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# Soluble CD23 Controls IgE Synthesis and Homeostasis in Human B Cells

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CD23, the low-affinity receptor for IgE, exists in membrane and soluble forms. Soluble CD23 (sCD23) fragments are released from membrane (m)CD23 by the endogenous metalloprotease a disintegrin and metalloprotease 10. When purified tonsil B cells are incubated with IL-4 and anti-CD40 to induce class switching to IgE in vitro, mCD23 is upregulated, and sCD23 accumulates in the medium prior to IgE synthesis. We have uncoupled the effects of mCD23 cleavage and accumulation of sCD23 on IgE synthesis in this system. We show that small interfering RNA inhibition of CD23 synthesis or inhibition of mCD23 cleavage by an a disintegrin and metalloprotease 10 inhibitor, GI254023X, suppresses IL-4 and anti-CD40-stimulated IgE synthesis. Addition of a recombinant trimeric sCD23 enhances IgE synthesis in this system. This occurs even when endogenous mCD23 is protected from cleavage by GI254023X, indicating that IgE synthesis is positively controlled by sCD23. We show that recombinant trimeric sCD23 binds to cells coexpressing mIgE and mCD21 and caps these proteins on the B cell membrane. Upregulation of IgE by sCD23 occurs after class-switch recombination, and its effects are isotype-specific. These results suggest that mIgE and mCD21 cooperate in the sCD23-mediated positive regulation of IgE synthesis on cells committed to IgE synthesis. Feedback regulation may occur when the concentration of secreted IgE becomes great enough to allow binding to mCD23, thus preventing further release of sCD23. We interpret these results with the aid of a model for the upregulation of IgE by sCD23. *The Journal of Immunology*, 2012, 188: 000–000.

**C**D23 is the low-affinity receptor for IgE on B cells. It is initially expressed as a 45-kDa type II membrane protein (membrane [m]CD23) containing a lectin head domain, harboring the IgE binding site, and a C-terminal tail in the extracellular sequence (1, 2). CD23 is assembled into a trimer (3), the predominant form in the B cell membrane, by way of an

$\alpha$ -helical coiled-coil stalk that links the three lectin head and tail domains to their transmembrane and cytoplasmic sequences (4). CD23 is expressed in two forms, CD23a and CD23b, resulting from alternative transcription initiation sites and differing only by six or seven amino acids in the intracellular N-terminal cytoplasmic sequence (5). CD23a is expressed on Ag-activated B cells, whereas CD23b expression is upregulated by IL-4 in allergic inflammation and is associated with elevated serum IgE (6–8). The anti-CD23 mAb lumiliximab downregulates IgE synthesis by human B cells in vitro (9) and reduced human serum IgE levels in a phase I clinical trial in patients with mild to moderate persistent allergic asthma (10, 11). This provides proof of principle that mCD23 is a valid target for therapy. Understanding the regulation of CD23 has the potential to inspire a more cost-effective interventional strategy.

It has long been known that CD23 negatively regulates the synthesis of IgE (12, 13). The most compelling evidence comes from CD23 knockout mice, which exhibit greatly increased levels of Ag-specific IgE after immunization (12, 13). IgE synthesis is also inhibited in human B cells by anti-CD23 Abs (9, 14, 15) or Ag–IgE complexes that bind to mCD23 (16). Neither free IgE nor Ab Fab fragments have this inhibitory activity, suggesting that cross-linking of mCD23 is required for the inhibition (15). These observations suggest that mCD23 may act in a negative-feedback mechanism on IgE synthesis.

mCD23 can be cleaved in the stalk region by the endogenous a disintegrin and metalloprotease 10 (ADAM10) to release a 37-kDa soluble fragment (soluble [s]CD23) both in vitro (17, 18) and in vivo (18–20). After the initial cleavage by ADAM10, sCD23 is susceptible to further cleavage by other proteases into fragments of various sizes (33, 29, 25, and 16 kDa), eliminating additional parts of the stalk and tail. These fragments lose the ability to independently form trimers but retain the ability to bind IgE, albeit at lower affinity (3). The 16-kDa fragment, termed derCD23, containing only the

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Abbreviations used in this article: ADAM10, a disintegrin and metalloprotease 10; CSR, class-switch recombination; Ct, threshold cycle; derCD23, 16-kDa fragment of soluble CD23 resulting from cleavage by group I allergen of *Dermatophagoides pteronyssinus*; eGLT, e germline transcript; m, membrane; MFI, mean fluorescence intensity; qPCR, quantitative PCR; s, soluble; sIgE, secreted IgE; siRNA, small interfering RNA; triCD23, recombinant trimeric soluble CD23.

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lectin domain and 10 aa of the tail, results from cleavage by the house dust mite allergen, group I allergen of *Dermatophagoides pteronyssinus* (21, 22). Whether sCD23, resulting from mCD23 cleavage, is involved in the positive regulation of IgE synthesis is not as clear as the negative regulation by mCD23. A number of studies have shown that sCD23 fragments either up- or down-regulate IgE synthesis in human B cells (23, 24), depending on their size and ability to form trimers (9, 25, 26). The use of selective ADAM10 inhibitors is considered a potential therapy for asthma, based on a recent preclinical trial in mice (19). Thus, sCD23, as well as mCD23, may be promising targets for therapy.

Human CD23 binds not only to IgE, but also to CD21 (CR2) (24), CD11b (CR3), CD11c (CR4) (27), the vitronectin receptor ( $\alpha_v\beta_3$ ) (28), and potentially other, as yet unidentified, proteins. However, IgE and CD21 are the only known ligands on mature B cells, with both binding sites distinct from each other and from the interface between the head domains in the CD23 trimer (29). Prior to the discovery of CD21 as the counterreceptor for CD23 on B cells (24, 30), CD21 was already well characterized as the receptor for the C3d fragments of complement that play an important role in the complement cascade and adaptive immunity (31, 32). In human CD23, the binding site for CD21 resides in the C-terminal tail (29). This tail is not present in murine CD23 (33), which may explain why sCD23 expressed in transgenic mice does not upregulate IgE during immunization, leaving only downregulation through mCD23 (13, 34, 35). sCD21, shed from cell membranes, is thought to inhibit IgE synthesis in human B cells by binding to free trimeric sCD23, thereby preventing the binding to mCD21 (26, 36). Abs against human CD21 modify IgE synthesis in anti-CD40-stimulated tonsil B cells in an epitope-dependent manner (24, 37). Hence, mCD21 is implicated in mediating the effects of sCD23 on IgE synthesis.

Due to the multiple forms of CD23, multiple ligands, and various activities of the different complexes, the mechanisms involved in IgE regulation by CD23 are still poorly understood. In the current study, we have focused on the positive regulation of IgE synthesis. In 2000, Mayer et al. (23) showed that IL-4- and anti-CD40-stimulated IgE synthesis in human PBMCs can be reduced by the addition of metalloprotease inhibitors. It is uncertain whether this is a direct effect on the B cells or whether it is due to inhibition of mCD23 cleavage and, therefore, sCD23 production. If sCD23 acts on B cells, are the stimulatory signals mediated by mCD21 or an unidentified counterreceptor?

To gain further insight into this question, we have stimulated purified human tonsil B cells with IL-4 and anti-CD40 and used either small interfering RNA (siRNA) to inhibit CD23 synthesis or an ADAM10 inhibitor (GI254023X) (38) to prevent cleavage of mCD23, leading to a reduction in sCD23 levels through two different mechanisms. We have followed the loss of mCD23 from the B cell surface, the appearance of sCD23 in the medium, and the expression and secretion of IgE as a function of time for up to 12 d by flow cytometry and ELISA. We have added a recombinant trimeric sCD23 (triCD23) to the ADAM10-inhibited B cells to test its ability to compensate for the reduction of endogenous sCD23. Finally, we have followed the expression of mIgE and mCD21 during the incubation of tonsil B cells with IL-4 and anti-CD40 and examined the effects of triCD23 binding to these ligands by confocal microscopy.

## Materials and Methods

### Isolation of human tonsil B cells

Following informed written consent, with ethical approval from Guy's Research Ethics Committee, we obtained human tonsils from donors undergoing routine tonsillectomies. The allergic status of the donor was determined by verbal communication with the parents at the time of

consent. Mononuclear cells were separated by density on a Ficoll gradient (GE Healthcare) and B cells isolated using 2-aminoethylisothiuronium bromide-treated SRBCs (TCS Biosciences). B cells were routinely >98% CD20<sup>+</sup> and <2% CD3<sup>+</sup>, as determined by flow cytometry (9).

### siRNA transfection

Total B cells were transfected with CD23 siGENOME SMARTpool siRNA (3  $\mu$ g) (referred to as CD23 siRNA) or ON-TARGETplus Non-targeting siRNA Pool #1 (3  $\mu$ g) (referred to as control siRNA; Thermo Scientific Dharmacon) using the Amaxa Human B cell Nucleofector kit and Nucleofector II Device (Lonza), according to the manufacturer's instructions. Transfection efficiency, 30 min after transfection, was determined to be 97% using a fluorescent nonfunctional siRNA (siGLO Red; Thermo Scientific Dharmacon). The efficiency of CD23 knockdown was quantified by quantitative PCR (qPCR).

### Cell culture

B cells were cultured in 24-well plates (Nunc) at  $0.5 \times 10^6$  cells/ml in RPMI 1640 with penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml), glutamine (2 mM; Invitrogen), 10% FCS (Hyclone; Perbio Biosciences), transferrin (35  $\mu$ g/ml), and insulin (5  $\mu$ g/ml) (Sigma-Aldrich). Cells were activated with IL-4 (200 IU/ml; R&D Systems) and anti-CD40 Ab (1  $\mu$ g/ml; G28.5; American Type Culture Collection) for up to 12 d. Mouse anti-human CD21 mAb (HB5; Santa Cruz Biotechnology) was added to cells at 0.1, 1, or 10  $\mu$ g/ml. Epitope analysis has shown this clone to inhibit CD23 binding to CD21 (30). The ADAM10 inhibitor GI254023X was purified on a CombiFlash Rf (Teledyne ISCO) system (column: RediSep Rf, 4 g silica; flow rate: 18 ml/min; solvent: acetonitrile;  $t_R$ : 10 min). HPLC analysis at 220 nm confirmed a purity of  $\geq 98\%$  (38). Cells were grown for 5 d to allow the upregulation of mCD23 and class-switch recombination (CSR) to IgE before addition of the inhibitor (1–15  $\mu$ M). Monomeric derCD23 (16145 Da) was made as previously described (29). IzCD23, made as previously described (9), was modified to produce the more stable trimer triCD23 (84414 Da), consisting of residues 131–321 of human CD23 prefixed by the trimerization motif (IAAIESK)<sub>4</sub> and expressed and refolded from inclusion bodies using the *Escherichia coli* vector pET151. This additionally provides N-terminal HIS<sub>6</sub> and V5 epitope tags and a TEV enzyme cleavage site that has been left uncleaved in the final product (M.W. Kao, J. Hunt, R.L. Beavil, M.N. Yahya, H.J. Gould, J.M. McDonnell, B.J. Sutton, and A.J. Beavil, manuscript in preparation). rCD23 proteins were dialyzed into PBS and sterile-filtered before addition to human B cell cultures. Concentrations were selected to be close to the calculated  $K_D$  value for trimeric CD23 binding to IgE ( $10^{-7}$  M) (9, 29) and used at a weight ratio of 1:3 (triCD23/derCD23) to maintain the same number of mIgE/mCD21 binding sites in each condition.

### qPCR

Total RNA was isolated from cells using RNeasy Mini kits (Qiagen), primed with oligo(dT) and random hexamers, and reverse transcribed using Superscript II (Invitrogen). qPCR was performed using TaqMan MGB gene expression assays (see below) and TaqMan Universal PCR Master Mix on a 7900HT real-time PCR machine (Applied Biosystems). Gene expression was normalized to an endogenous reference gene ( $\beta_2$ -microglobulin) and quantified by  $\Delta\Delta$  threshold cycle ( $C_t$ ) analysis (SDS 2.1 software): GAPDH, Hs02786624\_g1;  $\beta_2$ -microglobulin, 4310886E; CD23, Hs00233627\_m1;  $\epsilon$  germline transcript (eGLT) forward, 5'-CTGTCCAGGAACCCGACAGA-3', and reverse, 5'-TGCAGCAGCGGGTCAAG 3' with MGB probe 6FAM-AG GCACCAATG-MGB.

### Flow cytometry

B cells were stained for mCD23 expression with mouse anti-human CD23-FITC (1:50; DakoCytomation) and membrane ADAM10 expression with mouse anti-human ADAM10-PE (1:50; R&D Systems) and incubated on ice in the dark for 45 min. For intracellular staining, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 min, washed, and resuspended in permeabilization buffer (PBS, 0.05% Triton X-100, and 0.5% saponin [Sigma-Aldrich]) for 15 min. Goat anti-human IgE-FITC (1:500; Vector Laboratories) and monoclonal mouse anti-human IgG-APC (1:50; Miltenyi Biotec) were added and incubated on ice in the dark for 45 min. Collection of flow cytometry data was conducted using an FACSCalibur (BD Biosciences), with gating on live cells determined by forward versus side scatter, and events were analyzed using FlowJo software (Tree Star).

### Ig ELISA

Maxisorp plates (Nunc) were coated with polyclonal mouse anti-human IgE (1:7000; DakoCytomation) or polyclonal goat anti-human IgG (1:1000;

AbD Serotec) in pH 9.8 carbonate buffer (distilled water, 0.2 M NaCO<sub>3</sub>, and 0.2 M NaHCO<sub>3</sub>) overnight at 4°C. Unbound sites were blocked with 2% milk powder in PBS + 0.05% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. Supernatant samples were then added at appropriate dilutions and plates incubated overnight at 4°C. Human serum IgE (from 800 ng/ml; NIBSC) or IgG (from 200 ng/ml; Sigma-Aldrich) were used to construct standard curves. Binding was detected by mouse anti-human IgE-HRP (1:1000; DakoCytomation) or goat anti-human IgG-HRP (1:1000; Sigma-Aldrich) in 1% milk powder in PBS + 0.05% Tween 20 for 2 h at 37°C. The color reaction was developed with *o*-Phenylenediamine (Sigma-Aldrich) and analyzed at 492 nm using an automated plate reader (Titertek). Ig concentration was calculated from the standard curve using GraphPad Prism 5.03 software (GraphPad, San Diego, CA), with a minimum detectable concentration of ~2 ng/ml. IgE secretion (by day 12) ranged from 2–1255 ng/ml (mean 401 ± 71 ng/ml; *n* = 26). IgG secretion (by day 12) ranged from 113–2264 ng/ml (mean 365 ± 269 ng/ml; *n* = 8).

#### sCD23 ELISA

Human sCD23 EASIA ELISA kits (BioSource International) were used according to the manufacturer's instructions. Briefly, supernatants were added to microtiter plates precoated with a mixture of monoclonal anti-CD23 Abs, and anti-CD23-HRP was added for 2 h at room temperature. The color reaction was developed with tetramethylbenzidine and analyzed at 450 nm. The kit recognizes the 16-, 25-, 29-, and 37-kDa fragments of sCD23, with a minimum detectable concentration of ~200 pg/ml. sCD23 production (by day 12) ranged from 13–102 ng/ml (mean 58 ± 4 ng/ml; *n* = 33).

#### Confocal microscopy

Human tonsillar B cells were stimulated for 8 d with IL-4 and anti-CD40, harvested, and dead cells removed by density on a Ficoll gradient. A total of  $3 \times 10^5$  cells was stimulated with media alone, derCD23 (3 μM/48 μg/ml), triCD23 (1 μM/84 μg/ml), or anti-CD21 (10 μg/ml) at 37°C for 30 min. Cells were fixed with 4% paraformaldehyde, washed (with PBS + 0.05% Triton X-100), mounted onto poly-L-lysine-coated coverslips, and fixed again with 4% paraformaldehyde. Coverslip-mounted cells were stained with goat anti-human IgE-FITC (1:200; Vector Laboratories) and monoclonal mouse anti-CD21 (1:100; HB5 clone; Santa Cruz Biotechnology) for 1 h, washed, and secondary anti-mouse-Alexa 594 (1:500; Molecular Probes, Invitrogen) was added for 45 min. The nuclear stain Hoechst 33258 (1:20000; Molecular Probes, Invitrogen) was added for 10 min, cells were washed three times, and immunofluorescence visualized with an SP2 confocal microscope (Leica Microsystems).

#### Statistical analysis

Flow cytometry and ELISA data are shown relative to control-treated cells (either transfected with control siRNA or stimulated with IL-4 and anti-CD40 alone) to compensate for interdonor variation. Data from 7 out of 33 donors who failed to respond to IL-4 and anti-CD40, with undetectable levels of IgE expression and secretion by day 12, were excluded. Data are summarized as mean ± SEM. Statistical analysis was performed using ANOVA with Bonferroni correction, unless otherwise stated. A *p* value of < 0.05 was considered significant (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001). Significance to control conditions is indicated above data, and significance between two conditions is shown between data. Correlation analysis was performed using Spearman's rank correlation coefficient.

## Results

#### siRNA-induced inhibition of CD23 mRNA, mCD23, sCD23, and IgE secretion

Human tonsillar B cells were transfected with a pool of four siRNA duplexes, directed against CD23, and stimulated with IL-4 and anti-CD40 for up to 12 d. A significant inhibition of CD23 mRNA expression was observed following transfection with CD23 siRNA compared with control siRNA (Fig. 1A), but no effect was seen on the expression of a nontargeted gene, GAPDH (Fig. 1B). The maximum knockdown of CD23 (70.0 ± 0.1%) occurred between 18 and 24 h following transfection, after which CD23 mRNA levels began to recover to that of control siRNA-transfected cells.

Despite the short-term inhibition of CD23 mRNA shown in Fig. 1, mCD23 protein levels were reduced following transfection with CD23 siRNA. Fig. 2A shows a representative example of flow cytometric analysis of mCD23 levels from 18 h to 12 d following

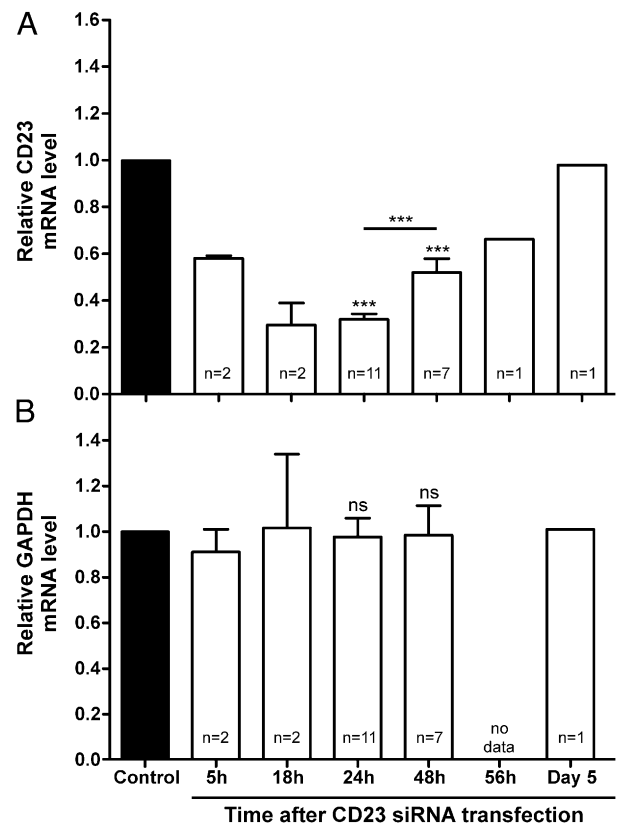
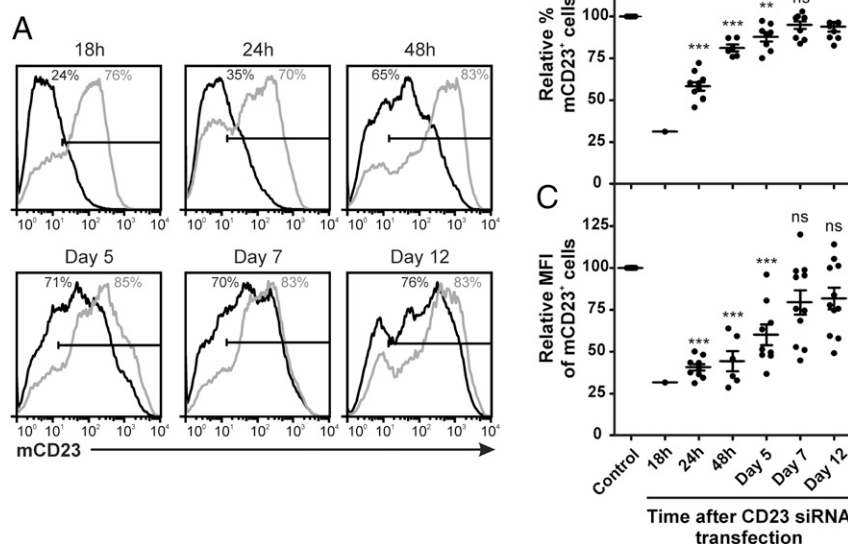


**FIGURE 1.** siRNA-induced inhibition of CD23 mRNA expression. Human tonsillar B cells were transfected with either control siRNA (black bars) or CD23 siRNA (white bars) and cultured for up to 5 d with IL-4 (200 IU/ml) and anti-CD40 (1 μg/ml). Cells were harvested at the times indicated, and qPCR was performed to quantify mRNA expression levels for CD23 (A) and GAPDH (B). Expression levels were calculated by  $\Delta\Delta C_t$  analysis, normalized against the endogenous reference gene  $\beta_2$ -microglobulin, and expressed relative to control siRNA-transfected cells at each timepoint (*n* = 11). \*\*\**p* < 0.001.

transfection with either control siRNA or CD23 siRNA. In control siRNA-transfected cells, mCD23 expression reached a peak on day 5 (88.4 ± 2.8%, *n* = 8). Fig. 2B shows that the percentage of mCD23<sup>+</sup> cells was reduced following transfection with CD23 siRNA, and this reduction remained statistically significant until day 7, although it was largely recovered by 48 h. The level of mCD23 expression on cells, as measured by the mean fluorescence intensity (MFI), was significantly lower on cells transfected with CD23 siRNA compared with control siRNA (Fig. 2C). This reduction also remained statistically significant until day 7, but the MFI was suppressed to a greater degree than the percentage of mCD23<sup>+</sup> cells and took longer to recover.

sCD23 is produced by cleavage of mCD23, initially by the membrane-bound metalloprotease ADAM10 (18). sCD23 was first detectable in the supernatant 4 d after transfection with either control siRNA or CD23 siRNA (data not shown). In accordance with the reduced mCD23 levels, sCD23 production decreased significantly following CD23 siRNA transfection (Fig. 3A). By day 12, sCD23 levels were, on average, 17.4% (± 5.7%) lower in supernatants from cells transfected with CD23 siRNA compared with control siRNA. No significant reduction was seen at the earlier time points of days 5 and 7. The expression level of ADAM10 was no different between control siRNA and CD23 siRNA-transfected cells at any time point during the culture (Supplemental Fig. 1), which reveals that the loss of ADAM10 was not responsible for the decrease in sCD23 levels.

**FIGURE 2.** Reduced mCD23 expression following CD23 siRNA transfection. **(A)** Human tonsillar B cells were transfected with either control siRNA (gray line) or CD23 siRNA (black line) and cultured for up to 12 d with IL-4 (200 IU/ml) and anti-CD40 (1  $\mu$ g/ml). mCD23 expression was analyzed by flow cytometry, and the percent of mCD23<sup>+</sup> cells (above isotype control as indicated by horizontal marker) is indicated on the histograms ( $n = 1$ , representative of 11). Multiple donor data, relative to control siRNA-transfected cells at each time point, are shown for percent mCD23<sup>+</sup> cells **(B)** and CD23<sup>+</sup> MFI **(C)**, at 18 h to 12 d following transfection ( $n = 11$ ). \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



Because transfection with CD23 siRNA successfully resulted in reduced sCD23 levels by day 12, we next investigated the association between reduced sCD23 production and IgE synthesis at this time point. Secreted IgE (sIgE) was first detectable in the supernatant 5 d after stimulation with IL-4 and anti-CD40 (data not shown). Fig. 3A also shows the relative levels of sIgE 5, 7, and 12 d after transfection with control siRNA or CD23 siRNA. Of the 10 donors analyzed for sCD23 production, 4 donors did not pro-

duce any detectable sIgE by day 12 and so were excluded from the analysis. CD23 siRNA-transfected cells secreted 47.4% ( $\pm 9.5\%$ ) less IgE than control siRNA-transfected cells (Fig. 3A). The extent to which sIgE levels were inhibited was not related to the amount of IgE the cells secreted. Fig. 3B correlates the level of inhibition of sCD23 production with the level of inhibition of IgE secretion. The positive correlation between sCD23 and sIgE is statistically significant ( $r = 0.94$ ;  $p = 0.0167$ ), as determined by

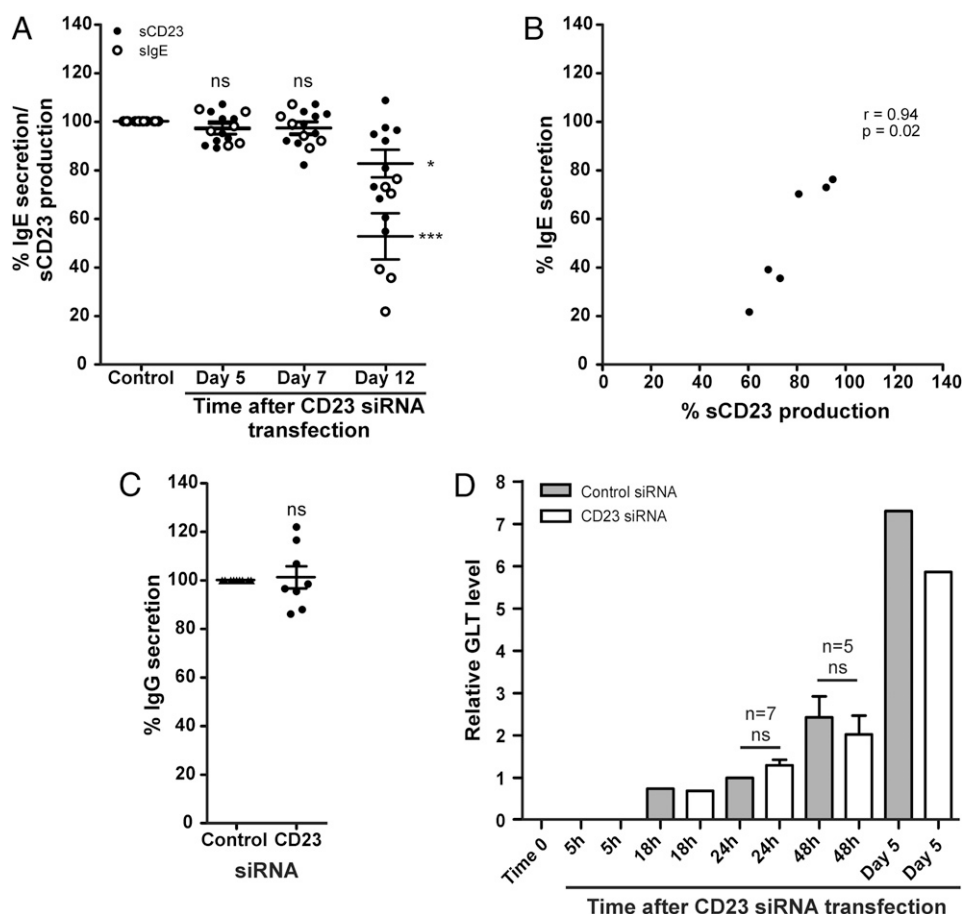


**FIGURE 3.** Inhibition of sCD23 production by CD23 siRNA correlates with reduced IgE secretion. Human tonsillar B cells were transfected with either control siRNA or CD23 siRNA and cultured for up to 12 d with IL-4 (200 IU/ml) and anti-CD40 (1  $\mu$ g/ml). **(A)** Supernatants were harvested on days 5, 7, and 12, and the percent inhibition of sCD23 production (●) ( $n = 10$ ) and IgE secretion (○) ( $n = 6$ ) were analyzed by ELISA, relative to control siRNA-transfected cells. **(B)** Correlation between inhibition of IgE secretion and inhibition of sCD23 production on day 12 ( $n = 6$ ). **(C)** Supernatants were harvested on day 12, and the percent inhibition of IgG secretion was analyzed by ELISA, relative to control siRNA-transfected cells ( $n = 8$ ). **(D)** qPCR was performed to quantify RNA levels for  $\epsilon$ GLT up to 5 d following transfection with either control (gray bars) or CD23 siRNA (white bars). Expression levels were calculated by  $\Delta\Delta C_t$  analysis, normalized against the endogenous reference gene  $\beta_2$ -microglobulin, and expressed relative to control siRNA-transfected cells at 24 h ( $n = 1$ , unless indicated otherwise). \* $p < 0.05$ , \*\*\* $p < 0.001$ .

Spearman's rank correlation coefficient ( $n = 6$ ). Importantly, CD23 siRNA transfection led to no significant changes in the levels of IgG secretion by day 12 (Fig. 3C). From day 7 onwards, following transfection with CD23 siRNA, mCD23 levels had returned to those of control siRNA-transfected cells (Fig. 2B, 2C). Thus, any CD23-mediated differences in IgE secretion between days 7 and 12 can be attributed to sCD23 rather than mCD23.

Fig. 3D shows qPCR analysis of  $\epsilon$ GLT, an early marker of CSR to IgE. No significant differences in  $\epsilon$ GLT levels were detected between control siRNA and CD23 siRNA-transfected cells at either 24 or 48 h following transfection, despite the inhibition of IgE secretion shown in Fig. 3A. Membrane and intracellular staining for IgE and IgG, analyzed by flow cytometry, showed no significant changes in the percentage of IgE<sup>+</sup> or IgG<sup>+</sup> cells between control siRNA and CD23 siRNA-transfected cells at days 7 or 12 (Supplemental Fig. 2). In addition, there were no differences in general cell viability (live cell gating determined by forward versus side scatter) or maturation (CD38 expression) between control siRNA and CD23 siRNA-transfected cells (data not shown).

#### ADAM10 inhibition with GI254023X reduces mCD23 shedding, sCD23 release, and IgE secretion

Because CD23 siRNA transfection reduced sCD23 through the early reduction of mCD23 expression, a different approach was taken that would reduce sCD23 levels through preventing mCD23 shedding, thus maintaining mCD23 levels. To achieve this, we used the ADAM10 inhibitor GI254023X. Human tonsillar B cells were stimulated with IL-4 and anti-CD40 for 5 d, after which GI254023X was added at 1–15  $\mu$ M. The addition of GI254023X did not lead to a significant increase in the percentage of CD23<sup>+</sup> cells, although a significant dose-dependent accumulation of mCD23 on the surface of CD23<sup>+</sup> cells (as measured by MFI) was observed by day 12 (Fig. 4A). With the addition of 10  $\mu$ M GI254023X, this was accompanied by a significant reduction in sCD23 production ( $28.3 \pm 9.9\%$ ) (Fig. 4B) and a significant reduction in IgE secretion ( $64.9 \pm 7.2\%$ ) (Fig. 4C), with no effect on IgG secretion (data not shown).

To assess the mechanism of action, GI254023X was added at days 8 or 10, in addition to day 5, and sCD23 and sIgE levels were determined at day 12. Fig. 4B and 4C show that when the inhibitor was added progressively later in the incubation period, the levels of both sCD23 and sIgE were higher, due to the shorter time period in which GI254023X could inhibit mCD23 shedding. However, inhibition of sCD23 production and IgE secretion could still be achieved even when GI254023X was added as late as day 10 in the incubation period. In further support of this association, Fig. 4D shows the relationship between sCD23 and sIgE levels, measured in the supernatant following 12 d stimulation with IL-4 and anti-CD40. The sCD23 concentrations are indicated in nanograms per milliliter, and also in micromoles, to facilitate comparison with the known  $K_D$  values of interaction with IgE and CD21 (see Discussion). The positive correlation between sCD23 and sIgE is statistically significant ( $r = 0.77$ ;  $p \leq 0.0001$ ), as determined by Spearman's rank correlation coefficient ( $n = 33$ ). There appear to be two phases in the relationship, separated by a threshold concentration of sCD23 (60 ng/ml/1.6  $\mu$ M), above which a steep rise in IgE secretion was observed.

#### Effect of exogenous sCD23 fragments on IgE secretion

We have previously shown that oligomeric sCD23 (1zCD23) above a certain threshold concentration upregulates, whereas monomeric sCD23 (derCD23) downregulates, IgE production from human tonsillar B cells (9). The IgE-potentiating ability of the more stable

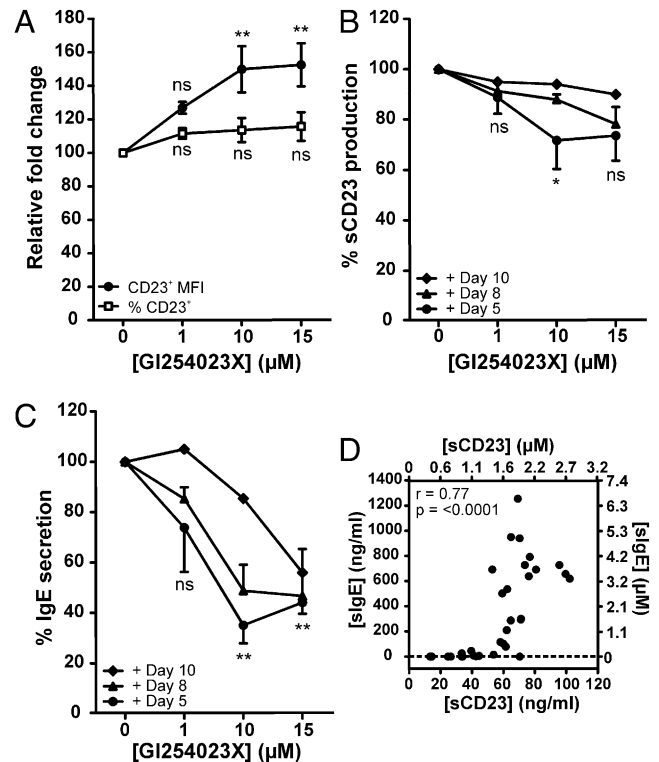


**FIGURE 4.** ADAM10 inhibition with GI254023X reduces mCD23 shedding, sCD23 release, and IgE secretion. Human tonsillar B cells were cultured for 12 d with IL-4 (200 IU/ml) and anti-CD40 (1  $\mu$ g/ml) in the absence or presence of varying concentrations of GI254023X, added on day 5 unless otherwise stated. (A) mCD23 expression was analyzed by flow cytometry on day 12 and the fold change plotted for the percent of mCD23<sup>+</sup> cells (□) and CD23<sup>+</sup> MFI (●), relative to cells cultured with IL-4/anti-CD40 alone ( $n = 6$ ). (B) GI254023X was added on days 5 (●) ( $n = 6$ ), 8 (▲) ( $n = 2$ ), or 10 (◆) ( $n = 1$ ), and supernatants were analyzed on day 12 for sCD23 (B) production and IgE secretion (C), relative to cells cultured with IL-4/anti-CD40 alone. (D) Correlation between sCD23 and sIgE levels after 12 d ( $n = 33$ ). Values are shown in nanograms per milliliter on the bottom x-axis and left y-axis and in micromoles on the top x-axis and right y-axis (1  $\mu$ M 190-kDa sIgE = 190 ng/ml; 1  $\mu$ M 37-kDa sCD23 = 37 ng/ml). \* $p < 0.05$ , \*\* $p < 0.01$ .

triCD23 was assessed by culturing B cells with IL-4, anti-CD40, and triCD23 (1  $\mu$ M) (added at day 0) for 12 d. Although not a naturally occurring fragment, triCD23 has been specifically designed to mimic the trimeric state of endogenous sCD23 when it is first cleaved off the membrane in allergic tissues. Western blot analysis showed triCD23 did not degrade into smaller fragments during the 12 d in culture (data not shown). Analysis by ELISA on day 12 showed a 200.0% ( $\pm 27.2\%$ ) increase in IgE secretion from cells cultured with triCD23 compared with IL-4 and anti-CD40 alone, whereas cells cultured with monomeric derCD23 produced significantly less IgE (Fig. 5A). These effects were isotype specific, as IgG secretion remained unaffected, and flow cytometric analysis showed triCD23 had no effect on the percentage of IgE<sup>+</sup> cells (Supplemental Fig. 3), suggesting that triCD23 preferentially promotes expression of the secreted form of IgE.

The ability of triCD23 to rescue the inhibition of sCD23 production and IgE secretion caused by addition of the ADAM10 inhibitor (GI254023X) was then investigated. As shown in Fig. 5B, IgE secretion increased 2.3-fold when GI254023X (5  $\mu$ M) was cocultured with triCD23 (1  $\mu$ M) compared with GI254023X alone.

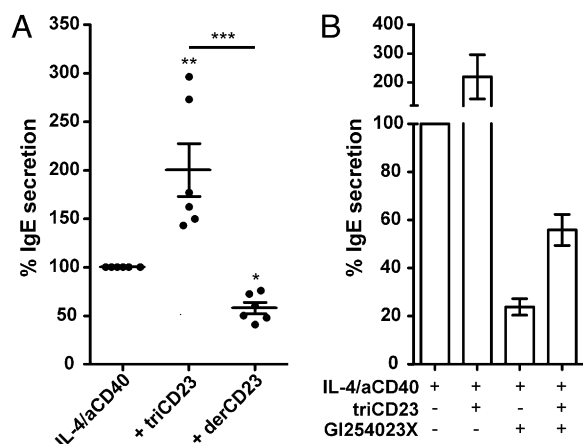


**FIGURE 5.** Effect of exogenous sCD23 fragments on IgE secretion. **(A)** Human tonsillar B cells were cultured for 12 d with IL-4 (200 IU/ml) and anti-CD40 (1  $\mu$ g/ml) alone, + triCD23 (1  $\mu$ M/84  $\mu$ g/ml), or + derCD23 (3  $\mu$ M/48  $\mu$ g/ml). Supernatants were analyzed on day 12 for IgE secretion, relative to cells cultured with IL-4/anti-CD40 alone ( $n = 6$ ). **(B)** Human tonsillar B cells were cultured for 12 d with IL-4/anti-CD40 alone, + triCD23 (1  $\mu$ M), + GI254023X (5  $\mu$ M), or + triCD23 and GI254023X. Supernatants were analyzed on day 12 for IgE secretion, relative to cells cultured with IL-4/anti-CD40 alone ( $n = 2$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### Trimeric sCD23 colocalizes mIgE and mCD21

Having established in this study that sCD23 was involved in the positive regulation of IgE, we next sought to identify the mechanism by which this may occur. Confocal microscopy was used to test the binding of triCD23 to mIgE and mCD21 and visualize the surface dynamics of these complexes. Human tonsillar B cells were cultured with IL-4 and anti-CD40 for 8 d to ensure sufficient time for the dual expression of mIgE and mCD21 (Supplemental Fig. 4)

and then stimulated for 30 min in the presence of media alone, derCD23, or triCD23. The *left* and *middle panels* of Fig. 6A show the uniform distribution of mIgE and mCD21 on the surface of B cells incubated with either media alone or monomeric derCD23, respectively. The *right panel* of Fig. 6A shows the redistribution and colocalization of mIgE and mCD21, following incubation with triCD23. Several distinct microclusters formed, and particularly strong capping of mIgE and CD21 is indicated on the merged image in the *bottom right panel* of Fig. 6A.

To further investigate the role of mCD21 in the sCD23-mediated regulation of IgE synthesis, tonsil B cells were cultured with increasing concentrations of an anti-CD21 mAb (HB5 clone) for 12 d. Fig. 6B shows the addition of anti-CD21 resulted in a dose-dependent decrease in IgE secretion. This was accompanied by an increase in mCD23 expression and a decrease in sCD23 production (data not shown). When cells were cultured with a combination of anti-CD21 (10  $\mu$ g/ml) and triCD23 (1  $\mu$ M), the IgE-stimulating effects of triCD23 were blocked. Fig. 6C shows confocal microscopy analysis of mCD21 expression. Total B cells were cultured with IL-4 and anti-CD40 for 8 d and then stimulated for 30 min in the presence of anti-CD21, triCD23, or anti-CD21 and triCD23. Distinct microclusters of mCD21 formed on the surface of cells stimulated with either anti-CD21 or triCD23. In the cells pretreated with anti-CD21 before stimulation with triCD23, the distribution of mCD21 was more uniform. Together, these data support the hypothesis shown in Fig. 7, whereby trimeric sCD23 molecules can bind both mIgE and mCD21 to stimulate IgE synthesis.

#### Discussion

The cytokines IL-4 and IL-13 induce the CSR to all Ig isotypes downstream from C $\mu$  in the H-chain locus on human chromosome 14, but can be replaced by other cytokines for switching to isotypes other than IgE. IL-4 upregulates CD23, so that the expression of CD23 and IgE are inextricably linked (25). The role of

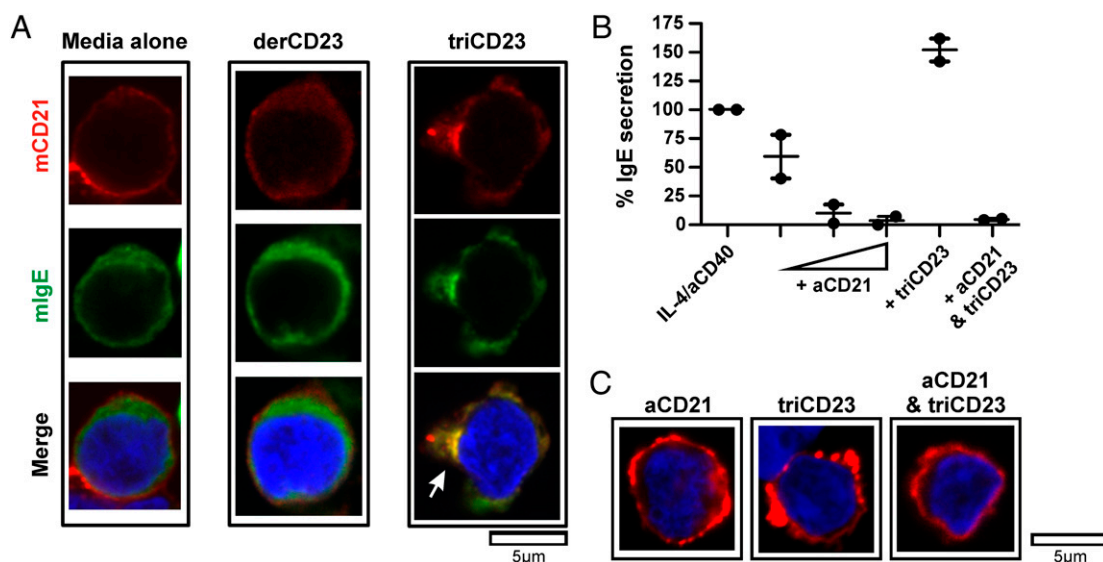


**FIGURE 6.** triCD23 colocalizes both mIgE and mCD21 to upregulate IgE secretion. **(A)** Human tonsillar B cells were cultured for 8 d with IL-4 (200 IU/ml) and anti-CD40 (1  $\mu$ g/ml) and stimulated for 30 min with media alone, derCD23 (3  $\mu$ M/48  $\mu$ g/ml), or triCD23 (1  $\mu$ M/84  $\mu$ g/ml). Cells were stained with mouse anti-CD21, plus secondary anti-mouse-Alexa 594 (red), anti-IgE-FITC (green), and the nuclear stain Hoechst (blue). Cells were visualized by confocal microscopy and images show a single field of view, with the *bottom panel* showing a three-color overlay. The white arrow indicates strong capping of mIgE with mCD21 ( $n = 1$ , representative of 3). Scale bar, 5  $\mu$ m. **(B)** Human tonsillar B cells were cultured for 12 d with IL-4/anti-CD40 alone, + anti-CD21 (0.1, 1, or 10  $\mu$ g/ml), + triCD23 (1  $\mu$ M), or + anti-CD21 (aCD21; 10  $\mu$ g/ml) and triCD23. IgE secretion was analyzed by ELISA on day 12, relative to cells cultured with IL-4/anti-CD40 alone ( $n = 2$ ). **(C)** Cells were cultured as described in (A) and stimulated with anti-CD21 (10  $\mu$ g/ml), triCD23 (1  $\mu$ M), or anti-CD21 and triCD23. Cells were stained for mCD21 (red) and the nucleus (blue), as previously described for (A), and visualized by confocal microscopy ( $n = 1$ ). Scale bar, 5  $\mu$ m.

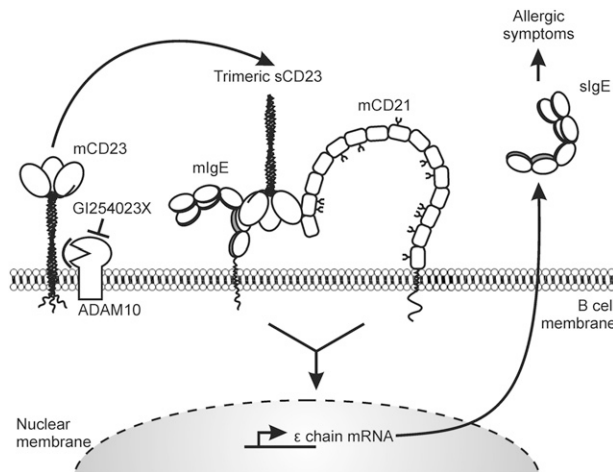


**FIGURE 7.** Proposed mechanism of IgE upregulation by trimeric sCD23. In this model, mCD23 is cleaved by ADAM10 to release trimeric sCD23, which coligates both mIgE and mCD21 on the surface of IgE-committed B cells to upregulate IgE synthesis, triggering the onset of allergic symptoms.

CD23 in the regulation of IgE in the human system appears to be more complex than in the mouse (2).

Negative regulation of IgE synthesis through mCD23 occurs in murine B cells *in vivo* (13, 39) and in human B cells *in vitro* (15). In human B cells, this requires the cross-linking of mCD23 (e.g., by Abs or Ag–IgE complexes). In the current study, we focused on a suggested mechanism by which sCD23 positively regulates IgE synthesis exclusively in human B cells (9, 29). In analyzing this mechanism, it is necessary to consider that there are multiple forms of sCD23, several potential ligands, and various complexes with different affinities and topological constraints associated with binding to their ligands in solution and particularly on cells.

sCD23 is released from cells by the action of an endogenous metalloprotease, identified as ADAM10 (17, 18), and accumulates in the supernatant of B cells following IL-4- and anti-CD40-stimulated CSR to IgE (23, 40, 41). In this study, we have used CD23 siRNA and an ADAM10 inhibitor (GI254023X) to confirm that mCD23 expression and cleavage to release sCD23 is required for the enhancement of IgE synthesis in primary human B cells.

Short-term inhibition of CD23 mRNA with siRNA led to a significant reduction in the frequency and MFI of CD23<sup>+</sup> cells until day 7 following transfection. By day 12, but not at the earlier time points of days 5 and 7, CD23 siRNA-transfected cells showed significantly reduced sCD23 production, which correlated with reduced IgE secretion, implicating sCD23 in the upregulation of human IgE synthesis (Figs. 1–3).

To further investigate the role of sCD23, ADAM10 was inhibited using GI254023X. It has been previously shown that inhibition of ADAM10 *in vivo* (20, 42), and the addition of GI254023X to human tonsil B cells (18), results in decreased sCD23 production. However, subsequent effects on IgE were not analyzed. In this study, addition of GI254023X resulted in increased mCD23 expression, reduced sCD23 production, and reduced IgE secretion. Inhibition of sCD23 production and IgE secretion could still be achieved when GI254023X was added progressively later in the incubation period, albeit to lesser extents, indicating that the ADAM10 inhibitor is regulating secretion of IgE by a postswitch event (Fig. 4). Because mCD23 is not cleaved, but is actually elevated in these conditions, the observed inhibition of IgE synthesis firmly points to positive regulation mediated through sCD23. Derepression of negative signaling through

mCD23 does not appear to play a major role. When sIgE binds to mCD23, it blocks metalloprotease cleavage and the release of sCD23 (16). However, this is not a complication in the present system, as the sIgE concentrations in the medium ( $<10^{-10}$  M) are well below the  $K_D$  of the IgE–mCD23 interaction. As in previous work with alternative matrix metalloprotease inhibitors and IL-4-stimulated human PBLs (23), we conclude that sCD23 is required to maintain IgE synthesis in human B cells.

We confirmed the findings from previous studies (9, 23, 26) that addition of recombinant trimeric sCD23 (triCD23) to primary human B cells enhances IgE synthesis (Fig. 5A). We now also show that when B cells are incubated with GI254023X in the presence of triCD23, a relief from GI254023X-mediated IgE suppression is observed (Fig. 5B), confirming the positive regulation of IgE synthesis by sCD23. However, the level of IgE synthesis was not fully restored to that of cells cultured only with triCD23. This might be explained by off-target effects of the ADAM10 inhibitor. Further studies are required to investigate this possibility.

mIgE and mCD21 are the prime candidates for mediating the stimulatory effects of sCD23. We show in this study that mCD21 expression declines in the first few days of incubation with IL-4 and anti-CD40 and reaches a plateau on day 5 (Supplemental Fig. 4). Loss of mCD21 is probably due to the shedding of soluble fragments (43), and further shedding may be prevented by the binding of sCD23 to mCD21 and mIgE. Although the concentrations of endogenous sCD23 (Fig. 4D) are far lower than the  $K_D$  for the 1:1 interaction with mCD21 or mIgE ( $K_D = 10^{-6}$  and  $10^{-8}$  M, respectively) (9, 29), the avidity effect of binding of three sCD23 heads as a trimer to multiple mCD21 and/or mIgE molecules may dramatically enhance binding affinity at the cell surface. Prior binding of sCD23 to mIgE, the stronger ligand, may enable the recruitment of mCD21 into a trimolecular complex. The CD23 binding site for the two N-terminal domains of CD21 lies in the C-terminal tail and is sufficiently distant from the IgE binding site to allow the simultaneous binding of both ligands in solution (29). Indeed, the observed capping of mIgE and mCD21 on B cells stimulated with triCD23 (Fig. 6A) reveals that there are likewise no topological constraints to prevent a trimeric CD23 molecule from coligating mIgE and mCD21 and forming the predicted multimolecular network on the cell surface (29). In cells in which CD23 binding to mCD21 is blocked with the addition of an anti-CD21 mAb, triCD23 is no longer able to increase IgE secretion (Fig. 6B).

Fig. 4D reveals that there is a remarkable relationship between the concentration of sCD23 and sIgE after incubation of tonsil B cells with IL-4 and anti-CD40. There appears to only be a slight increase in sIgE at low sCD23 concentrations, up to 60 ng/ml (1.6  $\mu$ M) (concentrations calculated for the 37-kDa fragment). However, above this threshold, we observed a steep rise in sIgE with increasing concentrations of sCD23. The relationship shown in Fig. 4D may reflect the avidity of sCD23 in the trimolecular complexes with mIgE and mCD21 at the surface of the fluid B cell membrane, as this curve exhibits cooperativity.

Earlier work has shown that the incubation of PBMCs with sCD23 stimulates ongoing IgE synthesis, rather than increasing CSR to IgE, which would require stimulation by IL-4 (or IL-13) (41). In our system, with IL-4 and anti-CD40 stimulation, we observed no difference in the expression of eGFP, an early marker for CSR, in CD23 siRNA- compared with control siRNA-transfected cells (Fig. 3D). Neither inhibition of sCD23 through siRNA transfection nor addition of recombinant triCD23 altered the proportion of IgE<sup>+</sup> or IgG<sup>+</sup> cells when measured by flow cytometry (Supplemental Figs. 2, 3B). Additional experiments

with tonsil B cells have shown CSR to occur in the first few days of the incubations (P.S. Hobson, unpublished observations). However, recombinant sCD23 can still increase IgE synthesis when added as late as day 9 in the incubations (M.R. Jutton, unpublished observations). It has also been shown that the addition of a metalloprotease inhibitor terminates incremental IgE synthesis after CSR has occurred (Fig. 4C) (23). Taken together, these experiments indicate an isotype-specific role for sCD23 in promoting IgE synthesis through a postswitch mechanism.

Fearon and Carter (31) showed that coligation of Ag-specific IgM and the CD19–CD21–TAPA complex on naive B cells by Ag, covalently linked to the C3d fragment of complement, stimulates B cell proliferation in the immune response. The effect of a blocking Ab against CD21 in vivo demonstrated the importance of this mechanism for a robust T cell-dependent immune response (44). The mechanism operates by synergistic signaling through IgM and CD21 to augment the expression of the B cell survival factors Bcl-X<sub>L</sub> and Bcl-2, respectively (45). CD23 expressed in a fibroblast cell line can mimic the activity of the Ag–C3d complexes in lowering the threshold of B cell proliferation by an anti-IgM (surrogate Ag) (46). However, it is not known whether mIgE can mimic mIgM in this mechanism. The cytoplasmic sequence of IgE, required for survival, differs from that of IgM (and other isotypes) (47), although mIgE is associated with the same signal transduction proteins (the  $\alpha$  and  $\beta$  subunits) as other isotypes (48). Evidently, mIgE has some capacity for signaling, but nothing is known about the signal transduction pathways.

It is informative to consider the sequence of events after placing the B cells into culture with IL-4 and anti-CD40. At first, sCD23 can only bind to mCD21, which may elicit a proliferative response (49). When IgE is expressed on the membrane, sCD23 appears to sequester the mIgE and mCD21 in raftlike structures (Fig. 6A) (29), which resemble the fate of cross-linked IgM–CD19–CD21–TAPA complexes in complement-enhanced IgM BCR activation (31, 50). Whether formation of an mIgE–sCD23–mCD21 complex would lead to similar functional consequences remains to be investigated.

In this study, two experimental approaches were taken to reduce sCD23 production in primary human B cells. Both techniques culminated in reduced sCD23 production and reduced IgE secretion, albeit through different actions on mCD23 (reduced expression or inhibition of cleavage). Each approach has its limitations, but they are different from each other, and the combined results conclusively demonstrate that sCD23 stimulates IgE synthesis in human B cells.

Our results suggest that sCD23 may be an active partner, rather than an innocent bystander, in regulating IgE synthesis and therefore a promising therapeutic target for allergic disease. Two current strategies, the anti-CD23 mAb lumiliximab, which showed efficacy in lowering IgE levels in asthmatic patients (10, 11), and metalloprotease inhibitors, already tested in mice (19), are aimed primarily at mCD23. Our results, and those from others (9, 23, 26, 41, 51), should encourage the rational design of inhibitors of sCD23 binding to its ligands for the treatment of allergy and asthma.

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## Disclosures

The authors have no financial conflicts of interest.

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**Figure S1. CD23 siRNA transfection has no effect on ADAM10 expression.** Human tonsillar B cells were transfected with either control siRNA (▲) or CD23 siRNA (●) and cultured for up to 12 days with IL-4 (200IU/ml) and anti-CD40 (1µg/ml). Membrane ADAM10 expression was analyzed by flow cytometry and the % of ADAM10<sup>+</sup> cells plotted (n=3).

**Figure S2. CD23 siRNA transfection has no effect on the percentage of IgE<sup>+</sup> or IgG<sup>+</sup> cells.** Human tonsillar B cells were transfected with either control siRNA (■) or CD23 siRNA (□) and cultured for up to 12 days with IL-4 (200IU/ml) and anti-CD40 (1µg/ml). IgE and IgG expression were analyzed by flow cytometry and the % of (A) IgE<sup>+</sup> cells or (B) IgG<sup>+</sup> cells plotted, relative to control siRNA-transfected cells at each timepoint (n=6).

**Figure S3. triCD23 has no effect on IgG secretion or the percentage of IgE<sup>+</sup> or IgG<sup>+</sup> cells.** Human tonsillar B cells were cultured for 12 days with IL-4 (200IU/ml) and anti-CD40 (1µg/ml) alone or + triCD23 (1µM). (A) Supernatants were analyzed on day 12 for IgG secretion, relative to cells cultured with IL-4/anti-CD40 alone (n=6). (B) IgE and IgG expression were analyzed by flow cytometry on day 12 and the % of IgE<sup>+</sup> or IgG<sup>+</sup> cells indicated on the plots (n=1, representative of 6).

**Figure S4. Differentiation of human B cells into mCD21<sup>+</sup>mIgE<sup>+</sup> cells over time.**

Human tonsillar B cells were cultured for up to 10 days with IL-4 (200IU/ml) and anti-CD40 (1μg/ml). mCD21 and mIgE expression were analyzed by flow cytometry and the fold change plotted for the % of (A) mCD21<sup>+</sup> (○) or mIgE<sup>+</sup> (Δ) cells (n=1, representative of 5) and (B) mCD21<sup>+</sup>mIgE<sup>+</sup> cells (□) (n=5), relative to Day 1.

