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Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFκB

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Abstract

Activation-induced cytidine deaminase (AID) is an inducible gene that plays an important role in class switch recombination, somatic hypermutation and gene conversion in B cells. We examined the regulation of AID gene expression in human and mouse B cells by IL-4 and CD40 ligation. IL-4 by itself and, to a much lesser extent, CD40 ligation induced AID mRNA expression in primary B cells. The two stimuli strongly synergized in inducing AID mRNA and protein expression. IL-4 induced STAT6 binding to a site in the 5′ upstream region of the AID gene, while CD40 ligation induced NFκB binding to two sites in that region. B cells from STAT6$^{-/-}$ mice failed to up-regulate AID in response to IL-4, while B cells from p50$^{-/-}$ mice were impaired in their ability to up-regulate AID in response to CD40 ligation and IL-4. These results suggest that signals delivered via CD40 that activate NFκB synergize with signals delivered via the IL-4 receptor that activate STAT6 to induce optimal AID gene expression.

Introduction

While B cells develop and mature, Ig genes undergo different molecular processes using recombination, mutation and rearrangement in order to broaden the diversity of antibody response. Early in B cell development, V(D)J recombination occurs to bring together V, D and J segments in different combinations, thus increasing the repertoire of antibodies (1). Later in B cell development, following antigen stimulation, nucleotide changes can be introduced to variable regions through a process known as somatic hypermutation (SHM) (2). In some species, like chicken and rabbits, gene conversion is the dominant mechanism for antibody diversification (2). In response to antigen, mature B cells can also alter their heavy chain usage by a unique DNA deletional recombination process known as class switch recombination (CSR) (3–5). This allows a change in the biological effector functions of the antibody, without altering its antigen-binding specificity.

While the molecular processes of V(D)J recombination have been well characterized (1), this is not the case for SHM and CSR. CSR occurs between tandemly repeated sequences within the switch region located upstream of each constant heavy chain gene allowing switching to downstream Ig classes (IgG, IgA and IgE) (5). Two distinct signals are essential for successful isotype switching. One signal is delivered by cytokines and causes the transcription of specific isotype C_H genes from promoters located upstream of the switch regions, targeting them for switch recombination (3,5,6). For instance, IL-4 targets C_ε and $C_\gamma 4$ in humans, and C_ε and $C_\gamma 1$ in mice. The second signal is provided by the interaction of B cell surface antigen CD40 and its ligand (CD40L/CD154), which is expressed on activated T cells, leading to deletional switch recombination (7). The role of CD40–CD40L interactions in isotype switching is impaired in patients with mutations in CD40L (8) and CD40 (9) as well as in CD40$^{-/-}$ (10,11) and CD40L$^{-/-}$ mice (12). Recently, interaction between BAFF and its homologue APRIL expressed by dendritic cells and their receptors on B cells have been

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shown to also provide second signal for CSR for human B cells (13) Finally, in mice lipopolysaccharide (LPS) provides the second signal to B cells to undergo class switching (6).

Activation-induced cytidine deaminase (AID) is expressed specifically in germinal center B cells (14). AID was identified by subtractive hybridization between the IL-4, transforming growth factor-β- and CD40-stimulated and unstimulated murine B cell lymphoma cell line, CH12F3-2, which is induced to switch to IgA secretion by these stimuli (14). The deduced amino acid sequence of AID shows homology to APOBEC-1, which is a catalytic subunit of the RNA editing complex for the apolipoprotein B mRNA (14). APOBEC-1 converts a cytosine at position 6666 in apolipoprotein B mRNA to uracil, creating a stop codon in the mRNA. This results in the expression of a truncated protein with a different function. The human APOBEC-1 and AID genes are closely mapped to chromosome 12p13 (15). The similarities between these two genes, the observation that recombinant AID has *in vitro* cytidine deaminase activity and the requirement for *de novo* protein synthesis for an event downstream to AID expression in CSR (14,16), have suggested that AID might be an RNA editing enzyme which can give rise to a protein that functions as a class switch recombinase. However, recent data has shown that AID catalyzes deamination of dC residues on single-stranded DNA *in vitro*, but not on double-stranded DNA, RNA–DNA hybrids or RNA (17,18).

A role for AID in CSR, SHM and gene conversion is supported by the observations that AID alone is able to induce CSR as well as SHM in transfected target genes in non-B cells like fibroblasts (19,20). The critical role of AID in CSR and SHM has been established by the observation that both are severely impaired in AID$^{-/-}$ mice (15) and in patients with autosomal recessive hyper-IgM syndrome (HIGM2) that carry mutations in the AID gene (21). We examined the mechanisms for AID gene expression and up-regulation in B cells. We provide evidence that signals delivered via CD40 that activate NFκB synergize with signals delivered via the IL-4 receptor that activates STAT6 to induce AID gene expression.

Methods

Preparation and culture of human B cells and B cell lines

Human peripheral blood mononuclear cells (PBMC) were purified from heparinized blood obtained with informed consent from healthy adults by density gradient centrifugation on Ficoll-Hypaque as described previously (22). B cells were isolated using the human B cell negative isolation kit (Dynal, Oslo, Norway). Human tonsils were obtained from patients undergoing tonsillectomy and minced into single-cell suspensions. B cells were isolated by E-rosetting followed by Ficoll-Hypaque centrifugation as previously described (23). Preparations consisted of >95% CD19$^+$ cells and <1.5 CD3$^+$ cells. The Epstein–Barr virus-negative, sIgM$^+$ human B cell lines BL-2 and BL-41 were maintained in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 10% heat-inactivated FCS (complete medium) in a 5% CO_2 atmosphere as previously described (24). PBMC, tonsil B cells and cell lines were cultured in complete medium for 4 days in medium alone and in the presence of IL-4 (5 ng/ml; R & D Systems, Minneapolis, MN), anti-CD40 mAb 626.1 (5 μg/ml; a kind gift of S. M. Fu, University of Virginia, Charlottesville, VA) and IL-4 + anti-CD40.

Preparation and culture of mouse B cells

The following mice strains were used: BALB/c, C57BL/6/129, IL-4Rα$^{-/-}$ (25), CD40$^{-/-}$ (10), STAT-6$^{-/-}$ mice (26) and p50$^{-/-}$ mice (27) and their littermate controls (BALB/c for STAT-6$^{-/-}$ and IL-4Rα$^{-/-}$; C57BL/6/129 for CD40$^{-/-}$ and p50$^{-/-}$ mice). B cells were purified from spleen cell suspensions using Dynabeads M-280 Streptavidin (Dynal) after incubation with a biotinylated antibody cocktail of Thy1.2 (53–2.1), Mac1 (M1/70), Gr-1 (RB6-8C5) and anti-CD43 (PharMingen, La Jolla, CA). Purified cells were suspended in RPMI containing 10% FCS, L-glutamine and 50 μM β-mercaptoethanol, and cultured in medium alone, IL-4 (10–20 ng/ml; R & D Systems), hamster IgM anti-mouse CD40 (anti-CD40-IgM; 1 μg/ml) and IL-4 + anti-CD40-IgM for 4 days. The purity of B cells was analyzed by FACS, using appropriate FITC- and phycoerythrin (PE)-conjugated antibodies directed to B (CD19) and T (CD3) cell lineage-specific antigen as previously described (7). Mouse preparations consisted of >95% CD19$^+$ cells and <1.5 CD3$^+$ cells.

Northern blot analysis

Total RNA was extracted from cells using TRIzol reagent, as described by the manufacturer (Gibco/BRL, Gaithersburg, MD) and 10–20 μg was subjected to northern blotting using ^{32}P-labeled AID and GAPDH probes. Human and mouse AID PCR products generated by RT-PCR were used as probes. For human AID, the following primers which amplified the entire coding region were used in the PCR reaction: forward 5′-gaggcaagaagacactctgg; reverse 5′-gtgacattcctgggaagttgc. To generate a mouse AID probe as well as human and mouse GAPDH probes by RT-PCR, PCR primers were used as described previously (8,25,29). Northern blot hybridizations were performed according to standard procedures. The intensities of the AID mRNA and GAPDH mRNA bands were assessed by densitometry and quantitated by using the NIH Image program 1.62. AID mRNA expression was calculated as the ratio of the intensity of the AID band to the GAPDH band.

Preparation of nuclear extracts and oligonucleotide probes for electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from 1×10^7 BL-2 and BL-41 cells, untreated or stimulated with IL-4 (5 ng/ml) and/or anti-CD40 mAb (5 μg/ml) for 30 min as described previously (25). Protein concentrations of the nuclear extracts were determined by the BCA protein assay (Pierce, Rockford, IL). The 3.9 kb 5′ upstream of the human AID gene mRNA initiation site were analyzed by the Transfac program. EMSA analysis was performed using a 22-bp oligonucleotide that spanned potential STAT6- and NFκB-binding sites in the 5′ upstream of the human AID gene. For STAT6, we used ttgtagTTCccaCAAttccc corresponding to bp –1334 to –1315 (capital letters refer to the core binding sequence). For the proximal NFκB site, we used tataaaGGGattTCCCcctgc corresponding to bp –1209 to –1188. For the distal NFκB site, we used

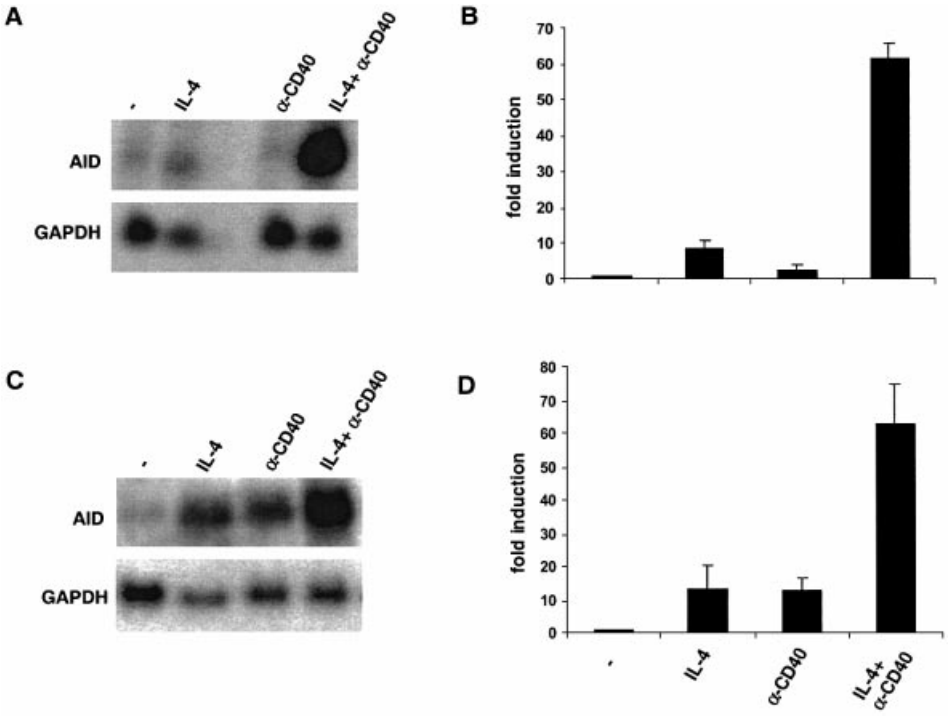

Fig. 1. Induction of AID mRNA expression in human peripheral and tonsil B cells. Northern blot analysis of a representative experiment and bar graph of mean ± SD fold induction of AID mRNA expression from three independent experiments using human blood B cells (A and B) and tonsil B cells (C and D). Cells were either left unstimulated or stimulated for 4–5 days with IL-4 (5 ng/ml), anti-CD40 (5 μg/ml) and IL-4 + anti-CD40; 20 μg of total RNA was loaded per lane. Filters were hybridized first with human AID cDNA and then with human GAPDH cDNA as loading control.

gggggtGGGtctTTCCcatgc corresponding to bp −1273 to −1253. Complementary oligonucleotide pairs were annealed, labeled and purified on polyacrylamide gels as described previously (25). Annealed double-stranded oligonucleotides were end-labeled using [^{32}P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase (Gibco/BRL). Free radioactivity was removed by chromatography over Sephadex G-25 columns (Amersham Biosciences, Uppsala, Sweden).

EMSA and supershift assays

EMSA and supershift assays for STAT6 and NFκB/Rel were performed as described previously (25). Briefly, for STAT6 analysis, nuclear extracts (5 μg) were added to binding buffer (10 mM Tris, pH 8, 0.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT), with poly(dI–dC) (Amersham Biosciences; 5 μg) and ^{32}P-labeled probe (100,000 c.p.m./lane), in the presence or absence of 100-fold excess competitors. NaCl and glycerol were adjusted to 0.1 M and 10% respectively. After a 30-min incubation on ice, the binding reactions were run on a 4% polyacrylamide gel (acrylamide:bisacrylamide, 30:1) in 0.5% TBE buffer (50 mM Tris, 50 mM boric acid and 1 mM EDTA, pH 8) and 4% glycerol at 30 mA for 2–3 h at 4°C. For NFκB/Rel analysis, the binding buffer contained 10 mM Tris, pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol. NaCl and glycerol were adjusted to 40 mM and 4% respectively. After a 30-min incubation on ice, binding reactions were run on a 6% polyacrylamide gel in 0.5% TBE buffer at 30 mA for 2–3 h at 4°C.

Supershifts were performed by pre-incubating nuclear extracts with the appropriate antisera (2–4 μg/reaction) for 30 min on ice before adding the probe. Consensus STAT6 oligonucleotide, rabbit polyclonal IgG against human STAT6 (sc-621 X), NFκB p50 (sc-114 X), NFκB p65 (sc-109 X), RelB (sc-226 X), c-Rel (SC-70) and control rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Consensus NFκB and Oct-1 oligonucleotides were purchased from Promega (Madison, WI).

Western blotting for AID

To investigate AID protein expression in IL-4- and/or anti-CD40-stimulated B cells western blot analysis was performed on lysates of 1–2 × 10^6 cells, using a rabbit antibody raised against a peptide derived from the C-terminus of AID (19).

Results

CD40 ligation synergizes with IL-4 in inducing AID expression in human primary B cells

We examined the expression of AID mRNA in human peripheral blood B cells (>95% CD19$^+$ cells) by northern blot analysis. There was very little or no detectable expression of AID in unstimulated B cells (Fig. 1A). Stimulation with IL-4 for 5 days induced weak expression of AID mRNA, which migrated as a single 2.9-kb band. CD40 ligation using anti-CD40 mAb induced weak or no AID mRNA expression. However, CD40 ligation strongly synergized with IL-4 in the induction of AID

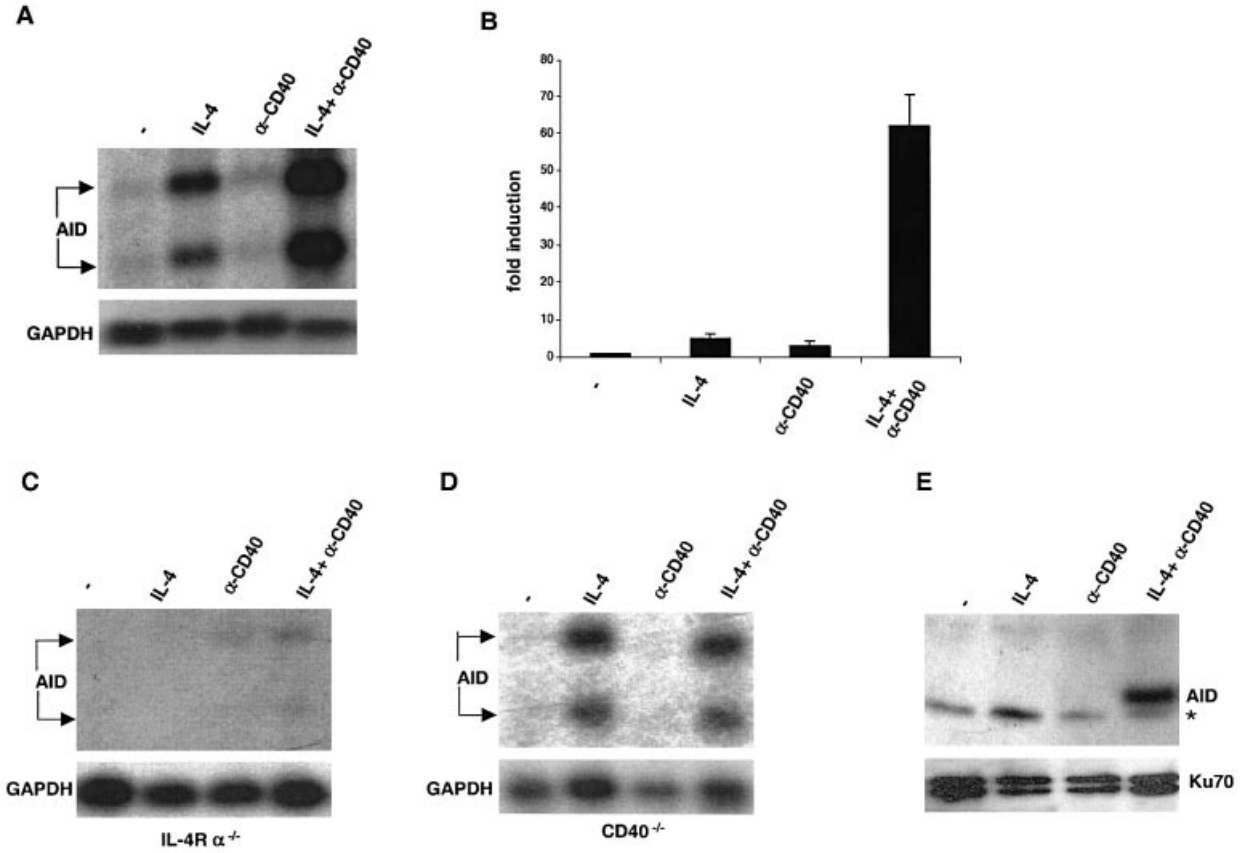

Fig. 2. Induction of AID mRNA and protein expression in murine splenic B cells. (A) Northern blot analysis of a representative experiment. Splenic B cells from BALB/c mice were either left unstimulated or stimulated for 4 days with IL-4 (10 ng/ml), anti-CD40 (1 μg/ml) and IL-4 + anti-CD40; 20 μg of RNA was loaded per lane. Filters were hybridized first with murine AID cDNA and then with murine GAPDH cDNA as a loading control. (B) Bar graph of mean ± SD fold induction of AID mRNA expression from three independent experiments. (C and D) Northern blot analysis of spleen B cells from IL-4Rα$^{-/-}$ mice and CD40$^{-/-}$ mice. (E) Western blot analysis of AID protein expression in lysates of spleen B cells from BALB/c mice. The band labeled with an asterisk migrating immediately below the AID band is a non-specific band, because it was not consistently observed and was sometimes detected in lysates from splenic B cells of AID$^{-/-}$ mice. The bottom panel shows Ku70 protein expression as loading control. Similar results were obtained in three experiments.

mRNA. The mean fold induction of AID mRNA expression of three independent experiments was 8.4-fold with IL-4, 2.5-fold with anti-CD40 and 61.5-fold with CD40 + IL-4 (Fig. 1B). Similar results were obtained in tonsil B cells (Fig. 1C), with the exception of a stronger induction of AID expression by anti-CD40 (12.7 ± 4.0-fold compared to 2.5 ± 1.5-fold for peripheral blood B cells). The mean fold induction of AID mRNA expression of three independent experiments was 13-fold with IL-4, 12.7-fold with anti-CD40 and 63-fold with CD40 + IL-4 (Fig. 1D). These results suggest that CD40 ligation synergizes with IL-4 in inducing AID gene expression in human primary B cells.

CD40 ligation synergizes with IL-4 in inducing AID expression in murine primary B cells

We examined whether CD40 ligation and IL-4 synergize in inducing AID gene expression in primary mouse B cells. The availability of mice with deletions of genes that may be important for the regulation of AID expression prompted us to examine AID induction in murine splenic B cells. We examined the expression of AID mRNA in purified splenic B cells from BALB/c mice. Northern blot analysis revealed that IL-4 and, to a much lesser extent, CD40 ligation induced expression of AID mRNA, which migrated as two bands, 2.4 and 1.2 kb in size (Fig. 2A). CD40 ligation strongly synergized with IL-4 in inducing AID mRNA expression. Figure 2(B) depicts the mean fold induction of AID mRNA expression of three independent experiments. These results confirm those obtained with human B cells.

LPS has been reported to induce AID expression in murine B cells (15). To ensure that IL-4, and not contamination by LPS, is responsible for induction of AID mRNA expression by the recombinant IL-4 preparation, we examined its effect on B cells from IL-4Rα$^{-/-}$ mice. Figure 2(C) shows that splenocytes from IL-4Rα$^{-/-}$ mice (26) failed to express AID mRNA following IL-4 stimulation. Furthermore, IL-4 failed to synergize with anti-CD40 in inducing AID mRNA expression in these cells (2.5 ± 1.0-fold increase with anti-CD40 stimulation versus 2.4 ± 1.0-fold increase with anti-CD40 + IL-4 in AID mRNA expression).

To examine whether IL-4 induction of AID depends on prior CD40 ligation *in vivo*, we examined splenic B cells from CD40$^{-/-}$ mice (10). Figure 2(D) shows the IL-4-induced

expression of AID mRNA in splenocytes from CD40$^{-/-}$ mice. As expected, anti-CD40 failed to induce AID expression and did not synergize with IL-4-mediated AID expression in these cells. These results suggest that IL-4 induces AID gene expression independently of CD40.

To examine if AID mRNA expression is accompanied by AID protein expression, we performed western blotting of lysates from splenic B cells using rabbit anti-AID antibody. AID protein expression was not detectable in unstimulated B cells. There was faint and not always detectable AID protein expression following IL-4 stimulation, and no detectable AID protein expression following CD40 ligation. IL-4 and CD40 ligation synergized to induce strong AID protein expression (Fig. 2E).

IL-4 induces STAT6 binding to a site in the 5′ upstream region of the AID gene

Phosphorylation and nuclear translocation of the transcription factor STAT6 following IL-4 stimulation has been shown to mediate many of the effects of IL-4-induced gene up-regulation (30). Analysis of the 3.9 kb 5′ upstream of the human AID gene revealed a single potential STAT-binding site at position –1328 to –1320 bp (TTCccaCAA). This site differs from the canonical STAT binding site (TTCxxxGAA) at only one nucleotide position. We used EMSA to examine whether IL-4 causes STAT6 binding to a synthetic oligonucleotide that corresponds to a 22-bp sequence in the AID gene that spans this site (–1334 to –1315 bp). We used the human B cell line BL-2 in these experiments because the preparation of nuclear extracts for EMSA requires relatively large numbers of cells. BL-2 was chosen because, among seven B cell lines tested (Raji, NALM6, Reh, BL-2, Ramos, BJAB and BL-41), it had negligible baseline AID mRNA expression and was up-regulated after IL-4 stimulation (Fig. 3A).

Figure 4(A) shows that addition of nuclear extract from IL-4-stimulated cells to the oligonucleotide derived from the AID gene gives rise to a retarded complex. This complex was inhibited by 100-fold excess of a cold 22-bp oligonucleotide that spans the consensus STAT6-binding sequence derived from the C_ε gene promoter (TTCccaaGAA), as well as by 100-fold excess of self-competitor (data not shown), but not by a 22-bp OCT-1 oligonucleotide (Fig 4A). To ascertain that the nuclear complex induced by IL-4 contained STAT6, we performed a supershift assay. Figure 4(B) shows that the complex was supershifted by a rabbit antibody to STAT6, but not by control rabbit IgG. These results suggest that IL-4-activated STAT6 binds to a 5′ STAT-like sequence in the AID upstream promoter region.

CD40 ligation induces NFκB binding to two sites in the 5′ upstream region of the AID gene

CD40 ligation causes nuclear translocation and activation of NFκB in B cells (31). Two potential NFκB sites were found in the 3.9-kb 5′ upstream region of the AID gene at positions –1203 to –1193 (GGGattTCCC) (proximal NFκB site) and –1267 to 1258 (GGGtctTTCC) (distal NFκB site) respectively. To examine if CD40 ligation induces binding to these sites, we used the BL-41 cell line because, among the seven B cell lines tested, it had low baseline AID mRNA expression, which was modestly up-regulated by IL-4, but strongly up-regulated upon addition of anti-CD40 (Fig. 3B).

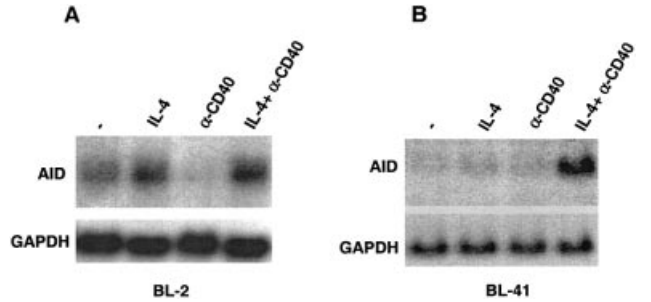

Fig. 3. Induction of AID mRNA expression in BL-2 and BL-41 human B cell lines. Northern blot analysis in the human B cell lines BL-2 (A) and BL-41 (B) following stimulation with IL-4 (5 ng/ml), anti-CD40 (5 μg/ml) and IL-4 + anti-CD40 for 4 days. Similar results were obtained in three experiments.

Nuclear extracts from unstimulated BL-41 cells bound to a 22-bp oligonucleotide that spanned the proximal (–1203 to –1193) putative NFκB site in the 5′ upstream region of the AID gene, giving rise to two retarded bands (arrows in Fig. 5A). CD40 stimulation caused the appearance of two additional slower migrating bands (stars in Fig. 5A). The intensity of bands increased following anti-CD40 + IL-4 induction. The entire complex of bands was inhibited by 100-fold excess of the Igκ gene enhancer NFκB consensus sequence (Fig. 5A), as well as by 100-fold excess of cold self-competitor, but not by the OCT-1 oligonucleotide (data not shown). To ascertain that the nuclear complex induced by CD40 ligation contains NFκB, we performed a supershift assay. Figure 5(A) shows that the entire complex of bands was supershifted by rabbit antibody to p50. The two inducible bands were supershifted by anti-p65, while no detectable shifts were seen with antibodies to RelB and c-Rel.

Nuclear extracts from unstimulated BL-41 cells also bound to a 22-bp oligonucleotide that spanned the distal (–1267 to 1258) putative NFκB site in the 5′ upstream region of the AID gene, giving rise to a retarded band, indicated by an arrow in Fig. 5(B). This band was specifically inhibited by 100-fold excess of self or NFκB consensus oligonucleotide, but not by a 100-fold excess of a 22-bp OCT-1 oligonucleotide (Fig. 5B). Furthermore, it was partially shifted with anti-p50 and anti-c-Rel. CD40 stimulation caused the appearance of a slower migrating band that was also specifically inhibited by an excess of cold self-competitor and by the NFκB consensus sequence. This inducible band was completely supershifted with antibodies to p50 and p65, and was partially blocked with anti-c-Rel, but not by anti-RelB or rabbit IgG (Fig. 5B). These results suggest that CD40 ligation induces NFκB binding to the 5′ sequences present in the AID gene upstream promoter region.

STAT6 and p50 are essential for IL-4 induction of AID gene expression

To examine the role of STAT6 in IL-4 induction of AID gene expression we analyzed purified splenic B cells from STAT6$^{-/-}$ mice and wild-type controls. Northern blot analysis shows that IL-4 induction of AID mRNA expression was completely abolished in B cells from STAT6$^{-/-}$ mice (Fig. 6A). The modest up-regulation of AID expression by anti-CD40 was compar-

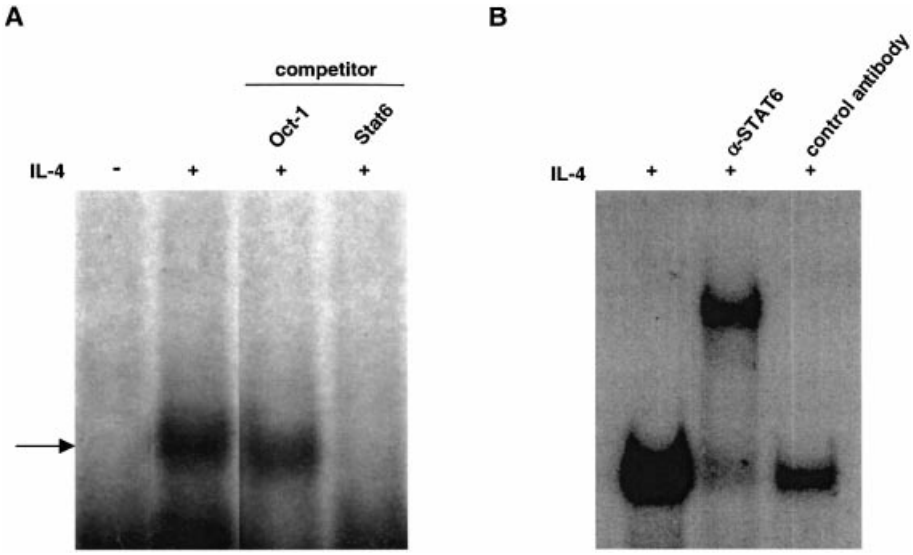

Fig. 4. IL-4 induces STAT6 binding to a site in the 5′ upstream regulatory region of the AID gene. (A) EMSA analysis of the binding of nuclear extracts from BL-2 cells to a ^{32}P-labeled 22-bp oligonucleotide probe that spans the putative STAT-binding sequence derived from the human AID gene. Cells were incubated in medium or stimulated with IL-4 for 30 min. The arrow points to the specifically retarded band. (B) A representative experiment of a supershift assay performed on nuclear extracts of IL-4-stimulated BL-2 cells using rabbit anti-human STAT6 antibody and control rabbit antibody. Similar results were obtained in three experiments.

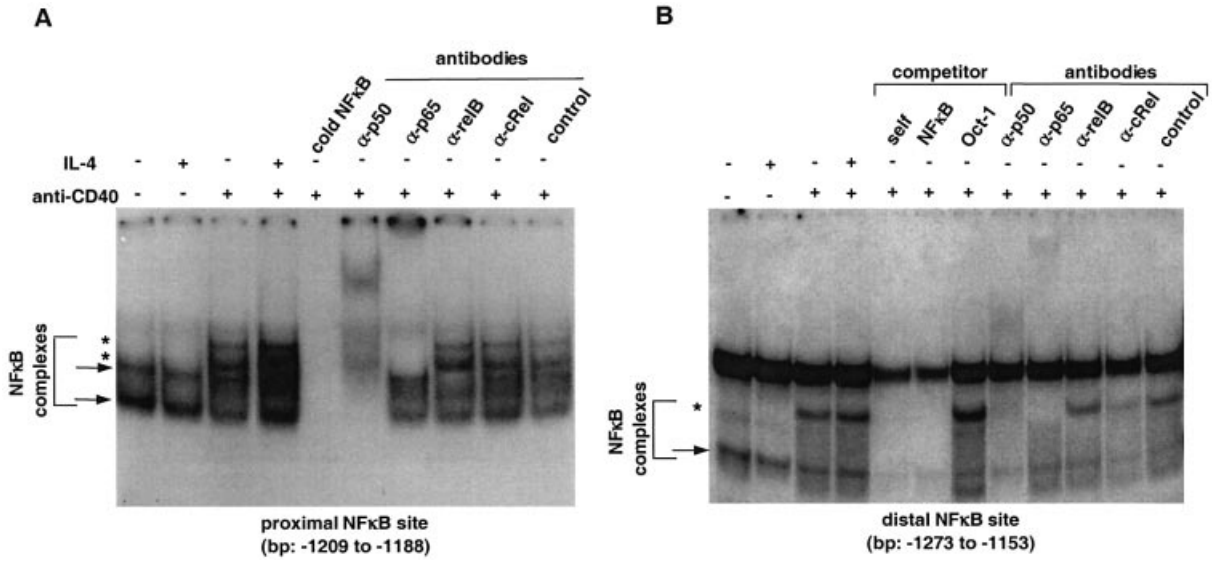

Fig. 5. CD40 ligation induces NFκB binding to two sites in the 5′ upstream regulatory region of the AID gene. EMSA and supershift analysis of the binding of nuclear extracts from BL-41 cells to the ^{32}P-labeled 22-bp oligonucleotide probes that span the two putative NFκB-binding sequences derived from the human AID gene. (A) Proximal NFκB site: –1203 to –1198 bp; (B) distal NFκB site: –1267 to –1258 bp. Arrows denote constitutive NFκB complexes. Asterisks denote induced NFκB complexes. The NFκB consensus sequence was derived from the Igκ gene enhancer. Similar results were obtained in three experiments.

able in B cells from STAT6$^{-/-}$ and wild-type controls (2.3 ± 0.6-fold in STAT6$^{-/-}$ B cells compared to 2.9 ± 0.9-fold in wild-type B cells, n = 3 each). Furthermore, IL-4 failed to synergize with anti-CD40 to up-regulate AID mRNA expression in STAT6$^{-/-}$ B cells. These results suggest that STAT6 mediates the induction of AID mRNA expression by IL-4.

Since the NFκB complexes that bound to the NFκB site that we identified in the AID 5′ upstream region contained p50, we used splenic B cells from p50$^{-/-}$ mice to examine the role of NFκB in CD40 up-regulation of AID gene expression. We compared the response of these mice to littermate controls on the C57BL/6/129 background. Northern blot analysis showed that the modest CD40 induction of AID mRNA expression in B cells from wild-type mice was absent in B cells from p50$^{-/-}$ mice. Unexpectedly, induction of AID by IL-4 was also not detectable in B cells from p50$^{-/-}$ mice. Synergy between CD40 ligation and IL-4 in up-regulating AID expression was markedly impaired, but not abolished, in these B cells (53 ± 3-fold in wild-type B cells versus 15 ± 4-fold in p50$^{-/-}$ B cells) (Fig. 6B). These results indicate that NFκB mediates the up-

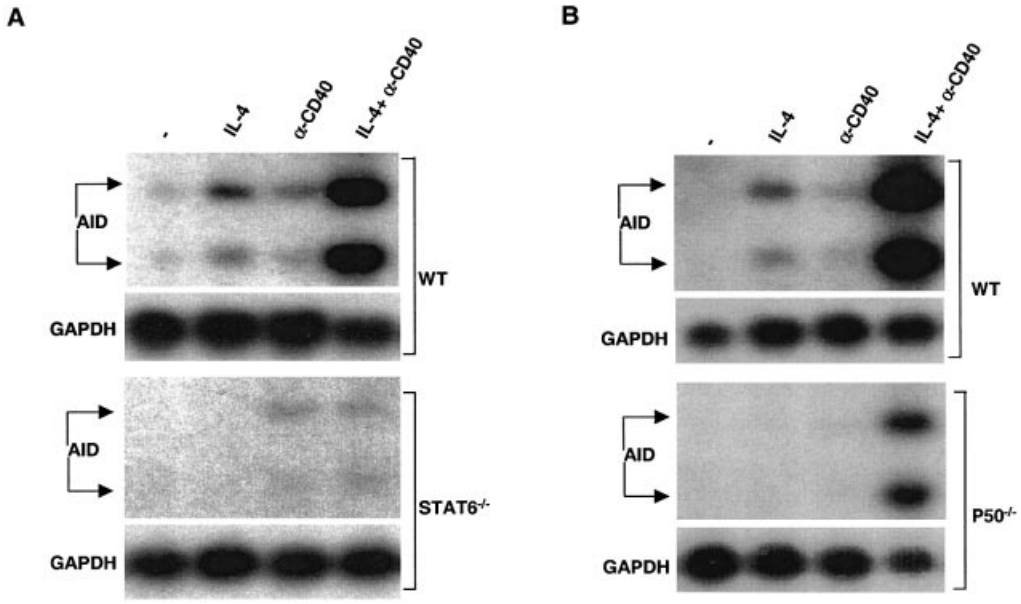

Fig. 6. AID mRNA expression in B cells from STAT6$^{-/-}$ and p50$^{-/-}$ mice. (A) Northern blot analysis of spleen B cells from (A) STAT6$^{-/-}$ mice and wild-type controls, and (B) p50$^{-/-}$ mice and wild-type controls. Similar results were obtained in three experiments.

regulation of AID mRNA expression by CD40 ligation and suggest that IL-4 induction of AID gene expression is dependent on baseline NFκB activity.

Discussion

In this study we provide evidence that signals delivered by CD40 ligation via NFκB synergize with signals delivered by the IL-4 receptor via STAT6 to induce AID gene expression in primary B cells.

Our results showed that IL-4 is able to induce modest AID mRNA expression in both primary human and mouse B cells (Figs 1 and 2). Induction of AID mRNA expression by IL-4 in primary human B cells is consistent with a recent observation by Zhou *et al.* (32). Induction of AID mRNA expression by IL-4 in murine B cells is consistent with the initial observations of Muramatsu *et al.* in the murine CH12-F3-2 B cell line (15). However, these authors did not observe detectable induction of AID gene expression by IL-4 in murine splenic B cells.

CD40 ligation using anti-CD40 mAb induced a weak or no detectable expression of AID mRNA in human blood B cells and in mouse splenic B cells (Figs 1 and 2). This is consistent with previous observations (15). In contrast, CD40 ligation induced strong AID expression in tonsil B cells. Tonsil B cells are activated *in vivo* by exposure to exogenous, particularly bacterial, antigens. This suggests that CD40 ligation may synergize with other signals delivered *in vivo* to tonsil B cells. These may include signals delivered by locally secreted cytokines, which include IL-4 (33), by antigens and superantigens through sIg molecules and MHC class II molecules respectively.

CD40 ligation also synergized with IL-4 to induce strong AID mRNA and protein expression in mouse primary B cells (8,19). As previously reported (15), northern blot analysis revealed two transcripts. Consistent with our previous observation (8), RT-PCR analysis using primers that hybridize to the 5′ and 3′ ends of the AID cDNA-coding region revealed a single product. In addition, western blot analysis with anti-AID antiserum revealed only a single band. This suggests that the two AID RNA transcripts detected by northern blot analysis differ in the length of the 3′ end by alternate splicing of heretofore undescribed exon or 3′ untranslated regions. We cannot, however, rule out the possibility that one of two transcripts represented the product of a related gene. Neither IL-4 nor CD40 ligation alone was able to induce detectable AID protein expression. The fact that IL-4 induced modest AID mRNA, but not protein, expression suggests that AID expression may be regulated at the post-transcriptional level. Although IL-4 by itself induces C_ε germline transcripts (GLT) in human B cells and $C_\gamma 1$ GLT in murine B cells, and CD40 ligation induces $C_\gamma 1$ GLT in murine B cells, these stimuli do not induce CSR to these isotypes (8,34). This suggests that synergy between IL-4 and CD40 may be required to achieve a threshold level of AID expression for CSR to proceed. In addition, synergy between IL-4 and CD40 ligation in inducing GLT may also be important for CSR. We cannot, however, rule out that CD40 ligation and IL-4 also synergize to induce the expression of additional factors that may be required for CSR. This is made less likely by the observation that strong expression of AID gene is sufficient to cause CSR in actively transcribed recombination substrates (20,21).

Using EMSA and supershift experiments we have demonstrated that IL-4 induces the binding of STAT6 to an oligonucleotide derived from the AID 5′ upstream region that spans a potential STAT-binding sequence. This sequence differs from the canonical STAT-binding site (TTCxxxxGAA) at the underlined single nucleotide position. Furthermore, four, rather than three, central nucleotides usually separate the invariant flanking nucleotides in STAT6-binding sites. The presence of only three central nucleotides in the STAT6-binding site of the AID gene makes it a candidate for also binding other STAT proteins that include STAT1, 3 and 5,

raising the possibility that signals that activate these STAT proteins may induce AID gene expression. We have found that IFN-γ, which is known to activate STAT1, is unable to induce detectable AID mRNA expression alone or in synergy with CD40 ligation (Dedeoglu, unpublished observations). However, IL-2, which activates STAT5, induces weak AID mRNA expression and synergizes modestly with CD40 ligation in inducing AID expression in B cells (Dedeoglu, unpublished observations). Also, IL-4 (35) and CD40 (36,37) have each been reported to activate STAT3 and STAT5 in B cells. However, CD40 ligation did not induce nuclear proteins that bound to STAT6 induced by IL-4 in the 5′ upstream region of the AID gene (Dedeoglu, unpublished observations). However, STAT3 and STAT5 proteins induced by CD40 ligation may bind to other sites in the AID promoter.

EMSA and supershift assays also suggested that CD40 ligation induces the binding of NFκB to two sites in the AID 5′ upstream region. The NFκB complexes bound at both sites contained p50, p65 and Rel-B. In addition, the complex that bound to the distal site also contained c-Rel. The composition of these NFκB complexes is consistent with that of NFκB complexes known to be induced by CD40, and that bind to the $C_\gamma1$, $C_\gamma3$ and C_ε promoters (38–40). Although we have not examined nuclear extracts for the presence of p52 following CD40 ligation, p52 is not normally activated by CD40 ligation (41,42). However, p52 is activated by BAFF, which was recently shown to induce AID and activate CSR (14), and may play a role in the induction of AID by BAFF.

In vitro binding of transcription factors does not necessarily indicate functional relevance. The importance of STAT6 and NFκB in induction of AID expression by IL-4 and CD40 was demonstrated in studies of STAT6$^{-/-}$ and p50$^{-/-}$ mice. IL-4 failed to induce AID expression in B cells from STAT6$^{-/-}$ mice, suggesting that STAT6 is essential for IL-4 induction of AID gene expression. This is supported by the recent observation that IL-4-dependent AID induction was inhibited by a dominant-negative STAT6 (32). Moreover, triggering of CD45 with anti-CD45 antibodies inhibited IL-4-induced AID expression, and this correlated with the ability of anti-CD45 to suppress IL-4-activated JAK1, JAK3 and STAT6 phosphorylations. These results suggest that IL-4 induced AID expression via the JAK/STAT6 signaling pathway (32).

The ability of CD40 ligation to induce AID expression and to synergize with IL-4 in AID induction in B cells was impaired in p50$^{-/-}$ mice. This suggests that although CD40 ligation activates a number of transcription factors, activation of NFκB is important for its ability to induce AID expression and isotype switching. The critical role of p50 and NFκB in the induction of AID gene expression by CD40 + IL-4 is consistent with the fact that CSR is impaired in mice deficient in p50 (43) and in patients with mutations in the IKKγ/NEMO component of the NFκB pathway (44,45). Surprisingly, IL-4 induction of AID gene expression was impaired in p50$^{-/-}$ mice, suggesting that it requires synergy between STAT6 and NFκB. Since IL-4 is thought not to activate NFκB, it is likely that STAT6 induced by IL-4 synergizes with baseline NFκB present in nuclei of unstimulated splenic B cells. A similar requirement for NFκB exists for IL-4 induction of $C_\gamma1$ and C_ε GLT (38,40,46). Finally, our results on STAT6$^{-/-}$ and p50$^{-/-}$ mice, together with the fact that the STAT6- and NFκB-binding sites we identified were the only candidate sites in the 3.9 kb of the 5′ region of the AID gene, suggest that these sites are important for AID expression.

STAT6 and NFκB have been previously shown to synergize in the induction of a number of genes (34,38,39,46), and a physical interaction between these transcription factors has been demonstrated (47). Therefore, synergy between STAT6 and NFκB is likely to underlie the synergy between IL-4 and CD40 ligation in inducing AID gene expression. Synergy between IL-4 and STAT-6 was diminished, but not abolished, in p50$^{-/-}$ mice. This suggests that NFκB/rel components, other than p50 activated by CD40, may play a role in these mice. We cannot, however, rule out a role of other transcription factors induced by CD40 ligation in B cells, such as AP-1, STAT3 and NFAT.

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Abbreviations

AID	activation-induced cytidine deaminase
CSR	class switch recombination
EMSA	electrophoretic mobility shift assay
GLT	germline transcript
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cell
SHM	somatic hypermutation

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