

B Cell Activation and Ig, Especially IgE, Production Is Inhibited by High CD23 Levels *in Vivo* and *in Vitro*¹

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The capacity of CD23 to regulate IgE production was evaluated in both an *in vitro* and an *in vivo* system. The decreased IgE response seen in CD23 transgenic mice was confirmed and observed to occur at all antigen doses used. In addition, purified B cells from the Tg animals in general exhibited lower IgE production when stimulated with CD40L and IL-4. To examine this down-regulating activity of CD23 an *in vitro* model system was developed. CHO cells were transfected with CD23, ICAM-1, or both CD23 and ICAM-1. ICAM-1 was chosen to enhance B cell–B cell interaction. Purified resting B cells were placed into culture with the mitomycin C-treated transfected or control CHO cells and activated with IL-4, IL-5, and CD40L-CHO. A dose-dependent decrease in IgE production was observed with increasing cell numbers of the CHO transfectants that expressed CD23. The effect lasted up to Day 3 of culture. B cell proliferation was also inhibited in a dose-dependent fashion by increasing numbers of CD23-expressing cells suggesting a potential effect of CD23 on B cell apoptosis. In contrast, ICAM-1-transfected or CHO control cells had minimal effects on either Ig production or B cell proliferation. While IgE production was inhibited up to 95% by high numbers of CD23-transfected CHO cells, some inhibition of IgG and IgM production was also seen. Finally, the mechanism of CD23-mediated inhibition of IgE production was compared with the inhibition in IgE production seen when B cells were coactivated with multivalent anti-IgD in conjunction with CD40L plus optimal IL-4. To this end we used RT-PCR to compare the relative levels of ϵ -germline transcripts in control cultures and cultures coactivated by anti-IgD, CD40L, and IL-5 or activated in the presence of high levels of CD23-expressing cells. CD22 was used as an internal standard since levels

change little with B cell activation. Coactivation strongly inhibited ϵ -germline transcript levels but the presence of CD23-expressing cells did not. Thus, coactivation potentially operates prior to isotype switching, while high CD23 coculture blocks either recombination or more likely B cell differentiation to high Ig producers stage. Our data support the hypothesis that IL-4 induces both IgE and a controlling agent for IgE, namely, CD23. © 1997 Academic Press

INTRODUCTION

The low-affinity receptor for IgE (Fc ϵ RII) was initially discovered in 1975 by Lawrence *et al.* (1) by using aggregated IgE on human peripheral blood mononuclear cells. Cloning studies (reviewed in 2) demonstrated that Fc ϵ RII is a type II integral membrane glycoprotein and that the 45-kDa B cell activation antigen designated as CD23 and Fc ϵ RII were the same protein (3). In humans two transcription initiation sites are used resulting in two forms of CD23, termed CD23a and CD23b (4). While this difference is only reflected in the first six cytoplasmic amino acids, the two isoforms have quite different expression characteristics. CD23a is present only on B cells and possibly follicular dendritic cells (5), but CD23b is expressed on a much wider number of hematopoietic cell types (reviewed in 2). In the mouse, only the CD23a isoform is expressed at detectable levels (6), although low amounts of CD23b message can be detected by RT-PCR (7).

CD23 has a somewhat unusual metabolic pathway. Upon arrival at the cell surface (8) and possibly also internally (9), CD23 is cleaved with the majority of the COOH-terminus being released. The initial fragment, termed sCD23 or IgE-binding factor (10), is 37 kDa (human) and is not N-glycosylated (11). The fragment is then further broken down to the size limit of 12–16 kDa, consisting of the lectin homology domain only (11, 12). Mouse CD23 is initially cleaved to 38 kDa and is then cleaved into smaller fragments, termed mouse soluble CD23 (msCD23) (13).

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The binding site of IgE for CD23 is on the C ϵ 3 domain (14, 15). The binding is Ca²⁺ dependent, but does not require carbohydrate as deglycosylation of IgE did not significantly influence the binding (16, 17). Mutations in the lectin domain change IgE binding, indicating that the interaction site of CD23 with IgE is in the lectin cassette (18, 19). The stalk region of the molecule has homology with tropomyosin and other similar molecules that exhibit α -helical coiled-coil motifs (20, 21). Such molecules are anticipated to form trimers and even higher oligomers have been reported for both the asialoglycoprotein receptor and the mannose-binding protein, two members of the C-lectin family (22, 23). Dierks *et al.* (24), using chemical crosslinkers, demonstrated that the intact form of mCD23 oligomerized to at least a trimeric form and also showed that the stalk repeat region mediated this oligomerization. In addition, the fact that the multimeric form was exclusively eluted from an IgE affinity column supported the hypothesis that the oligomerization resulted in a higher affinity for IgE, presumably via multipoint binding. This finding has led to a new model for CD23 and helps explain the dual affinity seen between IgE and CD23 (24). Unlike CD23, mouse sCD23 does not exhibit this oligomerization capacity and only interacts with IgE with a single low affinity ($10^6 M^{-1}$) (25).

Ever since its discovery, CD23 has been postulated to play a role in IgE regulation. The finding that CD23 is regulated by the same cytokines that induce IgE (IL-4, IL-13) (26, 27) potentially suggested that these cytokines are involved in the induction of isotype switching to IgE and induce a regulator for IgE, namely, CD23. The purpose of this study was to further investigate this issue. In the CD23-deficient mouse, one study (28) but not others (29, 30), reported an elevation of antigen-specific IgE after antigen/alum injection. An important result for this study was the finding that CD23 transgenic mice showed a decrease in IgE production. These CD23 transgenic mice have an increased expression of CD23 on both B and T cells (29). The results with the CD23 transgenics have been confirmed further in that the IgE suppression was also observed with purified B cells. In addition, an *in vitro* model was developed in which coculture of purified B cells with cells expressing high levels of CD23 was shown to suppress IgE production.

MATERIALS AND METHODS

Anti-IgD (AF3.33) was a gift of Dr. Fred Finkleman (31). For FACS analysis, the following antibodies were used. Hamster IgG anti-mouse CD40L (anti-gp39 or MR-1) (32) was a gift of Dr. R. Noelle, and phycoerythrin-conjugated or biotin-conjugated MR-1 was purchased from PharMingen (San Diego, CA). B3B4 is a rat IgG_{2a} anti-mouse CD23; the site of interaction is close but not identical to the site on CD23 that interacts

with IgE (33). This Mab was purified from the rat ascites. Fluorescein-labeled B3B4 was prepared in house or purchased from PharMingen. MK-1 is rat IgG anti-mouse ICAM-1 mab obtained from Dr. G. Burton (VCU) and cell culture supernatant was used for staining. Fluorescein-labeled goat F(ab')₂ anti-mouse IgG and fluorescein-labeled goat F(ab')₂ anti-rat IgG were purchased from Cappel (Durham, NC). CD40LT³ and M15 (mouse IgG₁ anti-trimer) were from Immunex Co. (Seattle, WA) (34). The preparation of CD23 transgenics under the control of the Thy1 promoter has been previously described (29). Transgenic animals were bred in our aseptic facility. BALB/C mice, C57BL/6 mice (Jackson Laboratory), and CD23 transgenics were all used between 6 and 12 weeks of age. All animals were kept in an accredited virus-free facility until used. FACS analysis was performed on a FACScan (Becton-Dickinson, San Jose, CA) using the Cyclops (Cytomation, Ft. Collins, CO) operating software.

Cytokines, cell lines, and B cell preparation. Recombinant mouse IL-4 was a generous gift from Dr. William Paul (NIH, Bethesda, MD); mouse IL-5 was purchased from R&D Systems (Minneapolis, MN). CHO-K1 or CHO/DHFR⁻ (35) were obtained from the ATCC and were maintained in supplemented DMEM or IMDM with nucleosides, respectively. A cell suspension of mouse splenic cells was first depleted of adherent cells by culturing for 30 min at 37°C in B cell medium (RPMI 1640 containing 10% heat-inactivated FCS, 10 mM Hepes, 10 mM Na pyruvate, $5 \times 10^{-5} M$ 2-mercaptoethanol, and 100 U/ml penicillin and streptomycin). Subsequently, B cells were purified by negative selection using anti-CD8, anti-CD4, and anti-Thy1.1 as described previously (25) and resting B cells were obtained by using discontinuous Percoll gradients (36); cells at the 66–70% interface were considered to be resting B cells and were used in the assays described.

Preparation of transfected cells. The preparation of CD40L-CHO permanent transfectants was described previously (37). Briefly, the cDNA for mouse CD40L was obtained by RT-PCR and inserted into the pCDNA1 expression vector. CHO/DHFR⁻ were cotransfected using CD40L-pCDNA1 and pSV2-DHFR (5/1 ratio) using the calcium phosphate procedure (38). Positive clones were identified by FACS using PE-MR-1 (PharMingen) and CD40L expression was further amplified using methotrexate. The positive line used herein is termed m40L-2 and is maintained in MEM- α containing 4 μM methotrexate. mRE2-1 (CD23-CHO)

³ Abbreviations used: Fc γ RI-CHO, CHO transfected with the mouse Fc γ RI cDNA; m40L-2, CHO transfected with mouse CD40L cDNA; CD40LT, recombinant CD40L trimer; mRE2-1, CHO transfected with mouse CD23; mIC2-1, CHO cells transfected with mouse ICAM-1; mICRE2-1, CHO transfected with both CD23 and ICAM-1.

were prepared as described previously using the glutamate synthetase system (pEE6/GS) (39) and were maintained in DMEM GS. Mouse ICAM-1 cDNA in pH β APr-1-neo was a gift of A. Brian (40). Transfection into CD23⁺ CHO (24) or CHO/DHFR⁻ (ATCC) was performed by electroporation using a Bio-Rad (Hercules, CA) gene pulser II. Briefly, 2×10^6 cells (200 μ l) were mixed with 20 μ g purified mouse ICAM-1 cDNA-pCDNAI/Neo, in electroporation buffer. The cells were pulsed two times with 350 V, 25 μ F, and cultured for 3 days in 75-cm² tissue culture flasks. On Day 3, cells were split one to four into a 10-cm culture dish containing 1 mg/ml geneticin. Visible colonies were evident after 10 days. Double expressing cells of mouse CD23 and mouse ICAM-1 were confirmed by FACS analysis and the clone used was termed mICRE2-1. The single expressing cells of mouse ICAM-1 was confirmed also by FACS analysis and the clone was termed mIC2-1. The transfected cell lines are collectively called "support cells" when cultured with B cells. When necessary, recloning was performed to maintain expression. A plasmid (mFc γ RI-pEE6) having the Fc γ RI cDNA (41) was a gift from Mark Hogarth (Heidelberg, Australia) and the Fc γ RI-expressing CHO were prepared using the glutamate synthetase system as described previously (37). Analysis of Fc γ RI expression was performed using FACS analysis with mouse IgG_{2a} and F(ab')₂-anti-mouse-IgG.

B cell activation. *In vitro* studies were performed using resting B cells from CD23⁺, BALB/C, or C57BL/6 mice. B cells were cultured with 10,000 U/ml IL-4, 5 ng/ml IL-5, and mitomycin-C treated m40L-2 (6000/well). In some experiments CD40LT (0.1 μ g/ml) and M15 (0.1 μ g/ml) were used instead of m40L-2. When cocultured with increasing numbers of support cells, 5000 B cells and 6000 m40L-2 cells were added along with 10,000 U/ml IL-4, 5 ng/ml IL-5. Support cells and m40L-2 cells were treated with mitomycin C to suppress their proliferation. Coactivation using Fc γ RI-CHO was performed as described previously (37). Briefly, purified B cells (5×10^4 /well) were added to a 96-well plate that contained Fc γ RI-CHO (6000/well) plus 1 μ g/ml anti-IgD (10.4.22 mab (42)) and the CD40L ligand trimer/M15 and IL-4 and IL-5 as above. For RNA isolation, one to five plates were set up under identical conditions and on Day 3 cells were collected and RNA isolated using the Trizol procedure as recommended by the manufacturer (Gibco, Grand Island, NY).

In vivo studies were performed by giving CD23-transgenic, BALB/C, and C57BL/6 mice a primary immunization by injecting antigen subcutaneously (10, 100, or 250 μ g DNP-KLH in an adjuvant consisting of 4 mg alum and 2×10^8 heat-killed *Bordetella pertussis*). After 2 weeks the mice were given a secondary immunization (10, 100, or 250 μ g DNP-KLH, 2 mg alum, and

1×10^8 heat-killed *B. pertussis*) in the footpads and subcutaneously.

Proliferation assays. Support cells (m40L-2, mRG1-1, mRE2-1, mIC-1, mICRE2-1, or CHO control) were collected by EDTA treatment and $1-3 \times 10^6$ cells were incubated with 25 μ g/ml mitomycin C for 30 min at 37°C in a foil-wrapped tube. Support cells were then washed 4 $\times$ by centrifugation and resuspended in B cell medium and the indicated cell number was added to a 96-well flat-bottomed culture plate (Costar). B cells, cytokines, and anti-IgD were added as indicated with a final volume of 200 μ l/well. On Day 3 after culture initiation, the wells were pulsed for 8 hours with 1 μ Ci/well [³H]thymidine (6.2 Ci/mMol, NEN, Boston, MA). Cells were then harvested using a PHD harvester (Cambridge Technology, Cambridge, MA) and counted in a Model 1220 LKB liquid scintillation counter. All samples were run in triplicate and data are expressed as CPM $\pm$ 1 SD.

Ig production analysis. IgM, IgG₁, and IgE production was determined from supernatants after 8 days of culture or serum from preimmune or immunized mice 9 days after the secondary immunization. All Ig analyses were performed by ELISA essentially as described previously (43). Alkaline phosphatase-labeled or unlabeled polyclonal anti-IgM and IgG₁ were obtained from Southern Biotech (Birmingham, AL). IgE assays utilized a pair of rat anti-mouse IgE monoclonals, R1E4 and B1E3, again as described previously (15). Antigen-specific IgE was determined by coating wells with 5 ng/ml B1E3 and R1E4. Plates were blocked with PBS containing 2% FBS and 0.02% Tween 20. Samples and standard (mouse IgE anti-DNP) were added next and then detected with biotinylated DNP-KLH followed by streptavidin-AP (Sigma) and *p*-nitrophenyl phosphate substrate (Sigma). All ELISA determinations were performed with a duplicate series of dilutions and read at dual wavelength (405/650 nm) with a V_{max} ELISA reader (Molecular Devices, Sunnyvale CA). Standard curves were run with the corresponding purified myeloma/hybridoma proteins and four-parameter analysis with Molecular Devices software. Dilution values that fell in the linear portion of the curve were used for analysis; the values shown represent duplicate samples determined at multiple dilutions $\pm$ 1 SE.

RT-PCR analysis. The primers for ϵ -germline and mRNA of CD22 were purchased from VCU DNA core. For ϵ -germline, the primers, upstream I ϵ exon primer (5'-GCACAGGGGGCAGAAGGAT-3') and downstream C ϵ 1 exon (5'-CGTTGAATGATGGAGGAT-3') were used; these are the same primers used by Warren *et al.* (44) and are specific for the ϵ -germline transcript. For mouse CD22, upstream primer 5'-AAACTCCAC-TACCAAGG-3' and downstream primer 5'-AGG-TGATCTTAGAATCTTCC-3' were used resulting in a

419-bp PCR product, from 650 to 1069 on murine CD22 cDNA (Genebank). RNA was prepared by the Trizol method and was used as template. RT-PCR was performed using the Geneamp RNA PCR kit (Perkin-Elmer, Branchburg, NJ). Briefly, reverse transcription reagent was prepared in PBS buffer, with 5 mM MgCl₂, 1 mM dGTP, 0.8 mM dATP, 1 mM dUTP, 1 mM dCTP, 1 U/ μ l RNase inhibitor, 1 U/ μ l MuLV reverse transcriptase, 2.5 μ M random hexamers, and different levels of RNA. This was adjusted to 20 μ l with dH₂O. The reverse transcription mixture was incubated according to the following schedule in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA). 10 min at 20°C, 15 min at 42°C, 5 min at 95°C, and then held at 4°C until the next step. After the RT step, PCR was performed. PCR reagent (1 $\times$ PCR buffer, 2 mM MgCl₂, 2.5 U Amplitaq DNA polymerase, 0.02 mM [³²P]dATP, 0.625 μ M upstream primer, and 0.625 μ M downstream primer) was prepared in 80 μ l. The total 100- μ l reagent was centrifuged and then PCR was performed with the following schedule: 95°C for 3 min, 15 cycles at 95°C for 45 sec, 54°C for 45 sec, and 72°C for 2 min; and then a final incubation of 72°C for 6 min was performed. The product was kept at 4°C until analysis by electrophoresis. For CD22, the downstream primer was used instead of random hexamer, for reverse transcription. The PCR product was applied to 6% vertical PAGE in TBE buffer. The samples were run at 60 V, 1 hr, and 100-bp ladder *M_r* standards were identified by EtBr staining. The gel was dried and analyzed with PhosphorImager 445SI (Molecular Dynamics).

RESULTS

Initial examination of the CD23 transgenic animals used in this study had found a suppression of IgE responses to both alum-injected antigen and parasite infection (29). To extend these results, animals were injected with three different levels of antigen (DNP-KLH) and the resulting IgE production is shown in Fig. 1. Since the transgenics are on a C57BL/6 $\times$ BALB/C background, control mice from both backgrounds were also analyzed. The preimmune IgE levels show a large difference in the amount of IgE produced by BALB/C mice compared to C57BL/6 and CD23 transgenic animals. This is probably attributed to differences between high-IgE and low-IgE responder mice. BALB/C animals are high responder mice and C57BL/6 are low responders (45). The CD23 transgenic animals also seem to be low responders like C57BL/6 mice. In confirmation of earlier results performed with a single dose of antigen (29), IgE responses were suppressed at all three doses of antigen in the transgenics and were lower than even the C57BL/6 animals. Antigen-specific IgE was also analyzed (Fig. 2) and the same trend was seen. The antigen-specific IgE ELISA gives relative

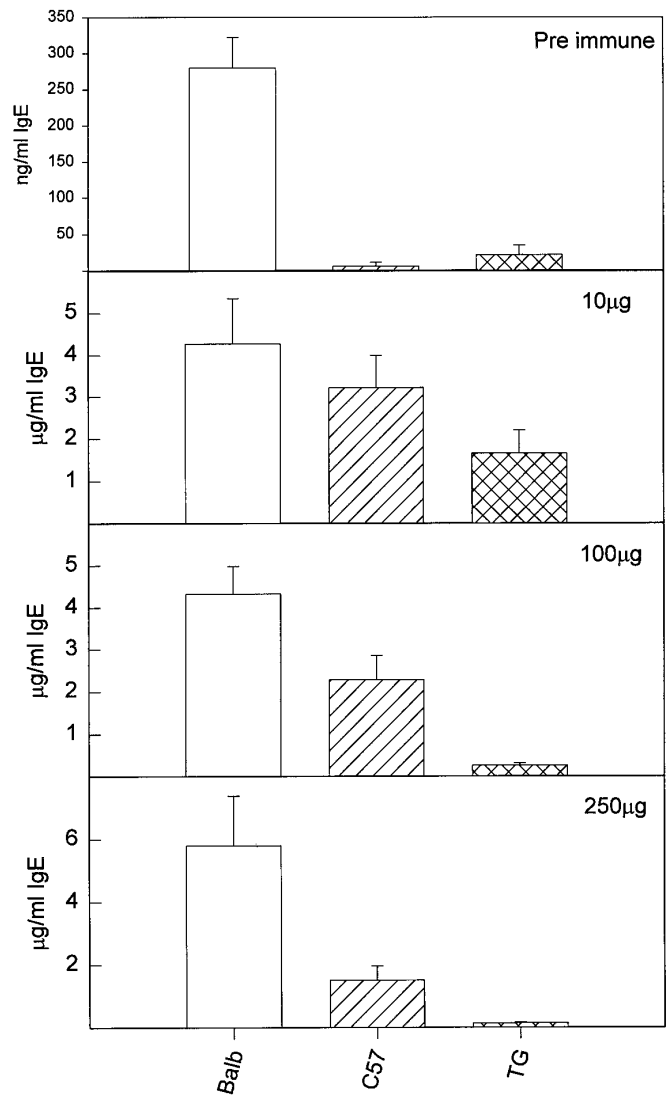


FIG. 1. Transgenic mice produce less IgE than either of the parental background strains to an antigen alum regimen. Preimmune IgE levels from BALB/C (open bars), C57BL/6 (striped bars), or CD23 transgenic mice (cross-hatched bars) and mice immunized with the indicated level of antigen (DNP-KLH) emulsified in alum plus *B. pertussis* are shown. Animals (four per group) were given two injections as indicated under Materials and Methods and 9 days after the secondary injection, blood was collected and analyzed for total IgE by ELISA.

amounts of IgE present in the samples. This is due to differing affinities for the antigen between the polyclonal serum IgE and the monoclonal IgE anti-DNP standard. Therefore, an exact amount of antigen specific IgE cannot be determined. Antigen-specific IgE is expressed as percentage of control animals. We next analyzed whether the transgenic animals had a decreased IL-4 or increased IFN- γ response, since these cytokines would be expected to have an enhancing or suppressing effect on IgE production, respectively. To do so lymph nodes were removed from immunized ani-

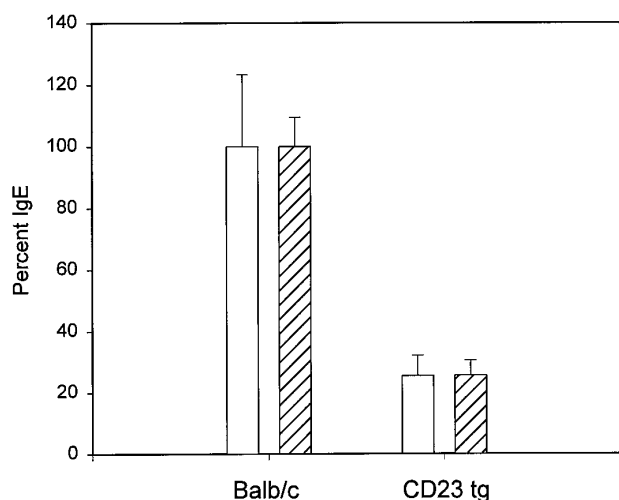


FIG. 2. Analysis of antigen-specific IgE produced by CD23 transgenic animals. Mice were immunized as in Fig. 1 and total (open bars) and antigen-specific (hatched bars) IgE were determined by ELISA 9 days after secondary immunization. IgE produced by CD23 transgenic animals is expressed as percentage of control mice. Absolute IgE values for total IgE in control mice was 5000 ng/ml.

imals and dispersed cells were cultured in the presence of increasing doses of antigen, similar to the procedure of McKnight *et al.* (46). After 3 days of culture supernatants were analyzed for IL-4 (CT4S proliferation (47)) or IFN- γ levels by ELISA. No significant differences in the production of either cytokine was evident, in comparing transgenic to control animals (data not shown).

Since levels of the two cytokines most heavily implicated in IgE regulation were not significantly affected, we next considered whether the B cells from the transgenic animals produced less IgE under optimal stimulation conditions. As reported elsewhere (37), the level of IgE produced in *in vitro* culture is highly dependent upon B cell levels. Once a critical threshold number of B cells are present, the IgE production is inversely proportional to B cell number. Thus, different numbers of B cells were stimulated with optimal levels of CD40L-trimer (34) plus IL-4 and IL-5 and the IgE production determined by ELISA. A total of six experiments of this type were performed where IgE production by B cells from BALB/C or C57BL/6 mice were compared to the levels produced by the transgenic B cells. Figure 3A shows a representative experiment of four of the six experiments performed and as can be seen, IgE production is lower when the source of B cells was the transgenic animals. The trend was seen at all input levels of B cells, arguing against the effect being due to a shift in the B cell dose-response curve. However, in two of the six experiments, the effect was of marginal significance. An example is shown in Fig. 3B. A possible explanation for the variation is the observation by Texido *et al.* (29) that transgene expression

resulted in only a marginal increase in CD23 expression on B cells and backcrossing with CD23 knockouts was required to confirm expression. This result is due to the choice of Thy-1 promoter for the CD23 transgene.

Development of an in vitro model for the effect of high-level CD23 on B cells. The results with the transgenic animals suggested that overexpression of CD23 inhibits the IgE response. This was further investigated by developing an *in vitro* model system utilizing support cells and m40L-2. This term support cells is used to indicate the transfected cells that were placed into culture with the B cells. Thus, modulation of CD40-dependent B cell activation by CD23 was investigated by adding the indicated number of either untransfected CHO or CHO transfected with CD23 (mRE2-1), ICAM-1 (mIC-1), or both ICAM-1 and CD23 (mICRE2-1). Figure 4 shows FACS analysis of ICRE2 cells with respect to CD23 expression and is compared to levels of CD23 on normal splenocytes. These same cells also express high levels of ICAM-1 by FACS analysis (data not shown). Based on this analysis, CD23 levels on support

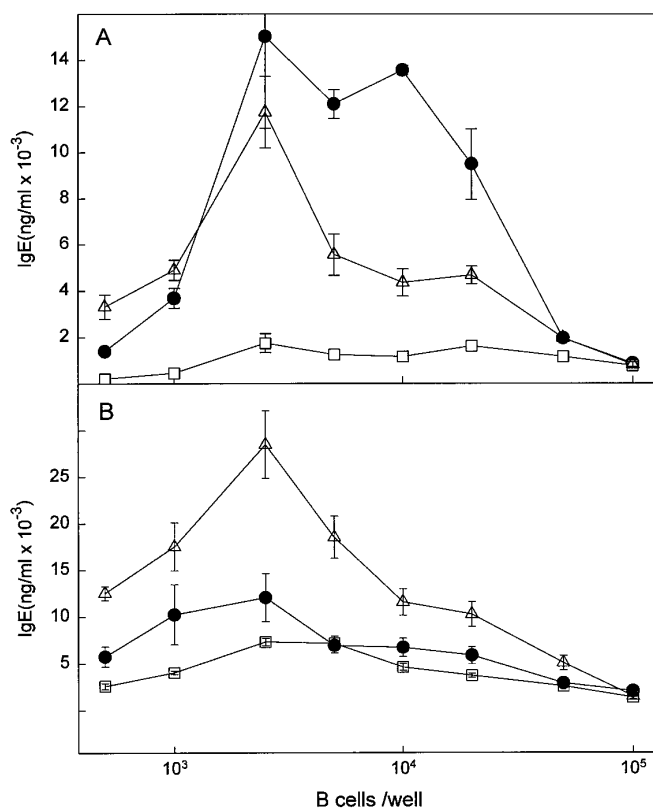


FIG. 3. Analysis of IgE production by CD40L-stimulated B cells from transgenic vs parental strains. B cells were purified from BALB/C (●), C57BL/6 (Δ), or CD23 transgenic (□) mice and stimulated in culture with recombinant CD40LT, IL-4, and IL-5 as indicated under Materials and Methods. After 8 days of culture, IgE levels in supernatants were determined by ELISA. Two of six experiments of this design are shown.

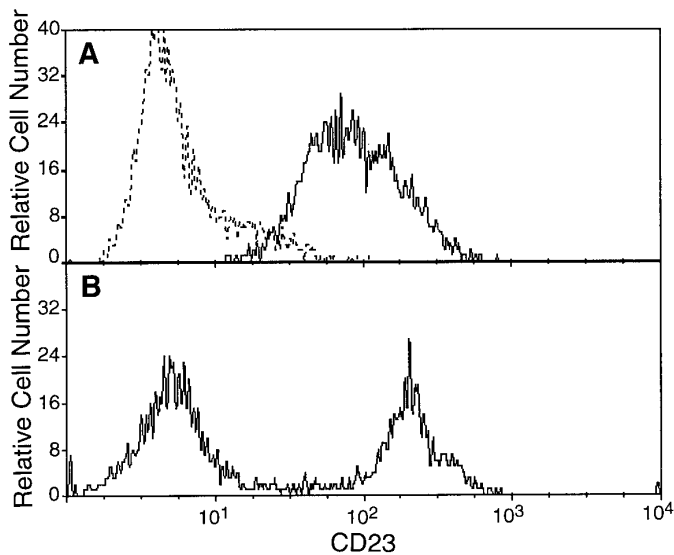


FIG. 4. Cell surface expression of CD23 by ICRE2 cells. ICRE2 (solid line) or untransfected CHO (broken line) (A) and total splenocytes (B) were stained with fluorescein-labeled anti-mouse CD23 to determine the amount of CD23 on the cell surface of these cells. Stained cells (1×10^6) were examined on a FACScan.

cells were equal to or higher than CD23 levels on normal splenocytes. CD23 levels on RE2 cells was similar to that shown for ICRE2 (data not shown). Figures 5A and 5B shows Ig production (8 days) from experiments of this design with either BALB/C or C57 B cells being used, respectively. Note that there was a dose-dependent decrease in IgE production, based on support cell concentration, especially when double-positive mICRE2-1 were used, and a smaller trend with mRE2-1. At higher support cell concentrations (12 and 24K, respectively), some decreased production of IgG₁ and IgM was also seen. The two experiments shown are representative of a total of eight of similar design. A common finding was the dose-dependent decrease in IgE production, with IgE production approaching zero at the higher doses of mICRE2-1 or mRE2-1 cells. Note also that little difference was seen between mRE2-1 and double-positive mICRE2-1 cells in one of the two experiments shown. While the reason for this is not clear, it is possible that ICAM-1 does show some enhancement of the CD23 signal. This is currently being investigated using lower expressing CD23 lines. From these data it is again evident that IgE is the most susceptible to inhibition, while higher doses of support cells reduce both IgG₁ and IgM as well. Note that high levels of CD23 expression always results in an increase in the inhibition seen. In a total of 10 experiments of this design, a consistent result was the indicated dramatic inhibition of IgE production followed by decrease in IgG₁ and IgM production at higher levels of CD23⁺ support cells. Single-positive (ICAM-1⁺) or CHO

alone had no effect at or below 6000/well and only modest effects at the higher concentration. The effects of control cells at the higher concentration are nonspecific and presumably related to media exhaustion.

Effect of CD23 on B cell proliferation. The generalized effect of high level CD23 cells on Ig production suggested that B cell proliferation may be influenced. In the experiments shown in Fig. 5, the initial B cell input was relatively low (10,000/well) in view of the increased IgE production seen at this level of B cell input (37). This aspect was investigated using a higher level of B cells/well and as can be seen (Fig. 6) both double-positive mICRE2-1 and mRE2-1 cells caused an evident dose-dependent decrease in B cell proliferation, while CHO cells had no effect on B cell proliferation. ICAM-1 transfectant also had no effect on B cell proliferation (data not shown). This result is reminiscent of the apoptotic effect of cocrosslinking CD23 and sIg (see Discussion).

Time course of inhibitory action. In order to determine the time course of the high-level CD23-induced effect, B cell cultures were initiated with CD40L, IL-4, and IL-5 and CD23 single-positive, double-positive, or control cells were added on different days. Data from a representative experiment are shown in Fig. 7. The data are consistent with respect to IgE production; namely, an inhibitory effect on IgE production was seen at least through Day 3 and the expected decrease in proliferation was seen with the addition of CD23⁺ cells on Day zero. IgG₁ and IgM production was also inhibited although the effect was largely gone by Day 2.

Examination of ϵ -germline transcript for inhibition by high level CD23. Our laboratory has recently described an additional inhibitory system for IgE production, namely, the coactivation of B cells by both anti-Ig and CD40L (37). Two general mechanisms can be suggested for the inhibitory effects observed on IgE production by the coactivation (37) and high-level CD23 model systems. Namely, the effects could be a result of the inhibition of isotype switching or alternatively could be the result of inhibiting terminal differentiation of the switched B cells to high Ig production at the plasma cell level. In order to differentiate between these two mechanisms the effects of the two systems on ϵ -germline transcript production was investigated. The production of a germline transcript is a good predictor of whether isotype switching will occur, and thus, an inhibition of germline transcript production would suggest that isotype switching is being inhibited. In contrast, no effect on germline transcript production would suggest that terminal differentiation is being inhibited.

The RT-PCR protocol used is based on a published procedure. Primers for the ϵ -germline transcript are as published (44) and are indicated under Materials and

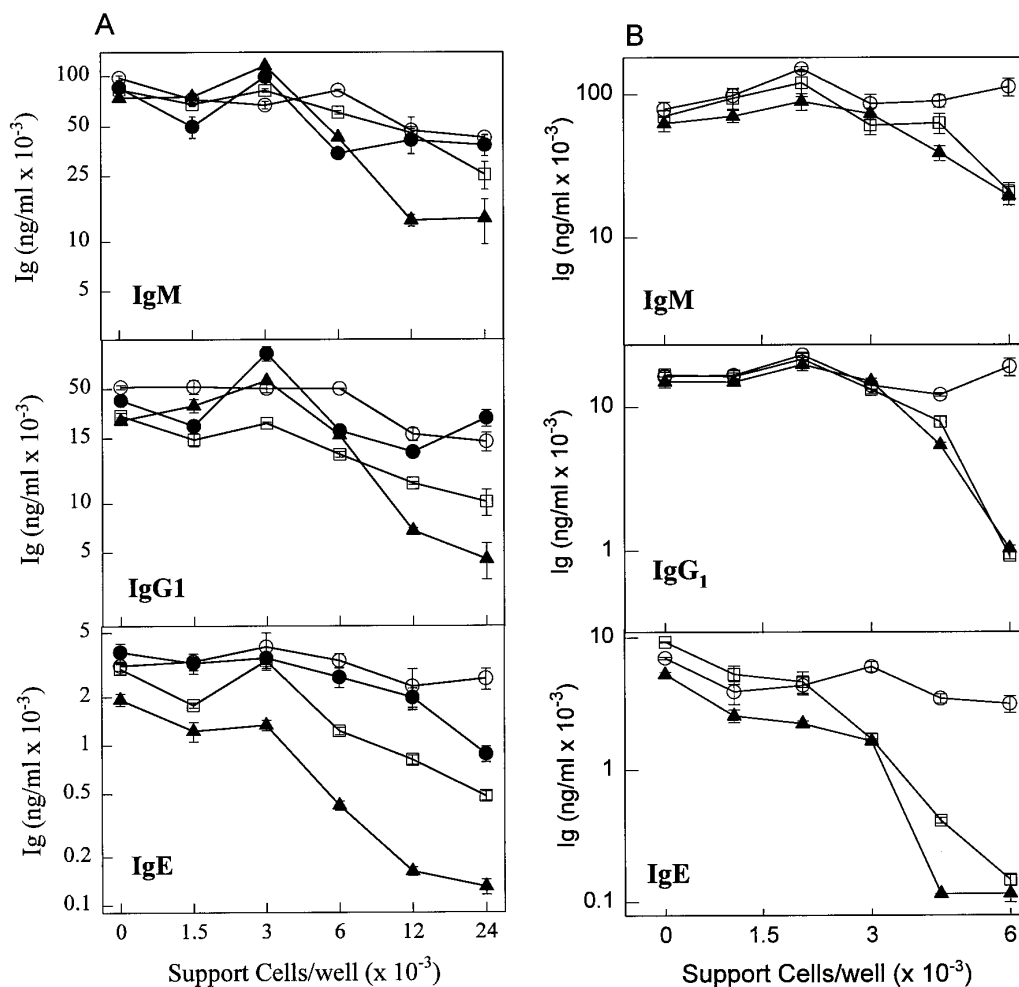


FIG. 5. Down-regulation of Ig (especially IgE) production by B cells cultured in the presence of high-level CD23. Purified resting B cells from BALB/C mice (A) or C57BL/6 mice (B) (10^4 /well) were placed into culture wells precoated with CHO ($\circ$), CD23-CHO ($\bullet$), ICAM-1-CHO ($\square$), or double transfectants (ICRE2) ($\blacktriangle$), at indicated number/well (termed "support cells"). Activation was induced using CD40L-CHO (6000/well) plus IL-4/5 as indicated under Materials and Methods. All CHO transfectants were treated with mitomycin C prior to use in experiments. Ig levels were determined by ELISA after 8 days of culture.

Methods. Initial attempts to use either hypoxanthine-guanine phosphoribosyl transferase (as used in 44) or actin-specific primers as the housekeeping control proved unsuccessful due to cross-reaction of the primer with the hamster counterpart from the support cells. For this reason, primers for CD22, a B-cell-specific protein that maintains a relatively constant expression level through different stages of B cell activation (48) were chosen. Primers for CD22 result in a 419-bp PCR product from murine CD22 cDNA (sequence obtained from Genbank). B cells were activated in 96-well tissue culture plates in the presence of either Fc γ RI-CHO and anti-IgD or are exposed to high CD23 as above. At the indicated time points, RNA was isolated by the Trizol method. RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin-Elmer) as described under Materials and Methods. Note that the PCR product is directly ^{32} P labeled by inclusion of [^{32} P]dATP in the

protocol. For the PCR step, 15 cycles were chosen with the objective of being on the linear portion of the PCR expansion curve and preliminary experiments in which different initial inputs of RNA were used demonstrated this to be the case. Namely, increasing band intensity for the PCR product was evident, while when 35 cycles of PCR were performed, this increase was not apparent (data not shown). Thus, we conclude, based on the Warren and Berton study (44) along with this analysis, that 15 cycles can be used to estimate relative ϵ -germline transcript levels.

Following either coactivation or coculture with high-level CD23, RNA from appropriately activated B cells or from control cultures was subjected to 15-cycle RT-PCR and the final PAGE gel was analyzed with a PhosphorImager. Figure 8 shows the results of an experiment of this design with C57BL/6 B cells and similar results were found using B cells from BALB/C mice

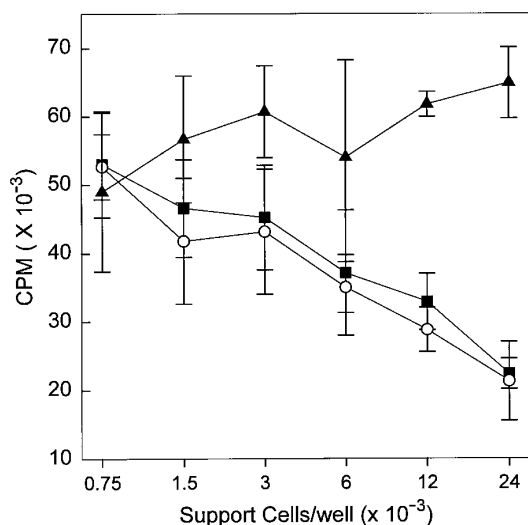


FIG. 6. B cell proliferation is inhibited by high CD23. BALB/C B cells (50,000/well) were cultured with the indicated number of CHO (▲), CD23-CHO (■), or double transfectant (ICRE2) (○). Activation was induced with CD40L-CHO as in the legend to Fig. 5 and all CHO cells were treated with mitomycin C before use. On Day 3, cells were pulsed for 8 hr with 1 μ Ci/well of [³H]thymidine and harvested as described under Materials and Methods in order to determine relative cell proliferation.

(data not shown). As can be seen, a clear suppression of ϵ -germline transcript levels is apparent after coactivation; however, those levels remain relatively constant in the presence of high-level CD23. The bottom part of Fig. 8 shows a bar graph analysis of the data normalized for the levels of CD22 that were present. The data lead to the conclusion that coactivation results in an inhibition of isotype switching, while high CD23 levels apparently inhibit the differentiation of switched B cells.

DISCUSSION

As discussed in the Introduction, CD23 was initially described as a B cell activation antigen on the one hand and a low-affinity receptor for IgE on the other. The hypothesis that CD23 was involved in IgE regulation was a natural outgrowth of the finding that B cell activation in conjunction with IL-4 induced both IgE and CD23 production—the hypothesis basically being that IL-4 induced both positive (STAT6) and negative (CD23) regulating elements for IgE. However, evidence to support this hypothesis was limited until recently. Indeed, the observed enhancing effect that CD23 has on antigen processing led to the opposite conclusion in that IgE–antigen complex were immunostimulatory (49), albeit this stimulation was not specific for IgE production itself. Other evidence in support of this hypothesis is the finding by Bonnefoy *et al.* (50) that anti-CD23 inhibited IgE production in human lymphocyte

culture and that a prolonged anti-CD23 treatment suppressed IgE in a rat model (51). In the mouse, one promising report that CD23 KO mice had increased IgE responses (28) has not been supported by other studies. However, as mentioned earlier, mice transgenic for CD23 do exhibit a decreased IgE response, both to parasite models (29) and to antigen/alum regimens (Fig. 1; Ref. 29).

The latter finding with the CD23 transgenic mouse led to the development of an *in vitro* system to determine the effects of high-level membrane CD23 on IgE production, again using the mouse system. The parameters of CD40L stimulation, either with transfected CHO cells (m40L-2) or soluble CD40LT had been developed in another study with anti-IgD costimulation (37). We first attempted to simply test the capacity of purified B cells to be stimulated to produce IgE. The results (Fig. 3) indicated a variability in that a significant dif-

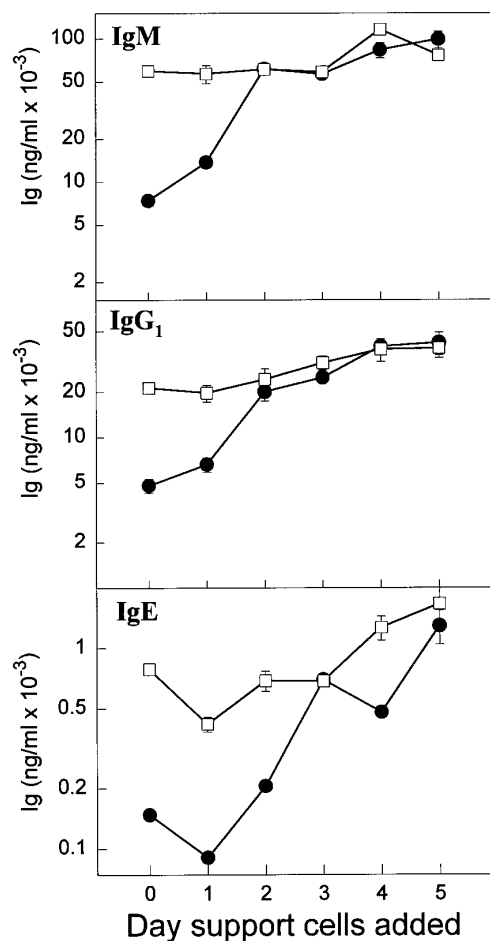


FIG. 7. Effect of delayed addition of high CD23 on Ig production. BALB/C resting B cells (10^4 /well) were placed into culture and activated with CD40LT and IL-4/5 as in the legend to Fig. 5. Support cells (1.2×10^4 /well) consisting of CHO (□) or double transfectant ICRE2 (●) were added on the day indicated. Eight days after culture initiation, Ig levels were determined by ELISA.

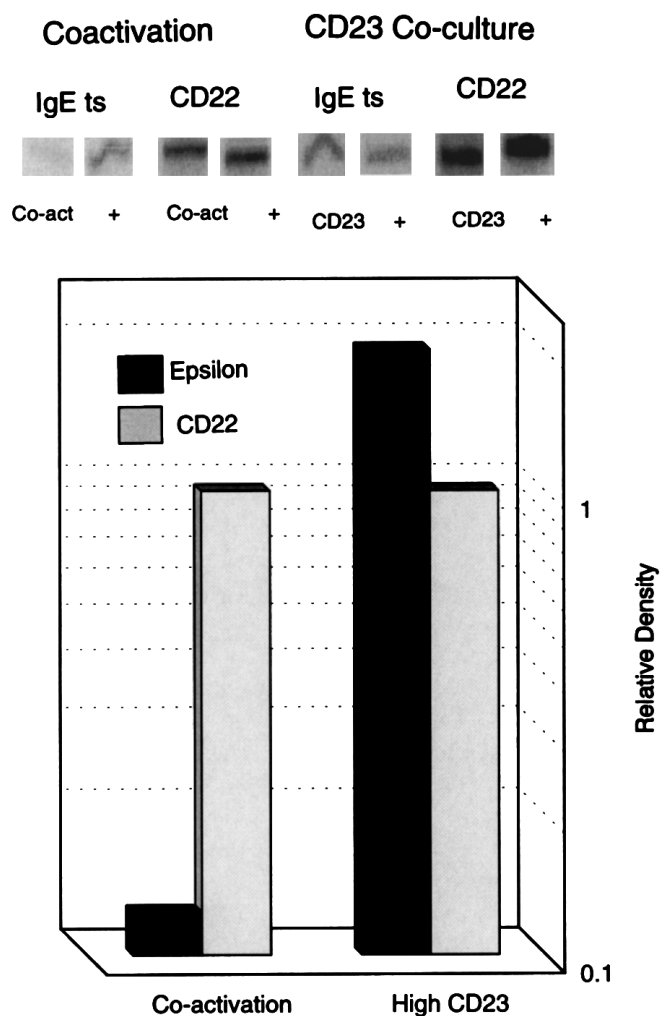


FIG. 8. Effect of high CD23 or coactivation on ϵ -germline production. Relative levels of ϵ -germline were determined by RT-PCR. B cells were coactivated with anti-IgD bound to Fc γ RI-CHO plus CD40L, as described under Materials and Methods and elsewhere (37), or by culture in the presence of high CD23 (ICRE2). Control plates contained identical numbers of untransfected CHO cells. On Day 3, RNA was isolated using the Trizol procedure. After the RT step and 15 cycles of PCR in the presence of [^{32} P]ATP, samples were applied to 6% PAGE and band intensities were determined on the dried gel using a PhosphorImager. To correct for differences in RNA isolation, RT-PCR for CD22 was performed on the same samples. The top of figure shows the RT-PCR bands for ϵ -germline and CD22, respectively, while the bar graph represents amounts of ϵ -germline normalized for CD22 recovery (CD22 adjusted to relative density of 1).

ference in IgE production was not always evident. Since transgenic CD23 expression on B lymphocytes was relatively low, this was considered the most likely explanation for this inconsistency. Thus, a new model system was derived where B cells were cocultured with support cells expressing high levels of CD23, ICAM-1, or both agents was developed. ICAM-1 was chosen due to its primary involvement in the high-level B cell clustering observed after activation in the presence of IL-4. It was

anticipated that while the ICAM-1/LFA interaction might not transmit a signal on its own, the enhanced clustering would amplify any CD23-specific events. The general observation from these studies was that when membrane CD23, either on mitomycin C-treated mRE2-1 or on mICRE2-1, was present, a dose-dependent down-regulation of IgE production was apparent. Indeed, at high CD23⁺ cell levels, a general down-regulation of Ig production ensued and, if the dose of B cells was increased so as to more accurately determine B cell proliferation, this proliferation was decreased also in concordance with CD23⁺ cell number. The finding that coculture with high-CD23-expressing cells down-regulates IgE relates to the transgenic model (29) where the support cells *in vivo* were T cells where CD23 expression was driven by the Thy-1 promoter. The results of the proliferation experiments as exemplified in Fig. 6 would argue for a general "down-regulation" activity for high-level CD23. Indeed this concept received further support in that IgG1 and even IgM production decreases at the higher level of support cells. However, the isotype that is most sensitive to this suppression is IgE. This is seen in that suppression occurs at a lower input of support cells and that there is a higher degree of suppression.

The decrease in B cell proliferation seen (Fig. 6) suggests the intriguing possibility that high-level CD23 may induce B cell apoptosis—clearly a subject for further study. In addition, the "ligand" or counter receptor that interacts with CD23 is not clear from these studies. One attractive hypothesis is that high-level CD23 interacts with sIgE on stimulated B cells and increases this apoptosis. In this sense, CD23 would be acting much like the models proposed for the anti-IgE antibody that block IgE synthesis (52). However, in spite of numerous attempts, it was not possible to demonstrate that any of the anti-CD23 reagents available to the laboratory (both mab and polyclonal reagents) influenced the activity of the CD23⁺ cells. Obviously, any antibody blocking would be epitope dependent—namely, the epitope for the antibody must either be close to or be the same as any site on CD23 that interacts with other ligands. Since both of the mab anti-CD23 (B3B4 and 2G8) are known to inhibit CD23-IgE interaction, this reduces the attractiveness of the sIgE as a target.

The second current candidate for a "CD23 ligand" is CD21, shown by the Bonnefoy lab to interact with CD23 (53). This same lab reported evidence that this interaction (in the human system) actively enhanced IgE synthesis (53). Clearly, the interaction of CD21 with TAPA-1 and CD19 in the "BCR" (54) suggests that an agent that interacts with CD21, simultaneous with anti-Ig (antigen-sIg), would enhance this stimulation. However, the interaction reported herein is via CD40L, not anti-sIg B cell stimulation, and the effects of cocross-linking of CD21 and CD40 are not known at present.

So, although no positive results for mouse CD23/CD21 interaction have been reported, this remains a possibility, as does another, as yet undiscovered alternate ligand. In this regard, the laboratory is attempting to prepare a soluble CD23 that exerts a similar biologic activity to the membrane form. Such a molecule would greatly enhance the capability for identifying alternate ligands.

Finally, regarding the mechanism for the IgE down-regulation by anti-IgD vs high-level CD23, kinetic analysis indicated that the CD23 effect, especially with respect to IgE inhibition, was available at least through Day 3 and even up to Day 5. In contrast the anti-IgD effect was largely gone by Day 3 (data not shown). This correlates with the investigation of the effect on ϵ -germline transcript production. While the role of these transcripts remain unclear, with the exception of IgD, germline transcripts in all cases precede the actual isotype switch. Analysis of ϵ -germline transcript levels clearly shows that while the anti-IgD system inhibits both IgE protein and ϵ -germline transcript levels, the high-level CD23 affects only the protein. Thus, both the kinetic and the transcript studies are consistent with an effect on terminal differentiation for the high-level CD23 system and inhibition of the isotype switch for the anti-IgD system.

In conclusion, this study demonstrates that high-level CD23 expression can modulate CD40L-induced B cell activation. These data suggest a rationale for why IgE is produced in relatively low levels. Stimulation of B cells with an activation agent, e.g., CD40L, in conjunction with IL-4 induces efficient isotype switching to IgE, but also induces high-level CD23, which in both transgenic animals and in *in vitro* models results in lower IgE production. Thus, this finding brings back the earlier-mentioned hypothesis that IL-4 induced both positive and negative regulatory elements for IgE.

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