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Regulation of *aicda* expression and AID activity: Relevance to somatic hypermutation and class switch DNA recombination

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Abstract

Expression and activity of activation-induced cytidine deaminase (AID) encoded by the *aicda* gene are essential for immunoglobulin (Ig) gene somatic hypermutation (SHM) and class switch DNA recombination (CSR). SHM and CSR unfold in general in germinal centers and are central to the maturation of effective antibody responses. AID expression is induced by activated B cell CD40 signaling, which is critical for the germinal center reaction, and is further enhanced by other stimuli, including interleukin-4 (IL-4) secreted from CD4⁺ T cells or Toll-like receptor (TLR)-activating bacterial and/or viral molecules. Integration of different intracellular signal transduction pathways, as activated by these stimuli, leads to a dynamic *aicda*-regulating program, which involves both positively acting *trans*-factors, such as Pax5, HoxC4, E47 and Irf8, and negative modulators, such as Blimp1 and Id2, to restrict *aicda* expression primarily to germinal center B cells. The phosphatidylinositol 3-kinase (PI 3-K), which functions downstream of activated B cell receptor (BCR) signaling, likely plays an important role in triggering the downregulation of *aicda* expression in post-germinal center B cells and throughout plasmacytoid differentiation. In B cells undergoing SHM and CSR, AID activity and, possibly, AID targeting to the Ig locus are regulated at a post-translational level, including AID dimerization/oligomerization, nuclear/cytoplasmic AID translocation and phosphorylation of the AID Ser38 residue by protein kinase A (PKA). Here, we will discuss the role of B cell activation signals, transcription regulation programs and post-translational modifications in controlling *aicda* expression and AID activity, thereby delineating an integrated model of modulation of SHM and CSR in the germinal center reaction.

Keywords

activation-induced cytidine deaminase (AID); B cell; class switch DNA recombination (CSR); enhanceosome; germinal center reaction; immunoglobulin (Ig); plasmacytoid differentiation; post-translational modifications; signal transduction pathways; somatic hypermutation (SHM); transcription factors

2. Introduction

The ability of vertebrates to mount specific and effective responses to a broad range of pathogens and develop long-term memory is underpinned by the broad diversity and exquisite specificity of adaptive immunity. During B cell development in the bone marrow of mammals, rearrangement of germline V (variable), D (diversity) and J (joining) gene segments in the immunoglobulin heavy (H) chain locus and V and J gene segments in the

Ig λ or Igk light (L) chain locus yields a highly diverse BCR repertoire, which is available for antigen recognition in the primary antibody response. Such V(D)J recombination events are dependent on the expression of the recombination-activating genes-1 (RAG-1) and RAG-2. The impact of antigen on the primary BCR repertoire leads to the antibody affinity maturation, i.e., emergence of high-affinity IgG, IgA and/or IgE antibodies through SHM and CSR, two unique genetic processes fostered by the specialized microenvironment of the germinal center¹. During the germinal center reaction, B cells are activated to undergo high-rate proliferation, SHM and CSR, positive selection of B cells expressing high-affinity BCR and, subsequently, differentiation to plasma cells and memory B cells. SHM inserts mainly point-mutations in Ig V(D)J DNA at a rate of approximate 10^{-3} change per base per cell division, which is about a million-fold higher than the spontaneous somatic mutation rate of the genome at large. CSR replaces the expressed IgH constant (C_H) exon cluster, as juxtaposed to the V_HDJ_H region, from the initial C_μ to a downstream C_γ , C_α or C_ϵ . The intervening DNA between the switch μ (S_μ) region, lying at upstream of C_μ , and a downstream S region, lying at upstream of the C_γ , C_α or C_ϵ exon cluster, is “looped-out” as a switch (S_μ -S) circle. While SHM depends on Ig V(D)J DNA transcription, CSR is dependent on the germline I_H - C_H transcription initiated by the I_H promoter that lies upstream of each S region^{2–6}.

Both SHM and CSR depend on expression of AID^{7,8}. AID shares significant sequence similarities with the RNA cytidine deaminase apolipoprotein-B editing complex catalytic subunit 1 (ApoBec1)⁹, the founding member of the recently annotated ApoBec/AID family of enzymes¹⁰. Many ApoBec/AID family proteins, including AID, are involved in innate antiviral response in the human and the mouse^{11,12} (Table I). In spite of the possibility that AID may deaminate cytidine in pre-RNA¹³, it is now generally accepted that AID acts as a DNA deaminase, deaminating dC residues to dU in single-stranded DNA, as existing transiently in the non-template DNA strand during V(D)J transcription or as the R-loop structure within transcribed S region repeats^{14–18}. Repair of dU by Ung-mediated base excision repair (BER) and Msh2-mediated mismatch repair (MMR) leads to SHM and CSR^{14–18}, as shown by studies in *ung*^{-/-}*msh2*^{-/-} double knockout mice¹⁹. Such DNA repair pathways, which are otherwise high-fidelity and critical for maintenance of genome integrity, are diverted by the SHM and/or CSR machinery to become mutagenic/recombinogenic through a subversion of the “polymerase switch”, by which the high-fidelity DNA polymerase (pol) δ and pol ϵ are replaced by error-prone translesion DNA synthesis (TLS) pol θ , pol η , pol ζ and Rev1 to effect faulty DNA synthesis^{20–30}.

As suggested by the induction of the *aicda* gene⁹ and TLS polymerase genes^{20,21,28} in B cells upon engagement of CD40 by its ligand, CD154, and BCR crosslinking, two crucial signals in germinal center B cell differentiation^{31,32}, multiple signal transduction pathways are activated and integrated to drive AID expression and many of the events that lead to SHM and CSR. However, the Ig locus- and B cell-specificity of SHM and CSR can be altered if appropriate and specific pathways are diverted or bypassed, as implied by the hypermutation in non-Ig transgenes and/or DNA recombination in plasmid substrates induced by enforced AID expression in fibroblasts^{33,34}. Here we will discuss recent advances in the understanding of specific regulation of *aicda* gene expression and AID activity in the context of their relevance to SHM and CSR. We will use the terms “AID expression” and “*aicda* expression” to discuss findings stemming from AID protein analyses and from RT-PCR- or microarray-based *aicda* transcript analyses, respectively.

3.1. AID INDUCTION BY B CELL SIGNAL TRANSDUCTION PATHWAYS

The *aicda* gene was identified in the murine B lymphoma cell line CH12F3-2 as specifically upregulated upon stimulation by CD154, IL-4 and tumor growth factor- β (TGF- β), three signals that induce CSR to IgA at a high rate in CH12F3-2 cells⁹. CD154:CD40 engagement

and IL-4 synergize to induce *aicda* expression in both human and mouse B cells^{9,35–38}. Such *aicda* induction may be further enhanced by BCR crosslinking, as effected by physically-linked antigenic determinants. AID expression, B cell proliferation and CSR are also induced or enhanced by microbial lipopolysaccharides (LPS) and/or DNA containing multiple unmethylated CpG motifs (CpG DNA), which activate TLR4 and TLR9 signal transduction pathways, respectively. It is conceivable that, *in vivo*, these pathogen-associated molecules activate intracellular signal transduction pathways that cross-talk with pathways activated by host immune system-generated B cell stimuli, such as CD154 and IL-4, to induce optimal AID expression, eventually leading to robust anti-bacterial/anti-viral immune response. Here, we will focus on the role of CD40, IL-4 receptor (IL-4R), BCR and TLRs in activating *aicda* expression in germinal center B cells *in vivo* or B cells stimulated to undergo germinal center-like differentiation *in vitro* (Figure 1). TGF- β induces *aicda* expression in CH12F3-2 B cells and is essential in mediating CSR to IgA^{31,39,40}, while inhibiting B cell proliferation and CSR to other Ig isotypes at a high concentration. TGF- β , in general, downregulates the activity of Stat6 and NF- κ B, two positively acting *trans*-factors for *aicda* expression (see below), and upregulates the activity of Id3, a negative *aicda* modulators⁴¹. However, in the mucosal environment, where B cells undergo CSR to IgA at a high frequency, TGF- β may trigger significantly different signaling events that lead to AID induction. Finally, expression of *aicda* is also induced by mucosal stromal cell- or dendritic cell (DC)-secreted B cell-activating factor of the tumour-necrosis factor (TNF) family (BAFF, BLyS) and a proliferation-inducing ligand (APRIL)^{42–45}, two B cell survival and activation factors that induce CSR to IgA *in vitro* and *in vivo*. Due to the space limitation, the induction of *aicda* and/or CSR by BAFF and APRIL through B cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI), two receptors common for BAFF and APRIL, and BAFF receptor (BAFF-R) (Figure 1) is not discussed here; readers are referred to these excellent reviews^{46–51} and original papers cited therein.

3.1.1. CD40—CD40 is a 48 kDa transmembrane glycoprotein constitutively expressed on the surface of B cells, DCs, macrophages, epithelial cells and activated T cells⁵². Like other members of the TNF family of receptors and ligands, CD40 trimerizes upon engagement by the trimeric CD154, a type II integral membrane TNF family protein transiently expressed on activated CD4⁺ T cell surface. In addition to the mouse CH12F3-2 B cell line, primary murine B cells^{9,35,38}, primary human B cells^{36,37} as well as human IgM⁺IgD⁺ B cell lines³⁶ all significantly upregulate *aicda* expression upon CD154:CD40 engagement *in vitro*. *In vivo*, CD154:CD40 engagement is essential to trigger the T-dependent germinal center reaction, as shown by impaired germinal center development in mice deficient in CD40 or CD154 or in patients with X-linked hyper IgM syndrome (X-HIGM), which is caused by a defective CD154 molecule (Table 2)^{53–57}. In *cd154*^{−/−} or *cd40*^{−/−} mice, serum levels of IgG1, IgG2a, IgG2b and IgE are severely decreased, similar to what occurs in X-HIGM patients, suggesting that the CD40 signaling is essential for CSR, which is, at least partially, due to the role of CD40 signaling in the AID induction. The normal or moderately decreased IgG3 level in *cd154*^{−/−} or *cd40*^{−/−} mice possibly reflects a T-independent B cell response triggered by chronic exposure to bacterial LPS, which induces both AID expression (see below) and CSR to IgG3.

In germinal centers, B cells proliferate vigorously, thus termed centroblasts, in a microanatomical region referred to as the “dark zone”, due to its high density of nuclei. Centroblasts transition from the dark zone into “light zone” centrocytes, which proliferate^{58,59}, but at a significantly lower rate. In the light zone, centrocytes undergo positive selection, as effected by the antigen driving the ongoing response, and plasmacytoid differentiation. As part of the germinal center reaction, SHM primarily occurs in centroblasts in the dark zone. The onset of SHM is earlier than that of CSR^{60,61}. In the

human, CSR likely initiates in centroblasts and unfolds to completion while centroblasts transition to centrocytes, as indicated by the ready detection of germline I_H-C_H transcripts in centroblasts, appearance of S circle DNA, a marker of newly completed S-S region DNA recombination, in centroblasts/centrocytes, abundance of mature $V_HDJ_H-C\gamma$ transcripts in centrocytes and, finally, the expression of surface IgG, IgA or IgE in centrocytes^{62–64} (Figure 2). In the mouse, *aicda* expression and $I\gamma 1-C\gamma 1$ germline transcription, two early and essential events for CSR to IgG1, as triggered by immunization with hapten 4-hydroxyl-3-nitrophenylacetyl conjugated to chicken γ globulin (NP-CGG), begin in early centroblasts, while $I\gamma 1-C\mu$ circular transcripts, as expressed from the excised $S\gamma 1-S\mu$ circle, appear later in the centroblasts/centrocytes⁶⁵.

Consistent with the unfolding of SHM and CSR processes, expression of AID in germinal centers initiates in early centroblasts, is maximal in full-blown centroblasts, significantly decreases in centrocytes and extinguishes in plasma cells (Figure 2). In early centroblasts, CD154:CD40 engagement activates a B cell transcription program, which involves paired box protein 5 (Pax5, also termed B cell-lineage specific activation protein, BSAP), interferon (IFN) regulatory factor 4 (Irf4) and Irf8, to initiate *aicda* expression. This transcription program likely requires *de novo* protein synthesis of those transcription factors, as suggested by the suppression of optimal *aicda* induction in CH12F3-2 B cells by cycloheximide, an inhibitor of the mRNA translation⁹. In the dark zone, few if any CD154-positive $CD4^+$ T cells can be detected⁶⁶, suggesting that the *aicda* regulation program, as triggered in early centroblasts, continues to be active and drives B cell division and differentiation (Figure 2). In human tonsil germinal centers, AID expression is predominant in the dark zone and limited in the light zone, which displays a rich follicular dendritic cell (FDC) network⁶⁷. In mouse spleen germinal centers induced by NP-CGG, AID is also preferentially expressed in the dark zone 14 days after immunization (our unpublished data). Downregulation of AID as centroblasts transition to centrocytes is likely mediated by changes in the expression level of transcription factors, i.e., decreased expression of positive *aicda* transcription regulators and increased expression of *aicda* negative modulators, in light zone B cells (Figure 2). The abated expression of AID, as well as its positive transcriptional regulators, likely results from insufficient signals B cells receive from $CD4^+$ T cells or FDCs, as localized in the light zone, to overcome the attenuation of the *aicda* transcription program (Figure 2). Insufficient T cell help could be caused by more transient T:B cell synapses, as compared to the T:B cell engagements involving early centroblasts^{59,68}, or a possible downregulation of CD154 expression in $CD4^+$ T cells, or both. Finally, the differential expression of AID in centroblasts and centrocytes, which display different proliferation rates, is consistent with the cell cycle-dependent regulation of *aicda* expression⁶⁹, i.e., a higher steady-state *aicda* mRNA level in B cells undergoing a higher than a lower rate of proliferation⁷⁰. The extinction of *aicda* expression during plasmacytoid differentiation is discussed later (see below).

The intracellular carboxyl-terminal tail of CD40 contains binding sites for different TNF α receptor-associated factors (Trafs). Traf6 binds to the KxxPx motif in the human CD40 and to the RQDPQE motif in the mouse CD40; Traf2 and Traf3 bind to the PxQxT motif in both human and mouse CD40. Depending on the Traf adaptor proteins that are recruited to clustered CD40 trimers, different signal transduction pathways are activated, leading to the differential activation of *trans*-factors, such as NF- κ B and p38. While a wildtype CD40 transgene fully rescued germinal center development, *aicda* expression and humoral immune responses in *cd40*^{-/-} mice, *cd40*^{-/-} mice carrying a CD40 transgene with mutations at both the Traf2/Traf3- and Traf6-binding sites (*cd40 Δ traf2/traf3 Δ traf6*) displayed severely impaired germinal center development, mimicking the effect of complete CD40 deficiency^{35,71}. However, the Traf2/Traf3- and Traf6-binding sites of CD40 were individually dispensable for B cell proliferation or germinal center formation, as shown by

similar *trans*-complementation studies in *cd40Δtraf2/traf3* and *cd40Δtraf6* mice⁷¹. *In vitro*, *cd40Δtraf6* B cells displayed a similar level of *aicda* induction by an anti-CD40 monoclonal antibody (mAb) as wildtype B cells. In contrast, *cd40Δtraf2/traf3* B cells displayed significantly impaired *aicda* induction and CSR efficiency upon stimulation by an anti-CD40 mAb, while *aicda* and CSR induction by LPS is apparently normal, suggesting that the CD40-Traf2/Traf3, but not the CD40-Traf6, signal transduction pathway plays a major role in CD40-mediated induction of *aicda* expression. The impaired *aicda* expression in anti-CD40 mAb-stimulated *cd40Δtraf2/3* B cells may indirectly result from the low proliferation rate of these B cells⁷¹, as suggested by the cell cycle-dependent induction of *aicda* expression⁷⁰.

In human B cells, the recruitment of Traf2 to the intracellular tail of CD40 and NF-κB activation are hampered upon engagement of CD30, a TNFR family protein expressed on the B cell surface, by its ligand CD153, which is expressed on the T cell surface^{31,32,72} (Figure 1). Expression of CD30 in human B cells is upregulated by CD154:CD40 engagement. In human tonsil germinal centers, CD30 and AID expression in the dark zone were mutually exclusive and, in CD30⁺ and AID⁺ B cells, expression of germinal center reaction-related transcription factors displayed a reciprocal profile⁶⁷. For instance, AID⁺ B cells were positive for the E-box protein E47 and negative for inhibitor of differentiation (Id2) and IRF4, while CD30⁺ B cells were positive for Id2 and IRF4 and negative for E47 (see below for the role of E47, Id2 and IRF4 in regulating *aicda* expression), suggesting that CD30 negatively modulates AID expression, likely through its repression of CD40-Traf2 signaling. CD154:CD40 engagement-mediated CD30 induction could be inhibited by BCR crosslinking⁷², suggesting that the BCR signaling plays an important role in relieving CD30-mediated suppression of *aicda* expression. Thus, the crosstalk between CD40- and BCR-activated signal transduction pathways is critical to for AID induction in germinal center B cells (Figure 2).

CD40-Traf signaling-mediated NF-κB activation depends on the phosphorylation and degradation of NF-κB inhibitor IκB. Upon recruitment to clustered CD40 tails, multimerized Traf adaptor proteins polyubiquitinate and activate the NF-κB essential modulator (NEMO) (also termed IκB kinase γ, IKKγ), a scaffolding molecule for the two other subunits of the IKK complex, IKKα and IKKβ. Missense mutations at Cys417 in the zinc finger domain of NEMO lead to the X-linked hyper-IgM syndrome with ectodermal dysplasia (XHM-ED) (Table 2)^{37,73,74}. In B cells from XHM-ED patients, the CD40-dependent NF-κB activation was abolished and CSR was impaired. However, induction of *aicda* was seemingly normal upon B cell stimulation by CD154 and IL-4³⁷, suggesting that NEMO activity is not essential for AID expression. Instead, an alternative NF-κB activation pathway, as mediated by NF-κB-inducing kinase (NIK) and IKKα, or a compensating Traf-dependent signal transduction pathway involving p38 likely leads to *aicda* induction in XHM-ED B cells. Among the five mammalian NF-κB proteins, p50 (NF-κB1), p52 (NF-κB2), Rel A (p65), Rel B and c-Rel, p50 seems to play a role in regulating *aicda* expression, as suggested by the partial impairment of *aicda* induction in *p50*^{-/-} mouse B cells stimulated with an anti-CD40 mAb and IL-4⁷⁵. Upon activation by an anti-CD40 mAb, p50 and Rel A, but not p52, c-Rel or Rel B, bound to a double-stranded oligodeoxynucleotide containing the two putative NF-κB sites, as identified in the upstream regulatory region of the *aicda* gene⁷⁵ (Table 3), suggesting that NF-κB directly binds to the *aicda* promoter and induces a basal transcription level that can be further raised by other B cell stimuli.

3.1.2. IL-4R—IL-4 is a pleiotropic cytokine produced by activated type 2 CD4⁺ T cells. By inducing both *aicda* expression and germline Iγ-Cγ transcription, IL-4 plays an essential role in regulating CSR to IgG4 and IgE in the human and CSR to IgG1 and IgE in the mouse⁷⁶. Murine B cells stimulated *in vitro* by CD154 or LPS proliferate at a high rate, but switch to

IgG2b and IgG3 at an efficiency at least two to five-fold lower than the switching efficiency to IgG1, as induced by CD154 or LPS together with IL-4, possibly reflecting a sub-optimal induction of AID in the absence of IL-4^{9,35,38}. The high-affinity binding of IL-4 to the 140-kDa α chain subunit of its receptor IL-4R (IL-4R α) causes heterodimerization of IL-4R α with the IL-4R γ common chain (γ c) subunit (Figure 1), an element shared by receptors for other cytokine, including IL-2, IL-7, IL-9 and IL-13⁷⁶. Upon IL-4R α and γ c heterodimerization, IL-4R α -associated receptor tyrosine kinase Janus kinase-1 (Jak1) and γ c-associated Jak3 autophosphorylate and/or reciprocally phosphorylate each other, leading to their own activation. Activated Jak1 and Jak3 in turn phosphorylate tyrosine residues in the cytoplasmic tail of IL-R α and γ c, respectively. Upon phosphorylation, the five conserved tyrosine residues in IL-4R α , Tyr497 (Y1), Tyr575 (Y2), Tyr603 (Y3), Tyr631 (Y4) and Tyr713 (Y5), become docking sites for downstream signaling molecules, which are then phosphorylated by Jaks and become activated⁷⁶. Phosphorylated Y1 recruits insulin receptor substrate (IRS) and Shc adapter proteins and plays a critical role in IL-4-stimulated B cell proliferation⁷⁷; phosphorylated Y2, Y3 and Y4 interact with the Src homology 2 (SH2) domain of the signal transducer and activator of transcription 6 (Stat6), which plays a major role in activating IL-4-induced gene expression, including *aicda* expression (see below); and finally, phosphorylated Y5 recruits Src homology-containing phosphatase 1 (Shp1), which dephosphorylates Stat6, thereby negatively regulating Stat6 signaling⁷⁸. How individual Tyr residues in IL-4R α contribute to AID induction and CSR to IgG and IgE remains to be determined. An alternative IL-4R (Type II), consisting of IL-13R α 1 and γ c, is also the receptor for IL-13, which controls the T_H2 response together with IL-4⁷⁹. The dual role of the IL-13R α 1/ γ c heterodimer as the type II IL-4R and IL-13R likely explain why IL-13, together with an anti-CD40 mAb, induced *aicda* expression⁸⁰, and why IL-13 induced CSR to IgG4 and IgE in primary human B cells co-cultured with CD4⁺ T cell membrane in an IL-4-independent fashion⁸¹.

Stat6 is one of the seven mammalian Stat proteins, all of which are latent in the cytoplasm before their phosphorylation at Tyr residues by Jak family kinases. Different Jaks and Stats are activated in response to different extracellular stimuli, such as IFNs, growth factors and ILs. Phosphorylated Stat6 disengages from IL-4R α and forms dimers that translocate to the nucleus, where they bind to DNA and direct gene transcription^{82,83}. In the mouse, Stat6 is critical for IL-4-induced B cell activation and CSR to IgG1 and IgE, but negatively regulates CSR to other IgG isotypes^{84–86}. Stat6-deficiency leads to a severely impaired *aicda* induction in murine B cells stimulated with an anti-CD40 mAb and IL-4⁷⁵. Stat6 signaling is negatively regulated by Shp1 phosphatase, which is recruited by IL-4R α Tyr713. In addition, the CD45 phosphatase, when induced by an anti-CD45 mAb, negatively regulates IL-4-induced *aicda* expression in human B cells, possibly through dephosphorylation of Jak1, Jak3 and Stat6⁸⁷. A gain-of-function polymorphic allele IL-4R α in the human, IL-4R α (Glu576Arg), is associated with hyper-IgE syndrome. This can be explained by the accelerated recruitment/activation or dimerization of Stat6 near the IL-4R α Tyr575 and/or suppressed dephosphorylation of Stat6⁷⁶, and would call for an analysis of AID induction in human B cells carrying this mutation. Similar to NF- κ B p50, Stat6 binds to a double-stranded oligodeoxynucleotide containing a canonical Stat-binding motif 5'-TTCnnn(n)GAA-3', as identified upstream of the *aicda* promoter (Table 3). However, whether NF- κ B and Stat6 directly bind to the *aicda* promoter region in B cells is unclear. NF- κ B and Stat6 seem to directly interact with each other and synergize in promoting transcription of IL-4-inducible reporter genes in human B lymphoma cells⁸⁸, suggesting NF- κ B and Stat6 synergistically bind to the upstream regulatory region (–1400 to –1200) of the *aicda* promoter (Table 3), thereby mediating the converging CD40– and IL-4R-activated signal transduction pathways for efficient *aicda* induction.

3.1.3. BCR—The BCR consists of IgH, IgL and the two intracytoplasmic signaling components, Ig α (CD79a) and Ig β (CD79b) (Figure 1). BCR signaling is required for early-stage B cell development and mature B cell activation and differentiation when antibody affinity maturation occurs^{89,90}. Upon crosslinking by antigen, most BCR molecules remain non-phosphorylated and are internalized by endocytosis, eventually resulting in the localization of the BCR-antigen complex into late endosomes for processing and antigen presentation⁹¹. A minor population of BCR molecules are phosphorylated at Tyr in Ig α and Ig β and retained by the lipid raft at the B cell surface, thereby initiating the assembly of the BCR signalosome, a macromolecular protein complex containing enzymes and adaptor/scaffolding proteins involved in pathways leading to Ca²⁺ response and NF- κ B activation^{91–93}. *In vitro*, BCR crosslinking by an anti-IgM F(ab')₂ fragment or an anti-IgD antibody conjugated to dextran (anti- δ dextran) mimics antigen-mediated BCR crosslinking. In human B cells, *aicda* expression is efficiently induced by co-stimulation of CD154 and anti-IgM F(ab')₂, two of the signals that are required for induction of germinal center-like differentiation, including SHM and CSR^{31,32,36}. Anti- δ dextran does not induce appreciable AID expression if used alone, but potentiates LPS-induced AID expression in primary murine B cells³⁸. *In vivo*, BCR signaling is essential for the T-dependent germinal center reaction in the spleen, but dispensable for *aicda* induction in gut-associated lymphoid tissues (GALTs), as suggested by studies in the BCR-deficient mouse strain D_HLMP2A⁹⁰. In D_HLMP2A mice, IgH expression was ablated and BCR signaling was mimicked by the Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A), which contains multiple domains that are functionally homologous to those in Ig α and Ig β ⁹⁰. LMP2A co-opted the lipid raft and functioned as a BCR surrogate to promote survival, development and maintenance in D_HLMP2A mouse B cells^{90,94}. D_HLMP2A mice displayed no germinal center formation in response to T-dependent antigens, likely due to the lack of antigen presentation and cognate T:B cell interactions, but displayed a number of germinal centers in GALTs, including Peyer's patches, comparable to their congenic Balb/C counterparts. In D_HLMP2A GALTs, *aicda* expression, SHM and CSR were seemingly normal, possibly as a result of chronic exposure of B cells to gut microbial flora that activate TLR signal transduction pathways⁹⁰. Thus, BCR signaling likely regulates *aicda* induction by enhancing the CD40-activated *aicda* transcription program and/or by relieving the inhibitory effect of CD30 (as discussed above) during the initiation of *aicda* induction (Figure 2).

During B cell activation, the threshold of BCR signal transduction is critically regulated by the BCR co-receptor CD19/CD21/CD81 complex (Figure 1), which is recruited by antigen-associated complement cleavage products C3d/C3dg and co-aggregates with crosslinked BCR⁹⁵. BCR crosslinking, together with co-engagement of CD21 and CD19, led to ready induction of *aicda* expression and SHM in the human Burkitt's lymphoma B cell line BL12⁹⁶. Translocation of CD19, as co-aggregated with crosslinked BCR, to the lipid raft activates PI 3-K signal transduction pathways by recruiting the p85 α regulatory subunit of PI 3-K through two CD19 Tyr residues, Y482 and Y513^{58,97,98}. In *cd19*^{-/-} mice expressing a transgenic CD19 with mutations in Y482 and Y513 (*cd19*^{-/-}TgY482F/Y513F), immunization with 2-phenyloxazalone coupled to chicken serum albumin (phOx-CSA) induced a seemingly normal germinal center development, centroblast proliferation and a significant recruitment of the V κ Ox1 gene, a V κ 4/5 family member used by 129/Sv mice for the phOx-specific antibody response, into the BCR repertoire. However, SHM frequency in the V κ Ox1 DNA in *cd19*^{-/-}TgY482F/Y513F mice is about half that in control wildtype mice⁵⁸, suggesting that AID might be expressed at a suboptimal level in the CD19 mutant mice. PI 3-K and its antagonistic enzyme PTEN likely play multiple roles in regulating *aicda* expression, CSR and plasma cell differentiation, as further discussed in the next section.

CD81, or its palmitoylated form, is necessary for the partitioning of co-aggregated BCR and CD19-CD21 into the lipid raft^{99–102}. CD81 is also a co-receptor for the hepatitis C virus (HCV) in both human hepatocytes and B cells. In the human B cell line Raji, binding of recombinant HCV E2 protein to CD81 led to Ig SHM, as underpinned by *aicda* induction, double-strand DNA breaks (DSBs) in V_HDJ_H DNA and induction of error-prone DNA polymerases¹⁰³, three hallmark events of SHM^{21,32,36}. Thus, E2 protein-activated CD81 induces *aicda* expression autonomously.

3.1.4. TLR—TLRs are pattern recognition receptors (PRRs) that recognize and bind microbial components, including LPS, flagellin, double-stranded RNA (dsRNA), CpG DNA and certain glycoproteins and lipoproteins^{104–106}. TLRs can be divided into two groups based on their subcellular localization, i.e., the transmembrane TLR1, TLR2, TLR4, TLR5, and TLR6, which belong to the IL-1R/TLR protein superfamily, and the intracellular TLR3, TLR7 and TLR9, which likely initiate signal transduction from acidic endosomes^{107,108}. All TLRs contain a Toll-IL-1R (TIR) domain, a protein-protein interaction module that recruits TIR-containing adaptor proteins to activated TLRs¹⁰⁵. In mammalian cells, there are at least four TIR adaptor proteins, including myeloid differentiation factor 88 (MyD88), TIR adaptor protein/MyD88 adapter like (Tirap/Mal), TIR domain-containing adapter inducing IFN- β /TIR domain-containing adapter molecule-1 (Trif/Ticam1) and Trif-related molecule/TIR domain-containing adapter molecule-2 (Tram/Ticam2). TIR-containing adaptor proteins, upon recruitment to TLRs, activate signal transduction pathways, thereby resulting in the activation of transcription factors, such as NF- κ B, p38 and IRFs¹⁰⁹. For instance, MyD88, possibly the best characterized TIR-containing adaptor proteins, activates the serine/threonine kinases IL-1R-associated kinase-1 (Irak1) and Irak4, which, in turn, recruit and activate Traf6. A Traf6-mediated activation of the IKK complex would lead to NF- κ B activation. A MyD88-independent TLR4 signal transduction pathway, as mediated by Trif, intersects with the MyD88-dependent pathway at the level of Traf6 and leads to the activation of the downstream NF- κ B and other transcription factors.

TLRs play an important role in directing efficient T-dependent adaptive immunity in the human and the mouse^{110,111}. TLR4, which is expressed in the mouse, but not in the human, together with membrane-anchored CD14 and extracellular MD-2 proteins, form the receptor for *E. coli* LPS¹¹² (Figure 1). When used together with T_H cytokines, such as IL-4, IL-5, IFN- γ and/or TGF- β , *E. coli* LPS stimulate primary murine B cells to undergo proliferation and efficient CSR *in vitro*. While LPS alone induces weak AID expression, it synergizes with IL-4 and/or BCR signals for effective AID induction^{9,38}. *In vivo*, LPS induce polyclonal B cell activation as a type 2 T-independent antigen. In addition, LPS, which are likely present in the form of the DC-captured immune complex, would bind to TLR4 expressed on mouse germinal center B cells, thereby enhancing DC-mediated BCR signaling events. It is conceivable that CD40, BCR and TLRs all contribute to the optimal AID induction in immune responses against bacterial infection (Figure 1), as suggested by the intrinsic defect of *myD88*^{-/-} B cells in generating effective antibody response to LPS-conjugated ovalbumin (LPS-OVA)¹¹⁰. However, the role of TLRs in the antibody response during natural bacterial infection and the relative contribution of MyD88-dependent and MyD88-independent pathways in LPS-induced AID expression remain to be determined.

TLR4-MyD88 pathway may also play a role in viral-induced AID expression. Human papillomavirus type 16 (HPV16) L1 virus-like particles (VLP) induce *aicda* expression and CSR in B cells through TLR4, as demonstrated by the lack of *aicda* induction in C3H/HeJ mice B cells, which carry a mutation in the TIR domain of TLR4. In addition, *aicda* transcription and secreted IgG levels were greatly decreased in HPV16-simulated *myD88*^{-/-} B cells, as compared to wildtype B cells¹¹³. HPV16 also synergizes with the CD40 signaling to further upregulate AID expression¹¹³, possibly reflecting the non-overlapping CD40-

Traf2/3 and TLR4-MyD88-Traf6 signal transduction pathways and their synergistic activation of NF- κ B. However, primary T-dependent responses *in vivo* and *aicda* expression in germinal center B cells were normal in MyD88-deficient mice upon infection with mouse polyoma virus¹¹⁴. Upon infection with Abelson murine leukemia retrovirus, in bone marrow B cells isolated from mice deficient in MyD88 or doubly-deficient in MyD88 and Trif, *aicda* was induced to a level comparable to that in wildtype bone marrow B cells¹¹⁵, suggesting a complex role of TLR-MyD88 in virus-induced AID expression *in vivo*.

Receptor-interacting protein (Rip) is a death domain kinase that is associated with Tnfr1 death receptor. Rip-deficiency in the mouse causes embryonic lethality that can be partially rescued by combined Tnfr1 deficiency. The interaction of Rip with Traf2 and Traf6 points to a possible role of Rip in CD40- and/or TLR4-mediated AID induction. Indeed, *rip*^{-/-}*tnfr1*^{-/-} murine B cells displayed more than 75% reduction of *aicda* expression and CSR to IgG and IgE upon stimulation with LPS and IL-4, as compared to *rip*^{+/-}*tnfr1*^{-/-} B cells. The comparable *aicda* induction in *rip*^{-/-}*tnfr1*^{-/-} and *rip*^{+/-}*tnfr1*^{-/-} B cells stimulated with anti-CD40 mAb and IL-4 suggests that Rip functions through Traf6, but not Traf2, in these B cells to activate the NF- κ B signaling¹¹⁶. The exact role of different NF- κ B proteins in LPS-mediated AID induction remains to be investigated in such knockout B cells⁷⁵.

Induction of *aicda* expression is also mediated by the intracellular TLR9, which is expressed in both the human and the mouse, in B cells stimulated with CpG DNA. CpG DNA is prevalent in bacterial, but not mammalian, genome¹¹⁷ and is a potent AID-inducing agent¹¹⁸. TLR9 is localized in the endoplasmic reticulum (ER) (Figure 1) and is recruited to lysosomes after acidification of endosomes and lysosomes, as agents that block endosomal acidification, such as chloroquine and bafilomycin A1, completely abrogate CpG DNA-induced signaling. TLR9 intracellular localization is critical for it to distinguish foreign DNA from self DNA, which can be released to the extracellular space from necrotic cells¹⁰⁷. TLR9 expression was upregulated in B cells stimulated by an anti-CD40 mAb or BCR crosslinking agents¹¹⁹. CD40 expression was upregulated in a time- and dose-dependent fashion in human B cells stimulated by CpG DNA, but not by control GpC DNA¹²⁰, suggesting that a reciprocal activation of CD40 and TLR9 exists to establish a positive feedback loop in regulating their expression. CpG DNA induced expression of TLR9 in B cells, thereby possibly further enhancing signaling¹¹⁹. Further, primary human B cells stimulated by CD154, BCR crosslinking agents and CpG DNA responded with a higher proliferation rate, a stronger *aicda* induction and a higher CSR efficiency than their counterparts stimulated by CD154 and BCR crosslinking only, thereby suggesting that TLR9 plays an important role in enhancing the CD40 and BCR signals that lead to AID induction and CSR¹¹¹. Finally, in human B cells, the TLR9 TIR domain, MyD88, Irak1 and Traf6, but not Traf2, were critical for CpG DNA and IL-10-induced *aicda* expression and CSR¹²¹.

In contrast to its enhancement of CD40- and BCR-induced CSR, CpG DNA suppressed IL-4-induced CSR to IgG1 and IgE in a TLR9- and MyD88-dependent fashion^{120,122,123}, possibly in a fashion that is independent of downregulation of AID expression. More likely, this specific CpG DNA-induced suppression of CSR is mediated by T-bet, a T-box family transcription factor that selectively downregulates germline I γ 1-C γ 1 and I ϵ -C ϵ transcription, and upregulates germline I γ 2a-C γ 2a and/or I γ 2b-C γ 2b transcription^{120,122,123}. Finally, that CpG modulates *aicda* expression through pathways independent of Id2, an E-box transcriptional suppressor that participates in the negative regulation of CD40- and IL-4 receptor-mediated *aicda* induction (see below), is suggested by the demonstration that the suppression of CSR to IgE by CpG DNA does not involve Id2¹²⁴.

3.2. TRANSCRIPTION PROGRAMS REGULATING *aicda* GENE EXPRESSION

In germinal center B cells, a characteristic gene expression profile arises that is distinct from that in naïve B cells, post-switched plasma cells or memory B cells. Although proteomic approaches are starting to be used to identify signature gene expression profiles, the prevalent approach in analyzing such profiles in germinal center B cells is quantitative RT-PCR and/or DNA microarray. These two approaches allow only for analyses of gene transcripts that show changes in steady-state level, while excluding analyses of post-translational modification. Due to this limitation, here we will discuss mainly the modulation of *aicda* expression by transcriptional regulators, whose own expression is regulated during the germinal center reaction.

A typical gene expression profile in cells undergoing differentiation consists of at least three hierarchical levels: (i) genes whose expression directly specifies major cellular functions, hence the term “terminal node” genes; (ii) genes that encode transcription factors which directly regulate terminal node gene expression; hence the term “proximal node” genes; and (iii) genes that encode “master switch” transcription factors that control expression of proximal node genes; hence the term “master switch node”. Intermediate level nodes between master switch and proximal nodes may exist (Figure 3).

In germinal center B cells where SHM and CSR unfold, *aicda* is a terminal node gene. In plasma cells that produce and secrete thousands of antibody molecules per second, terminal node genes include secretory pathway genes encoding factors involved in protein translocation across the ER membrane, protein folding and glycosylation in the ER and vesicle trafficking¹²⁵ (Figure 3). For some terminal node genes, such as *aicda*, the expression is highly specific; many other terminal node genes are expressed in a broad range of cell types/tissues, such as secretory pathway genes of plasma cells.

Proximal nodes genes are often expressed in multiple cell types or at different differentiation stages of the same cell lineage at fluctuating rates. For instance, *e2a*, an *aicda* proximal node gene in germinal center B cells (see below), is also preferentially expressed in B cell and T cell progenitors where *e2a*-encoded E-box protein E12 and/or E47 are essential for Rag1- and Rag2-mediated gene recombination¹²⁶. Proximal node genes function as signal “integrators”, i.e. their expression is induced by multiple signal transduction pathways, as activated by a combination of same stimuli that synergize to induce terminal node gene expression. In addition, several proximal node factors could be assembled into an “enhanceosome”^{127,128}, which determines the cell type- or cell stage-specificity in terminal node gene induction. For instance, CD154, IL-4 or BCR crosslinking each induces *aicda* expression at a basal level, but they synergize to potentiate *aicda* induction through upregulation of several proximal node transcription factors (see below) (Table 3), thereby restricting *aicda* expression primarily in germinal center B cells. Negative proximal node genes contribute to the specificity of terminal node gene expression in sequential differentiation stages. For instance, downregulation of AID in plasma cells, possibly effected by Id2 and B lymphocyte-induced maturation protein 1 (Blimp1) (Figure 3), would ensure the preservation of Ig sequences that are fixed to encode high affinity and class switched mature antibodies and prevent potential genotoxic effects of AID in plasma cells.

Master switch node genes regulate the expression of genes at different levels, including terminal node, proximal node and intermediate node gene. In germinal center B cells, the master node gene *pax5* directly regulates the expression of the terminal node gene *aicda* and an intermediate node gene B cell lymphoma 6 (*bcl6*). In fact, in addition to maintaining the B cell identity¹²⁹, Pax5 is critical for the germinal center reaction, which is, at least partially, due to its specific induction of *bcl6* and *aicda* in these cells. The linear cause-effect relationship of *pax5* and *bcl6*^{130,131} suggests that *bcl6* functions as a *de facto* master node

gene, although whether *bcl6* is a proximal node gene for *aicda* is unclear. An analogous master-intermediate node gene pair in plasma cells involves *prdm1* and *xbp1*, which encode Blimp1 (also termed positive regulatory domain containing protein 1, Prdm1) and X-box binding protein 1 (Xbp1), respectively (Figure 3). A unique feature of most master switch node genes is their dual functions as both transcription activators and suppressors, as exemplified by the intertwined gene expression signature files in germinal center B cells and plasma cells (Figure 3). In germinal center B cells, Pax5 directly induces *c-myc* and *aicda* expression, but suppresses *xbp1* expression¹³²; Bcl6 forms a co-repressor complex with MTA3 and Mi-2/NuRD to directly suppress *prdm1* expression¹³³. Conversely, in plasma cells, Blimp1 positively regulates *irf4* expression¹³⁴, but directly suppresses *pax5* expression, thereby relieving the inhibition of *xbp1* expression¹³⁵; Xbp1 regulates gene expression only positively, suggesting that it functions as an intermediate and/or proximal node gene, but not a *de facto* master node gene.

The reciprocal suppression between *pax5-bcl6* and *prdm1-xbp1* suggests the existence of a transitional state during which the *pax5-bcl6* axis is gradually downregulated while the *prdm1-xbp1* axis is upregulated¹³⁶. Recent evidence suggests that the downregulation of *pax5* precedes the initiation of *prdm1* expression¹³⁷, although the nature of events leading to the *pax5* downregulation is still unclear. In addition, Irf4 controls *prdm1* expression^{138,139}, suggesting that Irf4 plays an important role during the transition to plasma cells. A further, and possibly more drastic, downregulation of AID expression in this transitional state, as compared to the already decreased AID expression level in centrocytes (Figure 2), is possibly mediated by both Pax5 downregulation and expression of Blimp1. Here, we will review different levels of regulation, both positive and negative, of *aicda* expression, either direct or indirect, by Pax5, E47, HoxC4, Irf4, Irf8 and Blimp1, along the B cell germinal center reaction and plasmacytoid differentiation pathway (Figure 2).

3.2.1. Pax5—Pax5 is expressed from the pro-B cell to the mature B cell stage, being required to maintain B cell identity throughout the cell ontogeny¹²⁹. In addition to a highly conserved paired DNA-binding domain (PRD) and a partial homeodomain, Pax5 contains a transcription-activation domain and a transcription-repression domain, which mediate its dual transcriptional regulation activity. Pax5 controls the expression of many B lineage-specific genes, including *cd19*, *cd79a* and the B cell linker protein-encoding gene *blnk*, through binding to their promoters and/or other regulatory elements¹⁴⁰. In human and mouse germinal centers, the *pax5* transcription level is significantly upregulated in centroblasts¹⁴¹ (Figure 2), possibly as a result of activated CD40 and/or IL-4 receptor signal transduction pathways, as suggested by the efficient Pax5 induction in murine spleen B cells stimulated with an agonistic anti-CD40 mAb¹⁴². In mouse spleen B cells stimulated with LPS, *pax5* mRNA level increases by day 2, coincident with the initiation of *aicda* expression, and then decreases by day 4, with concomitant decrease of *aicda* expression^{38,141}. Enforced overexpression of Pax5 in a mouse pro-B cell line induces expression of the otherwise silent *aicda* gene, suggesting that Pax5 indeed regulates AID expression, possibly through its recruitment to Pax5-binding sites in the *aicda* promoter¹⁴³ (Table 3). The concomitant expression of Pax5 and AID is also kinetically associated with the expression of another transcription factor HoxC4 in mouse germinal center B cells as well as in *in vitro*-stimulated human and mouse B cells¹⁴⁴ (see below) (Figure 2). Indeed, optimal induction of the *aicda* promoter requires both Pax5 and HoxC4¹⁴⁴, suggesting that their simultaneous expression constitutes one of checkpoints that restrict AID expression to germinal center B cells, thereby avoiding its genotoxic effect in other cell types. Downregulation of expression of *pax5*, and, possibly, *hoxc4* by Blimp1 during the plasmacytoid differentiation would contribute to the downregulation of AID and termination of CSR, as suggested by the abrogation of CSR in B cells in which *pax5* expression is repressed by enforced Blimp1 expression. While stimulation of a subset of human chronic lymphocytic leukemia (CLL) B

cells by CD154 plus IL-4 induced expression of the full length *pax5* transcript and *aicda*, the expression of an alternative spliced form *pax5/ΔEx*, which encodes a Pax5 isoform without the activation domain but with a preserved suppression domain, was dampened¹⁴⁵. Both full length Pax5 and Pax5/ΔEx8 bound to the *aicda* promoter, suggesting that a balance between two isoforms plays a role in regulating *aicda* expression. Such a balance could be further compounded if Pax5/ΔEx8 precludes other trans-factors, such as E47 and HoxC4, from accessing the *aicda* promoter.

3.2.2. E47—The *e2a* gene, through differential splicing, encodes two transcription factor E12 and E47. Both E12 and E47 belong to a family of basic helix-loop-helix (bHLH) proteins known as E-proteins, which bind DNA at E-box sites. Similar to that of *pax5*, expression of *e2a* is essential for B cell development, maturation and activation. In mouse germinal centers, E47 expression level is the highest in dark zone B cells, reduced in the light zone and drastically decreased during plasmacytoid differentiation. This expression pattern is similar to that of Pax5, HoxC4 and AID, suggesting that E47, Pax5 and HoxC4 form the *aicda* enhanceosome that co-integrates signal transduction pathways to activate *aicda* expression (Figure 2). *In vitro*, the DNA-binding activity of E47 is induced in B cells activated by CD40, LPS, IL-4 and BCR crosslinking¹⁴⁶, the same signals that also induce *aicda* expression. E47 is recruited to the conserved region 2 (*cr2*) DNA in the first intron of the *aicda* gene through high-affinity binding to two E-box sites and transactivate the *aicda* promoter¹⁴⁷. E47 may also directly bind the *aicda* promoter at another E-box site, which is in proximity of both Pax5- and HoxC4-binding sites (Table 3), and synergize with Pax5 and HoxC4 in *aicda* induction.

The transactivation activity of E12 and E47 is antagonized by the Id family of transcriptional inhibitor proteins, including Id1, Id2, Id3 and Id4, all of which lack the basic domain essential for DNA binding. E-proteins that heterodimerize with Id proteins cannot bind to E-box sites, thereby failing to transactivate their target genes¹²⁶. In mature B cells, Id2 and Id3 are the two dominantly expressed Id proteins. Overexpression of Id3 in mouse splenic B cells results in a defective CSR, which can be partially rescued by enforced AID expression¹⁴⁷, suggesting that E47 mediates CSR by activating AID expression. Paradoxically, Id3 expression is induced by BCR crosslinking and Id3 is critical for BCR signaling-activated, but not CD40, IL-4 or LPS-activated, B cell proliferation¹⁴⁸. Thus, the Id3-mediated AID inhibition is independent of the B cell proliferation rate, suggesting that the downregulation of AID expression in the light zone is, at least partially, due to the Id3 expression in this area (Figure 2).

E47 expression is inhibited in plasma cells, possibly a result of decreased *e2a* gene expression, as effected by Blimp1¹³⁴. Blimp1 downregulates the expression of Id3 in plasma cells¹³⁴, suggesting that Id3 is not essential for the negative regulation of AID expression. In contrast, expression of another Id protein, Id2, may be indirectly upregulated by Blimp1 through the inhibition of Bcl6, a transcriptional suppressor of the *id2* gene¹⁴⁹. Id2 plays a role in the negative regulation of *aicda* expression, as suggested by the five-fold increase of the *aicda* transcript level in *id2*^{-/-} B cells stimulated with LPS plus IL-4, as compared to that in wildtype B cells¹⁴³. Conversely, overexpression of Id2 suppresses *aicda* induction, likely by antagonizing the transactivation activities of both E-proteins and Pax5¹⁴³. The inhibitory effect of Id2 can be overcome by enforced overexpression of Pax5, suggesting that in dark zone B cells, where Pax5 and E2A are highly expressed, the potential inhibition of AID expression by Id2 is suppressed.

3.2.3. HoxC4—Hox proteins are a family of transcription factors that specify anterior-posterior axis differentiation and regulate cellular differentiation and organogenesis during embryogenesis¹⁵⁰. All Hox transcription factors contain a helix-loop-helix homeodomain, as

encoded by a highly conserved 180-bp homeobox DNA sequence. *Hox* genes within each family are chromosomally clustered¹⁵¹ and their expression is temporally and spatially regulated^{150,152}. Among *Hox* genes, *hoxc4* is preferentially expressed in B cells in the human and the mouse and expression and activities of HoxC4 are regulated in different stages of B cell development and activation^{144,153,154}. *In vitro*, *hoxc4* expression is induced by the same stimuli that induce efficient *aicda* expression: (i) CD154 plus IL-4 in human B cells; (ii) CD154, IL-4 plus TGF- β in the mouse B cell line CH12F3-2; and (iii) CD154 or LPS plus IL-4 in primary mouse spleen cells¹⁴⁴ (Table 3). Conversely, in HoxC4-deficient B cells stimulated with CD154 and IL-4, *aicda* induction is significantly impaired, resulting, as compared to that in wildtype B cells¹⁴⁴, strongly suggesting that HoxC4 regulates *aicda* gene expression. The impact of HoxC4 on *aicda* expression is further emphasized by the severe impairment of SHM in HoxC4-deficient mice¹⁵⁵ and the significantly reduced CSR in *hoxc4*^{-/-} B cells, which can be rescued by enforced expression of AID¹⁴⁴.

The binding of Hox proteins to DNA through their homeodomain is inherently weak, but can be greatly enhanced by their interaction with transcription factors that are recruited to the same region in target genes, thereby effectively transactivating gene expression. For instance, HoxC4, together with Oct1 and/or Oct2 homeodomain transcription factors and OcaB co-activator, play a critical role in the induction of the IgH 3' hs1,2 enhancer in human B cells by binding to the evolutionarily conserved HoxC4 5' ATTT 3' site and composite HoxC4/Oct 5' ATTTGCAT 3' site¹⁵⁴. Likewise, an embedded HoxC4/Oct binding site 5' ATTTGAAT 3' in the *aicda* promoter is essential for *aicda* promoter induction by HoxC4¹⁴⁴ (Table 3), through the assembly of the HoxC4/Oct1/2/OcaB complex, as part of the *aicda* enhanceosome (Figure 1). While Oct1/2 are highly expressed throughout the germinal center reaction, expression of OcaB is highest in dark zone B cells, at least partly due to its upregulation by the CD40, IL-4R and BCR signaling¹⁵⁶, further emphasizing the synergistic *aicda* induction by the enhanceosome involving the HoxC4/Oct1/2/OcaB complex, Pax5 and E47. OcaB deficiency leads to impaired induction of *prdm1* transcription and concomitant de-repression of expression of the *pax5*, *bcl6* and *aicda* genes in cultured antibody-secreting cells¹⁵⁷, suggesting that OcaB may switch from a positive to a negative regulator of *aicda* expression during plasmacytoid differentiation.

3.2.4. Irf4 and Irf8—Irf4 (also termed Pip, ICSAT and LSIRF) and Irf8 (also termed IFN consensus sequence-binding protein, ICSBP) are two closely related members of the Irf transcription factor family. They bind to elements in promoters of IFN-inducible genes through a DNA-binding domain that contains five conserved Trp repeats. Irf4 and Irf8 interact with each other as well as other transcription factors, including SpiB, Pu.1, E47 and Stat6 to regulate cell proliferation, apoptosis and differentiation¹⁵⁸. Like HoxC4, Irf4 and Irf8 possess weak intrinsic DNA-binding activity and are only recruited to DNA as components of protein complexes, such as Pu.1-Irf4 and SpiB-Irf8, that bind to the Ets-Irf composite elements (EICE)¹⁵⁹.

Expression of Irf4 and Irf8 is restricted to B and T lymphocytes and other immune cells. In the mouse, Irf4 expression is high in bone marrow immature B cells, decreases in mature B cells, further decreases in centroblasts in germinal centers, and then increases again to become maximal in plasma cells^{160,161} (Figure 2). This expression pattern of Irf4 is consistent with an important role of Irf4 in initiating *aicda* expression in early centroblasts and CSR, as suggested by the impaired *aicda* induction and CSR in *irf4*^{-/-} B cells stimulated with CD154 or LPS plus IL-4, and impaired plasmacytoid differentiation in mice deficient in Irf4 expression in germinal centers^{138,139}. Irf4 likely regulates AID expression through other *trans*-factors, such as Pax5, which are induced in germinal center B cells but downregulated in plasma cells by Blimp1¹³⁹. Alternatively, Irf4 may suppress the expression of a putative transcription repressor of *aicda*, i.e. Id2, in germinal center B cells,

as suggested by the increased *id2* gene expression level observed in *in vitro* stimulated *irf4*^{-/-} B cells¹³⁸. Accordingly, Irf4 may regulate the expression and activity HoxC4 or other components of the *aicda* enhanceosome.

Irf4 and Irf8 signal transduction pathways are partially overlapping, as suggested by the complete block of the pre-B to B cell transition in the bone marrow of *irf4*^{-/-} *irf8*^{-/-} doubly-deficient mice but not in *irf4*^{-/-} or *irf8*^{-/-} mice¹⁶². Irf8 displays an expression pattern similar to that of Irf4, albeit with some difference, as it is expressed in bone marrow immature B cells, downregulated in peripheral mature B cells, maximally expressed in dark zone B cells and then downregulated in plasma cells¹⁶³ (Figure 2). Consistent with the expression and function of Irf8 in centroblasts, Irf8 likely binds to the *bcl6* or *aicda* promoter and possibly regulates their expression, as suggested by the significant reduction of *bcl6* or *aicda* transcripts in *irf8*^{-/-} B cells, as compared to wildtype B cells. Irf8 expression is negatively regulated by Blimp1 during plasmacytoid differentiation¹³⁴, either directly or through negatively regulated expression of *bcl6*, thereby contributing to the overall downregulation of *aicda* expression. A short stretch of the *aicda* promoter sequence 5'-ATTTTAAAA[AAAAGAAA]AAT-3' (Table 3) contains two HoxC4-bindings sites, which are in addition to the embedded HoxC4/Oct site discussed above, and one consensus Irf-binding site [AAxxGAAA]. HoxC4 and Irf8 may co-occupy this site to regulate *aicda* expression, in a fashion similar to the regulation of the Igλ enhancer by Pu.1/Irf4 complex through a composite EICE 5'-AAAAGG[AAGTAAA]CCA-3'¹⁵⁹.

3.2.5. Blimp1—As the master node gene controlling plasmacytoid differentiation, Blimp1 effectively inhibits gene expression of an array of transcription factors, including Pax5, E2A, Irf8, Pu.1, SpiB and Stat6¹³⁴, all involved in the positive regulation of *aicda* expression. Downregulation of these factors is partially responsible for the Blimp1-mediated *aicda* downregulation. Like the direct downregulation of Blimp1 target genes *bcl6*, *spiB* and *id3*, the downregulation of *aicda* expression cannot be rescued by the re-expression of Pax5, suggesting a direct suppression of the *aicda* promoter by Blimp1 through a putative Blimp1-binding site^{134,144} (our unpublished data, Figure 3). In addition, Blimp1 positively regulates expression of Id2 and Irf4, albeit indirectly, during plasmacytoid differentiation. As discussed above, an increased level of Id2 inhibits Pax5 and E2A activities. Although Irf4 upregulates *aicda* expression in early centroblasts, it does so indirectly, either through upregulating a positive intermediate *trans*-factor, such as Pax5, or through suppression of a negative intermediate factor, such as Id2. Both Irf4 activities can be overridden by the powerful transcriptional suppression effects of Blimp1. Finally, Blimp1 expression is also upregulated by Irf4, thereby turning Irf4 from a positive to a negative *aicda* regulator^{138,139}. In summary, Blimp1 may inhibit AID expression through at least three different mechanisms, by: (i) direct suppression; (ii) suppression of positive regulators, such as Pax5, E2A, Irf8 and possibly HoxC4; and, finally, (iii) induction of negative regulators, such as Id2. Blimp1-mediated negative regulation of AID expression is consistent with the increased CSR in *prdm1*^{-/-} cells stimulated with LPS or LPS plus IL-4¹⁶⁴.

During the germinal center reaction, Bach2, a transcriptional repressor, represses Blimp1 expression¹⁶⁵, thereby relieving Blimp1-mediated AID downregulation, as suggested by the striking reduction of *aicda* transcription and CSR in *bach2*^{-/-} B cells stimulated with LPS¹⁶⁶. The impaired CSR in stimulated *bach2*^{-/-} B cells could not be rescued by AID re-expression¹⁶⁶, suggesting that Bach2 also regulates expression of other genes critical for the germinal center reaction, SHM and CSR¹⁶⁶. How Bach2 is downregulated during plasmacytoid differentiation to allow Blimp1 expression is unclear.

The recent finding that *pax5* downregulation precedes the initiation of *prdm1* induction suggests that downregulation of *bcl6* is critical in this transitional stage, as Bcl6 is a direct

negative regulator of the *prdm1* gene. In addition, expression of the Bcl6 protein is regulated at a post-transcriptional/post-translational level, as implied by the ubiquitination and subsequent proteasome-dependent degradation of phosphorylated Bcl6, as induced by BCR crosslinking in spontaneously hypermutating Ramos B cells¹⁶⁷, suggesting that engagement of a somatically hypermutated and high-affinity BCR by antigen leads to decreased Bcl6 protein level. Finally, the balance between the Irf4 and Bcl6, the positive and negative regulator of the *prdm1* gene, respectively, likely determines how Blimp1 expression is initiated during plasmacytoid differentiation, as both Irf4 and Bcl6 bind to a conserved non-coding region of the intron 5 of the *prdm1* gene.

Impaired SHM results in failure to generate high-affinity BCR molecules, which, in turn, may result in a defective plasmacytoid differentiation. That Blimp1 expression is indeed driven by a high-affinity antigen-BCR interaction is demonstrated by studies using transgenic HyHEL10 mice, which express a high-affinity BCR specific for hen egg lysozyme (HEL)¹⁶⁸. Immunization with HEL led to normal germinal center formation and plasmacytoid differentiation of adoptively transferred HyHEL10 B cells, in the absence of mutations in the HyHEL10 transgene in plasma cells. In contrast, immunization with a mutated HEL (HEL^{3X}), which displays a 10,000-fold lower affinity for HyHEL10, resulted in an Tyr53 to Asp53 amino acid change in HyHEL10, which increases the affinity of HyHEL10 for HEL^{3X}, but not to HEL. Further, in B cells double-transgenic for HyHEL10 and *prdm1*^{+/GFP}, GFP, whose expression is driven by the *prdm1* promoter, was expressed almost exclusively in B cells that acquired a high affinity anti-HEL^{3X} HyHEL10, suggesting a critical role for BCR signaling in Blimp1 expression and AID downregulation.

One of the major pathways activated by BCR signaling is the PI 3-K pathway⁹⁸. In B cells deficient in Pten, reduced AID expression and impaired CSR could be partially rescued by a putative PI 3-K δ inhibitor, IC87114^{164,169}, suggesting a role of PI 3-K in downregulating AID expression/activity. The Pten deficiency-mediated CSR impairment could also be partially restored by Blimp1 deficiency¹⁶⁴, suggesting that *prdm1* expression is downstream of PI 3-K pathways. Increased CSR in *prdm1*^{-/-} B cells could be further enhanced by the PI 3-K inhibitor IC87114, suggesting a Blimp1-independent negative regulation of CSR by PI 3-K. In summary, PI 3-K signaling likely plays a double role in the germinal center reaction and plasmacytoid differentiation by: (i) enhancing AID induction, as triggered by BCR crosslinking in early centroblasts to initiate SHM and CSR; and (ii) activating Blimp1 and/or other factors to downregulate AID expression and extinguish CSR upon strong antigen-BCR interaction, i.e., crosslinking of hypermutated and high affinity BCRs, in the post-SHM stage.

3.3. POST-TRANSLATIONAL REGULATION OF AID ACTIVITY

AID undergoes a series of post-translational modification, such as dimerization/oligomerization, nuclear/cytoplasmic translocation and phosphorylation, all of which play important roles in regulating AID activity and function in SHM and CSR. The importance of AID post-translational modifications is further emphasized by the failure of enforced AID expression to induce SHM or CSR in transgenic mice¹⁷⁰. Here, we will discuss these AID post-translational modifications with an attempt to identify the physiologically active form of AID and the regulation of such specific AID form. Not only expression of the *aicda* gene is restricted, as effected by the assembly of the *aicda* enhanceosome, in germinal center B cells, and activity of AID is tightly controlled, as effected by its post-translational modification, an additional level of AID regulation is the alternative splicing of the primary *aicda* transcript(s). Two major *aicda* mRNA transcripts in activated mouse B cells result from differential polyadenylation⁹; and only one major *aicda* mRNA transcript is expressed in human B cell lines of germinal center origin, such as Ramos and Raji¹⁷¹. However, up to five alternative *aicda* mRNA molecules were identified in B cells from patients with

CLL^{172,173}. While putative AID mutants encoded by those transcripts await biochemical and functional characterization, it is clear that some of alternative *aicda* mRNA transcripts are implicated in the prognosis of CLL; and readers are referred to the original papers^{172,173}.

3.3.1. Dimerization/Oligomerization—In activated B cell nuclei, the single-stranded DNA deamination activity of AID existed in a broad fraction containing proteins or protein complexes of 20 to 70 kDa in size¹⁷⁴, suggesting a heterogeneity of AID oligomer and/or AID/co-factor complex. Indeed, recombinant AID expressed in *E. coli* or non-B mammalian cells formed dimers and/or oligomers¹⁷⁵. Exogenous AID expressed in HEK293 cells existed as an oligomer, or within a small protein complex, about 30–60 kDa in size, at a high salt concentration, i.e. 750 mM NaCl; at a low salt concentration, i.e. 100 mM NaCl, AID exists as a multimer, or within a large protein complex, about more than 500 kDa in size¹⁷⁶. Both the 500 kDa and the 30–60 kDa protein complexes displayed the dC deaminase activity¹⁷⁶. It is conceivable that the AID monomer, which is 20 kDa in size, forms a stable dimer/trimer or strongly interacts with a putative binding partner (or a complex of several smaller proteins) about 40 kDa in size. The 32 kDa subunit of the replication protein A (RPA) complex, which specifically binds to phosphorylated AID in B cells, but not to non-phosphorylated AID expressed in HEK293 cells¹⁷⁴ (see below), does not seem to be the putative partner. Recombinant AID with a streptavidin tag (StrepAID) expressed in *E. coli* migrated at about 80 kDa on an SDS-PAGE, reflecting the formation of a tetramer that is resistant to the denaturation by EDTA or β -mercaptoethanol. Such StrepAID tetramer could be disassociated into a monomer and a stable trimer upon treatment with EDTA together with strong denaturants, such as urea or guanidine, further emphasizing the ability of AID to form complex oligomers¹⁷⁷.

It is suggested that AID dimerization/oligomerization is essential for the processive deamination of cytidines in single-stranded DNA by AID^{178,179}. Structural analyses of human AID, as modeled using the solved crystal structure of human Apobec2, which share 33% identity and 44% homology overall and 75% identity and 96% homology in buried residues with AID, suggest that a β -strand (β -2 strand) encompassing amino acid residues from Ser41 to Asn51 in each AID monomer provides the major interface of the two monomers¹⁸⁰ (Figure 4). Two AID dimers may further form head-to-head tetramers, as suggested by the structure of Apobec2¹⁸⁰. AID with the double mutation Phe46Ala/Tyr48Ala in the β -2 strand displayed a 4-fold decrease in deaminase activity, likely due to the weakened, but not abolished, dimerization of this AID mutant¹⁸⁰. The extensive interface between the two AID β -2 strands suggests that a single amino acid mutation within the β -2 strand may result in only a partial loss of the deaminase activity and the residual AID activity may still be sufficient to mediate SHM and CSR. Amino acid residues between Gly47 and Gly54 have been shown to be important for AID dimerization and CSR¹⁷⁵, which is largely consistent with the modeled AID structure.

Several carboxyl-terminal truncated AID mutants isolated from HIGM2 patients displayed normal cytidine deaminase activity and effectively drive SHM in fibroblasts¹⁸¹, suggesting that defective CSR results from the inability of these AID mutants to interact with a critical CSR-specific cofactor(s). A likely role of the AID dimerization/oligomerization in mediating CSR is to interact with a dimer/multimer of the elusive CSR co-factor, as the interaction between monomeric AID and this putative CSR co-factor might be insufficient to target AID to the IgH locus. As such, the eight carboxyl-terminal amino acid residues would be essential for this protein-protein interaction, as suggested by the dominant negative activity of an AID mutant truncated at Arg190¹⁸¹. The formation of a dimer/oligomer consisting of both wildtype and this truncated AID would best explain the dominant negative activity of the AID mutant, as suggested by the intermediate HIGM2 phenotypes in patients that are heterozygous for the mutated allele¹⁸¹.

3.3.2. Nuclear/Cytoplasmic Translocation—AID is specifically expressed in germinal center B cells. Subcellular fractionation analyses of tonsil germinal center B cells demonstrated that AID is predominant in the cytoplasm¹⁸². As a small size protein of 20 kDa, which would otherwise freely diffuse through nuclear membrane pores, AID is either retained in the cytoplasm after translation or actively transported outside the nucleus. An efficient nuclear export would explain the low steady-state level of AID protein level in the nucleus, although this can also be explained by a non-mutually exclusive possibility that AID is subjected to a faster degradation in the nucleus. In cells treated with leptomycin B, which blocks nuclear transport by specifically inhibiting the ubiquitous soluble shuttle receptor chromosome region maintenance/exportin 1 (CRM1), AID localized in both nucleus and cytoplasm, suggesting that AID is actively transported out of the nucleus in a CRM1-dependent fashion^{183,184}. The AID carboxyl-terminal ¹⁸⁹LRDAF(R/K)(T/M)LGL/F¹⁹⁸ sequence is rich in Leu and hydrophobic Phe residues, thereby providing a putative nuclear export signal (NES) (Figure 4). Indeed, truncation of the carboxyl-terminal sequence or mutation of Phe198 to Ala led to nuclear localization of GFP-tagged AID, suggesting that this NES mediates the cytoplasmic localization of GFP-tagged AID and, likely, endogenous AID.

As a DNA or pre-RNA deaminase, AID would perform its function in the nucleus. It is possible that the low AID level in the nucleus is sufficient to exert its deamination activity in germinal center B cells. However, it is unlikely that the larger amount of AID localizing in the cytoplasm is turned over without expressing its activity, thereby raising the question of when and how AID is translocated from the cytoplasm into the nucleus. It is suggested that a bipartite nuclear localization signal (NLS) at the amino-terminus of human and mouse AID, together with the carboxyl-terminal NES, effectively regulates the dynamic shuttling of AID between the cytoplasm and the nucleus¹⁸³. However, the existence of such NLS has not yet been independently verified^{184,185}.

Although the molecular mechanism that governs the balance of AID nuclear import/export is unclear, it is conceivable that such a molecular mechanism provides B cells with two important functional features to: (i) hamper the highly active and mutagenic AID from accessing its substrate, DNA or pre-RNA, thereby preventing indiscriminate cytidine deamination, DNA damage and/or insertion of deleterious mutations, as suggested by the higher SHM rate brought by an AID mutant lacking the NES on an artificial DNA substrate^{183,184}; and (ii) generate a cytoplasmic AID reservoir, in which AID could be assembled with either SHM or CSR co-factors into a protein complex that, upon delivery of appropriate stimuli, can be promptly translocated into the nucleus and preferentially targeted to the Ig locus (Figure 5). Such a fast-kinetic nuclear localization of AID is suggested by the rapid nuclear accumulation of red fluorescent protein (RFP)-tagged AID when cells are treated with agents causing DSBs¹⁸⁵. It would be important to identify the stimulus that directs the nuclear localization of AID and analyze its role in regulating SHM and CSR during the germinal center reaction.

3.3.3. Phosphorylation—Recombinant AID displays deaminase activity on single-stranded, but not double-stranded, DNA substrates^{176,178}. When actively transcribed, double-stranded DNA can be effectively deaminated by phosphorylated AID, but not by non-phosphorylated AID^{174,176}, suggesting that phosphorylation plays an important role in targeting AID to its natural substrates, i.e., functionally single-stranded DNA, as occurring in actively transcribed Ig V(D)J and S regions. When expressed in HEK293 cells, AID is phosphorylated at a much lower degree, as compared to AID protein purified from *in vitro* activated mouse spleen B cells^{118,174,186} (Figure 4), suggesting a B cell-specific phosphorylation of AID. AID phosphorylation may explain the apparent size heterogeneity of the AID-containing complexes, ranging from 20 kDa to 70 kDa, in the B cell nucleus¹⁷⁴.

In activated B cells, AID can be phosphorylated at Ser38 and Tyr184^{118,174,186}. The functional meaning of AID Tyr184 phosphorylation is unclear. PKA-mediated phosphorylation of Ser38, which is located within a consensus PKA phosphorylation site RRxS^{118,186,187}, is critical for AID to interact with the RPA complex, which potentiates AID deamination of transcribed double-stranded DNA^{174,186,188–190}. Impaired CSR in *in vitro* stimulated *aicda*^{−/−} B cells can be rescued by wildtype AID, but not by an AID molecule carrying a Ser38Ala mutation. An AID Ser38Asp mutant, which likely mimics the constitutively phosphorylated AID at Ser38, is defective in rescuing CSR in *aicda*^{−/−} B cells^{118,174,186}, suggesting that a dynamic phosphorylation/dephosphorylation process is critical in regulating AID activity. Further, in CH12F3 mouse B cells treated with PKA inhibitors, CSR to IgA is reduced by five folds, as compared to similar cells untreated or treated with inhibitors of other kinases, such as PI 3-K. Conversely, in *in vitro* stimulated B cells that are deficient in PKA R1 α , one of the two inhibitory subunits of the PKA holoenzyme, and displaying a PKA kinase activity increased by four folds, CSR is significantly upregulated, further underlying the important role of PKA in regulating AID activity and CSR¹⁸⁷. Both Ser38Ala and Ser38Asp AID mutants display a reduced activity in mediating SHM of an artificial substrate, suggesting that AID phosphorylation at Ser38 is also critical in regulating the SHM process¹¹⁸.

Only a fraction of all AID molecules is phosphorylated at Ser38¹¹⁸. However, phosphorylated AID is enriched in the chromatin-associated nuclear fraction, as compared to the cytoplasmic and soluble nuclear fractions¹¹⁸, suggesting that phosphorylated AID is the active form of AID that exerts its DNA deaminase function in the nucleus. This selective enrichment of phosphorylated AID to chromatin likely results from either AID phosphorylation by PKA localizing in the nucleus or selective import of phosphorylated AID from the cytoplasm to the nucleus. Transiently expressed Flag-tagged AID specifically bound to S region DNA and to RNA polymerase II, thereby linking germline I_H-C_H transcription with AID targeting to S region DNA¹⁹¹. It is possible that phosphorylated endogenous AID preferentially targets V(D)J or S region DNA, as compared to non-phosphorylated AID. If AID phosphorylation and its nuclear localization are indeed linked, there may exist a phosphatase that dephosphorylates AID shortly after the dissociation of AID from V(D)J DNA and/or export of AID out of the nucleus (Figure 5). How PKA and, possibly, the putative AID phosphatase, in B cells are activated by CD40, IL-4R, BCR and/or TLR signals remains to be determined. Finally, if phosphorylation precedes AID dimerization/oligomerization, both phosphorylated and non-phosphorylated forms of AID would be present in the AID dimer/oligomer, which would be less active than an AID dimer/oligomer containing only the phosphorylated form. It is likely that AID dimerization/oligomerization occurs before phosphorylation and only a selective fraction of AID dimer/oligomers are phosphorylated (Figure 5).

4. Conclusions

The highly active nature and tightly regulated levels of AID in B cells account for the significant, albeit transient, burst of events leading to SHM and CSR in germinal centers. Restricted *aicda* expression in germinal center B cells is induced by specific stimuli from CD4⁺ T cells and/or invading microbial pathogens and is effectively downregulated by Blimp1 during plasmacytoid differentiation. AID induction is likely mediated by the formation of an enhanceosome consisting of specific transcription factors upregulated in centroblasts, including Pax5, HoxC4/Oct1/2/OcaB, E47 and Irf8. Blimp1 likely exerts its transcription repressor functions on multiple genes, thereby effectively shutting down *aicda* expression. A major fraction of AID is non-phosphorylated and confined to the cytoplasm, a compartment where AID cannot access DNA or pre-RNA, thereby avoiding its potential genotoxic effects. In spite of our knowledge about regulation of *aicda* expression and AID

activities, several important issues remain to be addressed, including: (i) identifying the dynamic composition of the *aicda* enhanceosome. To address this issue, a high-resolution proteomic approach would be required to analyze how expression of transcription factors, such as Pax5, HoxC4, E47, Irf4, Irf8 and Blimp1, changes from centroblasts in the dark zone to centrocytes in the light zone and then to plasmablasts, and systematically analyzing the role of these transcription factors in SHM and CSR; (ii) addressing how PKA and, possibly, a putative AID phosphatase, are regulated to modulate AID activity, SHM and CSR; (iii) defining the modulation of AID protein level and activity¹⁷⁰; and, finally, (iv) unraveling the mechanism(s) mediating the specific targeting of AID to the Ig locus, possibly as a result of multiple interactions between Ig *cis*-elements and germinal center-specific *trans*-factors.

Acknowledgments

Owing to space limitations, we could cite only a fraction of the papers relevant to the topic discussed in this review article. We apologize to the authors of the publications that are not cited here. This work was supported by NIH grants AR 40908, AI 45011 and AI 60573 to P.C.

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FIGURE 1.

Induction of *aicda* expression by activated B cell surface molecules. From left to right: (i) the TLR4-MD2-CD14 complex is the receptor for LPS or other bacterial glycoproteins; TLR9 is located in endosomes and recognizes CpG DNA from microbial pathogens. TLR4 and TLR9 associate with adaptor protein MyD88 or Trif through the Tir domain, depicted in yellow; (ii) the BCR complex containing BCR, Ig α and Ig β is crosslinked by physically-linked antigens. The CD19/CD21/CD81 co-receptor is activated by the C3d(g) complement cleavage product and modulates the BCR signaling threshold. CD81 is a co-receptor for HCV E2 envelope protein; (iii) cytokine receptors IL-4R and TGF- β R mediate *aicda* induction and CSR. IL-4R-triggered signaling can be inhibited by activated CD45; and (iv) among the TNF receptor family of B cell surface molecules, CD40 is engaged by CD154 expressed on CD4⁺ T cell surface and exists as a trimer at B cell surface. CD30, when engaged by CD153 expressed CD40⁺ T_H cell surface, negatively regulates CD40 signaling. TACI, BAFF-R and BCMA are receptors for BAFF and/or APRIL secreted by dendritic cells and can mediate CSR in B cells of mucosal areas. Activated intracellular signal transduction pathways likely converge to assemble an enhanceosome, which would include NF- κ B, Stat6, Pax5, E47, the Irf8/SpiB complex and the HoxC4/Oct1/2/OcaB complex, at the *aicda* locus to induce efficient *aicda* expression. The arrows depict the transcription initiation of *aicda* or other germinal center B cell-specific genes.

FIGURE 2.

Germinal center B cell differentiation, including SHM and CSR, stage-specific expression of AID, and positive regulators (depicted in blue) and negative modulators (depicted in gray) of *aicda* gene transcription. AID expression initiates in early centroblasts, is maximal in full-blown centroblasts, significantly decreases in centrocytes and extinguishes in plasma cells. The expression profile of AID and transcription factors in memory B cells is not shown here. The dual role of OcaB and Irf4 is dependent on different germinal center reaction stages. Most data are derived from studies in human tonsil or mouse spleen B cells and plasma cells. The expression profile of Id2 was not available and is deduced from those of Irf4 and Bcl6, two putative *id2* transcription regulators.

FIGURE 3. Downregulation of *aicda* expression by Blimp1 and B cell plasmacytoid differentiation. **(a)** Expression profiles of genes that regulate *aicda* expression in germinal center B cells (left panel) and plasmacytes (right panel). Positive regulators and negative regulators of *aicda* transcription at each stage of the germinal center reaction are depicted in solid green and red circles, respectively. Broken circles depict genes with a low steady-state expression level. Solid and broken arrows/lines indicate direct and indirect regulation of gene expression, respectively. In germinal center B cells, multiple transcription factors synergize to induce *aicda* expression. In plasmacytes, the transcription suppressor Blimp1 downregulates expression of both *aicda* and the positive regulators of *aicda* expression, such as *pax5*, *e2a* and *irf8* and upregulates negative regulators of *aicda* expression, such as *id2*. Irf4 switches from a positive to a negative regulator of *aicda* expression during plasmacytoid differentiation and its gradient-like expression pattern in light zone B cells is depicted as a light green circle. *c-myc* is one of the germinal center B cells-specific genes and *cd138* (*syndecan1*); other genes involved in protein folding and secretion are highly expressed in plasmacytes. **(b)** Blimp1-binding site in the promoter of its direct target genes: “*” denotes predicted Blimp1-binding sites and “*” denotes the consensus Blimp1-binding motif¹³⁵. Underlined nucleotides are present in all Blimp1-binding sites and likely contribute to Blimp1-DNA interaction that is essential for the transcription regulation activity of Blimp1.

FIGURE 4.

Predicted three-dimensional structure of the human AID dimer (Egest, Xu and Casali, unpublished data). The structure of amino acid residues 11 to 180 of AID is modeled, as based on the solved crystal structure of the human Apobec2 (PDB No: 2NYT) (33.3% identity and 44% homology). The structure of the carboxyl-terminal NES (amino acid residues 184–198) is modeled, as based on the solved crystal structure of the human p53 tetramerization domain (PDB No: 1C26) (33% homology), which contains a helical NES. The Pro 183 of AID likely hampers the formation of a long helix spanning carboxyl-terminal amino acid residues 168–198. Shown are the side chains of His56, Glu58, Cys87 and Cys90, which forms the active site in the AID cytidine deamination domain. His56, Cys87 and Cys90 coordinate with a Zn^{2+} ion, which is essential for the AID activity. The conformational change of the $\beta'1$ -turn from a loop to a hairpin, as shown in purple, likely controls the access of the AID active site to DNA/RNA substrates. The $\beta 2$ strand (amino acid residues Ser 41 to Asn 51) of each AID monomer mediates the dimerization. Shown is the side chain of Ser38, the PKA phosphorylation site.

FIGURE 5.

An integrated model of AID post-translational modifications. AID forms dimers/oligomers spontaneously. Most AID molecules localize in the cytoplasm, likely through active nuclear export, as effected by CRM1. Phosphorylation of cytoplasmic AID dimers by PKA would result in efficient nuclear import. After localizing to the nucleus, phosphorylated AID dimers binds to RPA and a putative AID cofactor, depicted as a dimer, to effect its DNA deaminase functions. Alternatively, PKA-mediated phosphorylation of cytoplasmic AID dimers increases AID binding to the putative cofactor before translocation into the nucleus, where the AID-cofactor complex binds to RPA and then deaminates dC in DNA. After deamination, AID is released, possibly dephosphorylated by a phosphatase and then exported out of the nucleus to replenish the cytoplasmic AID reservoir.

Table 1

Apobec/AID cytidine deaminase family of proteins in the human and the mouse

Group	Chromosomal location	Enzymatic activities	Putative function
Apobec1	hu Chr. 12 mo Chr. 6	C ⁶⁶⁶⁶ →U in ApoB mRNA ¹⁹² ; dC→dU in single-stranded DNA (weak) ¹²	Determines tissue-specific expression of the truncated ApoB protein ¹⁹²
Apobec2	hu Chr. 6 mo Chr. 17	Unknown; No deamination activity	Unknown
Apobec3 ^a			
Apobec3B	hu Chr. 22	Unknown	Inhibits HIV infection moderately
Apobec3C	hu Chr. 22	dC→dU in DNA (weak) ¹⁹³	Inhibits HIV infection ¹⁹³
Apobec3F	hu Chr. 22	dC→dU in nascent HIV minus-strand DNA; deamination-independent ^{12,194}	Inhibits HIV infection; Inhibits HBV pregenomic RNA reverse transcription ^{12,194}
Apobec3G	hu Chr. 22	dC→dU in nascent HIV minus-strand DNA; deamination-independent ^{12,194}	Inhibits HIV infection; Inhibits HBV pregenomic RNA reverse transcription ^{12,194}
Apobec3	mo Chr. 15	deamination-independent ¹⁹⁵	Inhibits MMTV infection ¹⁹⁵
AID	hu Chr. 12 mo Chr. 6	dC→dU in single-stranded DNA ^{178,179} ; dC→dU in transcribed double-stranded DNA ¹⁷⁴ ; C→U in putative pre-RNA ¹³ ; deamination-independent ¹⁹⁶	Mediates SHM and CSR ^{7,8} ; Limits infection by transforming retroviruses ¹⁹⁶

^aIn the human, there are at least eight Apobec3 group proteins, from Apobec3A to Apobec3H, and their genes are clustered; only the four best studied Apobec3 proteins are reported here. In the mouse, there is only one Apobec3 protein.

Table 2

Molecular basis of HIGM syndrome

Molecular deficiency	<i>aicda</i> expression	AID activity	Phenotypes
CD154 (CD40L)	Impaired	Not determined	Defective CSR; defective germinal center formation and SHM
CD40	Impaired	Not determined	Defective CSR; defective germinal center formation and SHM
NEMO ^a	Not affected	Not determined	Defective CSR; normal SHM; ectodermal dysplasia
AID ^b	Not affected	No AID protein expression; loss of deaminase activity; loss of binding to CSR cofactors ^c	Defective CSR; defective SHM ^c ; lymphoid hyperplasia; defective plasmacytoid differentiation
UNG	Not affected	Not affected	Defective CSR; altered SHM spectrum
ATM	Not affected	Not affected	Defective CSR; normal SHM; accelerated lymphomagenesis
MRE11-NBS1	Not determined	Not determined	Defective CSR; likely defective SHM; accelerated lymphomagenesis

^a NEMO deficiency leads to a defective NF-κB activation;

^b More than 20 AID mutations have been identified. They can be segregated into three groups: autosomal recessive type (AR-AID), AID with carboxyl-terminal mutations and AID with carboxyl-terminal deletions;

^c AID with carboxyl-terminal deletions cannot bind to putative CSR co-factors, but displays normal dC deamination activity and mediates SHM.

Table 3

Transcription factors and their putative binding sites in the murine *aicda* promoter^a and intronic enhancer region^b

Transcription factor	Inducing stimuli	Binding sites	Sequence	Reference
NF-κB	CD154; LPS/IL-4; BCR crosslinking	-1203 ~ -1193	5' GGGATTTCCTCC 3'	75
		-1267 ~ -1258	5' GGGTCTTTCC 3'	
Stat6	IL-4	-1327 ~ -1319	5' TTCCACAA 3'	75
Pax5	ND	-97 ~ -85	5' GGATACCTGGTGG 3'	143
		-68 ~ -57	5' GGGGAGGAGCCC 3'	
E47	LPS; CD154/IL-4; BCR crosslinking/IL-4	-105 ~ -100	5' CAGATG 3'	143,147
		+1642 ~ +1647	5' CAGCTG 3'	
		+1654 ~ +1659	5' CAGCTG 3'	
HoxC4/Oct HoxC4 ^c	CD154/IL-4; LPS/IL-4; CD154/IL-4/TGF-β	-20 ~ -13	5' ATTTGAAT 3'	144
		-169 ~ -166	5' ATTT 3'	
		-615 ~ -612	5' ATTT 3'	
		-600 ~ -597	5' AAAT 3'	
Irf8 Blimp1	Not determined	-607 ~ -600	5' AAAAGAAA 3'	163
	LPS; IL-2/IL-5; IL-6; BCR crosslinking/CD154/IL-4/ IL-5	-593 ~ -584	5' TTAGAGAATT 3'	136

^aThe promoter region is defined as the 1500 bp-DNA upstream the putative transcription initiation site⁹.

^bThe two putative enhancer regions within the first intron were previously^{143,147}; the *cr1* encompasses about 360 bp DNA (+300 ~ +660) and the *cr2* encompasses about 230 bp DNA (+1570 ~ +1800).

^cThere are six more putative HoxC4-binding site at the -900 ~ -400 region, ten more in the *cr1* and two more in the *cr2* region, respectively. The three HoxC4 sites as shown are close to the binding site of E47 or Irf8 and likely play a role in the assembly of the *aicda* enhanceosome.