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A thiol antioxidant regulates IgE isotype switching by inhibiting activation of nuclear factor- κ B

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The binding site for nuclear factor- κ B (NF- κ B) is present at the promoter region of the germline C ϵ gene, but there is little information on whether this factor is involved in regulating IgE synthesis by human B cells. Accordingly, we studied the role of NF- κ B in germline C ϵ transcription by using two human Burkitt's lymphoma B cell lines, DND39 and DG75. In both cell lines, n-acetyl-L-cysteine (NAC), a potent thiol antioxidant, inhibited the triggering of the nuclear expression of NF- κ B by IL-4 and by anti-CD40 monoclonal antibody. Although IL-4 activated signal transducers and activators of transcription (STAT) 6 in addition to NF- κ B, NAC treatment or the transfection of decoy oligodeoxynucleotides for NF- κ B or STAT6 only partly blocked IL-4-induced germline C ϵ transcription. However, these two decoy oligodeoxynucleotides together almost completely abrogated IL-4-induced germline C ϵ transcription. Of note, CD40-mediated enhancement of IL-4-driven germline C ϵ transcription was markedly decreased by NAC or by a decoy oligodeoxynucleotide for NF- κ B. The effect of NAC was also examined on deletional switch recombination underlying the isotype switch to IgE. NAC inhibited the generation of S μ /S ϵ switch fragments in normal human B cells costimulated with IL-4 and anti-CD40 monoclonal antibody. It also abolished IL-4-induced upregulation of CD40 but promoted upregulation of CD23. These results suggest that coordination of NF- κ B and STAT6 may be required for induction of germline C ϵ transcription by IL-4, and that CD40-mediated NF- κ B activation may be important in regulating both enhancement of germline C ϵ transcription and class switching to IgE. (*J Allergy Clin Immunol* 1997;100:S33-8.)

Key words: Antioxidant, nuclear factor- κ B, signal transducers and activators of transcription 6, IL-4, CD40, germline C ϵ transcription, deletional switch recombination, mature C ϵ transcription

Thiol antioxidants, including n-acetyl-L-cysteine (NAC), glutathione, and disulfiram, can inhibit activation of nuclear factor- κ B (NF- κ B) by cytokines, mitogens, or viruses in various types of cells.¹⁻³ Inactive

Abbreviations used

EBV:	Epstein-Barr virus
EMSA:	Electrophoretic mobility shift assay
G3PDH:	Glyceraldehyde 3-phosphate dehydrogenase
mAb:	Monoclonal antibody
NAC:	n-Acetyl-L-cysteine
NF- κ B:	Nuclear factor- κ B
PI:	Phosphatidylinositol
PKC:	Protein kinase C
STAT:	Signal transducers and activators of transcription

NF- κ B, which is present in a non-DNA-binding form in the cytoplasm, is a heterotrimer composed of three subunits (p50, p65, and I κ B). Phosphorylation and dissociation of I κ B results in translocation of the active p50-p65 dimer to the nucleus, in which active NF- κ B represents the DNA-binding activity. NAC is a potent antioxidant that acts upstream of I κ B phosphorylation. Several studies have demonstrated that I κ B is phosphorylated by at least the ζ isoform of protein kinase C (PKC ζ), which is activated by the lipid products of phosphatidylinositol (PI) 3-kinase.⁴⁻⁶ Our previous work showed that PI 3-kinase inhibitors, such as wortmannin and LY294002, inhibit the translocation of PKC ζ from the cytosol to the membrane fraction in a human Burkitt's lymphoma B cell line. In addition, PI3-kinase inhibitors abrogate nuclear expression of NF- κ B induced by IL-4 or by an agonistic anti-CD40 monoclonal antibody (mAb) in the same cell line.⁷⁻¹⁰

In addition to IL-4 or IL-13, induction of IgE synthesis in human B cells requires a second signal that is provided by CD40 ligand on activated T cells, mast cells, and basophils.¹¹⁻¹⁷ Ligation of CD40 on B cells enhances IL-4- or IL-13-driven germline C ϵ transcription and subsequently induces the deletional switch recombination that underlies isotype switching to IgE. This isotype switch leads to mature C ϵ transcription and IgE synthesis. The germline C ϵ promoter, which contains both an IL-4 response element and a CD40 response element, has been found to also possess the binding sites for some transcription factors, such as NF- κ B, NF-IL-4, signal

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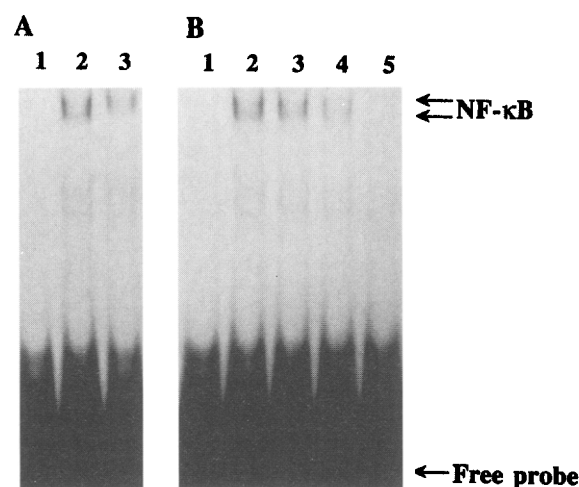


FIG. 1. Effects of LY294002 (**A**) and NAC (**B**) on activation of NF- κ B by IL-4 in DND39 cells. **A**, Cells were incubated with medium alone (lanes 1 and 2) or with 30 μ M LY294002 (lane 3) for 30 minutes, followed by stimulation with 250 U/ml IL-4 (lanes 2 and 3) for 1 hour. One of two comparable experiments is shown. **B**, Cells were incubated without (lanes 1 and 2) or with NAC at 10 (lane 3), 30 (lane 4), and 100 (lane 5) mmol/L for 30 minutes, followed by stimulation with 250 U/ml IL-4 for 1 hour. In each experiment, nuclear extracts were prepared and subjected to EMSA with a radiolabeled oligonucleotide probe containing the κ B enhancer sequence. One of three comparable experiments is shown.

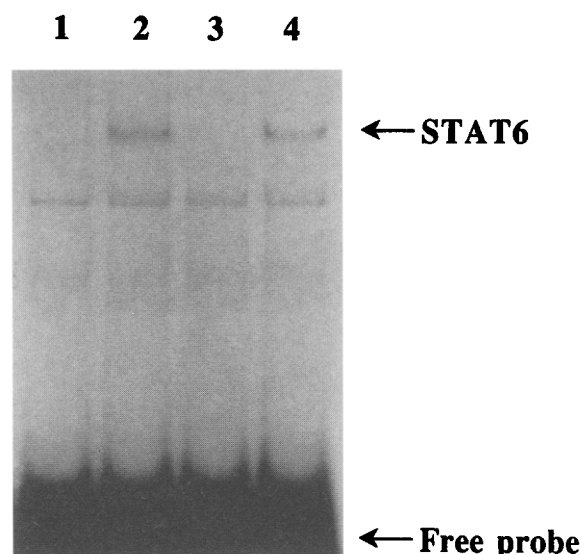


FIG. 2. Activation of STAT6 by IL-4 in DND39 cells. Cells were incubated without (lane 1) or with (lanes 2 to 4) 250 U/ml IL-4 for 1 hour. Nuclear extracts were prepared, incubated without any competitor (lanes 1 and 2) or with a 50-fold excess of unlabeled oligonucleotide competitor specific for STAT6 (lane 3) or NF- κ B (lane 4), and then subjected to EMSA with a radiolabeled oligonucleotide probe containing the STAT6 binding sequence. Representative results from one of three experiments are shown.

transducers and activators of transcription (STAT) 6, B cell-specific activator protein, and CCAAT/enhancer binding protein.¹⁸⁻²² The involvement of NF- κ B in germline as well as in mature C ϵ transcription has been reported in both rodents and humans.^{9, 10, 23, 24} For example, targeted disruption of the p50 subunit of NF- κ B results in inhibition of germline C ϵ transcription induced in mouse B cells by appropriate stimulation. In addition, NAC inhibits IgE production by human B cells costimulated with IL-4 and anti-CD40 mAb, which is accompanied by decreased mature C ϵ transcription.

In the present study, we investigated the involvement of NF- κ B in the molecular events leading to IgE synthesis by human B cells. Two Epstein-Barr virus (EBV)-negative Burkitt's lymphoma B cell lines, DND39 and DG75, were used for the analysis of germline C ϵ transcription, and peripheral blood B cells from normal donors were used to study class switching to IgE.

RESULTS AND DISCUSSION

NAC inhibits NF- κ B activation by IL-4 or by CD40 ligation in DND39 and DG75 cells

We have previously shown that stimulation of DND39 cells with IL-4 or anti-CD40 mAb results in activation of PI 3-kinase, translocation of PKC ζ , and nuclear expression of NF- κ B.⁷⁻¹⁰ Despite the similarities of these three events in IL-4 and CD40 signaling, some differences of the signal transduction components participating in PI 3-kinase activation were observed. For example, PI 3-kinase activity associated with Janus kinase 3 was increased after IL-4 stimulation, whereas Lyn kinase

contributed to CD40-mediated PI 3-kinase activity. The latter finding was also reported in Daudi cells,²⁵ but Lyn kinase was not involved in IL-4 signaling in DND39 cells.²⁶ Nevertheless, PI 3-kinase was involved in activation of PKC ζ by both IL-4 and CD40 ligation. Indeed, treatment of DND39 cells with wortmannin or LY294002 was shown to block PKC ζ translocation caused by IL-4 and by anti-CD40 mAb.^{8, 10} Moreover, we found that activation of NF- κ B by IL-4 was inhibited by LY294002 (30 μ M/L), as shown by electrophoretic mobility shift assay (EMSA) with a radiolabeled oligonucleotide probe containing the κ B enhancer sequence (Fig. 1, A). In addition, CD40-mediated induction of NF- κ B was inhibited by LY294002 (data not shown). These results indicate that IL-4 and CD40 ligation induce nuclear expression of NF- κ B at least via a mechanism dependent on the PI 3-kinase pathway. Regarding the stimulation of CD40, some members of the tumor necrosis factor receptor-associated factor family, which can be recruited to the cytoplasmic domain of CD40, are also involved in CD40 signaling linked to NF- κ B expression.^{27, 28} Thus multiple pathways are involved in CD40-mediated induction of NF- κ B.

Because NAC blocks the activation of NF- κ B by cytokines or mitogens in many cell types,¹⁻³ we examined its effect on the induction of NF- κ B activity by IL-4 and by CD40 ligation. DND39 cells were stimulated in the absence or presence of NAC with IL-4 or anti-CD40 mAb, and nuclear extracts from the cells were then subjected to EMSA. As expected, NAC (10 to 100 mmol/L) caused concentration-dependent inhibition of

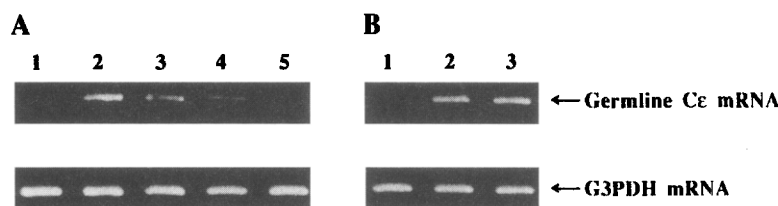


FIG. 3. Effects of decoy oligodeoxynucleotides for NF- κ B, STAT6, or EBNA-1 on IL-4-induced germline C ϵ transcription in DND39 cells. **A**, Cells without transfection (lanes 1 and 2), with transfection of the decoy oligodeoxynucleotide for NF- κ B (lane 3) or STAT6 (lane 4), or with transfection of the two decoy oligodeoxynucleotides for NF- κ B and STAT6 (lane 5). Untransfected and transfected cells were incubated without (lane 1) or with (lanes 2 to 5) 250 U/ml IL-4 for 48 hours. **B**, The decoy oligodeoxynucleotide for EBNA-1 was transfected (lanes 1 and 2) or was transfected (lane 3) into cells, and the cells were then incubated without (lane 1) or with (lanes 2 and 3) 250 U/ml IL-4 for 48 hours. In each experiment, total RNA was prepared, and the expression of germline C ϵ and glyceraldehyde 3-phosphate dehydrogenase mRNA was analyzed by reverse transcription-polymerase chain reaction. The reactions were all within the linear range. Representative results from one of three experiments are shown.

IL-4-stimulated NF- κ B expression (Fig. 1, *B*). Induction of NF- κ B by CD40 ligation was also similarly inhibited by NAC (data not shown). In both cases, almost complete abrogation of NF- κ B activity was obtained with an NAC concentration of 100 mmol/L. Similar results were obtained when DG75 cells were used (data not shown), and the cell viability at the end of culture was not affected by NAC at any concentration. Taken together, these results indicate that both LY294002 and NAC are effective in inhibiting the nuclear expression of NF- κ B induced by IL-4 and by CD40 ligation.

Coordination between NF- κ B and STAT6 is required for induction of germline C ϵ transcription by IL-4 in DND39 and DG75 cells

We have previously shown that treatment of DND39 cells with NAC reduces but does not abrogate IL-4-induced germline C ϵ transcription.¹⁰ This observation suggests that other transcription factor(s), in addition to NF- κ B, may be required for induction of germline C ϵ transcription by IL-4. In this context, it is interesting that the binding site for STAT6 is also present at the IL-4 response element in the germline C ϵ promoter. In keeping with previous reports that STAT6 is activated not only by IL-4 but also by IL-13,^{18, 29} we found that IL-4 activated STAT6 in DND39 cells, as identified by EMSA with a radiolabeled oligonucleotide probe containing the STAT6-binding sequence (Fig. 2). This was further confirmed by a specific competition assay with a 50-fold excess of unlabeled STAT6 competitor. On the basis of these findings, we studied the possible role of NF- κ B or STAT6 activation in IL-4-induced germline C ϵ transcription by using synthetic double-stranded oligodeoxynucleotides as "decoy" *cis*-elements to inhibit the binding of transcription factors (including NF- κ B and STAT6) to promoter regions of the germline C ϵ gene. Decoy oligodeoxynucleotides for STAT6, NF- κ B, or EBV-determined nuclear antigen (EBNA)-1 were transiently transfected into DND39 cells by electroporation. Transfection of decoy oligodeoxynucleotides for

NF- κ B or STAT6, but not of the decoy oligodeoxynucleotides for EBV-determined nuclear antigen, led to a decrease in IL-4-induced germline C ϵ transcription, as shown by reverse-transcription polymerase chain reaction (Fig. 3). In addition, transfection of the two decoy oligodeoxynucleotides for NF- κ B and STAT6 almost completely inhibited IL-4-driven germline C ϵ transcription. Similar results were obtained with transfection of DG75 cells with two such decoy oligodeoxynucleotides (data not shown). These findings, together with a recent report that B cell-specific activator protein plays no direct regulatory role in IL-4-induced germline C ϵ transcription,³⁰ strongly suggest that the coordinated action of NF- κ B and STAT6 may be required for induction of germline C ϵ transcription by IL-4.

NAC inhibits CD40-mediated enhancement of germline C ϵ transcription in IL-4-stimulated DND39 and DG75 cells

The germline C ϵ promoter has been shown to contain a CD40 response element that is distinct from the IL-4 response element.¹⁸⁻²² Although the CD40 response element also has a binding site for NF- κ B-related factor, it is generally accepted that germline C ϵ transcription is inducible by IL-4 or IL-13, but not by CD40 ligation. In contrast to this view, CD40 ligation rather than IL-4, has been reported to induce germline C ϵ transcription in EBV-transformed B cell lines.³¹ However, our results showed that DND39 or DG75 cells, which are EBV-negative, did not express germline C ϵ transcripts in response to stimulation with anti-CD40 mAb (Fig. 4, *A*). In these cells, CD40 ligation caused marked enhancement of IL-4-driven germline C ϵ transcription. This finding is consistent with previous reports that CD40 ligation and IL-4 have a synergistic effect on germline C ϵ transcription in normal human B cells.^{11, 14, 32, 33}

The observation that NAC inhibited NF- κ B activation induced by IL-4 and by CD40 ligation, led us to test whether NAC could modulate CD40-mediated enhance-

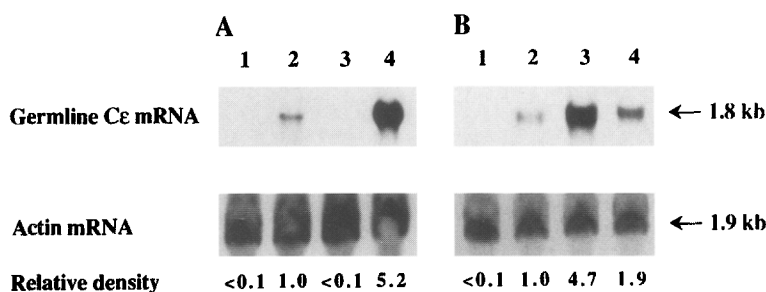


FIG. 4. CD40-mediated enhancement of IL-4-induced germline C ϵ transcription (**A**) and inhibition of its enhancement by NAC (**B**) in DND39 cells. **A**, Cells were incubated without stimulators (lane 1) or with 250 U/ml IL-4 (lane 2), 1 μ g/ml anti-CD40 mAb (lane 3), or both IL-4 and anti-CD40 mAb (lane 4) for 48 hours. **B**, Cells were incubated without NAC (lanes 1 to 3) or with 30 mmol/L NAC (lane 4), followed by incubation without IL-4 (lane 1) or with 250 U/ml IL-4 (lane 2) or with both IL-4 and anti-CD40 mAb in the absence (lane 3) or presence (lane 4) of 30 mmol/L NAC for 48 hours. In each experiment, total RNA was prepared and subjected to Northern blot analysis by using a probe specific for human C ϵ 2-4. The hybridized membrane was then stripped and rehybridized with the β -actin probe. The relative density of the germline C ϵ mRNA in lane 2 of each panel is set at 1.0. Representative results from one of three experiments are shown.

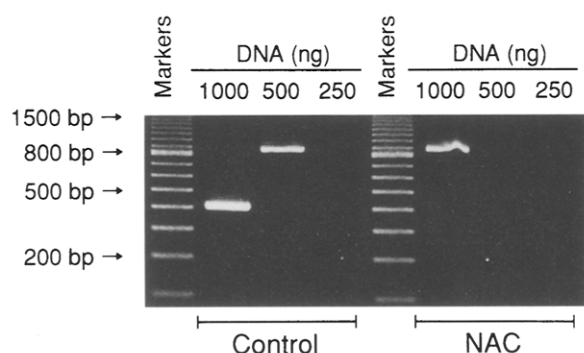


FIG. 5. Effect of NAC on the generation of S μ /S ϵ switch fragments in normal B cells costimulated with IL-4 and anti-CD40 mAb. B cells were costimulated in the absence or presence of 10 mmol/L NAC with 100 U/ml IL-4 and 1 μ g/ml anti-CD40 mAb for 7 days. Total DNA was prepared, serially diluted, and then amplified by the nested polymerase chain reaction. No fragments were amplified from the DNA of B cells stimulated with either IL-4 or anti-CD40 mAb (data not shown). One of two comparable experiments is shown. bp, base pairs.

ment of IL-4-driven germline C ϵ transcription in DND39 cells. Treatment with NAC (30 mmol/L) resulted in abrogation of the enhanced expression of germline C ϵ transcripts, as determined by Northern blot analysis (Fig. 4, B). This inhibition was not the result of toxicity, because NAC did not affect cell viability at the end of culture. Although NAC only reduced IL-4-induced germline C ϵ transcription,¹⁰ it had the ability to decrease CD40-mediated upregulation of such transcription. Similar inhibition was obtained in DG75 cells and in DND39 cells transfected with a decoy oligodeoxynucleotide for NF- κ B (data not shown). These results suggest that CD40-mediated NF- κ B activation plays an important role in enhancing IL-4-driven germline C ϵ transcription.

NAC inhibits CD40-mediated induction of switch recombination in IL-4-treated B cells

NAC has been shown to inhibit IgE synthesis and mature C ϵ transcription in normal human B cells costimulated with IL-4 and anti-CD40 mAb.^{9, 23} Because mature C ϵ transcription results from isotype switching to IgE,^{11, 12, 32, 33} we examined whether NAC could influence deletional switch recombination in normal B cells costimulated with IL-4 and anti-CD40 mAb. The nested primer polymerase chain reaction was used to amplify recombined S μ /S ϵ regions on DNA isolated from stimulated B cells, as described in detail elsewhere.^{12, 32} NAC (10 mmol/L), which induces about 70% inhibition of IgE synthesis without affecting cell viability,⁹ inhibited the generation of S μ /S ϵ switch fragments (Fig. 5). This finding suggests that NAC inhibits CD40-mediated deletional switch recombination, possibly by interfering with NF- κ B activation.

IL-4 upregulates the surface expression of CD23, CD40, HLA-DR, and IgM on normal B cells. We therefore determined whether NAC could modulate these four effects of IL-4. Flow cytometry revealed that NAC (10 mmol/L) decreased CD40 to below the constitutive level of expression, whereas it markedly increased CD23 expression (Fig. 6). However, the expression of HLA-DR and IgM was not significantly affected by NAC. These observations suggest that at least the two different signal transduction pathways for IL-4 participate in upregulating the expression of B cell markers. One is sensitive to NAC and may act selectively on CD40-specific events downstream of the common pathway leading to both CD23 and CD40 upregulation. It is therefore likely that inhibition of CD40 upregulation by NAC may result in accumulation of CD23. The other pathway is insensitive to NAC and may take part in HLA-DR and IgM upregulation. This concept is in keeping with previous reports that IL-4-induced upregulation of CD23 and IgM expression as well as that of

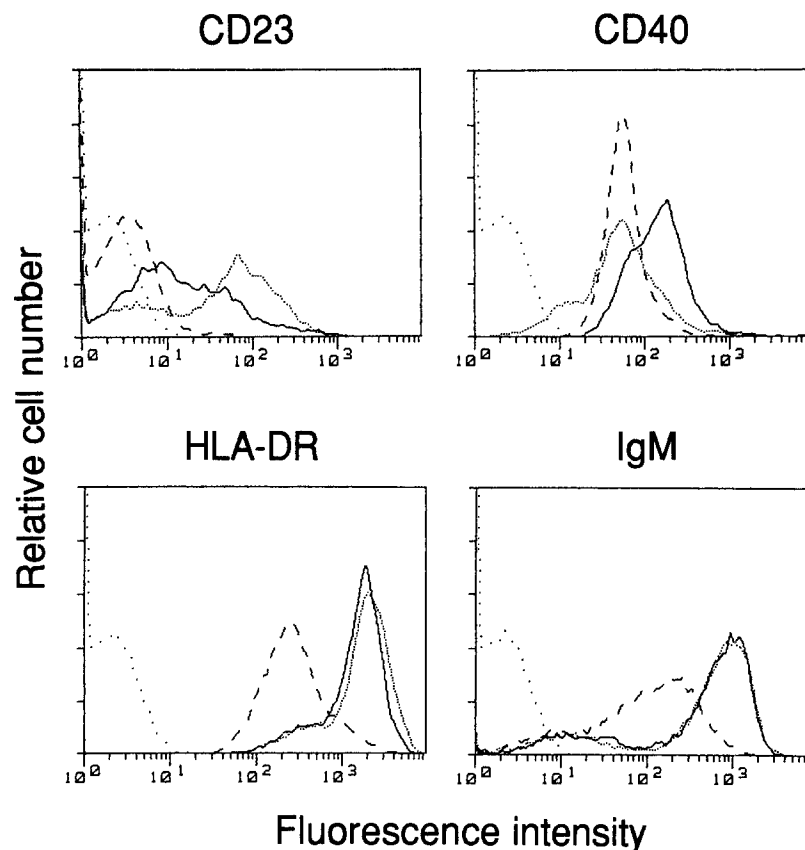


FIG. 6. Effect of NAC on IL-4-induced upregulation of surface CD23, CD40, HLA-DR, and IgM expression on normal human B cells. B cells were cultured with medium alone (---) or were stimulated with 100 U/ml IL-4 in the absence (—) or presence (.....) of 10 mmol/L NAC for 48 hours. The cells were stained with biotinylated anti-CD23 mAb plus phycoerythrin-conjugated streptavidin or with anti-CD40, anti-HLA-DR, and anti-IgM mAbs labeled with fluorescein isothiocyanate or phycoerythrin. IL-4-stimulated B cells were also labeled with isotype-matched control mAbs (-·-·-). The stained cells were analyzed with a flow cytometer. Representative results from one of three experiments are shown.

CD23 and IL-4 receptor expression is controlled independently in B cells.^{34, 35} In addition, it is possible that NF- κ B activation induced by IL-4 may also contribute to CD40 upregulation. Together, these results suggest that NAC may inhibit IgE synthesis in B cells costimulated with IL-4 and anti-CD40 mAb by targeting NF- κ B-dependent steps in the messenger systems for both IL-4-induced CD40 upregulation and CD40-mediated deletional switch recombination.

CONCLUSION

This study demonstrates that there are many similarities between NAC and a decoy oligodeoxynucleotide for NF- κ B with respect to the regulation of germline C ϵ transcription in DND39 and DG75 cells, and that NAC inhibits the generation of S μ /S ϵ switch fragments in normal B cells costimulated with IL-4 and anti-CD40 mAb. These findings indicate that nuclear expression of NF- κ B contributes to the molecular events leading to IgE synthesis by B cells.

Examination of NF- κ B activity in more detail suggests that coordinated action of NF- κ B and STAT6 is required for IL-4-induced germline C ϵ transcription, whereas CD40-mediated NF- κ B activity is involved in both enhancement of germline C ϵ transcription and induction of deletional switch recombination responsible for subsequent mature C ϵ transcription and IgE synthesis. Although the two signal transduction pathways for IL-4 and CD40 operating through NF- κ B interact at the level of the germline C ϵ promoter, NF- κ B activation triggered by either IL-4 or by CD40 ligation does not cause isotype switching to IgE, mature C ϵ transcription, or IgE synthesis. Recent studies have shown that in addition to PKC ζ activated through the lipid products of PI 3-kinase, some members of the tumor necrosis factor receptor-associated factor family, which are directly associated with the cytoplasmic domain of CD40, mediate CD40 signaling that is linked to NF- κ B activation.^{27, 28} Accordingly, further work is needed to identify and characterize the

members of this family and of the NF- κ B family that play an important role in CD40-mediated deletional switch recombination in B cells.

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