagen). Transformed bacteria were grown in LB (EM Biosciences) and induced at OD_{600}=0.8 with 2 mM IPTG for 3 hours at 37°C. Bacteria were lysed with B-PER (Pierce), and lysate was pre-cleared on Q-Sepharose FF (Amersham Biosciences) and loaded onto phosphocellulose P11 resin (Whatman). StrepAID eluted from P11 at 0.4 M KCl. Purified strepAID was loaded onto MonoQ (Amersham Biosciences) and eluted as tetramer at 0.5 M KCl.

Human AID constructs AID wt, coreAID (amino acids 1-158) and CTD (amino acids 158-198) were cloned into the pET-28b vector (Novagen) carrying a hexahistidine tag on C-terminus and transformed into Rosetta bacterial strain

(Novagen). Transformed bacteria were grown in LB (EM Biosciences) and induced at OD_{600}=0.8 with 1 mM IPTG over night at 16°C. Bacteria were lysed by high pressure (French Press) and DNA was sheared by sonication. The lysates were spun down at 14,000 rpm for 30 minutes at 4°C and supernatants were loaded onto the Co^{2+} resin (Talon). Column was washed with 30 mM imidazole, and AID-his proteins were eluted with 150 mM imidazole. Further purification was achieved by size exclusion chromatography on Sdex200 column (Amersham Biosciences).

For all protein analysis, I used 4-20% gradient tris-glycine gels by Biorad (161-1123). Coomassie staining was performed Simply Blue Safe Stain by Invitrogen (LC6060), according to manufacturer's instructions. Silver staining was performed by Silver Stain (ICN, 800665), according to manufacturer's instructions.

2.3 Electromobility Shift Assay (EMSA) and UV Cross-Linking

Oligonucleotide substrate (100 ng) was labeled by T4 polynucleotide kinase (NEB) using 0.1 mCi γ-P^{32}-ATP (Amersham). Binding reactions (1 ng substrate and 200 ng strepAID in total volume of 10 μL; 10 mM Hepes pH 7.6, 10% glycerol, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM DTT) were incubated for 40 minutes at room temperature.

For EMSA the entire reactions were loaded on 4-20% polyacrylamide gels (Bio-Rad Laboratories) and electrophoresed in tris-glycine running buffer at 100

V on a Bio-Rad Laboratories Mini-Protean apparatus until the bromophenol blue dye front had reached the bottom of the gel.

For UV cross-linking reactions were placed on ice 1.5 cm away from the light source and exposed for 10 minutes at 254 nm in a Stratagene UV Stratalinker. Samples were then heated to 95°C for 5 minutes in 1% SDS before loading the 4-20% polyacrylamide gels electrophoresed at 200V with 0.1% SDS in the running buffer. Gels were dried, and images were stored on phosphor screens and analyzed on STORM or Typhoon Phosohoimagers with ImageQuant software.

Substrates:

ssRNA: aUCGgaauguaugagaauaagaagagauaaugaauaauagaa

ssDNA: aTCGgaatgtatgagaatagaagagataatgaataatagaa

dsDNA (hp):

aTCGgaatgtatgagaatagaagagataatgaataatagaattctattattcattatctcttctattctcatacattc CGAt

2.4 Deamination Assay – Thin Layer Chromatography (TLC)

All deamination assays were done in a buffer containing 10 mM Hepes pH 7.6, 100 mM KCl, 5 mM EDTA at 30°C. The radiolabeled substrates (CTP, dCTP, RNA and DNA) were incubated with protein (cdd, strepAID, Apobec-1 or BSA) for 1 hour. Reactions were extracted with phenol:chloroform (Sigma) and digested with P1 nuclease (Roche). Resulting mononucleotides were loaded onto a TLC

plate (PEI cellulose; Merck), and plates were developed in a solvent system containing isopropanol:hydrocloric acid:water (7:2:1) for 12 hours. Plates were exposed to phospho-storage screens (Amersham Biosciences), which were scanned by Typhoon imager and analyzed using ImageQuant software (Molecular Dynamics).

2.5 Deamination Assay – Single Nucleotide Primer Extension (SNuPE)

All deamination assays were done in a buffer containing 10 mM Hepes pH 7.6,100 mM KCl, 5 mM EDTA at 30°C. However, AID was able to deaminate DNA in a range of conditions: pH 7.6-9.0, 50 – 100 mM KCl and 23-37°C. Ten nanograms of gel-purified oligonucleotide substrates were incubated with 100-200 ng AID for 30 minutes. A tenth of reaction was used for primer extension with a thermophilic Klenow fragment Bst Polymerase (NEB). Briefly, a 25 μL reaction containing 1 ng template, 1 ng primer and Bst buffer was incubated at 70°C for 1 minute, before addition of 0.5 units Bst. The reaction temperature was first lowered to the appropriate primer annealing temperature for 10 seconds and then raised to 65°C for 1 minute to allow for extension. Reaction was terminated by the addition of sample loading buffer, heated to 95°C and loaded on 14% urea denaturing gel (Sequagel, National Diagnostics). Gels were dried and exposed to phosphor storage screens (Amersham Biosciences), which were scanned on Typhoon imager and analyzed using ImageQuant software (Molecular

Dynamics). All RNA oligos were custom synthesized by Dharmacon, Inc.; DNA oligos were made by FisherOligos.

Substrates:

ACCint: agtaaatgaaACCgaatgtatgagaatagaagagataatga

ACCend: aACCgaatgtatggagaatagaagagataatgaataatagaa

TCGint: agtaaatgaaTCGgaatgtatgagaatagaagagataatga

TCGend: aTCGgaatgtatgagaatagaagagataatgaataatagaa

Primers:

PrExACC: atctcttctattctcatacattcG ($T_{annealing}$ = 58°C)

PrExTCG: atctcttctattctcatacattcC ($T_{annealing}$ = 57°C)

2.6 Transcription-based Assay for AID Activity

100 μL in vitro transcription reactions containing: 20 mM TrisHCl pH 7.9, 20 mM KCl, 5 mM $MgCl_2$, 2 nM ptac-KanL94P plasmid, 10 nM RNAP (Epicentre), 100 μM rNTPs and 20 nM AID were incubated at 37°C. Plasmid DNA was purified by Qiagen MinElute to yield 10 microliters of DNA in water, which was electroporated into UDG-deficient *E.coli*. Bacteria were plated on spectinomycin or spectinomycin/kanamycin/IPTG containing plates. The ptacKanL94P plasmid was obtained from Dr. Michel Nussenzweig.

2.7 Class Switch Recombination In Vitro and Rae-1 Detection

Resting B cells are isolated from mouse spleens by negative selection using αCD43 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. They were plated at concentration of 500,000 cells/mL in a 24-well plate (Fisher), and stimulated to switch with LPS (25 μg/mL) and Il-4 (5 ng/mL) (both from Sigma). Isotype switching was measured by surface expression of IgG_1 and IgG_3 by FACS (BD Biosciences). Biotinylated αIgG_1 (553441) and αIgG_3 (553401) antibodies were used as the primary staining, and SA-PerCP (340130) was used as the secondary antibody (all from BD Biosciences Pharmingen). Data were analyzed by FlowJo software (FlowJo). αRae-1 primary antibody (MAB17582) or isotype control (MAB0006) were used for the primary staining for Rae-1 expression, followed by αRat-PE (F0105) (all from R&D Systems, Inc.).

2.8 NK Cytotoxicity Assay

NK cytotoxicity CyToxiLux® Plus (OncoImmunin, Inc.) was used according to manufacturer's protocol. Briefly, stimulated B cells were taken at the appropriate time point (48, 60 and 80 hours post stimulation) and labeled with FL-2 fluorescing dye. 200,000 labeled B cells (target cells, t) were incubated with NK cells (effector cells, e), freshly purified from congenic mice spleens by NK cell isolation kit (Miltenyi Biotec, 130-090-864), in increasing ratios (t:e = 1:5, 1:10 and 1:20) for 1.5 hours. Caspase substrate was added to the reactions for 30

minutes. The level of B cell killing was measured as function of caspase substrate cleavage, indicated by fluorescence in the FL-1 channel.

2.9 Immunization and Germinal Center B Cell Isolation

Sheep red blood cells (CS1342; Colorado Serum Company) were diluted 1:10 with HBSS (GIBCO) and used to immunize mice (200 μL/mouse). Ten days post immunization, mice were sacrificed and spleens and lymph nodes were harvested. Germinal center B cells were sorted on FACSAria or FACSVantage cell sorters (BD Biosciences), after staining with GL7-FITC (553666) and Fas-PE (554258) antibodies (both from BD Biosciences Pharmingen). DNA was extracted by standard methods and translocations were detected as previously published (99).

CHAPTER 3. AID IS A ssDNA DEAMINASE *IN VITRO*

AID has been shown to be necessary for SHM and CSR, and it was initially postulated to be a functional cytidine deaminase acting on RNA (61). Further studies have indicated that AID may act on DNA directly, as ectopic overexpression of this protein results in dramatically increased levels of mutation in non-germinal center B cells (68), non-B cells (69), and even *Echerichia coli* (70). However, this effect could be achieved through a number of different mechanisms. First, AID could be deaminating cytidine, dCTP or CTP, thus altering the composition of cellular nucleotide pools, and even subtle pool alterations can lead to mutagenesis in bacteria (117). Second, AID could act on RNA, and overexpression of AID could lead to promiscuous mRNA editing, as reported for overexpressed Apobec-1 (118). Finally, AID could deaminate DNA directly. In fact, parallel work has convincingly identified uracil DNA glycosylase (UDG) as a factor in somatic hypermutation (79, 80): ung would remove the uridine product if cytidine is deaminated within the DNA molecule itself.

3.1 Recombinant Human AID Is a Tetramer

I took a biochemical approach to confirm that AID is indeed an active cytidine deaminase and to determine which was the preferred deamination substrate for AID *in vitro*. A wild type and a catalytic site mutant (E58Q/C87,90A) human AID were cloned, carrying a small strep-tag on N-terminus. Both proteins were overexpressed in *E.coli* and subsequently purified based on their affinity to

bind phosphocellulose (P11) – a DNA mimic resin, that was known to successfully bind various deaminases (119). AID eluted from P11 resin with a peak at 0.4 M KCl to relative purity as shown by silver stain (Figure 3a).

To verify the identity of purified proteins, strep-AID wt and mutant were boiled in SDS loading buffer, ran on a denaturing gel and Western-blotted using two different reagents – streptavidin and αAID antibodies. In both cases, strep-AID wt and mutant proteins migrated with a predicted size of a tetramer (Figure 3a). The tetramer was extremely stable, and could only be broken up by harsh denaturants, such as Urea, Guanidine HCl, and EDTA (Figure 3b). These experiments suggest that strep-AID is a tetramer when purified on a DNA mimic resin. Indeed, many other deaminases are multimers (dimers, trimers and tetramers), and many of them are stable on denaturing gels, such as Apobec-1 and BSD (120-122).

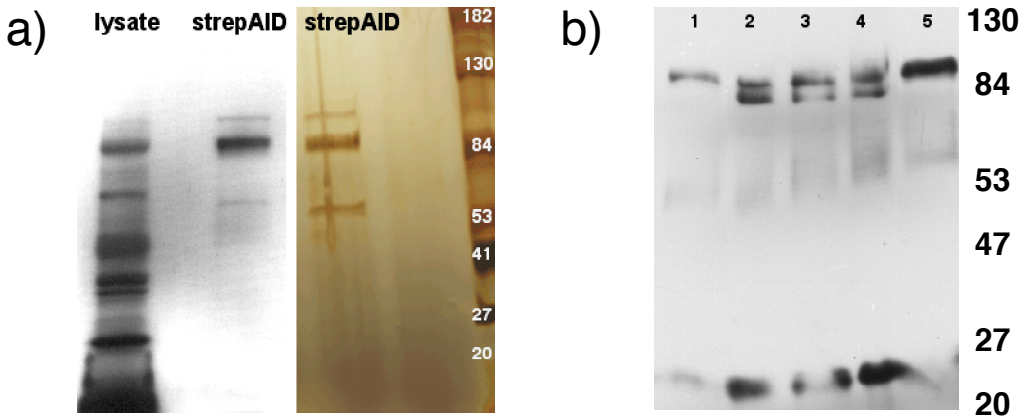

Figure 3. Purified recombinant AID behaves as a stable tetramer. a) Purified recombinant AID runs with a predicted size of a tetramer on a denaturing gel, as shown by αAID western blot (left panel). The protein was nearly homogeneous as shown by silver stain (right panel). b) Western blot that shows that strepAID tetramer can dissociate into trimer and monomer upon boiling in buffers containing strong denaturants and EDTA (lanes 2-4), but not in standard sample buffer (lane 1) or EDTA alone (lane 5). Lanes 2-4 show samples boiled in 50% formamide and 50 mM EDTA (lane 2), 0.8 M Urea and 50 mM EDTA (lane 3), or 25 mM Tris (pH 8), 10 mM EDTA, 2% SLS (lane 4). Protein size markers are shown on the right.

3.2 AID Does Not Deaminate Free CTP or dCTP

Many known and well described cytidine deaminases are metabolic (123, 124), acting on free bases such as CTP and dCTP. To find if recombinant strepAID can deaminate CTP (or dCTP), the protein was tested in thin layer chromatography (TLC) assay (125). Briefly, protein was incubated with radiolabeled substrate (CTP or dCTP) and reactions were resolved on TLC plate. When strepAID was incubated with α-P^{32}-CTP or α-P^{32}-dCTP for one hour no conversion to α-P^{32}-UTP or α-P^{32}-dUTP was detected (Figure 4a). In contrast, cdd, a metabolic cytidine deaminase from *Bacillus subtilis*, deaminated either substrate almost quantitatively (Figure 4a). The same was observed in a spectrophotometric assay (126). As expected, strepAID did not convert CTP/dCTP to UTP/dUTP even after one hour, whereas cdd deaminated about 75% of the substrate within minutes (Figure 4b). This further strengthens the finding that free CTP or dCTP cannot serve as strepAID substrate *in vitro*.

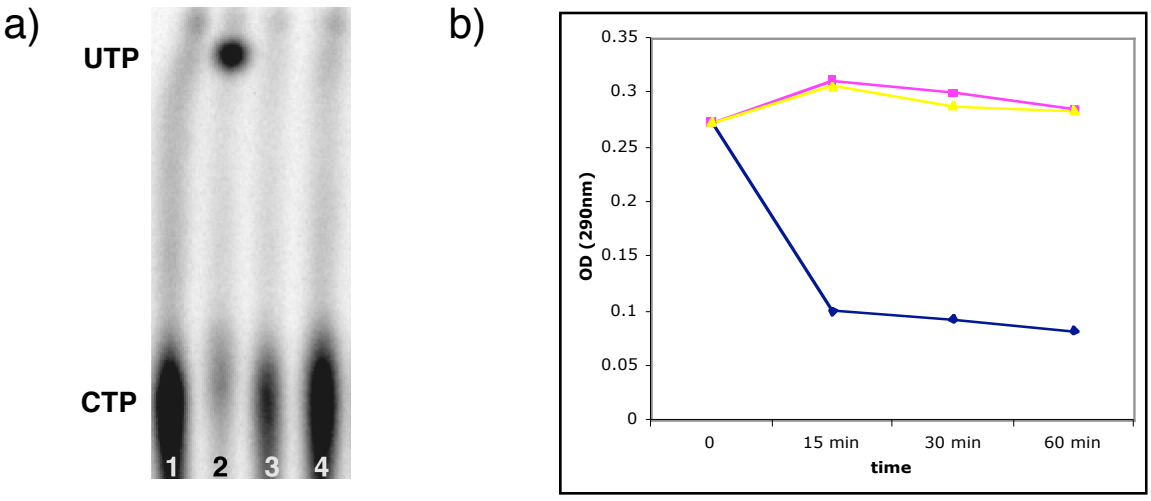

Figure 4. StrepAID does not deaminate CTP. a) StrepAID (lane 3) cannot deaminate CTP in standard thin layer chromatography assay, as compared to metabolic deaminase, cdd (lane 2). Apobec-1 (lane 4) didn't deaminate CTP. BSA (lane 1) was used as negative control. b) StrepAID (yellow) and mutant AID (pink) did not deaminate CTP in spectrophotometric assay, while cdd (blue) quickly converted CTP to UTP.

3.3 AID Binds Single Stranded Oligonucleotides In Vitro

After eliminating free bases, I considered other possible substrates such as oligomeric nucleotides. To determine if strepAID can bind RNA and DNA, a standard electromobility shift assay (EMSA) was performed. Indeed, strepAID bound single stranded RNA and DNA, but not double stranded DNA (Figure 5a). Binding to ssDNA was robust, as it could withstand a 25-fold excess of poly-dIdC or tRNA competitors (Figure 5b). StrepAID bound oligonucleotides in the same manner, regardless of presence or absence of cytidine within the sequence (data not shown). Additionally, whereas wt strepAID bound both ssRNA and ssDNA, mutant strepAID only showed binding to RNA (Figure 5b). This is consistent with findings that mutants of better studied cytidine deaminases, such as Apobec-1, lose substrate binding as well as catalytic activity when the cysteine residues of the active site are replaced by other residues (127).

When binding reactions were UV-irradiated, the resulting protein-DNA complex migrated on SDS containing gels with the approximate size of a strepAID tetramer (Figure 6). More specifically, strepAID only cross-linked to ssDNA, but not to dsDNA, or DNA:RNA hybrids (Figure 6). Mutant strepAID did not cross-link to ssDNA, but did cross-link to RNA (Figure 6). Taken together, the EMSA and UV cross-linking data suggest that ssDNA is a preferred substrate for strepAID. This residual binding of mutant AID to RNA may be nonspecific and due to the highly basic charge of the protein.

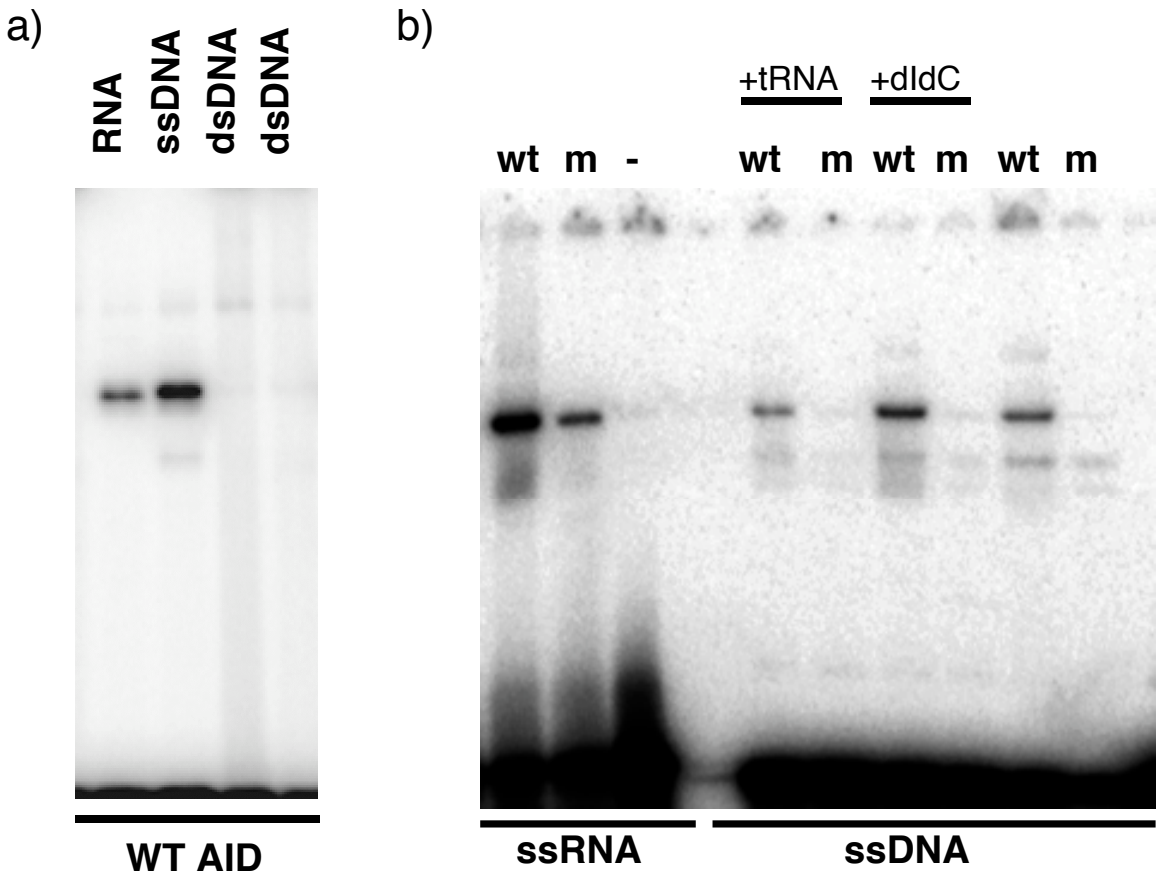

Figure 5. Recombinant strepAID preferentially binds ssDNA. a) EMSA: AID can bind ssDNA and RNA, but does not bind dsDNA. b) EMSA: active site mutant of AID (m) retains some ability to bind RNA compared to wt, but cannot bind ssDNA. Binding to ssDNA is resistant to 25 fold excess of tRNA or polydIdC.

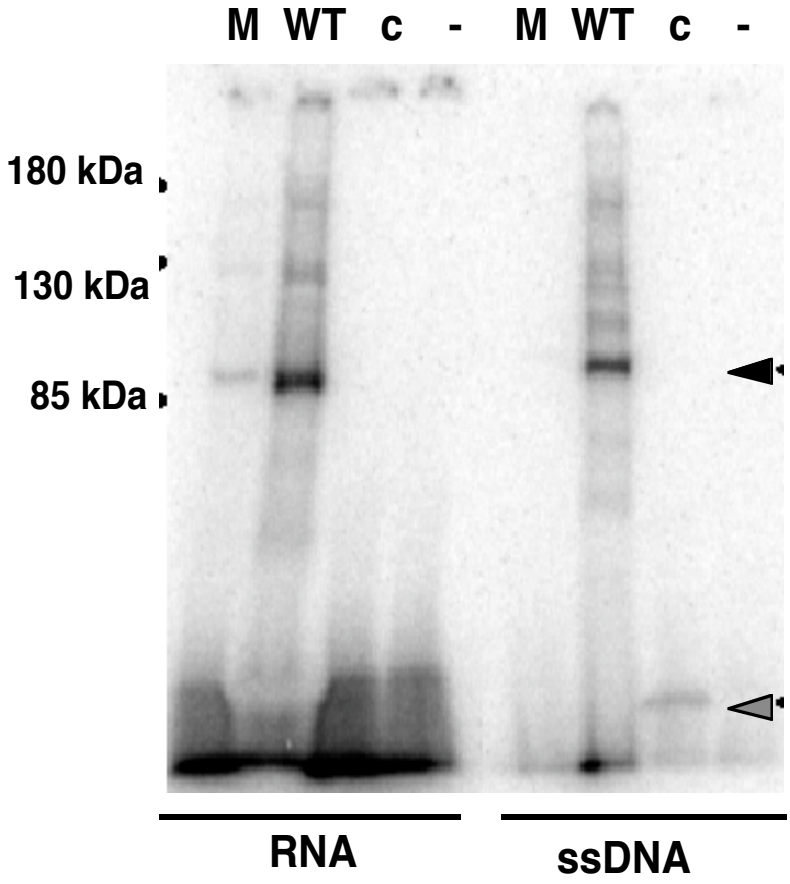

Figure 6. Recombinant strepAID uv-crosslinks to ssDNA and RNA. AID (wt) can be uv-crosslinked to RNA and ssDNA, and the cross-linked species run with a predicted size of a tetramer (black arrow). Mutant AID (m) can be uv-crosslinked to RNA, and the bacterial cytidine deaminase cdd can be weakly crosslinked to ssDNA (grey arrow).

3.4 AID Does Not Deaminate RNA

To determine if AID could deaminate RNA, the protein was tested in a standard thin layer chromatography (TLC) assay (125). Briefly, strepAID and control proteins (BSA, Apobec-1) were incubated for one hour with a synthetic RNA molecule, containing a single α-P^{32}-labeled cytidine. The reactions were phenol-extracted, digested by P1 nuclease, and the resulting mononucleotides were loaded onto the TLC plate. The plate was developed to resolve cytidine from its deamination product, uridine, by their difference in polarity: the more polar cytidines are bound more tightly to the plate and migrate more slowly than less polar uridines. Two different RNA substrates were tested: one containing the labeled cytidine close to the 5' end of the molecule, and another with the labeled cytidine embedded within the molecule.

In this assay, strepAID was not able to deaminate a single cytidine found at 5' end of an RNA oligomer, comparable to the BSA negative control (Figure 7). In contrast, Apobec-1, an RNA-editing deaminase, converted this cytidine into uridine with great efficiency (Figure 7). When a different RNA oligomer, carrying an internally labeled cytidine, was tested, strepAID remained inactive, whereas Apobec-1 retained its activity (Figure 7). Despite binding to RNA, strepAID was not able to deaminate two different RNA substrates in the TLC assay. It is possible that strepAID could deaminate some specific RNA, or that it could only deaminate cytidines within certain sequence context. However, based on these findings, RNA is not a good substrate for strepAID *in vitro*.

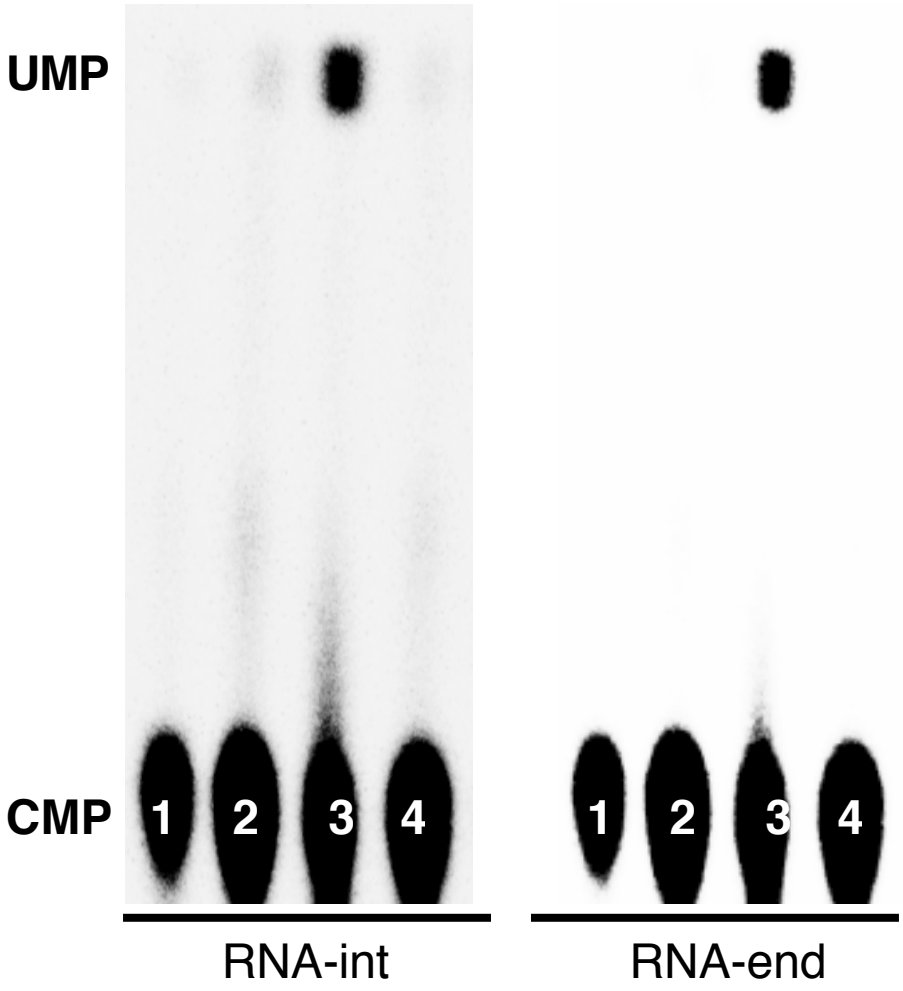

Figure 7. AID does not deaminate RNA in vitro. StrepAID (lane 4) did not convert CTP to UTP when embedded in a short RNA molecule (left panel). Apobec-1 deaminates RNA with great efficiency (lane 3). BSA (lane 1) and cdd (lane 2) did not deaminate RNA. The same is shown in the right panel, except that cytidine was closer to the 5' end of the RNA molecule.

3.5 AID Deaminates ssDNA In Vitro

To finally ascertain that strepAID can not only bind but also deaminate ssDNA, the protein was first tested in a standard TLC assay, as for RNA above. In this case, a DNA substrate containing a single α-P^{32}-labeled cytidine was incubated with strepAID for one hour, and the reaction processed as previously described. StrepAID converted cytidine to uridine embedded in a DNA oligomer (Figure 8). Although the deamination levels were low, they were consistent when the assay was repeated several times.

Since the level of deamination observed was very low, I sought to confirm this finding in an independent assay. In a single nucleotide primer extension assay (SNuPE), a primer that stops just short of potential deoxyuridine is annealed to DNA and extended by a single radioactive nucleotide. Under stringent annealing conditions, DNA polymerases such as Taq or Bst (thermostable Klenow fragment) only extend the primer if the radioactive nucleotide available precisely complements the template (128). The reaction is then analyzed on a sequencing gel, and the products are visualized by autoradiography (Figure 9a).

Figure 8. AID deaminates ssDNA *in vitro*. StrepAID (lane 2) deaminates ssDNA, whereas BSA (lane 1) and mutant AID (lane 3) do not.

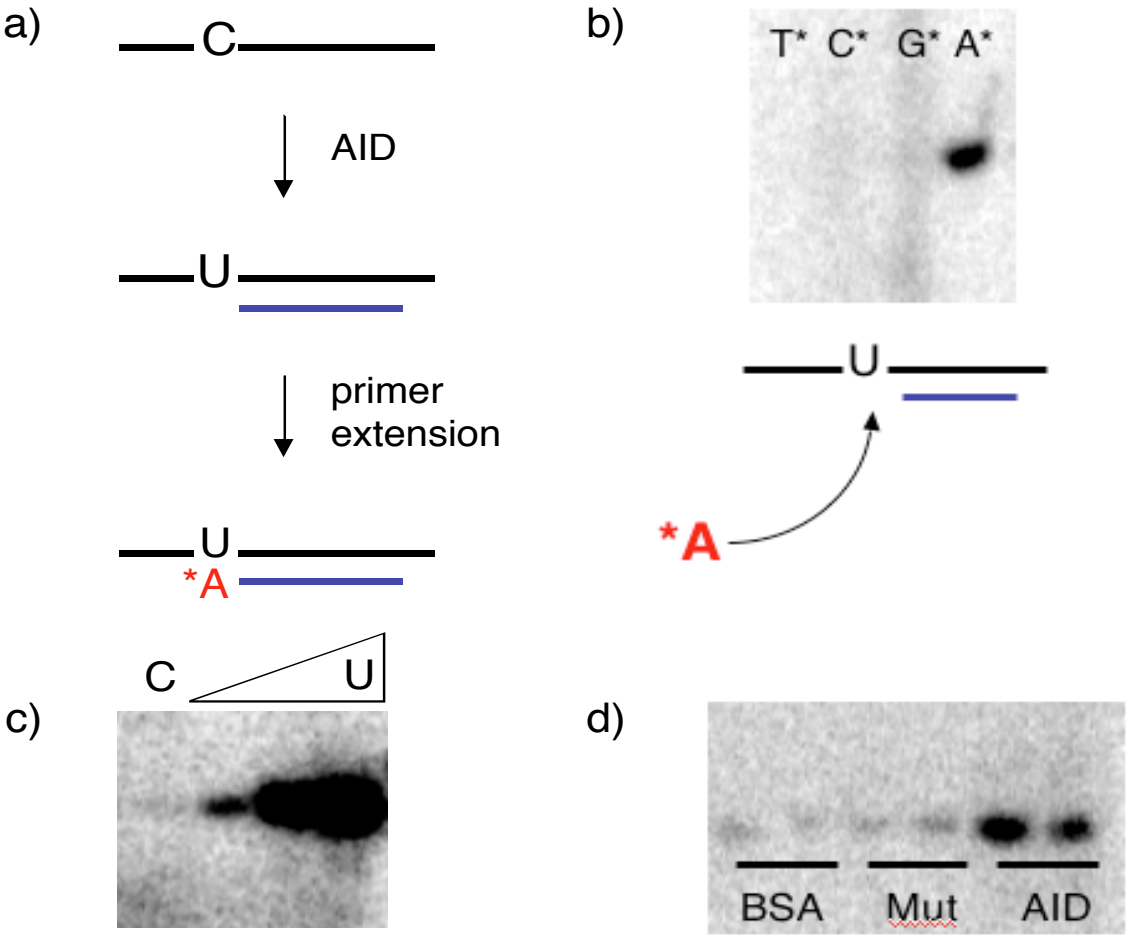

Figure 9. Recombinant strepAID deaminates ssDNA. a) Schematic of SNuPE reaction used to detect dC to dU conversion. b) The SNuPE reaction is specific: given the template with a uridine at a known position, Bst will only extend the primer with the addition of the complementary base (A*) and not any other nucleotide (C*, T*, G*). c) U containing oligo serves as calibration curve – the increasing signal corresponds to more uridine. d) BSA and mutant AID do not deaminate DNA. StrepAID deaminates ssDNA. All shown in duplicates.

To show that this assay is highly specific, a DNA oligonucleotide that contained a single deoxyuridine within the sequence was tested in a SNuPE reaction. Both Taq and Bst polymerases could extend the appropriate primer when α-P32-dATP was present, but not in the presence of any other radiolabelled nucleotide (Figure 9b). Furthermore, the primer could be extended with α-P32-dATP only when deoxyuridine-containing oligo was used as a template, but not when the template contained one of the other nucleotides (data not shown).

To determine the sensitivity of this assay, a series of template dilution SNuPE reactions was performed. Briefly, the template containing deoxyuridine was diluted by increasing concentrations of template in which the deoxyuridine was replaced by deoxycytidine. The SNuPE assay consistently detected the deoxyuridine-containing oligo when it comprised as little as 1% of the total template pool (Figure 9c). Hence, this assay allows for specific and sensitive detection of deoxyuridine within DNA.

To measure the ability of strepAID to deaminate ssDNA, the protein was incubated with an artificial oligonucleotide containing a single deoxycytidine close to the 5' end, and C-to-U conversion was detected by SNuPE assay. StrepAID consistently deaminated ssDNA whereas the mutant protein failed to do so (Figure 9c). The levels of cytidine deamination ranged anywhere from 1% to 10%, as assessed in comparison to standards containing uridine (Figure 9c). On

top, strepAID could not deaminate dsDNA, as it would be predicted by lack of binding to double stranded oligomers (Figure 9c).

Although strepAID showed very low levels of deamination activity, this reaction occurred very quickly (within 10 minutes), and over a range of conditions. Namely, strepAID deaminated ssDNA at varying salt concentrations (50-150 mM), range of pH conditions (7.6-9.0) and temperatures (25-37°C), and it could tolerate moderate levels of EDTA (5-10 mM). Taken together, these data strongly suggest that strepAID is an active cytidine deaminase, and it is able to convert cytidine to uridine within single stranded DNA swiftly and under a range of *in vitro* conditions.

CHAPTER 4. AID DEAMINATES DNA IN CONTEXT OF TRANSCRIPTION

Single stranded DNA is not readily available in vivo, raising a question: where and when can AID find its substrate in a germinal center B cell? Single stranded DNA may become transiently available during transcription, in which case it would be predicted that AID activity must be tightly coupled to the transcriptional apparatus, as previously envisioned (129). The first experiments to support this notion were done in *E.coli* that have been transfected with two plasmids: one carrying the AID gene under an inducible promoter, and the other plasmid containing a mutated kanamycin resistance gene KanL94P under a different inducible promoter. It was shown that induction of AID expression together with the induction of transcription of KanL94P gene can lead to reversion of L94P mutation and kanamycin resistance (130).

4.1 In vitro Transcription Provides ssDNA Substrate for AID

To show that transcription does not require additional *E. coli* proteins to target deamination by AID, the ptac-KanL94P plasmid was incubated with recombinant AID, *E.coli* RNAP and NTPs *in vitro*. After deproteinization, reactions were electroporated into ung- E. coli cells which were plated on both counting plates (containing spectinomycin) and selection plates (containing spectinomycin, kanamycin and IPTG). The mutation frequency was then

calculated as $kan^R spec^R$ colonies (selected)/$spec^R$ (total transformed) (Figure 10a).

In vitro incubation of ptac-KanL94P with AID alone did not appreciably increase the frequency of mutation of the kan^R gene (Figure 10b). The frequency was slightly increased with addition of RNAP to the reaction, but it only reached the in vivo levels in the context of transcriptional elongation after the addition of ribonucleoside triphosphates. This is an underestimate of the actual frequency of the AID-mediated mutations, since to be selected, clones carrying the deaminated plasmids must be able to grow in the presence of kanamycin.

The catalytic site mutant AIDC90H was also tested in this assay. As expected, AID^{C90H} showed no activity in this assay (Figure 11).

4.2 AID Activity Maps to the Predicted Catalytic Site

To further characterize AID, I used secondary structure prediction algorithms in conjunction with three-dimensional threading (3D-PSSM, (131)) to delineate the core domain of AID (coreAID) comprised of amino acids 1-158, and the C-terminal domain (CTD) comprised of amino acids 158-198. To confirm that activity resided with the core domain the kan^R gene was transcribed with *E. coli* RNAP in the presence of AID wt, coreAID or the CTD (Figure 11). Predictably, the CTD produced only background mutation in the presence of RNAP. However, the coreAID was just as active as the full length protein (Figure 11). These data suggest that the small his tag on the c-terminus of AID does not interfere with its

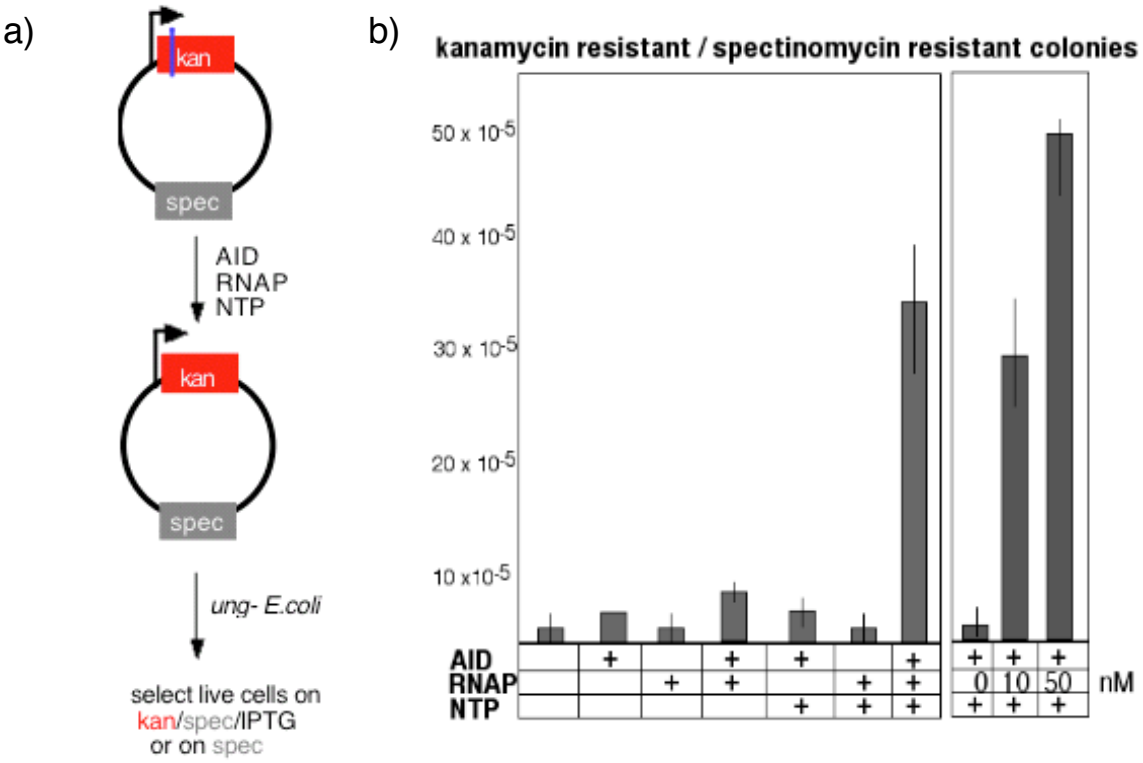

Figure 10. *In vitro* transcription by *E.coli* RNAP allows AID access to plasmid DNA. a) Schematic of transcription-based AID assay. b) In vitro transcription/deamination assay. AID is only able to deaminate the plasmid when ribonucleotides are added to the reaction.

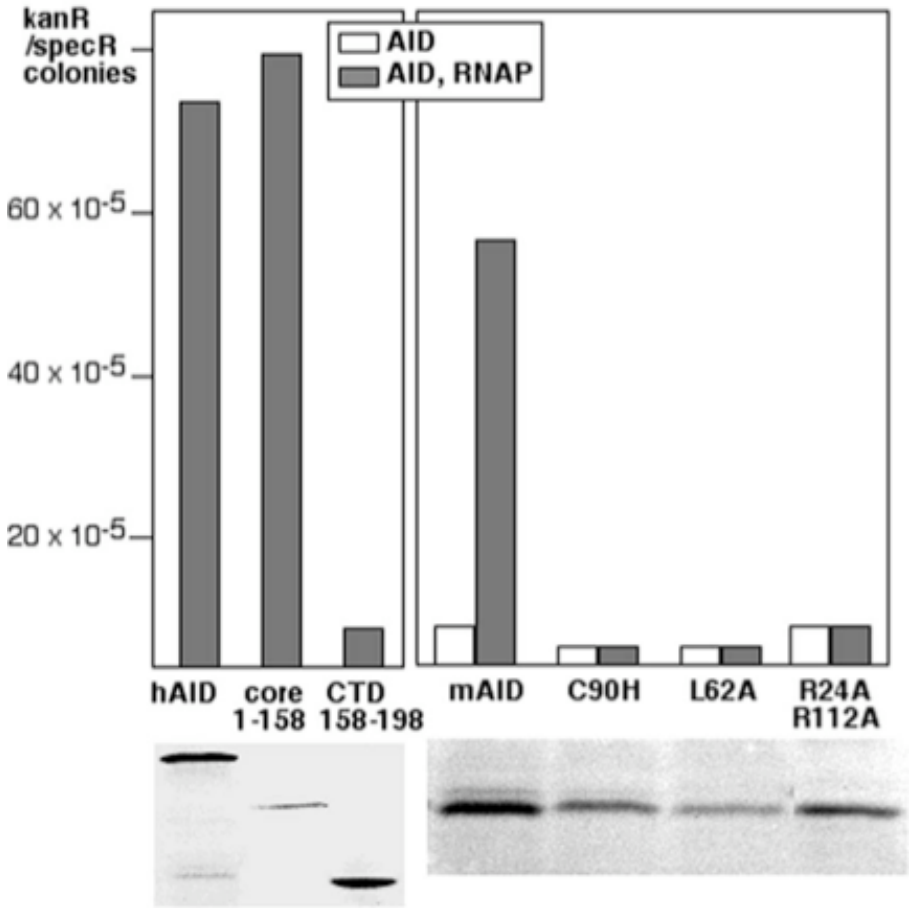

Figure 11. Delineation of AID domains. CoreAID mutates ssDNA, but CTD fails to increase the level of mutation (left panel). Mouse AID (mAID) deaminates the ptac-KanL94P plasmid when it is transcribed (grey) and not in the absence of transcription (white). Active site mutant (C90H) as well as other mutants (L62A and R24,112A) did not mutate the plasmid. Western blots shown in the bottom.

function, and a single mutation within the catalytic site renders the protein inactive.

This result clearly distinguishes two domains of AID. The core domain contains the active site responsible for the deamination of cytidine, as described for other cytidine deaminases. The c-terminal domain does not contribute to catalytic activity of the protein, and its main function is probably in subcellular localization, as shown by other groups (132, 133). As both the full length and the core protein show the same levels of activity, it can be concluded that the C-terminal domain does not contribute to substrate recognition and deamination in this assay.

Overall, recombinant AID was shown to be active, and it is the first enzyme within the family of cytidine deaminases shown to act on DNA, specifically single stranded DNA. Although its main role is to introduce beneficial mutations in the Ig variable region and induce switching of Ig isotypes, AID activity has to be tightly controlled (134-136) in order to prevent DNA damage it would induce if deaminating random cytidines within DNA.

CHAPTER 5. NK CELLS ELIMINATE B CELLS HARBORING IGH/C-MYC TRANSLOCATIONS

One of the possible outcomes of aberrant AID targeting and DNA repair is ever so rare *in vivo* appearance of AID-dependent IgH/c-myc translocations. The low frequency of the translocations can be ascribed to stringent regulation of resolution and repair of DNA breaks, but also to clearance of abnormal cells that escape from this pool.

I sought to determine if NK cells play role in targeting and clearance of B cells carrying potentially malignant IgH/c-myc translocations.

5.1 Ex Vivo Splenic B Cells Upregulate Rae-1 Upon AID-Induced DNA Damage

It has been shown previously that several types of genotoxic stress upregulate the NKG2D family of NK ligands on the surface of damaged cells which are then cleared by NK cells (115). AID induces double stranded DNA breaks within switch regions during CSR. I sought to determine whether this type of AID-induced DNA damage, i.e. a single regulated double stranded break, can upregulate activating ligands for NK cells, such as Rae-1.

Splenic B cells were purified from Balb/cByJ $AID^{+/+}$ and $AID^{-/-}$ mice and stimulated to undergo CSR with LPS/Il-4 *in vitro*. As expected, only AID wt and not AID-deficient B cells switched the Ig surface isotypes to IgG_1 and IgG_3 as shown by FACS analysis (Figure 12). In addition, switching AID wt B cells

upregulated Rae-1 with peak expression at 60 hours post stimulation, while AID-deficient B cells failed to do so (Figure 12 and 13). Not only is Rae-1 upregulated only on B cells that are switching in culture, but also the peak of upregulation coincides with the expression of AID in these cells. This shows that Rae-1 upregulation depends on the presence of AID in stimulated B cells *in vitro*. I conclude that AID-induced DNA damage can upregulate Rae-1, while $AID^{-/-}$ B cells that have been stimulated to undergo CSR fail to do so under the same conditions.

5.2 Rae-1^{+} B Cells Can Be Targeted And Eliminated by NK Cells

Upregulation of Rae-1 due to genotoxic stress and its expression on surface of the cells allows for recognition by NK cells. If Rae-1 on a target cell engages with NKG2D on the effector NK cell, the target cell can be eliminated by NK cell. To determine if Rae-1^{+} B cells can be targeted and eliminated by NK cells, I used an NK cytotoxicity assay. Briefly, splenic B cells were stimulated to switch with LPS and Il-4 *in vitro*. At a particular time point (48, 60 or 80 hrs post stimulation), stimulated B cells were labeled with a red fluorescent die (FL-2) and incubated with increasing ratios of NK cells (1:5, 1:10 and 1:20). The reaction was then incubated with colorless caspase substrate that starts to fluoresce in FL-1 channel upon cleavage by caspases from apoptotic B cells, and the toxicity is assayed by shift in fluorescence in FL-1 channel (Figure 14).

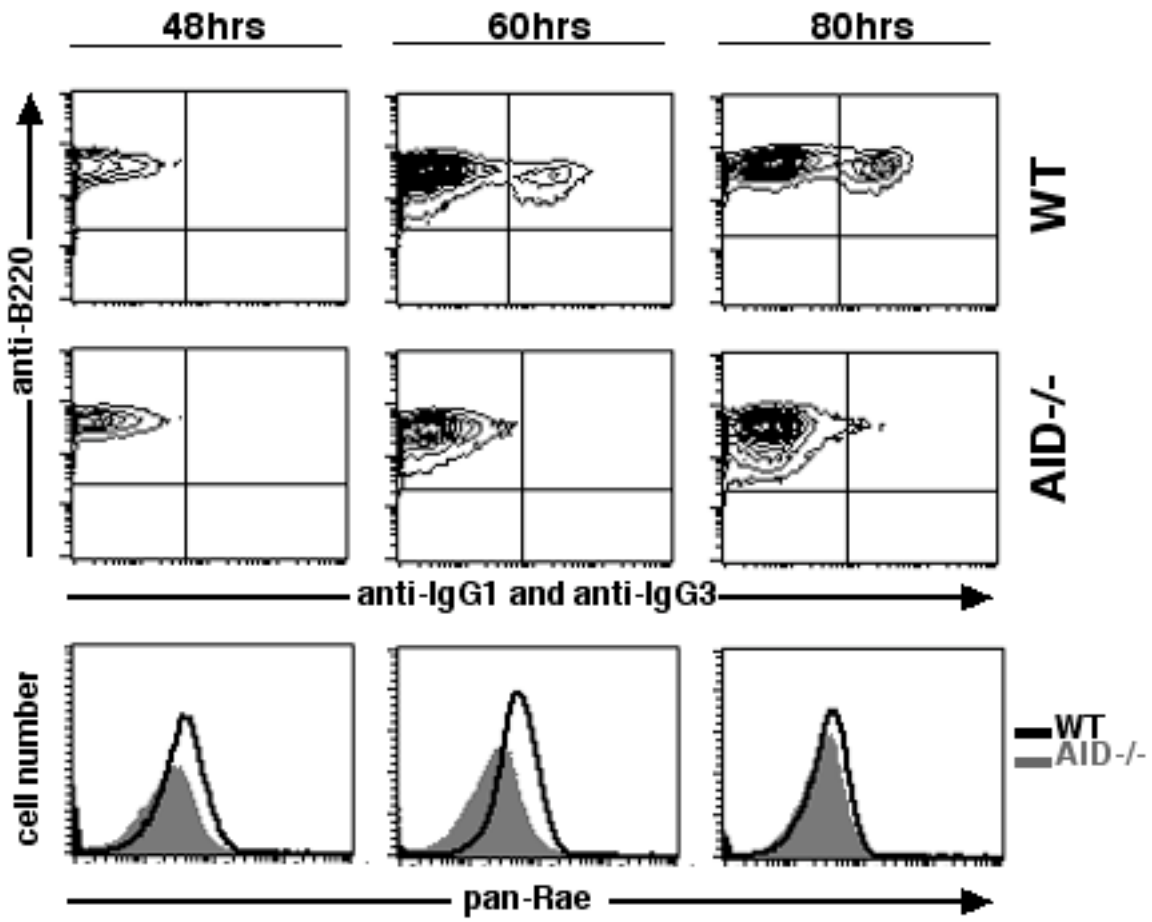

Figure 12. B cells undergo CSR *in vitro* upon LPS/Il4 stimulation and upregulate Rae-1. CSR can be detected by FACS analysis: wt B cells switch from IgM to IgG_1 and IgG_3 (top panel), whereas $AID^{-/-}$ B cells fail to do so (middle panel). Bottom panel shows upregulation of Rae-1 at the same time points: wt (black) and $AID^{-/-}$ (grey).

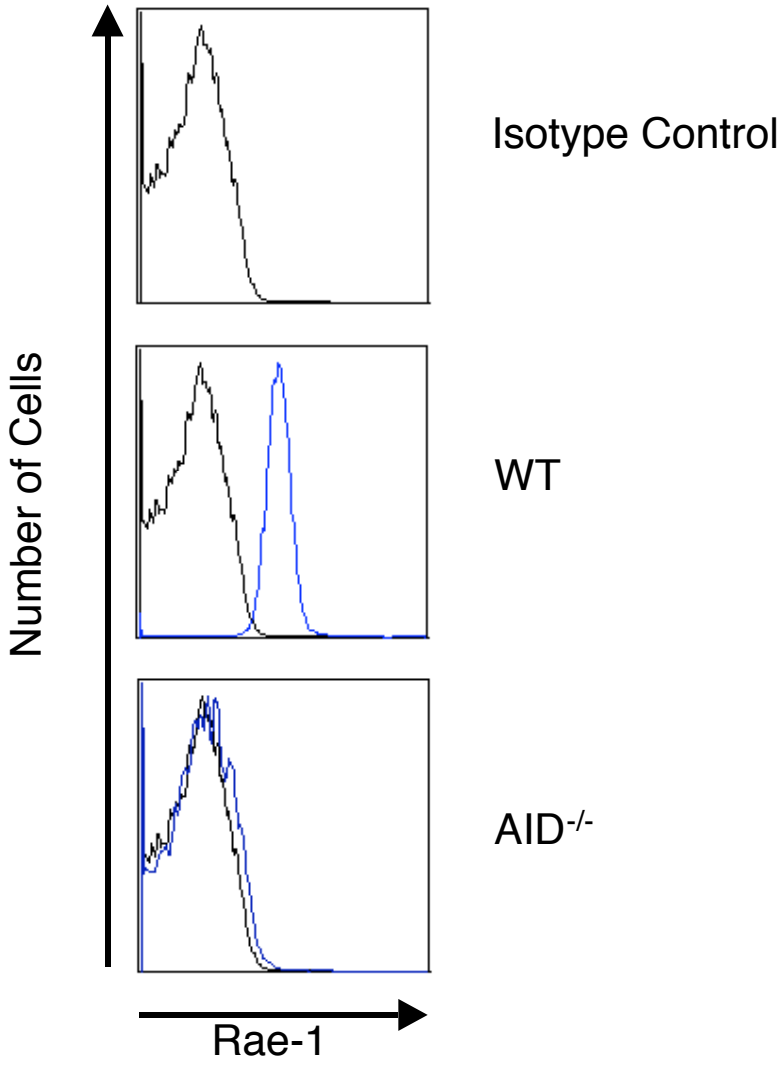

Figure 13. Rae-1 is upregulated at 60 hrs. B cells were stimulated to undergo CSR in vitro, and assayed for Rae-1 upregulation (blue) at various time points. The maximum Rae-1 upregulation was achieved at 60 hours after stimulation in wt B cells. $AID^{-/-}$ B cells failed to upregulate Rae-1. Isotype control is shown in black.

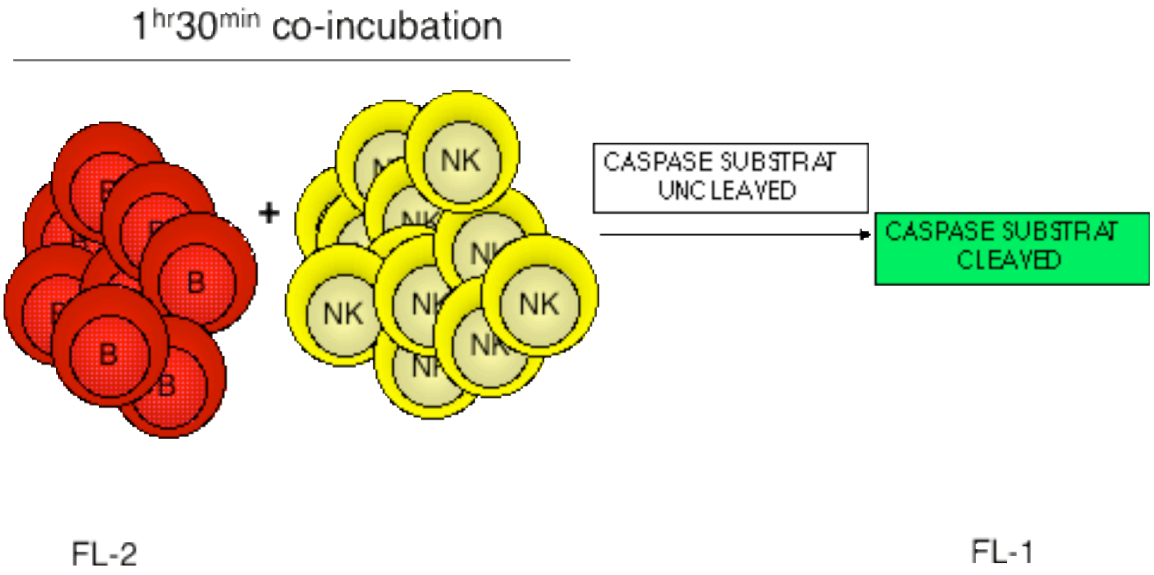

Figure 14. NK cytotoxicity assay. Stimulated B cells are labeled with FL-2 fluorescing dye and incubated with NK cells for 1.5 hours. Colorless caspase substrate is added for 30 minutes. B cell apoptosis is indicated by FL-1 fluorescence once the caspase substrate has been cleaved.

At 48 hours, while Rae-1 is still not upregulated in switching B cells, NK cell are unable to induce apoptosis, even when present in large excess (Figure 15). At 60 hours, NK cells were able to target and eliminate AID wt Rae-1$^+$ B cells (Figure 15). At high ratios of NK cells to B cells, the dying B cells comprised about 50 percent of the total B cell pool, suggesting highly effective NK targeting. This coincided with peak upregulation of Rae-1 on stimulated B cells in culture. At a later time point, at 80 hours, when Rae-1 is no longer detected on the surface of switching B cells, NK cells were not able to induce apoptosis in B cells (Figure 15). Therefore, upregulation of Rae-1 on B cells stimulated with LPS/Il-4 in vitro renders them susceptible for NK targeting and killing.

5.3 NK Cell Mediated B Cell Apoptosis Is Blocked by aRae-1 Antibody

To determine if NK cell mediated B cell apoptosis was directly due to Rae-1 surface expression, switching B cells were pre-incubated with either αRae-1 antibody or an isotype control. Apoptosis was prevented only when B cells were pre-incubated by specific αRae-1 antibody and isotype control did not have an effect (Figure 16). Hence, switching B cells can be targeted and eliminated by NK cells *in vitro* due to the expression of Rae-1 on their surface.

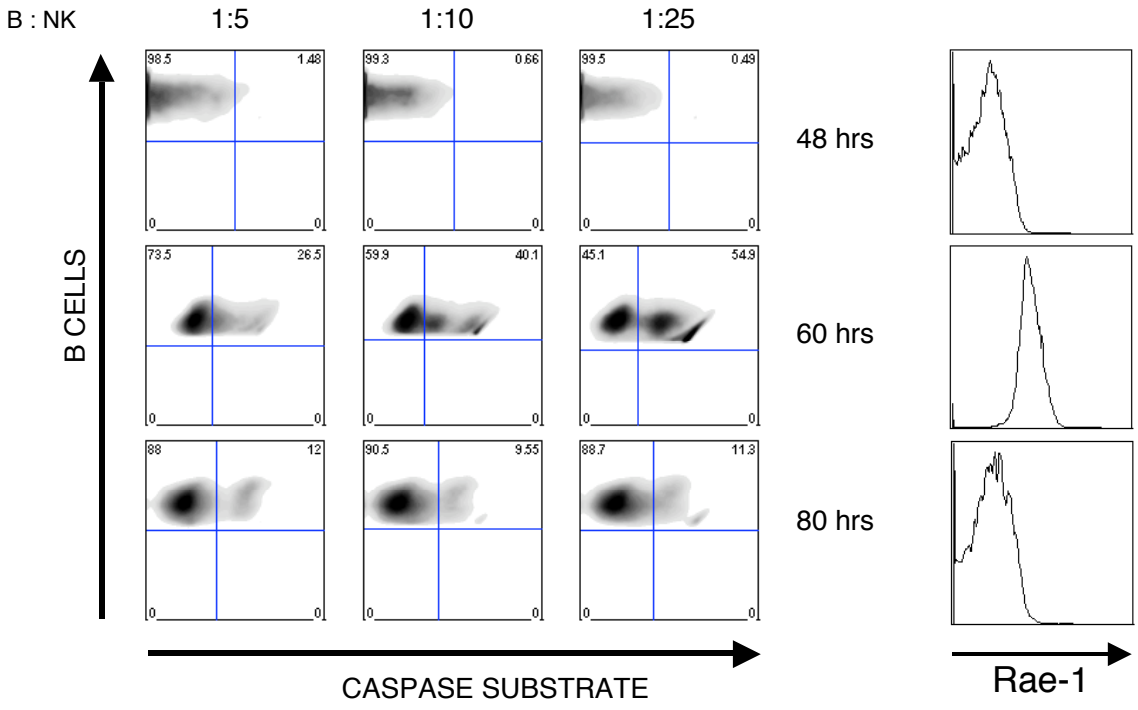

Figure 15. NK cells mediate B cell apoptosis *in vitro*. B cells were stimulated to undergo CSR in vitro and coincubated with increasing number of NK cells at various time points: 48 hours (top panel), 60 hours (middle panel) and 80 hours (bottom panel). Maximum B cell apoptosis was recorded at 60 hours (54.9%). Corresponding Rae-1 upregulation on B cells is shown on the right.

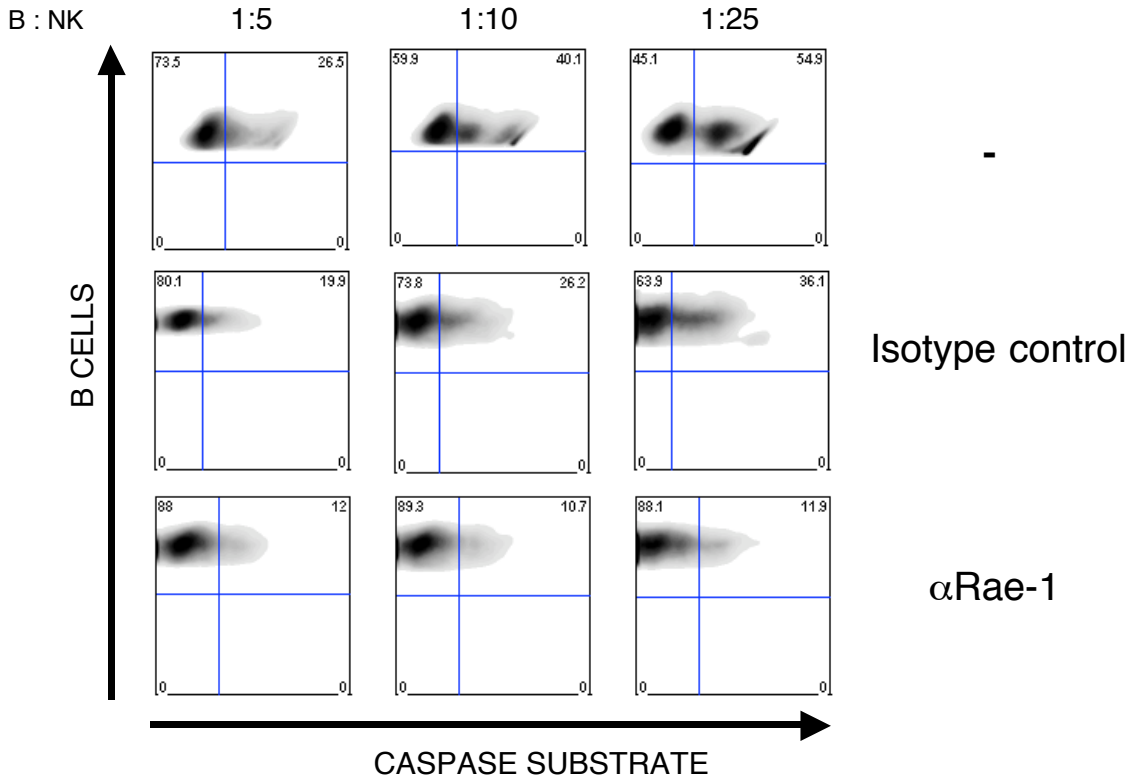

Figure 16. αRae antibody blocks NK mediated B cell apoptosis at 60 hours. B cells were stimulated to undergo CSR in vitro and incubated with isotype control (middle panel) or αRae-1 antibodies (bottom panel) before being subject to NK cytotoxicity assay. Only αRae-1 and not the isotype control blocked the NK mediated B cell apoptosis (compare to top panel).

5.4 IgH/c-myc Translocations Are Detected in p53$^{-/-}$ Mice In Vivo

Having established that AID-mediated lesions during CSR can lead to NKG2D ligand expression on the surface of *ex vivo* switching B cells, I wanted to know whether these cells may be cleared by NK cells *in vivo*.

IgH/c-myc translocations have been detected in switching B cells *in vitro* though rarely, and their frequency is vastly increased in the absence of tumor suppressors such as p53, ATM etc. (100). However, there are no reports of detection of such translocations in switching or mutating (i.e. GC) B cells *in vivo*.

Consistent with the rarity of IgH/c-myc translocations *in vitro* CSR cultures, I could not detect a single translocation in over $2x10^6$ germinal center B cells isolated from spleens 10 days post immunization with sheep red blood cells (data not shown). This was not discouraging, as translocations are very rarely detected even in wt B cells.

In contrast to wild type B cells, p53-deficient B cells have a much higher frequency of translocations *in vitro* (100). However, no reports have been made on the occurrence of IgH/c-myc translocations in p53-deficient animals *in vivo*. Therefore, I first asked if I could detect such translocations after immunization *in vivo*. In fact, IgH/c-myc translocations were detected in 1 in 100,000 germinal center B cells from spleens (Figure 17), with a total of $1x10^6$ cells assayed.

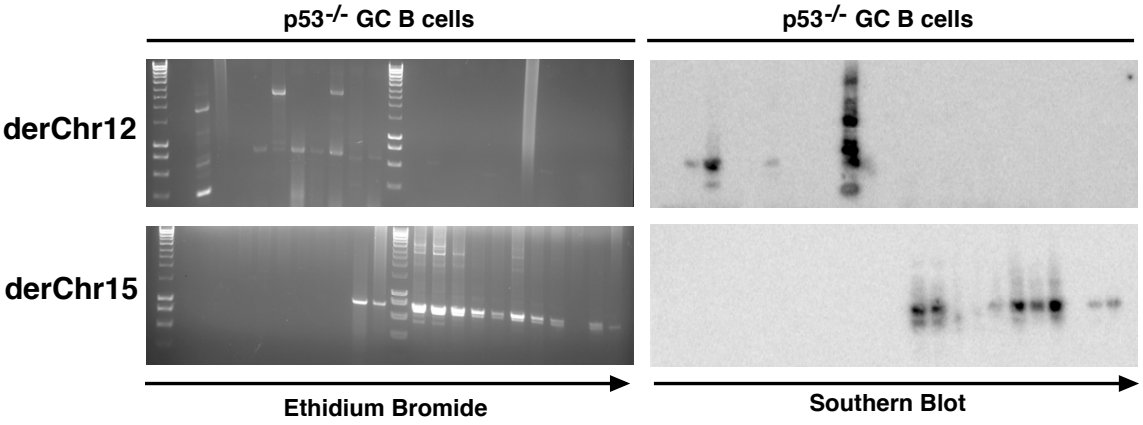

Figure 17. IgH/c-myc translocations are detected in germinal center B cells of p53-deficient mice. Left panel shows translocations on a ethidium bromide stained gel, whereas the right panel shows the southern blot of the same gel (c-myc probe). The results were identical when IgH probe was used. Top panel shows derivate chromosome 12, and the bottom panel shows derivative chromosome 15.

These translocations differed from those reported *in vitro*: they were less diverse in size, clustering at around 1 kb, with very few outliers. This is consistent with an earlier report that while in unimmunized mice translocations ranged in size from 1.9 to 7.5 kb, in immunized mice that size range was compressed to 2.1 to 4.7 kb (137). The translocations I observed varied in sequence (Figure 18), suggesting that particular translocation hotspots are positively selected *in vivo*.

Having detected IgH/c-myc translocations in germinal center B cells *in vivo*, I wondered whether p53-deficient cells carrying these translocations also express Rae-1 on their surface and if so, could they be accessed and targeted by NK cells? To test whether Rae-1 was expressed in GC B cells *in vivo*, I immunized mice with sheep red blood cells (sRBCs), a potent immunogen. At several time points after immunization the mice were sacrificed and their splenocytes collected and stained with antibodies against the germinal center B cell marker GL7 and against Rae-1. I found that GC B cells did in fact express Rae-1 on their surface in an AID-dependent manner (Figure 19a). When purified, the same cells were also targeted by NK cells in cytotoxicity assay *ex vivo* (Figure 19b). Hence, germinal center B cells upregulate Rae-1 on their surface and can be targeted and eliminated by NK cells ex vivo, suggesting a role of NK cells in suppression of translocation frequency *in vivo*.

If NK cells can target and eliminate germinal center B cells in vivo, then depletion of NK cells from $p53^{-/-}$ animals might increase the frequency of cells with translocations.

derChr.	Clone number	c-myc	IgH
12	3	CCAGTCTCTGAGAGGGCATT	AATTATCTAGACTTATTTCG
12	17	GGGATTACCTTTTGCGTTTG	TTTCAGTTAAGTGTATTAGT
12	7	AAGGGAGGTGTCTCTTATTA	AAACTGAGGTGATTACTCTG
15	8	GAAAGGGGAGTGGTTCAGGA	GTAAGGAGGGACCCAGGCTA
15	32	GAGGGAATTTTTGTCTATTT	AGTTTAAAATATTTTTAAAT
15	25	TGACTTGGGGGAAACCAGAG	AATTATCTAGACTTATTTCG

Figure 18. Breakpoint sequences of representative IgH/c-myc chromosomal translocations in p53-deficient mice.

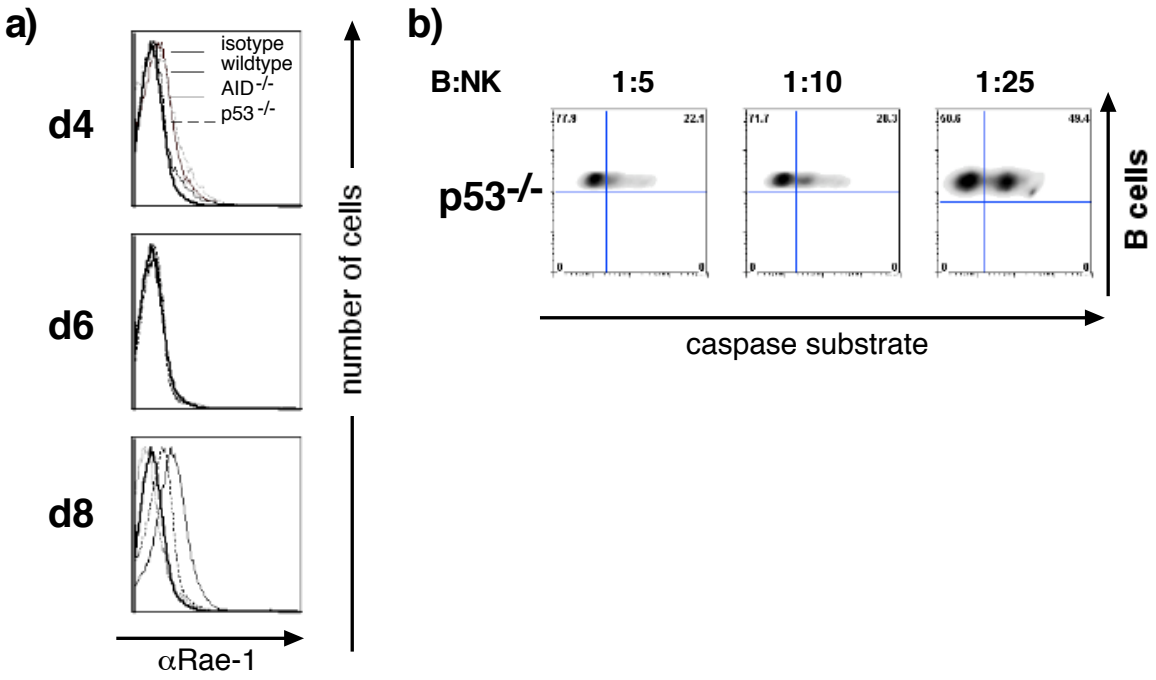

Figure 19. Germinal center B cells upregulate Rae-1 and are eliminated by NK cells *ex vivo*. a) Upregulation of Rae-1 in germinal center B cells from wild type mice (thin line) or $p53^{-/-}$ mice (dashed line) 8 days after immunization. Rae-1 is not upregulated in $AID^{-/-}$ mice (grey line). All compared to isotype control (thick line). b) Germinal center B cells from $p53^{-/-}$ mice can be targeted and eliminated by NK cells in standard cytotoxicity assay.

However, when I depleted $p53^{-/-}$ mice of NK cells I did not observe such an increase (data not shown). Furthermore, the same NK depletion protocol applied to wild type mice did not raise the frequency of translocations to detectable levels (data not shown). My data strongly suggest that the germinal center is a protected environment, which shields cells carrying translocations. Indeed, recent two-photon imaging experiments of NK cell interactions in the lymph node suggest that NK cells form a largely fixed network localized in the DC and T areas of the lymph node but excluded from the B cell areas (138).

CHAPTER 6. DISCUSSION

6.1 AID And the Adaptive Immune Response

A unique feature of the immune system is its ability to adaptively respond to antigenic encounter by altering the antigen receptor specificities in T and B cells. While VDJ recombination alone provides for more than a million T and B cell receptors, B cells can undergo additional diversification to allow for perfect recognition of soluble antigens. This additional diversification is achieved through two reactions: the variable region is changed through somatic hypermutation and the constant regions are exchanged in class switch recombination. Although they both occur in response to antigen, SHM and CSR seemed mechanistically quite distinct. Surprisingly, the recent discovery of AID has linked these two processes, as mice and humans deficient in AID neither mutate their antibodies nor switch isotype.

The discovery of AID opened up the field of antibody diversification and B cell biology in general, leading to some quite exciting findings. First, AID was shown to be a DNA-editing cytidine deaminase, and as such it was the first enzyme in the family of cytidine deaminases to cause direct mutations in DNA. Second, although the AID-induced DNA mutations are beneficial to the host and only arise in response to antigen activation of B cells, it was shown that AID-induced DNA damage can be detrimental to cells, leading to chromosomal translocations and tumorigenesis.

6.2 AID Is a DNA Editor

Prior to the discovery of AID in 1999 (62), all characterized cytidine deaminases fell into two categories: metabolic (119, 120) or RNA-editing (127). AID was shown to convert free cytidine to uridine in solution in vitro (62). It was possible to imagine that this would skew the cell nucleotide pools, which would then cause misincorporation of nucleotides within replicating V genes, ultimately leading to high levels of mutation in SHM. This hypothesis never gained much support, and I have shown that AID cannot deaminate free cytidine *in vitro*. The activity on free cytidine that was originally described in this early paper was later attributed to some contamination of a different deaminase in the protein preparation (61).

Due to its homology to APOBEC-1, as well as their proximity on chromosome 12p13, it was first postulated that AID would act on RNA (61), possibly on two different mRNAs, which when edited would translate into two distinct proteins, one of which would be necessary for SHM and the second one for CSR. This offered an elegant explanation for the fact that one enzyme, AID, was indispensable for two distinct molecular processes. Despite considerable efforts, the specific RNA target has never been identified.

The early studies showed that AID can mutate non-germinal center B cells (68), non-B cells (69) and even E. coli (70), together suggesting that AID is the only B cell specific factor required for mutation. At about the same time, a parallel line of work emerged, studying the role of UDG in immunoglobulin diversification

events. Initially it was shown that inhibiting UDG with a specific inhibitor in DT40 chicken cell lines abrogated gene conversion (79), an antibody diversification process also dependent on AID (139), but not discussed here in details. This finding that AID and UDG might act in the same genetic pathway was further strengthened when it was shown that somatic hypermutation is skewed and class switch recombination is severely abolished in $UDG^{-/-}$ mice (80).

During my thesis work I showed that purified recombinant AID acts on DNA directly (140), and more specifically, that *in vitro*, it binds and edits only ssDNA. At the same time, three other groups have also shown that AID deaminates ssDNA using different approaches (130, 141, 142). Therefore, it was convincingly shown that AID is a DNA deaminase that acts on ssDNA *in vitro*.

This finding brought attention to one main question: where and when is the ssDNA accessible *in vivo*? I hypothesized, as did others, that *in vivo*, single stranded DNA is probably accessed during active transcription. Indeed, AID has been shown to bind to the transcription elongation complex *in vitro (143)*. After this initial characterization of AID as the first DNA-editing cytidine deaminase, others have been described. One example is Apobec-3G (144, 145), which deaminates HIV-1 DNA as it is being reverse-transcribed in the cytoplasm of infected cells. Newly synthesized viral DNA is digested and cleared, thus preventing the integration of viral genome into the host genome and establishment of permanent infection.

The mutations induced by AID in variable or switch regions are beneficial for the host. However, if the only requirements for acquiring these mutations are presence of AID and active transcription, an important question clearly arises: how is AID targeted to the appropriate locus at the appropriate time? And if this restriction fails, how is AID-induced DNA damage repaired? There are as yet no complete answers to these questions, but there are examples of AID mistargeting, for instance, in mutating B cells, mutations are introduced in genes other than Ig, such as bcl-2 (146, 147). In switching B cells, AID mistargeting, in combination with anomalous repair, can lead to chromosomal translocations (99, 100).

6.3 Regulation of AID

Currently it is accepted that AID is DNA-editing enzyme, but it is still not clear how its activity is regulated in B cells in order to minimize genomic damage. AID appears to be a rate-limiting factor for mutation, as overexpression of the protein increases the levels of SHM (148, 149). Therefore, the levels of AID protein expression, its localization within the cell and other contributing factors are important in regulating AID activity.

6.3.1 AID Expression

AID expression seems to be strictly confined to B cells, and especially germinal center B cells (62). Obvious stimuli that induce AID expression are

those that induce SHM in human lymphoma cell lines (IgM-CD19-CD21 crosslinking) and signals that induce CSR in splenic B cells *ex vivo* (a combination of LPS, IL-4, CD40 and TGFβ). However, it is not clear which pathways are responsible in delivering these signals to the nucleus and inducing AID expression. Transcription factors of the E2A family, such as E2-2 and E47, bind 2 E-boxes within the first intron of AID, thus playing a role in AID expression. Other transcription factors, such as Pax5 and Id2 also have binding sites within the AID promoter region and are important in regulation of levels of AID in the cell (150). Overexpression of Pax5 induced AID expression in pro-B cells, whereas overexpression of Id2 causes a decrease in AID expression, which ultimately leads to diminished CSR.

AID is also upregulated in pro-B cells infected by a retrovirus (151), but this line of work will not be discussed in detail here, first because how a retrovirus induces AID expression is not understood and second, because the activity of AID as a host response to viral infection is not restricted to the Ig locus and therefore is qualitatively different than its activity during CSR and SHM.

6.3.2 AID Localization

Although AID is very small molecule that could easily diffuse through the nuclear pore, it was first reported to be found mainly in the cytoplasm (149), which would prevent it from accessing DNA and causing indiscriminate mutations. Later studies have shown that AID carries a nuclear export signal

within the last ten amino acids (132, 133), and it is thought to be actively transported out of the nucleus. At the same time, it was reported that AID contains a nuclear localization signal (NLS) located at its N-terminus (133). However, this putative NLS does not appear to be autonomous, since it cannot direct a large protein into the nucleus, while it also tolerates mutations of crucial positively charged residues such as arginine and lysine (132, 152). Hence, despite the presence of this putative NLS, how AID enters the nucleus remains unclear.

Keeping AID out of the nucleus, and thus far from its target, is an important way of restricting its action. Nevertheless, this still does not explain how AID finds the appropriate locus (IgH), and how it distinguishes the variable region from the constant region. In fact, it was shown that, when AID is overexpressed in human lymphoma cell line, it is randomly mutagenic until cells are specifically stimulated for hypermutation, focusing the mutations to IgH variable locus (153). This was the first tangible demonstration of the existence of reaction-specific AID partner proteins.

6.3.3 AID Co-factors

Many groups have unsuccessfully tried to identify AID partners that would explain this preferential, locus-specific, targeting. The first protein to be co-precipitated with AID was RNA Polymerase II (154), but the relevance of this

interaction was largely dismissed, since AID activity correlates with transcription, and the two enzymes are found in close proximity during SHM and CSR.

A couple of other proteins have also been described as potential AID partners. Cascalho and colleagues have shown that AID can interact with DNA-dependent protein kinase catalytic subunit (DNA-PKcs). However, this interaction requires the presence of DNA as a cofactor, arguing against a direct interaction of these two DNA binding proteins and thereby diminishing the relevance of DNA-PKcs as a true partner (155). At the same time, Neuberger and colleagues found that mdm2, an oncoprotein that shuttles between nucleus and cytoplasm, can interact with AID in a yeast two-hybrid screen. However, they went on to show that mdm2 is dispensable for antibody diversification, hence undermining the relevance of this interaction as well (156).

A third putative AID partner protein is replication protein A (RPA) (157). RPA is a trimeric complex, involved in replication, recombination and repair. The 32 kDa subunit of RPA was shown to interact with AID, and it was sufficient to target AID to *in vitro* transcribed substrates harboring SHM motifs and enhance the mutation rates of such targets. As a single stranded DNA binding protein, RPA probably stabilizes the single stranded DNA as substrate for AID. Curiously, RPA also interacts with UNG (158), and it is possible that it is important for immediate recruitment of UNG and other repair proteins to the newly formed U:G mismatches.

As initially characterized, the AID-RPA interaction was dependent on posttranslational modification of AID: only phosphorylated AID was able to bind RPA, and this interaction was specific to B cells (157). Non-phosphorylated AID can still deaminate ssDNA, but RPA-dependent deamination of actively transcribed dsDNA substrate is markedly reduced (134). AID was shown to be phosphorylated in B cells, at the protein kinase A (PKA) consensus site serine-38 (134-136). However, Nussenzweig and colleagues also found that AID is heavily phosphorylated in 293T cells, presumably by PKA, a ubiquitous enzyme, thereby calling into question the original claims of phospho-AID targeting to the V regions of the Ig locus through interaction with RPA. They also found that phosphorylated AID has much higher deamination activity (135) and that in contrast to 293T cells only a small fraction of AID is phosphorylated in B cells, and that possibly this form of AID accounts for the majority of SHM and CSR (135). Therefore, low levels of phosphorylation may provide a way of keeping most of the AID in the cell in an inactive, thus harmless form.

Given the uncertainty with the requirement for phosphorylation for the AID-RPA interaction, the actual role of phosphorylation is now unclear. It is possible that phosphorylation is important for AID stability and turnover in the cell, perhaps playing a role in degradation. Phosphorylation might also be essential in AID shuffling between nucleus and cytoplasm.

Finally, it is important to mention that PKA itself has been implicated in control of B cell receptor signaling (159) and NFκB function (160). By controlling

AID activity as well, PKA may play a major role in integrating the signals from antigens and antibody diversification events.

6.4 AID Mistargeting Can Lead To Malignancies

When targeting mechanisms fail, mutations can be found not only in the variable region of the immunoglobulin locus, but also in number of other genes such as BCL-6 (161), CD95 (162) and many others. The mutation frequency of the non-immunoglobulin genes is 50-100 fold lower than that of the Ig variable region (162), but the pattern of mutation remains the same and the same hotspots are preferentially targeted. When these aberrant mutations deregulate targeted proto-oncogenes and tumor suppressor genes, they can cause lymphomagenesis.

BCL-6 is a zinc-finger transcriptional repressor necessary for germinal center formation and plays a role in B cell lymphoma pathogenesis (163, 164). While it is one of the most common genes unintentionally mutated during SHM, it is also very often involved in chromosomal translocations in follicular lymphoma and diffuse large B cell lymphoma (165). Recent findings have shown that deregulation of BCL-6 leads to repression of tumor suppressor gene p53, thus suppressing a DNA damage-induced response in germinal center B cells (166). However, physiologic levels of BCL-6 in B cell lines protect them from DNA damage-induced apoptosis. Taken together, these data illustrate two important points. First, BCL-6 plays an important role in germinal center B cells undergoing

SHM and CSR by allowing them to tolerate the physiologic AID-induced DNA breaks that accumulate during these reactions. Second, deregulated, overexpressed BCL-6 contributes to cell transformation by silencing p53 repressor pathway.

Mistargeting of AID in context of class switch recombination can lead to translocations, which once again lead to generation of lymphomas. One of the best characterized case of AID-induced chromosomal abnormalities are IgH/c-myc translocations, which lead to plasmocytomas in mice (167-172) and Burkitt's lymphoma in humans (173-181).

IgH/c-myc translocations require AID, as shown in $AID^{+/+}$ Il-6 tg Balb/cByJ mice treated with pristein *in vivo* (99). Thus, the translocations seemed to be AID-induced. However, another study showed that, in a slightly different system, translocations were AID-independent, and that presence of AID probably induced secondary mutations that promoted survival of B cells already carrying AID-independent translocation (182). However, IgH/c-myc translocations were reproduced in an *in vitro* system in which B cells overexpressing AID were induced to undergo CSR and no selective pressure is present (100). Furthermore, the induction of translocations was dependent of AID catalytic activity and UNG activity, suggesting that switch region breaks in CSR and IgH breaks that lead to translocations can be generated by a common mechanism involving these two enzymes. Moreover, double stranded DNA breaks within IgH switch regions seem to be sensed by different proteins: p53 and p19 seem to

play an important role in the pathway eventually leading to chromosomal translocations and malignancy.

Therefore, chromosomal translocations involving IgH represent aberrant switching reactions: they are AID-dependent, and they often involve IgH switch regions (83, 86, 88). Indeed, the probability of such translocations occurring *in vivo*, in an animal or human constantly exposed to antigenic stimulation so that B cells are constantly undergoing CSR, is far greater than the observed frequency of B cells with such translocations actually emerging from the germinal center and becoming transformed and malignant. So, the question arises: why are IgH/c-myc translocations infrequently observed *in vivo*? Or, from a different point of view: what keeps the frequency of B cells with IgH/c-myc translocations at low levels *in vivo*? Or, what eliminates these B cells *in vivo*?

6.5 Role of NK Cells in Maintenance of Healthy B Cell Population

Most DNA damage is harmful and potentially malignant: therefore a cell has many ways of sensing this damage, repairing it or signaling for help. Could it be that switching B cells are sensing AID-induced DNA damage? I showed that *ex vivo* switching B cells do in fact upregulate Rae-1, which is normally upregulated upon genotoxic stress. Rae-1 is a ligand for NKG2D, an activating receptor found on NK cells. Thus, by upregulating Rae-1 these B cells signal their damaged genome to be recognized and killed by NK cells *in vitro*. This drastic form of repair is exactly what I observed *in vitro*.

Germinal center B cells are undergoing class switch recombination, and I showed that they are also capable of upregulating Rae-1 eight days after immunization. Furthermore, purified germinal center B cells can be targeted and eliminated by NK cells *ex vivo*, as I showed in a standard cytotoxicity assay. This result strongly suggested that NK cells could be important suppressors of frequency of translocations *in vivo*.

6.6 Control of Frequency of Translocations In Vivo

In vitro data suggest that IgH/c-myc translocations occur in rather high frequency in repair deficient background (100). For example, *in vitro* translocations occur with about 30% frequency in $p53^{-/-}$ B cells (100), and I have observed them with at least 10% frequency in p53-deficient mice *in vivo*. However, p53-deficient mice rarely succumb to B cell lymphomas, and it is not clear what factors are at work maintaining healthy B cell population in these animals.

A clear link has been established between the genetic background of animals and their predisposition to IgH/c-myc associated disease (137). In the susceptible BALB/cAn strain plasmocytomas can be induced by intraperitoneal injection of chemical carcinogens such as pristine in approximately 60% of animals, whereas DBA/2 mice are completely resistant to tumor developments (183). Nevertheless, no specific genes or functions have been defined as important in controlling the frequency of translocations *in vivo*.

I wondered if NK cells are important for maintenance of healthy B cell population *in vivo*. I could detect IgH/c-myc translocations in p53-deficient mice in vivo upon immunization with sheep red blood cells with about 10% frequency. These translocations differed from those reported in vitro (100): they were less diverse in size, clustering at around 1 kb, with some outliers bigger in size. However, this was consistent with a report that in mice immunized with cholera toxin, translocations were detected with size range of 2.1 to 4.7 kb, whereas they ranged from 1.9 to 7.5 kb in unimmunized mice, still rare (137).

Somatic hypermutation and class switch recombination are physiological landmarks of antigen-driven germinal center response, and the risk of genetic lesions (and thus translocations) is greatly enhanced. In fact, T cells that do not undergo such genetic rearrangements as CSR give rise to 10-20 times less lymphoproliferative disease compared to B cells (184). Therefore, tight checkpoints are in place which regulate positive and negative germinal center selection, saving the useful B cell repertoire and eliminating the harmful cells. The fact that translocations arising in immunized animals are less diverse in size could be due to this stringent regulation and selection.

Are NK cells involved in suppression of these translocations *in vivo*? Mice lacking NK cells do not show higher frequency of translocations. However, the fact remains that NK cells are able to clear Rae-1$^+$ B cells *in vitro*, and the question arises whether NK cells can access switching B cells *in vivo* within germinal centers. Together, my data suggest that, despite of fairly high frequency

of translocations and the co-existent upregulation of Rae-1 in germinal center B cells, NK cells are not able to access their targets *in vivo*. Indeed, a recent study showed that NK cells are not found within the B cells areas *in vivo* (138). However, one cannot exclude the possibility that NK cells still play an important regulatory role in animals in which switching and mutation of Ig genes occur outside of designated areas (i.e. germinal centers), such as MRL^{fas} mice (185).

6.7 AID – Friend or Foe?

AID plays an important role in adaptive immune response, allowing a B cell to evolve its antigen receptor, i.e. antibody, as required. Somatic hypermutation and class switch recombination are efficient reactions, temporally and spatially restricted to the immunoglobulin locus. The creation of a fit antibody with high affinity for the antigen plays a crucial role in maintenance of a healthy organism. However, when the control mechanisms fail, AID activity can lead to chromosomal abnormalities and pathogenesis. The mechanism that regulates AID targeting and restriction remains unknown, yet one can only imagine that balance between perfect targeting and perfect repair will be central to the explanation of the both the beneficial and the harmful effects of the activation-induced cytidine deaminase.

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