

Essential role of Id2 in negative regulation of IgE class switching

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Serum concentrations of immunoglobulin E (IgE) in normal circumstances are kept much lower than those of other Ig isotypes to avoid allergic reactions. B cells lacking Id2 have increased E2A activity, which leads to specific enhancement of germline transcription of the immunoglobulin ϵ locus. As a consequence, Id2-deficient B cells undergo class switch recombination (CSR) to IgE at a much higher frequency than wild-type B cells. In contrast, Id2 is induced in wild-type B cells by transforming growth factor- β 1 (TGF- β 1) and suppresses IgE CSR. Our results provide evidence for the inhibitory and selective role of Id2 in IgE CSR in response to TGF- β 1. Id2 might act as molecular safeguard to suppress IgE CSR to prevent serious complications such as allergic hypersensitivity during the normal course of immune responses.

Allergic hypersensitivity disorders are characterized by an excessive immunoglobulin E (IgE) response. High serum IgE concentrations have been reported in other human diseases such as hyper-IgE syndrome, AIDS and Omenn's syndrome¹. In normal individuals, IgE expression is tightly regulated and serum IgE concentrations are much less abundant than those of IgM, IgG and IgA. Antigen-activated B cells require signals through either lipopolysaccharide (LPS) or CD40 ligand (CD40L) to induce class switch recombination (CSR), which exchanges the IgM heavy chain constant region for that of another isotype. Such signaling induces the CSR machinery-related molecules such as activation-induced deaminase (AID)². CSR is blocked in AID^{-/-} mice². CSR occurs between switch (S) regions located 5' to every constant region gene of the Ig heavy chain except for δ . Prior to recombination, the target gene is specifically transcribed from I promoters located upstream of the S regions³⁻⁵. Recent studies that used an artificial construct containing two S regions have demonstrated that this transcription of an unrearranged gene, termed germline transcription (GLT), is quantitatively correlated with CSR efficiency⁶. Targeting isotype specificity of CSR is regulated by the cytokines interleukin 4 (IL-4), interferon- γ (IFN- γ) and transforming growth factor- β 1 (TGF- β 1) through the control of GLT of the target gene(s)^{7,8}.

For example, IFN- γ induces the GLT of γ 2a, whereas TGF- β 1 induces those of γ 2b and α . On the other hand, IL-4 enhances the CSR to IgG1 and IgE through the activation of signal transducer and activator of transcription 6 (STAT6). Accordingly, IL-4^{-/-} and STAT6^{-/-} mice show highly deficient IgG1 and IgE production⁹⁻¹². Numerous studies have indicated that IL-4 stimulates GLT of the γ 1 and ϵ heavy chain loci and induces CSR to both isotypes³⁻⁵, but switching to IgE CSR occurs much less frequently¹³⁻¹⁶. Thus, active molecular mechanisms appear to suppress IgE CSR to prevent serious complications during the normal course of the immune response.

We have established a mouse line deficient in Id2 that is a member of the Id protein family, negative regulators of basic helix-loop-helix (bHLH) transcription factors such as E2A proteins (E12 and E47) and MyoD^{17,18}. The mutant mice lack lymph nodes and Peyer's patches and exhibit a greatly reduced population of natural killer (NK) cells, although splenic architecture is normal, exhibiting T cell and B cell compartments and distinct germinal centers¹⁹. We also found that Id2^{-/-} mice secrete increasing amounts of IgE in sera²⁰. Here we show that B cells from Id2^{-/-} mice preferentially switch to IgE. As a result of Id2 deficiency, the activity of E2A proteins, which bind E-boxes in the ϵ germline promoter, was highly augmented and this elevated E2A

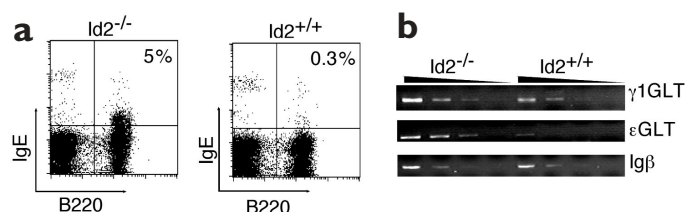


Figure 1. Enhanced IgE class switching in the spleen. (a) Class switch analysis by surface expression of IgE in Id2^{-/-} spleen immunized after 7 days. Data from one of three representative experiments is shown. Percentages indicate the proportions of IgE⁺ cells among B220⁺ cells. (b) RT-PCR of γ 1 and ϵ GLT in spleen 6 days after immunization. One of three independent experiments is shown. Ig β was used as internal control for RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts.

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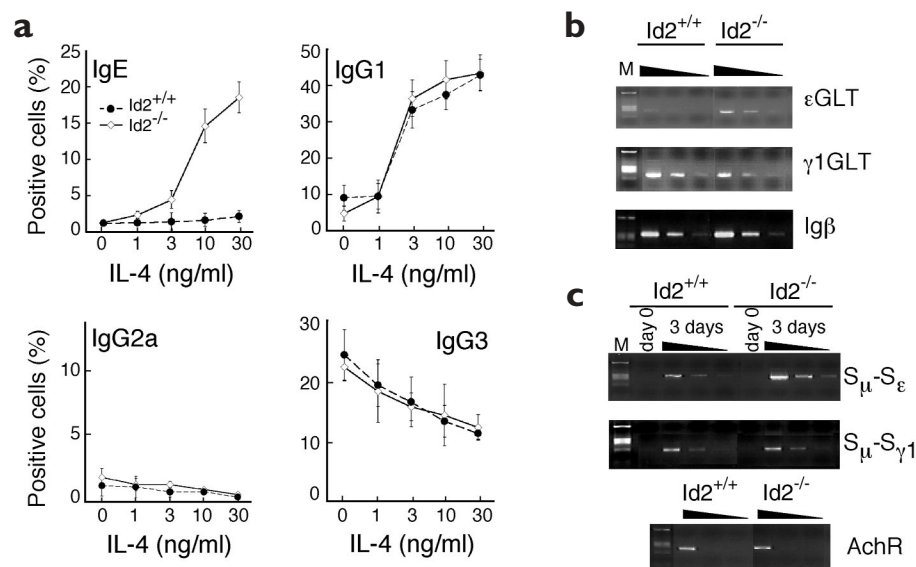


Figure 2. Enhanced IgE class switching in Id2^{-/-} B cells. (a) Class switch analysis by surface expression of each Ig in Id2^{-/-} B cells cultured for 4 days. All assays were independently performed five times. Mean $\pm$ s.e.m. data are shown. (b) RT-PCR of γ 1 GLT and ϵ GLT in B cells after 3 days of culturing with 3 ng/ml of IL-4 plus 10 μ g/ml of LPS. One of three independent experiments is shown. Ig β was used as internal control for RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. (c) Quantitation of S μ -S ϵ and S μ -S γ 1 recombination by DC-PCR in B cells cultured under the same conditions described in b. One of three independent experiments is shown. Acetylcholine receptor (AChR) was used as internal control for DC-PCR. Fivefold serial dilutions of DC-DNAs were amplified for the indicated products.

activity was the primary cause of enhanced ϵ germline transcripts and CSR. Id2 expression was induced by TGF- β 1, thus offering an explanation as to why TGF- β 1 can negatively regulate CSR to IgE. Finally, we demonstrated that inhibition of IgE CSR by TGF- β 1 was lost in Id2-deficient mice. Our results provide evidence that Id2 acts to maintain low serum concentrations of IgE in response to TGF- β 1.

Results

Enhanced IgE CSR in Id2^{-/-} mice

Mice deficient for Id2 secrete increased amounts of IgE in sera compared to wild-type (WT) mice although the mechanism is unclear²⁰. We therefore analyzed membrane-bound IgE (mIgE) expression of B cells in Id2^{-/-} mice immunized intraperitoneally by nitrophenol (NP)-conjugated chicken gamma globulin (CGG) with alum. mIgE⁺ B cells accumulated in the spleens of Id2^{-/-} mice as compared with Id2^{+/+} mice having the same genetic background (Fig. 1a). The number of mIgG1⁺ cells was slightly elevated in Id2^{-/-} mice (data not shown). These results suggested that the substantial elevation of serum IgE observed in Id2^{-/-} mice resulted from enhanced class switching to IgE *in vivo*. We examined, by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assays, germline transcript expression directed by the I promoters of γ 1 and ϵ , which precedes and is essential for CSR upon B cell stimulation. Id2^{-/-} spleen cells expressed higher amounts of γ 1 and

ϵ germline transcripts than Id2^{+/+} spleen cells, although the difference in expression was more pronounced for ϵ transcripts (Fig. 1b). No obvious differences in expression of germline transcripts of other isotypes were detected between Id2^{-/-} and Id2^{+/+} spleen cells (data not shown).

Enhanced IgE CSR of Id2^{-/-} B cells

The elevated ϵ germline transcript expression in Id2^{-/-} splenic B cells raised the possibility that the underlying B cell defect may directly contribute to enhanced class switching to IgE. To investigate the role of Id2 in CSR to IgE, we analyzed the frequency of mIg⁺ B cells of each isotype. Splenic B cells were isolated and stimulated *in vitro* with LPS and IL-4 to induce CSR to IgG1 and IgE. Flow cytometric analysis demonstrated that higher percentages of B cells were positive for mIgE expression in Id2^{-/-} mice than in control WT mice at every concentration of IL-4 used to induce CSR. This increase was dependent on the concentration of IL-4 (Fig. 2a). Culture supernatants of Id2^{-/-} B cells contained substantially higher amounts of IgE than those of the control littermates at each IL-4 concentration examined (data not shown). In contrast, Id2^{-/-} B cells induced under these conditions exhibited no difference in their expression of other Ig isotypes (IgG1, IgG2a and IgG3) either on the cell surface (Fig. 2a) or in culture supernatants (data not shown). Thus, enhanced class switching occurred exclusively to the IgE locus in Id2^{-/-} B cells in response to

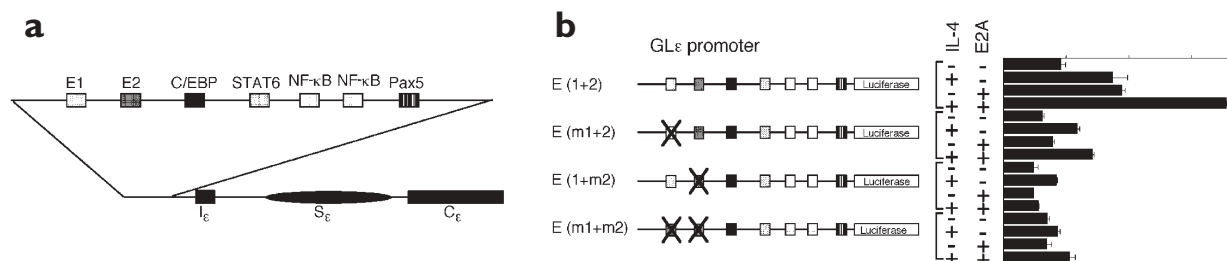
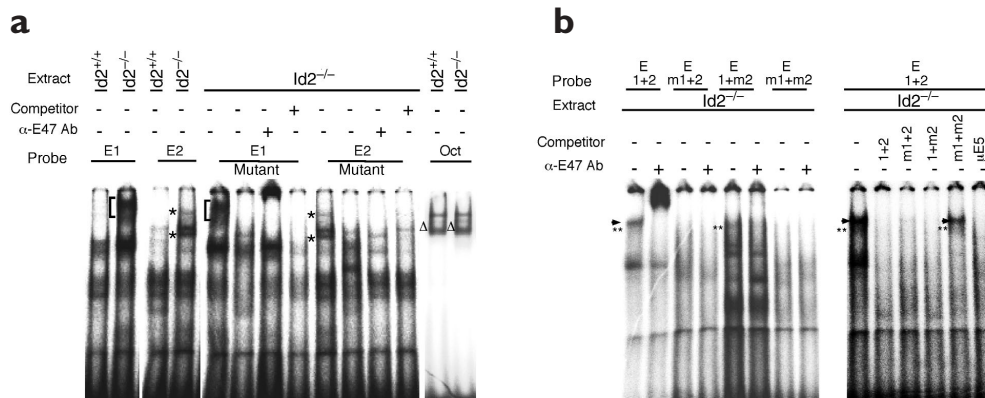


Figure 3. Transactivation of the GL ϵ promoter by E2A transcription factors. (a) Schematic representation of the GL ϵ promoter. (b) BaF/3 cells were transiently transfected with WT and E-box-mutated GL ϵ promoter-luciferase constructs in the absence or presence of 20 μ g of E2A expression plasmid and cultured with or without 20 ng/ml of IL-4. The total amount of DNA used for each transfection was adjusted to 40.5 μ g by the addition of pCMV empty vector. Each firefly luciferase activity was expressed as relative luminescence units normalized to sea pansy luciferase activity. All assays were independently performed three times in duplicate. m1, mutation of E1-box. m2, mutation of E2-box.

Figure 4. Binding of the GL ϵ promoter by E2A transcription factors. (a) EMSA of whole cell extracts from splenic B cells from Id2^{-/-} and control mice treated with 20 μ g/ml of LPS plus 3 ng/ml of IL-4 for 3 days with oligonucleotides containing a single E-box (E1 or E2) present in the GL ϵ promoter. Mutant, the mutant probe of E1 or E2 box. Oligonucleotides containing single octamer consensus sequence were used as control probes. (b) EMSA of whole cell extracts from splenic B cells from Id2^{-/-} and control mice treated as described in a with oligonucleotides containing two E-boxes (E1+E2). m1, mutation of E1 box. m2, mutation of E2 box. μ E5, the E2A binding sequence existed in a heavy chain intron enhancer. Asterisks, arrows and brackets indicate the E2A-containing complexes. Triangles indicate Oct-containing complexes.



LPS and IL-4. These results also indicated that the signaling through IL-4 in general was not enhanced in Id2^{-/-} B cells^{3,4,13} because the CSR to IgG1 was not affected. Selective enhancement of ϵ GLT by IL-4 was also observed in Id2^{-/-} cells (Fig. 2b). In addition, digestion-circularization PCR (DC-PCR) assays²¹, which allow the direct measurement of DNA rearrangements of an Ig locus, demonstrated biased class switching to IgE in Id2^{-/-} splenic B cells (Fig. 2c). These results indicated that enhanced class switching to IgE in Id2^{-/-} B cells was due to a direct effect of increased ϵ GLT¹⁶. However, we failed to detect differences between Id2^{-/-} and Id2^{+/+} splenic B cells in the expression of BCL-6, which suppresses, and Pax5, which enhances, both ϵ GLT and IgE CSR^{22,23} (data not shown).

Transactivation of GL ϵ promoter by E2A

Because Id2 is a negative regulator of E protein function^{17,18}, enhanced activity of E2A products may directly promote and enhance ϵ GLT in response to IL-4 in Id2^{-/-} B cells. The putative promoter-enhancer region of the GL ϵ transcript contains two E-boxes, the consensus binding sites for E2A proteins in addition to a consensus binding-site for STAT6 that mediates the signaling of IL-4 (Fig. 3a). To test the above possibility, we subcloned the 230 bp GL ϵ promoter region into a pGL3 plasmid and analyzed the promoter activity by the luciferase assay in a pro-B-like cell line, BaF/3²⁴. E47, an E2A gene product, induced transactivation from the wild-type promoter and IL-4 synergistically augmented this activity (Fig. 3b). On the other hand, deletion of one or two E-boxes present in the promoter region abolished the E47 responsiveness and the synergistic effects of E47 and IL-4 (data not shown). Promoter constructs containing mutations in E1 and/or E2 gave similar results, further

suggesting that the E2 site is critical to IL-4 and E47 responsiveness and that the E1 site is required for full activation (Fig. 3b). These results indicated that E proteins play a key role in the induction of ϵ GLT in response to IL-4.

Binding of GL ϵ promoter by E2A

To determine whether E2A binds to the two E-boxes in the GL ϵ promoter, we performed electrophoretic mobility shift assays (EMSA) using GL ϵ promoter oligonucleotides bearing E1 and/or E2 sites. Whole cell extracts prepared from B cells activated with LPS and IL-4 were tested for E2A binding activity to these nucleotides. E2A-containing complexes were detected by both probes having E1- or E2-boxes (Fig. 4a). Whole cell extracts from Id2^{-/-} B cells contained increased E2A binding activity (Fig. 4a). There was no difference in the binding activity of an unrelated transcription factor, Oct, between extracts from Id2^{-/-} and WT B cells (Fig. 4a). Using a probe bearing both the E1 and E2 (E1 + E2) E-boxes, we observed a large complex specific to this probe (Fig. 4b). This large complex was supershifted by the addition of anti-E2A and disappeared by the addition of specific competitors containing at least one WT E-box, but not by that containing only the mutated E-boxes (Fig. 4b). These results were consistent with reporter gene assays that showed a requirement for both E-boxes for full activation of the GL ϵ promoter.

Ectopic expression of Id2 suppresses IgE CSR

To determine whether the reintroduction of Id2 protein can suppress the enhanced class switching to IgE observed in Id2^{-/-} B cells, we employed a retrovirus-mediated approach to introduce Id2 into Id2^{-/-} B cells. Id2

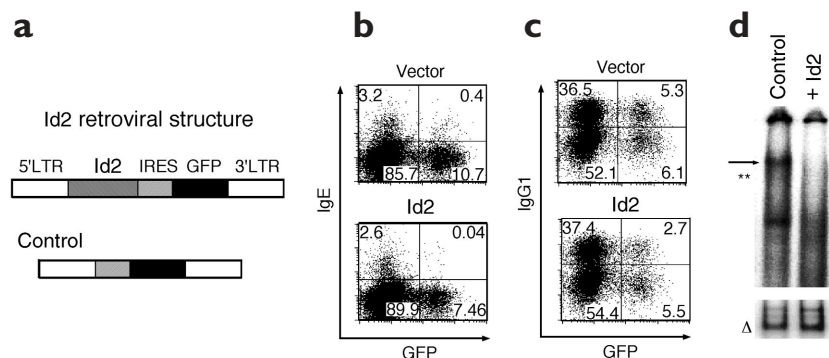
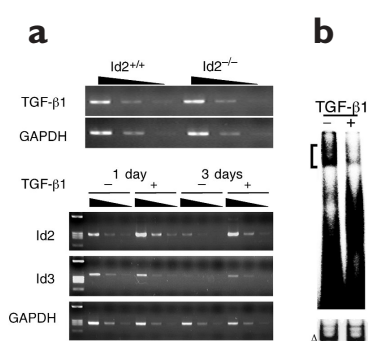


Figure 5. Reintroduction of Id2 protein restores enhanced IgE class switching. (a) Schematic of the Id2 retrovirus construct. (b) Splenic B cells from Id2^{-/-} mice were infected with either GFP-Id2 or control virus and cultured for 3 days with 10 μ g/ml of LPS plus 3 ng/ml of IL-4. Cells from these cultures were analyzed by flow cytometry for the expression of GFP and IgE. Data from one of three representative experiments are shown. (c) Cultured cells described in b were analyzed by flow cytometry for expression of GFP and IgG1. Data from one of three representative experiments is shown. (d) (Upper panel) Whole cell extracts prepared from the GFP-positive cells as described in b,c were analyzed by EMSA with the E1 + E2 site as a probe. (Lower panel) Control data for Oct binding of these extracts are shown. The E2A-containing complexes are indicated by arrow and asterisks. The Oct-containing complexes are indicated by triangles.

Figure 6. Id2 is induced by TGF- β 1 in splenic B cells. (a) RT-PCR of TGF- β 1 mRNA in the spleen 6 days after immunization. One of three independent experiments is shown. GAPDH was used as internal control for RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. (b) RT-PCR of Id2 and Id3 in B cells at the indicated times in cultures with or without 2 ng/ml of TGF- β 1 in the presence of 20 μ g/ml of LPS and 1 ng/ml of IL-4. One of three independent experiments is shown. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. (c) (Upper panels) EMSA of whole cell extracts from splenic B cells treated under the same conditions as in **b** for 24 h. (Lower panels) Data for Oct binding of these extracts are shown. The E2A- and Oct-containing complexes are indicated by bracket and triangles, respectively. One of three independent experiments is shown.



cDNA was cloned into the pMXIRES-GFP vector²⁵, which allowed Id2 expression to be monitored by green fluorescent protein (GFP) (Fig. 5a). B cells were infected with the virus by spin infection²⁶ and analyzed for cell-surface expression of IgG1 and IgE after 3 days of culture with LPS plus IL-4. As shown in Fig. 5b, Id2-transduced GFP-positive cells showed substantially reduced numbers of IgE-positive cells (3.6% IgE-positive cells in the control population as compared with 0.5% in the Id2-expressing population). Although Id2 transduction also suppressed IgG1 expression, the effect was not as pronounced (46% IgG1-positive cells in the control compared with 33% in the Id2-expressing population; Fig. 5c). To confirm that the expressed Id2 functioned as a dominant-negative regulator of E2A products, whole cell extracts were prepared from GFP-positive cells selected by fluorescence-activated cell sorting and analyzed by EMSA. As expected, E2A DNA binding complexes were lost in extracts from Id2-transduced cells, whereas binding complexes of the unrelated transcription factor Oct were unchanged (Fig. 5d). When the same experiment was performed with the use of Id2^{+/+} B cells to force increased Id2 expression, all class switching, including that of IgG1 and IgE, was completely blocked, as previously described²⁷ (data not shown). These data indicated that the quantity of active E2A products determines class switch recombination, particularly to IgE.

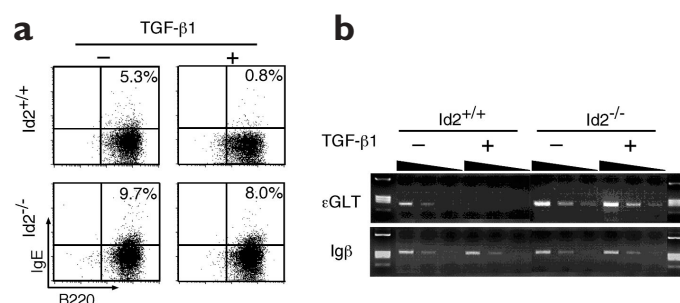


Figure 7. Inhibition of IgE CSR by TGF- β 1 is mediated by Id2. (a) FACS analysis of mIgE⁺ cells cultured for 4 days with or without 2 ng/ml of TGF- β 1 in the presence of 20 μ g/ml of LPS and IL-4 (30 ng/ml for Id2^{+/+} and 3 ng/ml for Id2^{-/-}, to roughly adjust the number of IgE⁺ cells without TGF- β 1). Data from one of three representative experiments is shown. (b) RT-PCR of ϵ GLT in B cells cultured as in **a**. One of three independent experiments is shown. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. Ig β was used as internal control for RT-PCR.

TGF- β 1 inhibits IgE CSR through Id2

TGF- β 1, a pleiotropic cytokine, inhibits IgE CSR *in vitro*^{28,29} and mice deficient for TGF- β 1 display a hyper-IgE phenotype³⁰. We investigated the relationship between Id2 and TGF- β 1 with respect to the regulation of IgE CSR. RT-PCR demonstrated no obvious difference in the expression of TGF- β 1 in splenocytes between each genotype, suggesting that Id2 does not affect the expression of TGF- β 1 (Fig. 6a). We examined the expression of Id2 by RT-PCR in wild-type splenic B cells after treatment with TGF- β 1 in the presence of LPS and detected a more than roughly fivefold increase of Id2 mRNA expression induced by the addition of TGF- β 1 from 24 h onward (Fig. 6b). In contrast, Id3 showed only a marginal induction after 3 days (Fig. 6b). Consistent with the increase of Id2 expression, in B cells treated with TGF- β 1 for 24 h, the activity of binding to the E1 + E2 probe of the GL ϵ promoter was only approximately 10% of that found in untreated B cells, whereas that to an unrelated probe, Oct, was unchanged (Fig. 6c). We analyzed the effect of TGF- β 1 on IgE CSR in the absence of Id2. Splenic B cells from Id2^{-/-} mice and their WT littermates were cultured with TGF- β 1 in the presence of LPS plus IL-4, and the IgE CSR of each sample was analyzed by assessing the expression of mIgE after 4 days of stimulation. TGF- β 1 strongly suppressed the percentage of IgE-positive cells in Id2^{+/+} B cells (from 5.3% to 0.8%), as reported^{28,29}. In contrast, TGF- β 1 had no effect on IgE expression in the Id2^{-/-} B cells (from 9.7% to 8.0%, Fig. 7a). TGF- β 1 influenced the expression of ϵ GLT, as shown in Fig. 7b by semiquantitative RT-PCR analysis with cDNAs from the same samples as in Fig. 7a. In contrast to the effect in Id2^{+/+} B cells, ϵ GLT was not repressed by TGF- β 1 in Id2^{-/-} B cells (Fig. 7b). These results demonstrated that Id2 was a direct effector induced by TGF- β 1 that can suppresses IgE CSR.

Discussion

We have demonstrated here that the IgE locus is a direct target of E2A in activated B cells. Moreover, we have shown that Id2 actively and specifically suppresses IgE CSR in response to TGF- β 1 through antagonizing E2A activity. Previously, it has been demonstrated that TGF- β 1 induces Id2 and Id3 in early B cell progenitors and inhibits their growth and survival. At the pro- and pre-B cell stages, Id3 induction is more prominent than that of Id2³¹. In mature B cells, in contrast, Id2 induction predominates, as shown here. Thus, differential induction of Id genes is one of the mechanisms that may determine the different *in vivo* functions of Id proteins, which are involved in many steps of cell proliferation and differentiation. Besides E2A, Id2 also interacts with Pax5³², which is essential for the normal development of B cells³³ and inhibits its DNA binding function. This function of Id2, antagonizing Pax5 activity, can work in activated B cells in which CSR takes place (unpublished data). However, the inhibitory effect of Id2 on Pax5 activity is less prominent than on E2A activity. These observations indicate that the augmentation of Pax5 activity might also contribute to enhanced IgE CSR in Id2^{-/-} B cells *via* the Pax5-binding sequence within the GL ϵ promoter as previously described²².

Other than Id2, which we have discussed here, a few other gene products have been reported to have effects on IgE CSR. Enhanced IgE CSR was observed in BCL-6^{-/-} B cells²³, in which BCL-6 acts as a negative regulator of STAT6. Because STAT6 also activates γ 1 GLT, enhancement of IgG1 CSR also occurred in these cells. These data principally indicate that the augmentation of IL-4 signal transduction, which induces γ 1 and ϵ GLT in B cells through the activation of transcription factors including STAT6, is one of the mechanisms for inducing the

hyper-IgE phenotype. In accordance with this notion, skewed T helper type 2 (T_H2) differentiation, which produces IL-4, also contributes to the hyper-IgE phenotype. In contrast, Id2 is the specific inhibitor of CSR to IgE but not IgG1. These results also provide a rationale to explain how the low plasma concentrations of IgE are tightly regulated *in vivo* and why IL-4 differentially affects CSR to IgG1 or IgE in B cells. TGF- β 1 has been shown to have an anti-inflammatory as well as immunosuppressive effects on many cell types³⁴. Consistent with this, uncontrolled inflammatory and autoimmune responses were observed in mice lacking TGF- β 1³⁵. Here, we have shown another important function of TGF- β 1 *in vivo* is to reduce IgE CSR by inducing the expression of Id2 in B cells. This scenario is consistent with the hyper-IgE phenotype observed in TGF- β 1^{-/-} mice. Our results suggest that Id2 acts as an inhibitor of IgE CSR by quenching the activity of E proteins and reducing ϵ GL transcription, which is positively correlated with CSR efficiency¹⁴. Id2^{-/-} mice are in a T_H2-dominant state and display elevated serum concentrations of IgG1 and IgE, with a more prominent elevation of IgE²⁰.

Our analysis provides insights into the fundamental question of how the concentration of IgE is kept much lower than those of other isotypes at the molecular level. By the system in which TGF- β -induced Id2 specifically reduces CSR to IgE without effects on that to other isotypes, the type 2 immune response could progress to produce antibodies that appropriately keep the IgE concentration low. This reduction of IgE CSR by Id2 seems to work as a molecular safeguard to prevent serious complications, including allergic disorders such as anaphylaxis, hay fever, food allergy and asthma. Defects in this system, conversely, could lead to hyper-IgE disorders, including immunodeficiency¹. Among the syndromes that are accompanied by high IgE concentration, genetic defects such as mutations in coding or regulatory sequences of the gene encoding Id2 might be found. Signaling from TGF- β 1 to induce Id2 expression in B cells could also be defective in allergic disorders. Drugs that enhance Id2 expression and/or signaling from TGF- β 1 might be applicable for the treatment or prevention of allergy. Analysis of the gene encoding Id2 in such disorders and development of drugs targeting this system would be promising future subjects.

Methods

Mice. Id2^{-/-} mice on the 129/Sv genetic background were maintained under specific pathogen-free conditions and used at 7 to 10 weeks of age. Procedures involving animals and their care were conducted according to the guidelines for animal treatment at the Institute of Laboratory Animals, Kyoto University.

Preparation of cells and *in vitro* cell culture. For the *in vitro* class switching study, purified splenic B cells were obtained and stimulated as described previously³⁶. Briefly, single-cell suspensions of spleen cells were prepared, and red blood cells were removed by hypotonic lysis. T cells were depleted by treatment with the monoclonal antibodies (mAbs) anti-Thy-1 (30H12), anti-CD4 (RL172.5) and anti-CD8 (3.155) followed by complement lysis. Further purification to remove IgG1- and IgE-positive cells was performed with MACS (Miltenyi Biotech, Bergisch Gladbach, Germany).

Flow cytometric analysis. The following antibodies were used for staining: fluorescein isothiocyanate (FITC)-anti-mouse B220 and biotin-anti-mouse IgE (both from Pharmingen, San Diego, CA); biotin-anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL); and allophycocyanin-streptavidin (Molecular Probes, Eugene, OR). All analyses were performed with a FACSCalibur (Becton Dickinson, Mountain View, CA).

PCR. Total RNAs were extracted from splenocytes and cultured splenic B cells with TRIzol (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions, and then quantitated at A₂₆₀. Oligo(dT)-primed cDNAs were prepared with reverse transcriptase. For semiquantitation, 50 ng of cDNAs were serially diluted and subjected to PCR amplification with the following primer pairs: 5' Iy1, 5'-CAGCCTGGTGTCAACTAG-3'; 3' Cyl, 5'-GCAAGGGATCCAGAGTCCAG-3'; 5' Ie, 5'-GCAGAAGATGGCTTCCAATAAGAACAGT-3'; 3' Ce, 5'-TCGTGAATGATGGAGGATGTGTCACGT-3'; 5' Ig β , 5'-CTGTGCTGTCTCCATGC-3'; 3' Ig β , 5'-G ATGAGGAGGGTCTGGATCA-3'; 5' Id2, 5'-AGCATCCCCCA GAACAAGAAGTG-3'; 3' Id2, 5'-ATCGTCTTGGCCAGGTGTCGTTCT-3'; 5' Id3, 5'-

CCTCTCTATCTCTACTCTCCAACA-3'; 3' Id3, 5'-TGACCAGCGTGTGCTAGCTCTTC A-3'; 5' GAPDH, 5'-CCATCACATCTTCCAGGAG-3'; 3' GAPDH, 5'-CCTGCTTACCA CCTTCTTG-3'; 3' TGF- β 1, 5'-CTGTCCAACTAAGGCTCGC-3'; and 3' TGF- β 1, 5'-GT ATTCCGTCTCCTTGGTTC-3'. DC-PCR was performed as described previously¹⁵. All the PCR products were resolved electrophoretically in 2% agarose gels and visualized by ethidium bromide staining. These bands were quantitated with a Bio-image analyzer (Fijix BAS2000, Fuji Photo Film, Tokyo, Japan). All the PCR data are representative of three independent experiments.

Retroviral infections. Retrovirus was produced by transfecting the ecotropic Phoenix packaging cell line³⁷ with pMXIRES-GFP or pMXIRES-GFP-Id2 plasmids. After overnight stimulation with LPS, purified splenic B cells were spin-infected with the virus-containing supernatant in the presence of 10 μ g/ml of DOTAP (Roche Molecular Biochemicals, Mannheim, Germany) and cultured with fresh complete RPMI medium plus cytokines.

ϵ germline transcription promoter analysis. GL ϵ promoter was amplified from C57BL/6 genomic DNA with the following 5'primers with 3' eproR, 5'-AAAAGATCTCCTGTGC AGGCTCCAGGCGTGTG-3' primer; 5' eproF/E (1+2), 5'-AAAAGATCTACCAGAGTG TGCAGGTGTGTCTCC-3'; 5' eproF/E(d1+2), 5'-AAAAGATCTGTGTCTCCTAGAAAG AGGCCT-3'; 5' eproF/E(d1+d2), 5'-AAAAGATCTAGACCCCACTGTGCCTTAGTC-3'; and 5' eproF/E(m1+2), 5'-AAAAGATCTACCAGAGTGTGTTAACTTGTCTCC. The BglII sites used for cloning are underlined. PCR-amplified fragments were digested with BglII. For construction of the mutant plasmids, named E(1+m2) and E(m1+m2), *in vitro* mutagenesis was performed according to the manufacturer's protocol (Stratagene, La Jolla, CA) with the template constructs E(1+2) or E(m1+2). Primers for mutagenesis (top and bottom primers): E (m2), 5'-AAGAGGCCTTTAACTAGACCC-3' and 5'-GGGTCTAGT-TAAAGGCCTT-3'. BaF/3 cells were washed twice with PBS, mixed with plasmid DNA, in 400 μ l of PBS, and electroporated at 960 μ F, 600 V/cm. The total amount of DNA added to 4 $\times$ 10⁶ cells was adjusted to 40.5 μ g, which contained 20 μ g of reporter construct, 0.5 μ g of tk-sea pansy control plasmid and 20 μ g of E2A expression plasmid or empty plasmid. Transfectants were harvested after 18 h of incubation with or without IL-4 (Peprotech, London, UK). Luciferase assays were performed as described previously³⁸. Firefly luciferase activity was normalized by Renilla luciferase activity.

Whole cell extracts of splenic B cells were prepared after incubation with LPS (Sigma, St. Louis, MO) plus IL-4 (Peprotech) and protein amounts quantified with Micro BCA protein Assay Reagent (Pierce, Rockford, IL). Double-stranded oligoprobes were end-labeled with T4 polynucleotide kinase (Takara, Japan) and purified with a G50 sepharose column. Whole cell extracts and labeled oligoprobes were used in a gel-shift assay as described previously³⁹. Briefly, labeled probes (50 fmol) were incubated with 2 μ g of poly(dI)•poly(dC) (Amersham Pharmacia, Buckinghamshire, UK) and 60 μ g of protein equivalents of whole cell extract in a total volume of 20 μ l. After incubation at room temperature for 30 min, the samples were loaded onto a 5% polyacrylamide gel that had been prerun for 30 min at room temperature at 120 V in 0.25 $\times$ TBE (Tris-borate EDTA). Samples were electrophoresed at 70 V for 2.5 h and then at 100 V for 30 min. Gels were dried at 65 $^{\circ}$ C for 30 min and exposed to imaging plates (Fuji Photo Film). Exposed gel images were visualized with a BAS 2000 imaging analyzer (Fuji Photo Film). Competitor oligonucleotides were added in a 100-fold excess. Probes used for EMSA are as follows (top and bottom oligos): E1, 5'-GACCAGAGTGTGACGGTGTCT-3' and 5'-GAGACACCTGCACACTCTGGT-3'; mutant E1, 5'-GACCAGAGTGTGTTAACTTCT-3' and 5'-GAGAAGTTAAACACTCT GGT-3'; E2, 5'-GAGGCCTCACCTGAGACCCCA-3' and 5'-AGTGGGGTCTCAGGT GAGGCCT-3'; mutant E2, 5'-GAGGCCTTTAACTAGACCCCA-3' and 5'-AGTGGGGTCT TAGTTAAAGGCCT-3'; E(1+2), 5'-TTGACCAGAGTGTGACGGTGTGTCTCCTAG AAAGAGGCTCACCTGAGACCCCA-3' and 5'-CAGTGGGGTCTCAGGTGAGGC CTCTTTCTAGGAGACACACCTGCACACTCTGGT-3'; E(m1+2), 5'-TTGACCAGA GTGTGTTAACTTGTCTCCTAGAAAGAGGCCTCACCTGAGACCCCA-3' and 5'-CAG TGGGGTCTCAGGTGAGGCCTCTTTCTAGGAGACAAGTTAAACACTCTGGT-3'; E(1+m2), 5'-TTGACCAGAGTGTGACGGTGTGTCTCTAGAAAGAGGCCTTTAAAC TAGACCCCA-3' and 5'-CAGTGGGGTCTAGTTAAAGGCCTCTTTCTAGGAGACACA CCTGCACACTCTGGT-3'; E(m1+m2), 5'-TTGACCAGAGTGTGTTAACTTGTCTCC TAGAAAGAGGCCTTTAACTAGACCCCA-3' and 5'-CAGTGGGGTCTAGTTAAAGGC CTCTTTCTAGGAGACAAGTTAAACACTCTGGT-3'; μ E5, 5'-TCGAAGAACC TGCAGCAGCT-3' and 5'-TAGAGCTGCTGCAGGTGTTCTT-3'; Oct, 5'-TGTCGAATG CAAATCACTAG-3' and 5'-TTCTAGTGATTGCAATCGA-3'. All the bands were quanti- tated with a Bio-image analyzer (Fijix BAS2000). α -E47Ab used for super-shift experi- ments was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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Competing interests statement

The authors declare that they have no competing financial interests.

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