

SOLUBLE CD23 MONOMERS INHIBIT AND OLIGOMERS STIMULATE IGE SYNTHESIS IN HUMAN B CELLS

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The low-affinity IgE receptor, CD23, is implicated in IgE regulation and the pathogenesis of allergic disease. CD23 is a type II integral membrane protein, comprising a lectin "head", N-terminal "stalk" and C-terminal "tail" in the extracellular sequence. Endogenous proteases cleave CD23 in the stalk and the tail to release soluble fragments that either stimulate or inhibit IgE synthesis in human B cells. The molecular basis of these paradoxical activities is not understood. We have characterized three fragments of CD23, monomeric derCD23, monomeric exCD23 and oligomeric lzCD23. We show that the monomers inhibit and the oligomer stimulates IgE synthesis in human B cells after heavy-chain switching to IgE. CD23 fragments could be targets for therapeutic intervention in allergic disease.

The low-affinity IgE receptor, CD23, is implicated in the pathogenesis of autoimmunity and allergic disease. Levels of soluble CD23 are greatly elevated in rheumatoid arthritis (1,2), SLE and Sjögren's syndrome (3), and the expression of CD23 is enhanced both as membrane and soluble forms in allergic patients (4,5). Strikingly, levels of CD23 are reduced following immunotherapy (6,7). An anti-CD23 antibody, lumiliximab, dramatically reduced IgE levels in patients with allergic asthma in phase I clinical trials

(8), and is currently in clinical trials for the treatment for chronic lymphocytic leukemia (9). CD23 is thus a promising target for alleviating a wide range of inflammatory conditions, including allergic disease.

CD23, a 45 kDa type II integral membrane protein, is unique amongst Ig receptors in belonging to the animal C-type lectin rather than Ig superfamily (10,11). The extracellular sequence comprises the lectin "head", N-terminal "stalk" and C-terminal "tail". The designation "stalk" refers to the predicted capacity of this sequence to form an α -helical coiled-coil or "leucine zipper" (12). Accordingly, protein-protein cross-linking experiments demonstrated that this sequence allows the self-association of CD23 into trimers and higher oligomers (13,14). In addition to its role as an IgE receptor, CD23 also binds to CD21, CD11b, CD11c and the vitronectin receptor (15-17).

Human CD23 is cleaved by endogenous proteases, initially at a specific site near the base of the stalk, to release a fragment of 37 kDa (18). The enzyme responsible for this cleavage appears to be the metalloproteinase ADAM10 (19,20). Other enzymes cleave the 37 kDa fragments further up the stalk and at a site within the tail to produce progressively smaller fragments containing the head and a portion of the tail (18). A 16 kDa fragment, containing only the lectin domain and 10 amino acids of the tail,

results from the digestion of human CD23 by the house dust mite allergen *Der p* I (21). In addition to the soluble fragments, there are two forms of CD23 that can be expressed in the cell membrane. CD23a and CD23b differ by 6/7 amino acids at the N-terminus of the cytoplasmic sequence, and have different modes of regulation, cell-type expression and functions (22,23). CD23a is constitutively expressed in activated B cells, while CD23b expression is stimulated by IL-4 in a wide range of human cells, including B cells.

Membrane CD23 performs a variety of functions, including IgE antibody-dependent presentation of antigens in murine (24) and human (25) B cells, feedback inhibition of IgE synthesis in murine B cells (26) and IgE antibody-dependent killing of tumor cells by human monocytes (27). Soluble fragments of CD23 both positively and negatively regulate IgE synthesis in human B cells (reviewed in 28, 29). Fragments larger and smaller than 25 kDa have appeared to exert opposite effects on IgE synthesis. The present work demonstrates that the critical parameter is the structure, not the size, of CD23 fragments.

We have expressed three recombinant fragments of human CD23, "derCD23", mimicking the fragment released by the *Der p* I protease, "exCD23", representing the entire extracellular sequence of CD23, and "lzCD23", the extracellular sequence with an isoleucine zipper (30) attached to the N-terminus to stabilize the oligomeric structure (31,14). We have examined the relationship between the oligomeric structure of the fragments, their kinetics of interaction with IgE, and the effects on IgE synthesis in tonsillar B cells following the induction of class switching to IgE by IL-4 and anti-CD40.

Experimental Procedures

Reagents

IgE Fc fragment Cε2-4 was produced and purified as described in (32). Humanized anti-CD23 antibody, Lumiliximab, was kindly provided by M. Reff (Biogen-IDEC).

Production of derCD23, exCD23 and lzCD23

Recombinant derCD23 comprised amino acids Ser156–Glu298 (MW 16,789), including the lectin domain and 16 amino acids (Asp283–Glu298) of the C-terminal tail of human CD23. ExCD23 comprised amino acids Asp48–Ser321 (MW 30,991), including the entire tail region. DerCD23 and exCD23 were produced and purified as previously described (33). lzCD23 comprised amino acids Trp45–Ser321 attached to the isoleucine zipper (MW 37,638). lzCD23 was produced and purified using the protocol described previously by (31) with the following alterations. lzCD23 was cloned into pet24a vector (Novagen), complete EDTA-free protease inhibitor cocktail tablets (Roche) were added to the refold buffer (1M GnHCL, 2mM EDTA, 100mM (NH₄)₂SO₄, 0.4M arginine, 1:2mM reduced/oxidized glutathione, 50mM CHES) to prevent degradation. A sample was analyzed by 12% SDS-PAGE, transferred to nitrocellulose (Schleicher and Schuell), probed with 10 µg/ml anti-CD23 (BU38 clone) and revealed with 1/2000 anti-mouse HRP (Dako) and ECL (Pierce) to check the purity and integrity of the protein. The oligomeric fragment was further purified by gel filtration using a Superdex-200 column (Pharmacia), using PBS as the running buffer. Fractions containing the oligomer were pooled, concentrated to approximately 250 µg/ml and stored at -80°C. All CD23 proteins contained <0.1U/ml endotoxin measured by a commercial chromogenic limulus amebocyte lysate assay (Cambrex).

Kinetics of Interaction with IgE-Fc Cε2-4

Surface plasmon resonance was used to measure the kinetics of interaction of der, ex and lzCD23 with IgE Fc (Cε2-4). All experiments were performed at 25°C on an automated instrument (Biacore 2000; GE Healthcare). The methods for derCD23 and exCD23 were outlined previously (33). A similar protocol was used for determining the kinetics of interaction of lzCD23 with Cε2-4. 500-700RU biotinylated Cε2-4 was immobilized to a streptavidin coated chip. DerCD23 or lzCD23 was injected over the chip in duplicate at a variety of concentrations (1000nM, 500nM, 250nM and 125nM) at a

flow rate of 20 $\mu\text{L}/\text{min}$ in HBS/2mM CaCl_2 with an association phase of 6min and dissociation phase of 8min. Regeneration of the chip was performed using 30s pulses of 10mM EDTA in HBS and 0.2M glycine pH2.2, followed by a 120min recovery.

Sedimentation equilibrium studies

Analytical ultracentrifugation studies were performed using a Beckman XL-A analytical ultracentrifuge as described previously (34). ExCD23 and derCD23 were dialyzed into TBS/ 2mM CaCl_2 , and spun at 4°C and 17,500, 20,000 and 24,000 RPM (derCD23) or 10,000, 14,000 and 17,000 (exCD23). Data was analyzed using the Beckman analysis software running under Microcal Origin v3.78 in terms of a single ideal solute to obtain the buoyant molecular mass, $M(1-\bar{v}\rho)$. Residuals were calculated by subtracting the best fit of the model from the experimental data. In all cases a random distribution of the residuals around zero was noted as a function of the radius. To determine the molecular mass of the CD23 fragments, values for partial specific volume were calculated from the sequence using SEDNTERP (<http://www.rasmb.bbri.org/>), and solvent density was used as previously described (34, 35). lzCD23 was prepared in TBS/2mM CaCl_2 /0.05% Sodium azide, loaded at 3 concentrations with A280 of 0.8, 0.6 and 0.4, and spun at 11,000, 9,500 and 8,000 RPM. Data could not be analyzed using a single ideal species model. The buoyant molecular mass of a monomer was calculated based on the solvent density of 1.00709 and partial specific volume of 0.7110 calculated from the sequence, again using SEDNTERP. Using this value, all three concentrations were simultaneously fitted to a range of models using Sigmaplot as described previously (35). These included self association up to hexamers, and also mixtures of non-interacting species.

DerCD23 and exCD23 were also studied in complex with Cε2-4. lzCD23 could not be studied in this way as it is not a monodisperse protein. Samples were dialyzed into TBS/2mM CaCl_2 /0.05% sodium azide, and then mixed in 1:1, 2:1 and 1:2 ratios of

CD23:Cε2-4 (as described previously for a similar fragment of CD23 binding to Cε3-4 (35)). Data were collected at 10,000, 12,000 and 14,000 RPM for derCD23:Cε2-4 and 7,000, 8,500 and 10,000 RPM for exCD23:Cε2-4. The data for all three loading ratios were simultaneously fitted to a range of models in Sigmaplot, using experimentally determined buoyant molecular mass values of 18085 ± 350 Da for the derCD23, 29990 ± 906 Da for the exCD23, and 19700 ± 800 for the Cε2-4. As previously demonstrated for a similar monomeric fragment of CD23 binding to Cε3-4 (35), a 1:1 and 2:1 interaction model fits the derCD23:Cε2-4 data well with randomly distributed residuals. Attempts were made to fit the exCD23:Cε2-4 data with this and a number of other models (e.g. considering exCD23 as a monomer, dimer or trimer), but none was found that adequately describes the data obtained.

Isolation and Culture of Human B Cells

Human B cells were isolated from tonsillar tissue from donors undergoing routine tonsillectomies (ethical approval from GKT Hospital Trust). Tonsillar mononuclear cells were separated by density on a ficoll gradient. B cells were then isolated using AET treated sheep red blood cells. B cells were routinely >98% CD20 positive and <2% CD3 positive as determined by flow cytometry. B cells were cultured in 24 well plates (Nunc) at 0.5×10^6 cells/ml in RPMI with penicillin (100 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), 2mM glutamine (Invitrogen), 10% fetal bovine serum (Hyclone, Perbio Biosciences Ltd), transferrin (35 $\mu\text{g}/\text{ml}$) and insulin (5 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich). Cells were activated with 1 $\mu\text{g}/\text{ml}$ anti-CD40 antibody (G28.5, ATCC) and 200 IU/ml of IL-4 (R&D Systems) for up to 10 days.

Immunoglobulin ELISA

IgE: Maxisorp plates (Nalge Europe Ltd.) were coated with polyclonal mouse anti-human IgE (DakoCytomation) diluted 1/7000 in carbonate buffer, pH 9.8, for 16 h at 4°C. Unbound sites were then blocked with 2% marvel in PBS/0.05% Tween (PBS-T) for 30 min at room temperature. Samples were added at appropriate dilutions to ensure that at least

three readings were within the linear portion of the standard curve and the plates were incubated for 16 h at 4°C; NIP-IgE (JW8/5/13, ECACC, UK) was used to construct a standard curve. IgE binding was detected by mouse anti-human IgE conjugated to HRP (DakoCytomation) diluted 1/1000 in 1% Marvel PBS-T for 4 h at room temperature. The color reaction was developed with OPD (Sigma-Aldrich Company Ltd.). Sensitivity was 10 ng/ml. It was possible that recombinant CD23 interferes with the detection of IgE by ELISA. We investigated this by incubating CD23 fragments with NIP-IgE at 37°C, employing the same culture conditions as in the experiments with B cells. IgE was then measured using the specific IgE ELISA.

IgG: Maxisorp plates were coated with polyclonal goat anti-human IgG (Oxford Biotechnology Ltd.) diluted 1/1000 in carbonate buffer, pH 9.8, for 16 h at 4°C. Unbound sites were then blocked with 2% Marvel in PBS-T for 30 min at room temperature. Samples were added at appropriate dilutions as described previously and the plates were incubated for 16 h at 4°C; total IgG (Sigma-Aldrich) was used to construct standard curves. IgG binding was detected by goat anti-human IgG HRP (Sigma-Aldrich) diluted 1/1000 in 1% Marvel PBS-T for 4 h at room temperature. The color reaction was developed with OPD (Sigma-Aldrich). Sensitivity was 4 ng/ml.

Polyacrylamide gel electrophoresis and Western blotting

Purity and apparent molecular weight of CD23 fragments were determined by 12% SDS-PAGE under reducing conditions. Protein was visualized by coomassie blue stain. Western blotting was used to confirm CD23 identity, using anti-CD23 (BU38 clone) and anti-mouse IgG HRP. Bound antibodies were visualized by enhanced chemiluminescence (Pierce).

Statistics

One tailed, paired student's *t* tests were used to calculate *P* values, significance <0.05.

RESULTS

Self-association of derCD23, exCD23 and lzCD23.

The CD23 fragments were prepared as described in experimental procedures. Each of the three fragments exhibited a principal species of the expected size on SDS gel electrophoresis (Fig. 1a), and was confirmed to be CD23 by Western blotting (data not shown). The CD23 fragments were studied by sedimentation equilibrium in the analytical ultracentrifuge. Fig. 1c and d shows the data obtained for derCD23 and exCD23. The data for both fragments could be fitted using a single ideal species model, giving residuals randomly distributed around zero, revealing the fragments to be monomeric. The molecular masses calculated were 18085 ± 350 Da for the derCD23 and 29990 ± 906 Da for the exCD23. The structure of derCD23 has been determined by NMR spectroscopy (33) and found to be monomeric at 1.7 mg/ml (100 μ M), although a small proportion of oligomer could be detected at extremely high concentration, 20 mg/ml (1.2 mM). ExCD23 exhibited the propensity to form dimers and trimers at 1 mg/ml (32 μ M) in sucrose gradient sedimentation velocity experiments (36). Taken together, these results suggest that the lectin head alone (derCD23), and the head plus the stalk (exCD23) both have the potential for self-association at sufficiently high concentrations.

To compare the activity of exCD23 as a monomer and an oligomer in our assays, we expressed exCD23 with an isoleucine zipper attached to the N-terminus to generate lzCD23 (31) (calculated molecular mass for a monomer, 37,638 Da). The isoleucine zipper was previously characterized as a trimeric α -helical coiled-coil by X-ray crystallography (30). As shown in Fig. 1b, lzCD23 eluted from a size exclusion column as a monodisperse protein with an apparent molecular mass of approximately 203 kDa. However, the presence of a 15 nm α -helical coiled-coil stalk, extended to 20 nm by addition of the zipper (4.8 nm), would lead to a highly asymmetric structure and possibly an over-estimation of its size by gel filtration. To

obtain an unambiguous determination of the size of lzCD23, we used sedimentation equilibrium in the analytical ultracentrifuge (Fig. 2). The data could not be fitted to a species of a single size (data not shown) and therefore data from three concentrations were fitted simultaneously to a wide range of self-association and mixture models. The residuals for the best fit are shown (Fig. 2b); this is for a monomer-trimer-hexamer equilibrium, and yields residuals randomly distributed about zero. In addition, fits for monomer-trimer equilibrium (Fig. 2c), and a non-interacting mix of monomers, trimers and hexamers (Fig. 2d) are shown for comparison, and these are clearly poorer fits to the data, with systematic residuals in both cases. The same result was obtained using data collected at two other speeds and at a lower range of concentrations (data not shown). The effective K_d values for the monomer to trimer, and monomer to hexamer equilibria are 3.5 μM and 14.4 μM respectively, which, for a total concentration of 12.5 μM lzCD23, the highest concentration examined, leads to calculated values of 3.4 μM for monomeric, 3.0 μM trimeric, and 2.3 nM hexameric lzCD23. The proportion of lzCD23 molecules as hexamer is thus extremely low, but their contribution is essential to achieve a good fit to the data. The fraction of lzCD23 molecules in the trimeric form is clearly substantial at all concentrations tested: 73% at 12.5 μM , 68% at 9.3 μM and 60% at 6.4 μM total concentration.

Stoichiometry of the interaction of CD23 fragments with C ϵ 2-4.

To investigate the interaction of derCD23 and exCD23 with IgE-Fc (C ϵ 2-4) we used sedimentation equilibrium as previously described for a similar monomeric CD23 fragment binding to C ϵ 3-4 (35). Fig. 3a shows the data for the derCD23:C ϵ 2-4 interaction fitted to the same 1:1 and 2:1 interaction model used in Shi et al (35), and it is clear that this is a good fit to the data. Using a larger fragment of IgE-Fc thus has no effect on the stoichiometry of the interaction.

The interaction between exCD23 and IgE-Fc is more complex, even though the exCD23 alone is also monomeric. Fig. 3b

shows the same 1:1 and 2:1 model fitted to the data, and it is clearly a poor fit. Despite trying an extensive range of other models, we were unable to find one that fits these data. It is clear however that the complex (or mixture of complexes) formed is larger than would be expected for the 1:1 and 2:1 interaction, implying that exCD23 must be oligomerising upon binding to the IgE-Fc.

Kinetics of interaction of CD23 fragments with C ϵ 2-4.

To investigate the influence of oligomer formation on the kinetics of interaction with IgE, we immobilized the IgE-Fc (C ϵ 2-4) on a Biacore sensor chip and exposed this surface to derCD23 or lzCD23 (Fig. 4). Surface plasmon resonance was measured as a function of time to measure the rate of association with C ϵ 2-4 and, after stopping the flow of CD23, to measure the rate of dissociation of the complex. As shown in Fig. 4a, the interaction of derCD23 with C ϵ 2-4 was monophasic and exhibited relatively fast on and fast off rates and micromolar ("low") affinity ($K_D = 1000$ nM). In contrast, lzCD23 exhibited biphasic kinetics ($K_D \sim 2000$ nM and 100 nM) (Fig 4b). Previous analysis of exCD23 yielded binding curves and association constants similar to those of lzCD23, ($K_{DS} \sim 1000$ nM and 40 nM) (33).

Inhibition of IgE synthesis by anti-CD23.

Having characterized all three CD23 fragments using biophysical techniques, we next investigated their biological activity with respect to IgE synthesis. Firstly, as a control, we examined the antibody, lumiliximab, an anti-CD23 antibody produced as a potential therapy for allergic asthma. Results from *in vitro* studies clearly showed that lumiliximab blocked the production of IgE in human PBMC (37,38). To determine whether CD23 was involved in IgE synthesis in a purified B cell system we investigated the outcome of blocking CD23 with lumiliximab. Lumiliximab, added with IL-4 and anti-CD40 to tonsillar B cells, significantly inhibited IgE synthesis by an average of -46% (range -32 to -57%) (Table 1). Clearly, CD23 is involved in IgE production from purified tonsillar B cells. It is not known, however, whether the

antibody acts by binding to membrane CD23 or its soluble fragments.

Effects of derCD23, exCD23 and lzCD23 on IgE synthesis.

Preliminary experiments were carried out to determine the concentrations of CD23 fragments required for their maximum positive or negative effects on IgE synthesis in our system. In all cases maximum activity was observed at concentrations equal to or greater than 100 ng/ml (data not shown). We therefore used 100 ng/ml in subsequent experiments. It is notable that 100 ng/ml (6 nM for derCD23, 3 nM for exCD23 and 0.8 nM for lzCD23), is 2-3 orders of magnitude below the observed K_D values of for the interaction of these fragments with Cε2-4. B cells stimulated with anti-CD40 and IL-4 for 10 days produced variable amounts of IgE, depending on the donor (Fig. 5 and Table 2). Addition of derCD23 at 100 ng/ml significantly inhibited IgE synthesis. The percentage inhibition varied between donors, but was unrelated to the level of IgE synthesis. Of 8 donors tested, 6 demonstrated inhibition of IgE by derCD23 (mean = -24%; range -59 to +10%). Addition of lzCD23 at 100 ng/ml to the tonsil B cells, stimulated with anti-CD40 and IL-4, significantly upregulated IgE in 6 out of 8 donors (mean +99%, range -41 to +446%) (Fig. 5, Table 2). The level of stimulation, like that of inhibition by derCD23, varied between donors, but was not related to that of IgE production. Addition of exCD23 at 100 ng/ml had no significant effect on anti-CD40 and IL-4 stimulated IgE synthesis (mean -3%, range -38 to +41%) (Fig 5, Table 2). Since CD23 binds to both secreted IgE and membrane IgE it was possible that recombinant CD23 interferes with the detection of IgE by ELISA. This was tested by competitive ELISA and found not be the case (data not shown). It was also possible that CD23 fragments might be degraded during the lengthy incubations with B cells. Western blot analysis of CD23 in parallel cultures revealed that no degradation occurred (data not shown). A further consideration is that incubation of the B cells with IL-4 up-regulates the expression of CD23 and the release of endogenous soluble fragments. In fact we observed variable

concentrations (30-50 ng/ml) of soluble CD23 in the culture medium after incubation of B cells with IL-4 and anti-CD40 in the absence of added CD23 fragments (results not shown), but this is at least 50% less than the concentrations of the recombinant CD23 fragments added to the cultures.

Effects of derCD23 and lzCD23 on IgG production.

To investigate whether the effect of soluble CD23 on IgE production was isotype specific or a general effect on immunoglobulin levels, we analyzed IgG production in the presence of derCD23 and lzCD23. Neither fragment, added to human B cell cultures with anti-CD40 and IL-4, had a significant effect on the production of IgG (Table 3).

DISCUSSION

This is the first study to compare the effects of structurally well-characterized monomeric and trimeric human CD23 fragments upon IgE synthesis in human B cells. We have demonstrated that derCD23, containing the head domain of CD23, is a monomer, exCD23, with the head and stalk, is also a monomer, whereas lzCD23, which differs from exCD23 only by the attachment of an isoleucine zipper at the N-terminus, forms an equilibrium mixture of monomers and trimers. The use of sedimentation equilibrium in the analytical centrifuge determines the self-association state of the three CD23 fragments unambiguously.

We have studied the kinetics of binding of these fragments to IgE-Fc. The interaction of derCD23 is monophasic with "low" affinity ($K_D \sim 1000$ nM), whereas the interactions of both exCD23 and lzCD23 are biphasic with "low" and "high" affinity components ($K_{DS} \sim 1000$ and 100 nM). This biphasic kinetic behavior is similar to that observed for IgE binding to membrane-bound murine CD23, which also exhibits "low" and "high" equilibrium binding affinities with similar K_D values (13). Protein-protein cross-linking revealed that membrane-bound CD23 can form trimers (13,39) and thus the interpretation of the biphasic kinetics is that

the interaction with a single CD23 head domain accounts for the “low” affinity component, while interaction with the trimer (and engagement of two head domains) accounts for the “high” affinity component of binding to IgE. Since we have shown that exCD23 is monomeric, yet displays biphasic kinetics, we propose that oligomerization of exCD23 occurs upon binding to IgE-Fc. The analytical ultracentrifugation data for exCD23:Fc ϵ 2-4 complexes reported here support this proposition.

We have previously shown by protein-protein cross linking studies of a fragment similar to exCD23 in solution, that it has the capacity to form trimers (39). Furthermore, we have shown here that the C ϵ 2-4 fragment of IgE-Fc binds two molecules of derCD23. This provides a molecular mechanism to explain the ligand-induced oligomerization. When C ϵ 2-4 was added to exCD23 in the analytical ultracentrifuge, we observed the formation of high molecular weight complexes, consistent with oligomerization of the exCD23 and formation of extended aggregates with the divalent IgE-Fc. It was not possible to define the size distribution of these extended complexes, but the analytical ultracentrifugation data show clearly that although exCD23 and derCD23 are both monomeric when unliganded, they behave very differently in the presence of IgE-Fc, exCD23 forming large aggregates, while derCD23 forms only 1:1 and 2:1 complexes (CD23:IgE-Fc).

Most importantly, we have correlated the structure and IgE interaction kinetics of the CD23 fragments with their effects on IgE synthesis. The results clearly show a correlation between the capacity of fragments to form trimers and the stimulation of IgE synthesis in this system. DerCD23 inhibits, exCD23 has no apparent effect and lzCD23 stimulates IgE synthesis. The apparent lack of activity of exCD23 is probably due to competition between stimulatory trimeric and inhibitory monomeric fragments binding to IgE on the B cells. Furthermore, the observed effect was specific to IgE, since no change in IgG synthesis was seen.

CD23 has two ligands expressed on IgE class-switched B cells, IgE (40) and CD21

(41). While IgE binds to a site in the lectin head, CD21 binds to the C-terminal tail, and NMR studies revealed that monomeric derCD23 is able to bind IgE and CD21 simultaneously (33). Murine CD21 has been shown to mediate the signals of oligomeric C3dg and related fragments of the C3 component of complement in stimulation of the immune response (42). Pierce and co-workers further demonstrated that this is accompanied by the formation of signaling platforms in the B cell membrane (43). Signaling through IgM and the CD19/CD21 complex stimulates the synthesis of Bcl-x_L and Bcl-2, respectively, and these two signals act synergistically to promote B cell survival and the production of antigen-specific antibodies in the immune response (44). Co-ligation of IgM and CD21 on human B cells leads to the rescue from Fas-induced apoptosis through the induction of Bcl-2 (45). CD23 binding to CD21 and IgE in IgE-switched B cells might be analogous to antigen-C3dg complexes binding to IgM and CD21 in unswitched naïve B cells, thus promoting IgE synthesis in the allergic response (Fig. 6a).

In this present study we have demonstrated that lzCD23 upregulates IgE synthesis from human B cells, and we propose that lzCD23 may act by co-ligating IgE and CD21 on IgE-switched B cells (Fig. 6a). In fact, trimeric CD23 could form a large array of such complexes (shown schematically in Fig. 6d), which could act as a signaling platform, similar to that observed for IgM and CD21 (43). The extended complexes formed between dimeric IgE-Fc and trimeric CD23 (Fig. 6d) are consistent with the observation of large complexes formed between Fc ϵ 2-4 and exCD23 in the analytical ultracentrifuge. In contrast, derCD23 was found to inhibit IgE synthesis, and the mechanisms responsible may be understood by taking into account the presence of endogenous CD23. Clearly, endogenous CD23 is involved in IgE synthesis induced by CD40-ligation and IL-4, since anti-CD23 (lumiliximab) blocks IgE synthesis. DerCD23 may therefore act by blocking the binding of endogenous trimeric soluble CD23 to CD21 and IgE (Fig. 6b), limiting the size of the signaling platform induced by the trimeric

soluble CD23, since derCD23 only forms 2:1 complexes with IgE-Fc (Fig. 6c), and thereby preventing up-regulation of IgE synthesis.

Further support for this model comes from earlier work, employing less fully characterized CD23 fragments, which showed that soluble fragments of CD23 target on-going IgE synthesis from committed B cells (28,46-48). These observations are consistent with a role for membrane IgE in the regulation of IgE synthesis. Membrane IgE is associated with the same $\alpha\beta$ signaling subunits as IgM (49), and cytoplasmic sequence of the ϵ -chain is required for the survival of IgE-expressing B cells (50). A role for CD21 has also been established. Antibodies that bind to the same region of CD21 as CD23 stimulate IgE synthesis, whereas antibodies that bind to other regions do not (15,51). Experiments are underway to discover how the interaction of

CD23 with IgE and CD21 upregulates IgE synthesis.

In conclusion, our biophysical studies provide insight into the biological effects of different fragments of human CD23 that have puzzled earlier workers. We have for the first time demonstrated the crucial importance of the oligomeric nature of human CD23 fragments in the regulation of IgE synthesis in isolated B cells. These results have implications for the design of agents to prevent the release of soluble CD23 from cells, or to modulate the oligomeric state and thus the activity of soluble CD23 fragments, in the treatment of allergic disease. As exemplified by lumiliximab, the inhibition of soluble CD23 fragments binding to membrane IgE and CD21 on B cells is a promising strategy for dampening the allergic response.

REFERENCES

1. Huissoon, A. P., Emery, P., Bacon, P. A., Gordon, J., and Salmon, M. (2000) *Scand. J. Rheumatol.* **29**, 154-159
2. Ribbens, C., Bonnet, V., Kaiser, M. J., Andre, B., Kaye, O., Franchimont, N., de, G. D., Beguin, Y., and Malaise, M. G. (2000) *Clin. Exp. Immunol.* **120**, 194-199
3. Bansal, A., Roberts, T., Hay, E. M., Kay, R., Pumphrey, R. S., and Wilson, P. B. (1992) *Clin. Exp. Immunol.* **89**, 452-455
4. Aberle, N., Gagro, A., Rabatic, S., Reiner-Banovac, Z., and Dekaris, D. (1997) *Allergy* **52**, 1238-1242
5. Yanagihara, Y., Sarfati, M., Marsh, D., Nutman, T., and Delespesse, G. (1990) *Clin. Exp. Allergy* **20**, 395-401
6. Kljaic-Turkalj, M., Cvoriscec, B., Tudoric, N., Stipic-Markovic, A., Rabatic, S., Trescec, A., Gagro, A., and Dekaris, D. (1996) *Int. Arch. Allergy Immunol.* **111**, 188-194
7. Tanaka, A., Ohashi, Y., and Nakai, Y. (1999) *Ann. Otol. Rhinol. Laryngol.* **108**, 193-200
8. Rosenwasser, L. J. and Meng, J. (2005) *Clin. Rev. Allergy Immunol.* **29**, 61-72
9. Ferrajoli, A., Faderl, S., and Keating, M. J. (2006) *Expert. Rev. Anticancer Ther.* **6**, 1231-1238
10. Kikutani, H., Inui, S., Sato, R., Barsumian, E. L., Owaki, H., Yamasaki, K., Kaisho, T., Uchibayashi, N., Hardy, R. R., Hirano, T., and . (1986) *Cell* **47**, 657-665

11. Ludin, C., Hofstetter, H., Sarfati, M., Levy, C. A., Suter, U., Alaimo, D., Kilchherr, E., Frost, H., and Delespesse, G. (1987) *EMBO J.* **6**, 109-114
12. Beavil, A. J., Edmeades, R. L., Gould, H. J., and Sutton, B. J. (1992) *Proc. Natl. Acad. Sci. U. S. A* **89**, 753-757
13. Dierks, S. E., Bartlett, W. C., Edmeades, R. L., Gould, H. J., Rao, M., and Conrad, D. H. (1993) *J. Immunol.* **150**, 2372-2382
14. Chen, B. H., Ma, C., Caven, T. H., Chan-Li, Y., Beavil, A., Beavil, R., Gould, H., and Conrad, D. H. (2002) *Immunology* **107**, 373-381
15. Aubry, J. P., Pochon, S., Gauchat, J. F., Nueda-Marin, A., Holers, V. M., Graber, P., Siegfried, C., and Bonnefoy, J. Y. (1994) *J. Immunol.* **152**, 5806-5813
16. Lecoanet-Henchoz, S., Plater-Zyberk, C., Graber, P., Gretener, D., Aubry, J. P., Conrad, D. H., and Bonnefoy, J. Y. (1997) *Eur. J. Immunol.* **27**, 2290-2294
17. Hermann, P., Armant, M., Brown, E., Rubio, M., Ishihara, H., Ulrich, D., Caspary, R. G., Lindberg, F. P., Armitage, R., Maliszewski, C., Delespesse, G., and Sarfati, M. (1999) *J. Cell Biol.* **144**, 767-775
18. Letellier, M., Sarfati, M., and Delespesse, G. (1989) *Mol. Immunol.* **26**, 1105-1112
19. Weskamp, G., Ford, J. W., Sturgill, J., Martin, S., Docherty, A. J., Swendeman, S., Broadway, N., Hartmann, D., Saftig, P., Umland, S., Sehara-Fujisawa, A., Black, R. A., Ludwig, A., Becherer, J. D., Conrad, D. H., and Blobel, C. P. (2006) *Nat. Immunol.* **7**, 1293-1298
20. Lemieux, G. A. et al. The low affinity IgE receptor (CD23) (2007) *J. Biol. Chem.* **282**, 148236-14844
21. Schulz, O., Laing, P., Sewell, H. F., and Shakib, F. (1995) *Eur. J. Immunol.* **25**, 3191-3194
22. Yokota, A., Kikutani, H., Tanaka, T., Sato, R., Barsumian, E. L., Suemura, M., and Kishimoto, T. (1988) *Cell* **55**, 611-618
23. Gould, H. J., Beavil, R. L., Reljic, R., Shi, J., Ma, C. W., Sutton, B. J., and Ghirlando, R. (1997) IgE Homeostasis: is CD23 the Safety Switch ? In Vercelli, D., editor. *IgE Regulation: Molecular Mechanisms*, John Wiley & Sons Ltd.,
24. Kehry, M. R. and Yamashita, L. C. (1989) *Proc. Natl. Acad. Sci. U. S. A* **86**, 7556-7560
25. Pirron, U., Schlunck, T., Prinz, J. C., and Rieber, E. P. (1990) *Eur. J. Immunol.* **20**, 1547-1551
26. Yu, P., Kosco-Vilbois, M., Richards, M., Kohler, G., and Lamers, M. C. (1994) *Nature* **369**, 753-756

27. Karagiannis, S. N., Wang, Q., East, N., Burke, F., Riffard, S., Bracher, M. G., Thompson, R. G., Durham, S. R., Schwartz, L. B., Balkwill, F. R., and Gould, H. J. (2003) *Eur. J. Immunol.* **33**, 1030-1040
28. Delespesse, G., Sarfati, M., Wu, C. Y., Fournier, S., and Letellier, M. (1992) *Immunol. Rev.* **125**, 77-97
29. Gould, H. J., Sutton, B. J., Beavil, A. J., Beavil, R. L., McCloskey, N., Coker, H. A., Fear, D., and Smurthwaite, L. (2003) *Annu. Rev. Immunol.* **21**, 579-628
30. Harbury, P. B., Kim, P. S., and Alber, T. (1994) *Nature* **371**, 80-83
31. Kelly, A. E., Chen, B. H., Woodward, E. C., and Conrad, D. H. (1998) *J. Immunol.* **161**, 6696-6704
32. Young, R. J., Owens, R. J., Mackay, G. A., Chan, C. M., Shi, J., Hide, M., Francis, D. M., Henry, A. J., Sutton, B. J., and Gould, H. J. (1995) *Protein Eng* **8**, 193-199
33. Hibbert, R. G., Teriete, P., Grundy, G. J., Beavil, R. L., Reljic, R., Holers, V. M., Hannan, J. P., Sutton, B. J., Gould, H. J., and McDonnell, J. M. (2005) *J. Exp. Med.* **202**, 751-760
34. Ghirlando, R., Keown, M. B., Mackay, G. A., Lewis, M. S., Unkeless, J. C., and Gould, H. J. (1995) *Biochemistry* **34**, 13320-13327
35. Shi, J., Ghirlando, R., Beavil, R. L., Beavil, A. J., Keown, M. B., Young, R. J., Owens, R. J., Sutton, B. J., and Gould, H. J. (1997) *Biochemistry* **36**, 2112-2122
36. Reljic, R. (1996) The interaction of CD23 with CR2 and its functional consequences.
37. Nakamura, T., Kloetzer, W. S., Brams, P., Hariharan, K., Chamat, S., Cao, X., LaBarre, M. J., Chinn, P. C., Morena, R. A., Shestowsky, W. S., Li, Y. P., Chen, A., and Reff, M. E. (2000) *Int. J. Immunopharmacol.* **22**, 131-141
38. Yabuuchi, S., Nakamura, T., Kloetzer, W. S., and Reff, M. E. (2002) *Int. Immunopharmacol.* **2**, 453-461
39. Beavil, R. L., Graber, P., Aubonne, N., Bonnefoy, J. Y., and Gould, H. J. (1995) *Immunology* **84**, 202-206
40. Spiegelberg, H. L. (1984) *Adv. Immunol.* **35**, 61-88
41. Pochon, S., Graber, P., Yeager, M., Jansen, K., Bernard, A. R., Aubry, J. P., and Bonnefoy, J. Y. (1992) *J. Exp. Med.* **176**, 389-397
42. Fearon, D. T. and Carter, R. H. (1995) *Annu. Rev. Immunol.* **13**, 127-149
43. Pierce, S. K. (2002) *Nat. Rev. Immunol.* **2**, 96-105
44. Roberts, T. and Snow, E. C. (1999) *J Immunol* **162**, 4377-4380

45. Mongini, P. K., Jackson, A. E., Tolani, S., Fattah, R. J., and Inman, J. K. (2003) *J Immunol* **171**, 5244-5254
46. Sarfati, M., Rector, E., Rubio-Trujillo, M., Wong, K., Sehon, A. H., and Delespesse, G. (1984) *Immunology* **53**, 207-214
47. Sarfati, M., Rector, E., Wong, K., Rubio-Trujillo, M., Sehon, A. H., and Delespesse, G. (1984) *Immunology* **53**, 197-205
48. Sarfati, M., Rector, E., Sehon, A. H., and Delespesse, G. (1984) *Immunology* **53**, 783-790
49. Venkitaraman, A. R., Williams, G. T., Dariavach, P., and Neuberger, M. S. (1991) *Nature* **352**, 777-781
50. Achatz, G., Nitschke, L., and Lamers, M. C. (1997) *Science* **276**, 409-411
51. Flores-Romo, L., Cairns, J. A., Millsum, M. J., and Gordon, J. (1989) *Immunology* **67**, 547-549

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Purification and analytical ultracentrifugation of derCD23 and exCD23. Purified CD23 fragments were analyzed by SDS-PAGE and size exclusion chromatography. (a) 12% reducing SDS-PAGE stained with Coomassie blue: lane 1 derCD23, lane 2 exCD23 and lane 3 lzCD23. (b) Purified lzCD23 was analyzed by size exclusion chromatography using a Superdex-200 column. A single peak at 15 min corresponding to 203 kDa was observed. (c) and (d) The CD23 fragments were studied by sedimentation equilibrium in the analytical ultracentrifuge, representative data is shown and was collected at (c) 24,000 RPM for derCD23 and (d) 10,000 RPM for exCD23. Residuals show both proteins fit to a single monomeric species, with molecular mass of 18085 ± 350 Da for the derCD23 and 29990 ± 906 Da for the exCD23.

Fig. 2. Analytical ultracentrifugation of lzCD23. The data shown was collected at 11,000 RPM and 3 loading concentrations with an A280 of 0.8 (circles), 0.6 (squares) and 0.4 (triangles). A shows the data curves simultaneously fitted to a monomer-dimer-trimer equilibrium model (with the triangles offset by +0.1 absorbance units for clarity). This gives an excellent fit to the data: the residuals (B) are randomly distributed around zero in all cases. C and D show the residuals for other models fitted to the same data, and it is clear that both the monomer-trimer model and the mixture of non-interacting monomers, trimers and hexamers give systematic residuals and therefore do not fit the data. Despite trying a wide range of other possible fits (data not shown), the monomer-trimer hexamer fit was the best at all speeds and when the experiment was performed at lower concentration.

Fig 3. Analytical untracentrifugation of der and exCD23 interacting with Cε2-4. The data were collected at 10,000, 12,000 and 14,000 RPM for derCD23:Cε2-4 and 7,000, 8,500 and 10,000

RPM for exCD23:Cε2-4 at ratios of CD23:Cε2-4 of 1:1, 2:1 and 1:2. The data curves shown are simultaneous fits for a 1:1 and 2:1 interaction model. This gives a good fit for derCD23 (a) with residuals randomly distributed around zero. In contrast exCD23 (b) clearly does not fit this model.

Fig. 4. SPR sensorgrams of derCD23 and lzCD23 interacting with Cε2-4. Serial dilutions of 1000nM (-), 500nM (-), 250nM (-) and 125nM (-) of (a) derCD23 and (b) lzCD23 were injected over biotinylated Cε2-4 immobilized to a streptavidin coated sensor chip. After an association phase of 6 minutes, the dissociation was measured for 8 minutes. Data was analyzed using BIAevaluation software and the fits are shown for each binding curve. The residuals for the fits are shown below each sensorgram.

Fig. 5. Soluble CD23 fragments influence IgE production. Human tonsillar B cells were cultured for 10 days with 1μg/ml of anti-CD40 and 200IU/ml IL-4, with or without CD23 fragments, derCD23 (circles), exCD23 (triangles) and lzCD23 (squares) for 10 days. IgE was measured in cell supernatants by specific ELISA.

Fig. 6. Proposed mechanisms of IgE regulation by soluble CD23. Panel (a) Model of trimeric soluble CD23 cross-linking IgE and CD21 on the B cell surface, leading to the formation of large networks, as shown in panel (d), and the upregulation of IgE synthesis. Panel (b) DerCD23 competition with trimeric soluble CD23 for binding to IgE and CD21, preventing the formation of a network, and thus the upregulation of IgE synthesis. Panel (c) IgE (Cε3 dimer shown in black) binding to two derCD23 heads (hatched), which can also bind to CD21 (domains D1D2 shown in white), in a defined complex, that cannot form larger aggregates. Panel (d) Trimeric soluble CD23 molecules (shaded) binding up to three IgE molecules (one or two only are shown) and CD21 (D1D2), leading to the formation of large networks of CD23-IgE-CD21. It is this event that we propose leads to the upregulation of IgE synthesis by trimeric soluble CD23 observed in this study. This model satisfies our present knowledge of the stoichiometry of the complexes that can be formed between these molecules.

Table 1 Lumiliximab (anti-CD23) at 1µg/ml inhibits IgE production from tonsillar B cells stimulated with anti-CD40 1µg/ml and IL-4 200IU/ml for 10 days

	IgE ng/ml (+/-s.d.) n≥3	
	no addition	Anti-CD23
Exp 1	37.3 +/- 4.5	17.9 +/- 1.3 p = 0.001 -48%
Exp 2	420.8 +/- 88.6	287 +/- 73.9 p=3.2 x 10 ⁻⁵ -32%
Exp 3	1487.3 +/- 528.2	642.9 +/- 14.1 p=2.3 x 10 ⁻⁵ -57%
Exp 4	351.9 +/- 64.1	177.5 +/-18.9 p=1.1 x 10 ⁻⁷ -49%

Table 2 IgE production from tonsillar B cells stimulated with IL-4 (200IU/ml) and anti-CD40 (1µg/ml) in the presence or absence of soluble CD23 for 10 days.

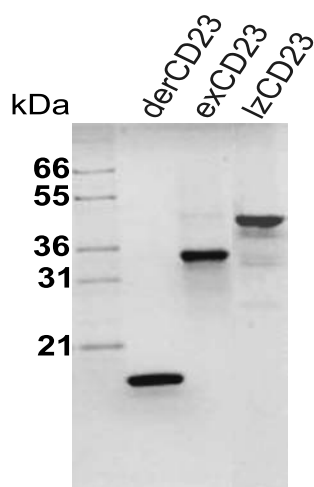
IgE ng/ml (+/- s.d.)				
	No addition	derCD23	exCD23	lzCD23
Exp 1	1527 +/- 420	622 +/- 94 p = 5 x 10 ⁻⁸ -59%	1654 +/- 731 p = 0.3 +8%	2016 +/- 578 p = 0.01 +32%
Exp 2	48 +/- 7	35 +/- 1 p = 0.004 -27%	48 +/- 2 p = 1 0%	262 +/- 22 p = 4 x 10 ⁻⁸ +446%
Exp 3	114 +/- 10	61 +/- 4 p = 1 x 10 ⁻⁷ -46%	ND	174 +/- 9 p = 6 x 10 ⁻⁷ +53%
Exp 4	405 +/- 46	452 +/- 18 p = 0.05 +10%	573 +/- 47 p = 5 x 10 ⁻⁵ +41%	652 +/- 145 p = 5 x 10 ⁻⁷ +61%
Exp 5	444 +/- 23	268 +/- 4 p = 2 x 10 ⁻⁷ -40%	276 +/- 18 p = 2 x 10 ⁻⁷ -38%	262 +/- 8 p = 9 x 10 ⁻¹³ -41%
Exp 6	85 +/- 4	63 +/- 1 p = 2 x 10 ⁻⁹ -26%	55 +/- 3 p = 4 x 10 ⁻⁸ -35%	272 +/- 26 p = 0.05 +220%
Exp 7	86 +/- 7	75 +/- 5 p = 0.01 -13%	76 +/- 3 p = 0.009 -12%	79 +/- 7 p = 0.04 -8%
Exp 8	243 +/- 34	259 +/- 41 p = 0.2 +7%	267 +/- 42 p = 0.2 +10%	271 +/- 40 p = 0.2 +12%

Table 3 IgG production from tonsillar B cells stimulated with IL-4 (200IU/ml) and anti-CD40 (1µg/ml) in the presence or absence of soluble CD23 for 10 days.

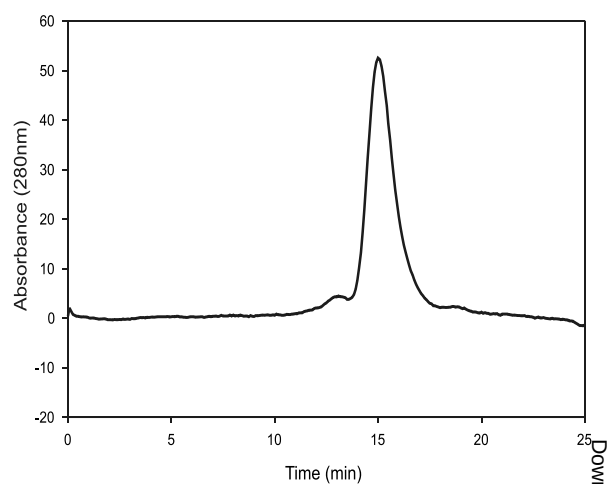
	IgG ng/ml (+/- s.d.)		
	no addition	derCD23	lzCD23
Exp 1	220+/- 53	271 +/- 35 p = 0.2 +23%	246 +/- 52 p = 0.3 +12%
Exp 2	37 +/- 6	39 +/- 3 p = 0.3 +4%	37 +/- 2 p = 0.5 0%
Exp 3	84 +/- 8	68 +/- 2 p = 0.0004 -19%	89 +/- 2 p = 0.1 +6%
Exp 4	48 +/- 6	48 +/- 6 p = 0.5 0%	43 +/- 2 p = 0.1 +2%

Figure 1

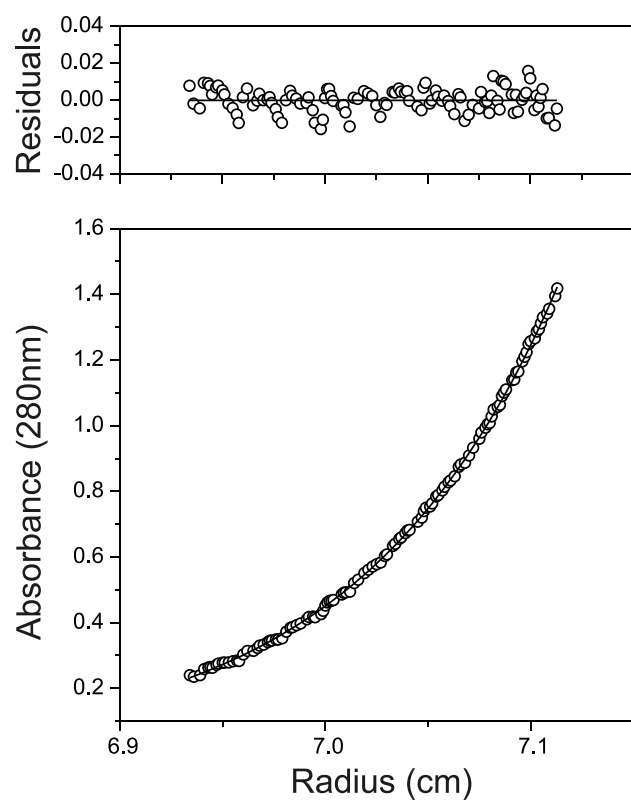
(a)



(b)



(c)



(d)

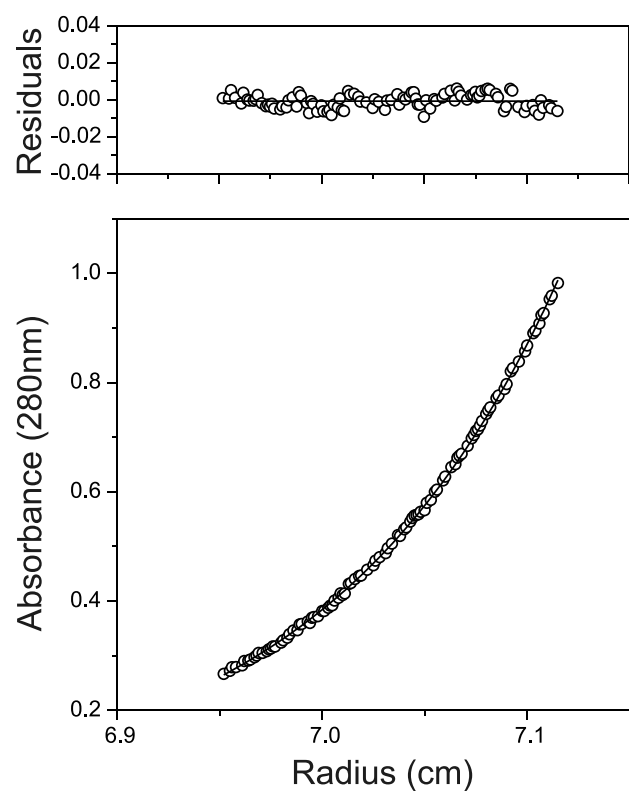


Figure 2

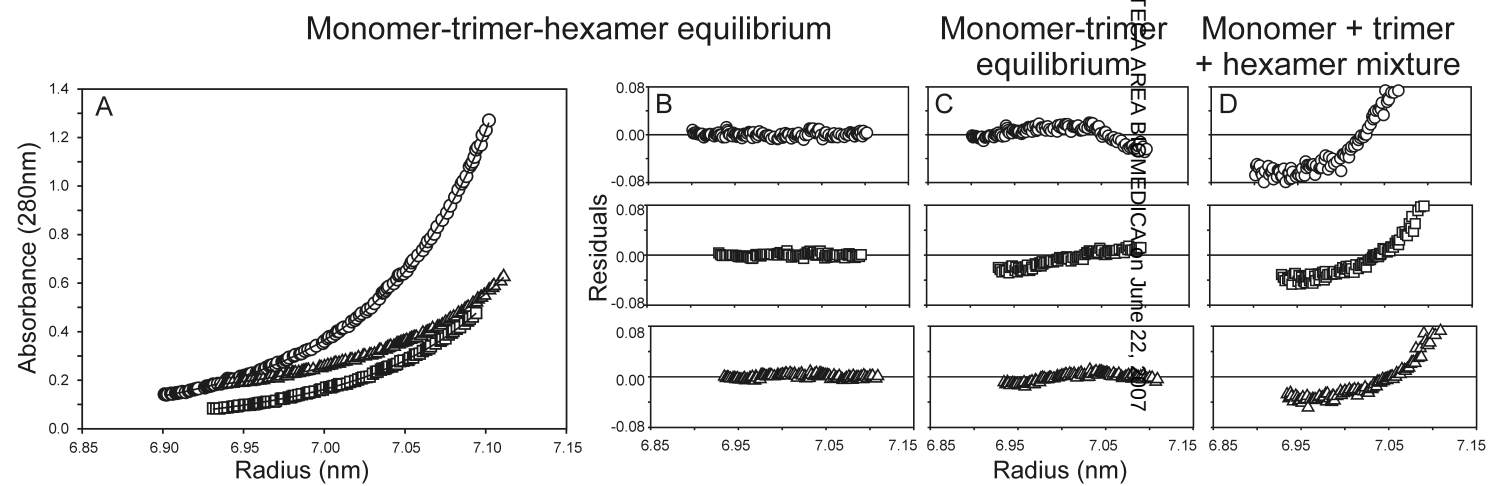
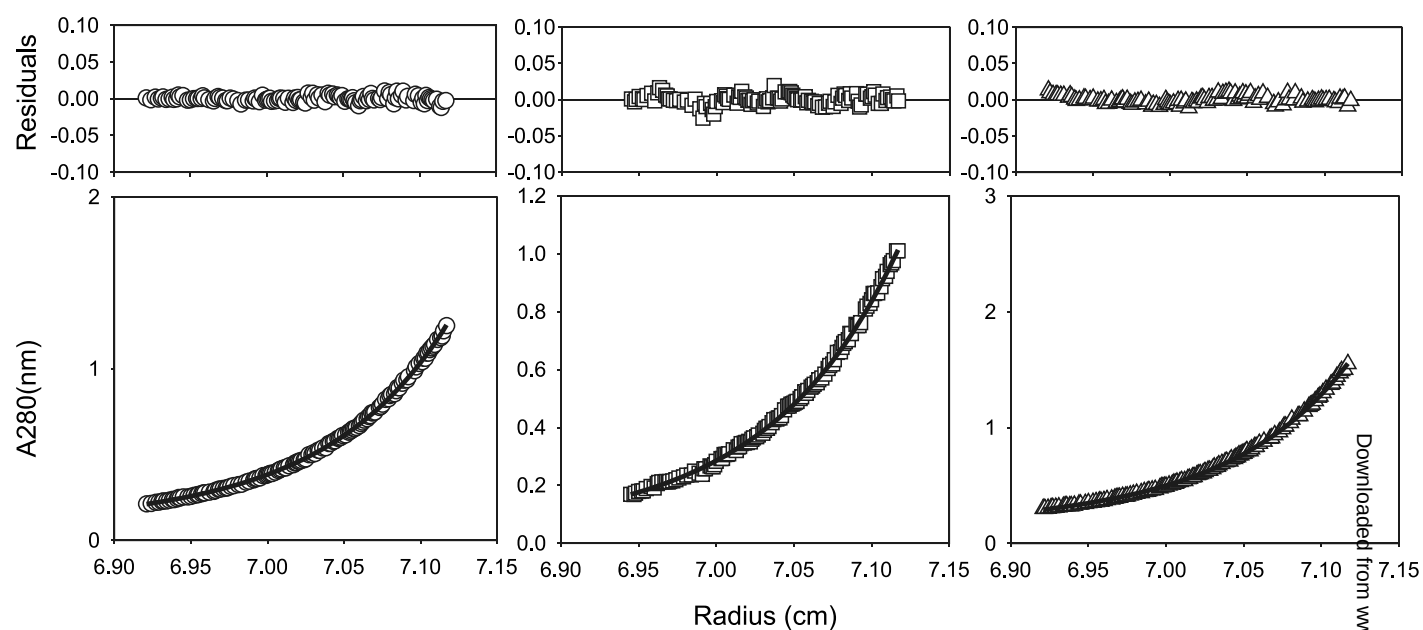
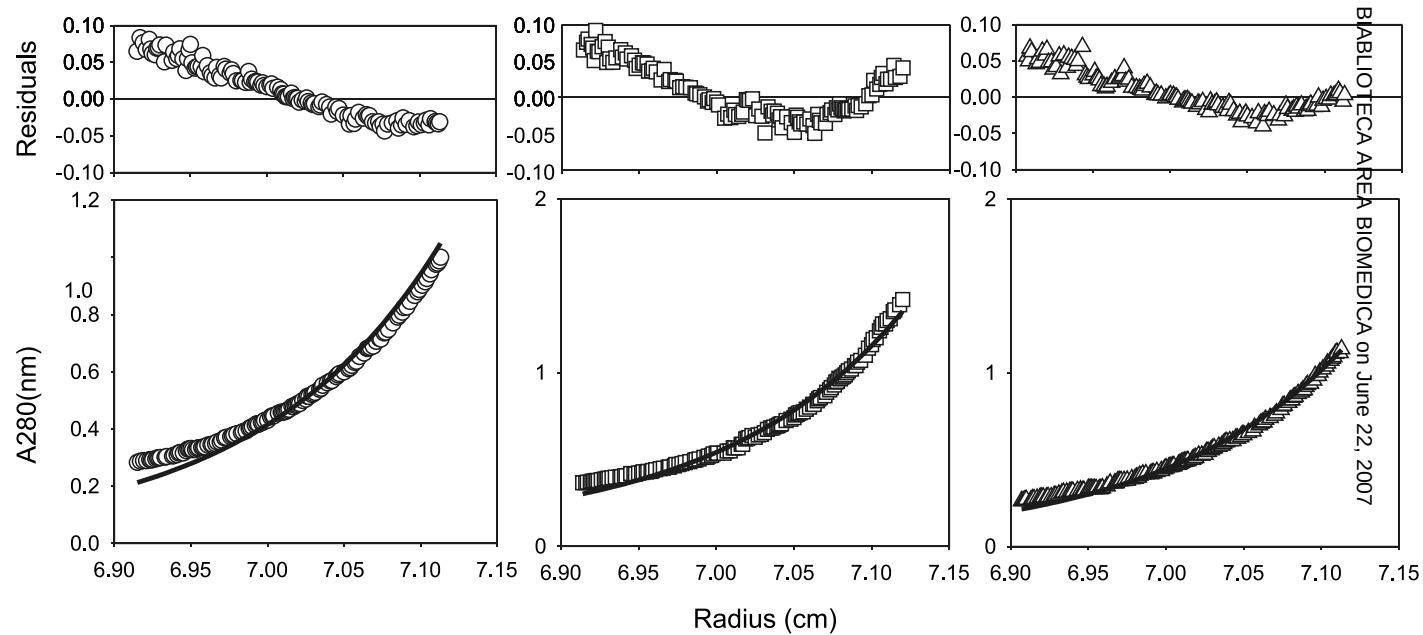


Figure 3

(a) derCD23 binding to C ϵ 2-4



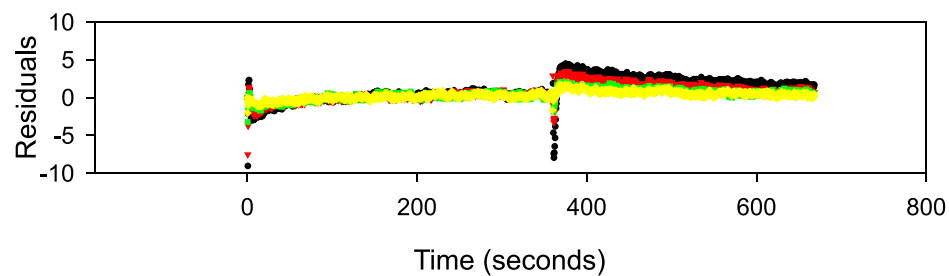
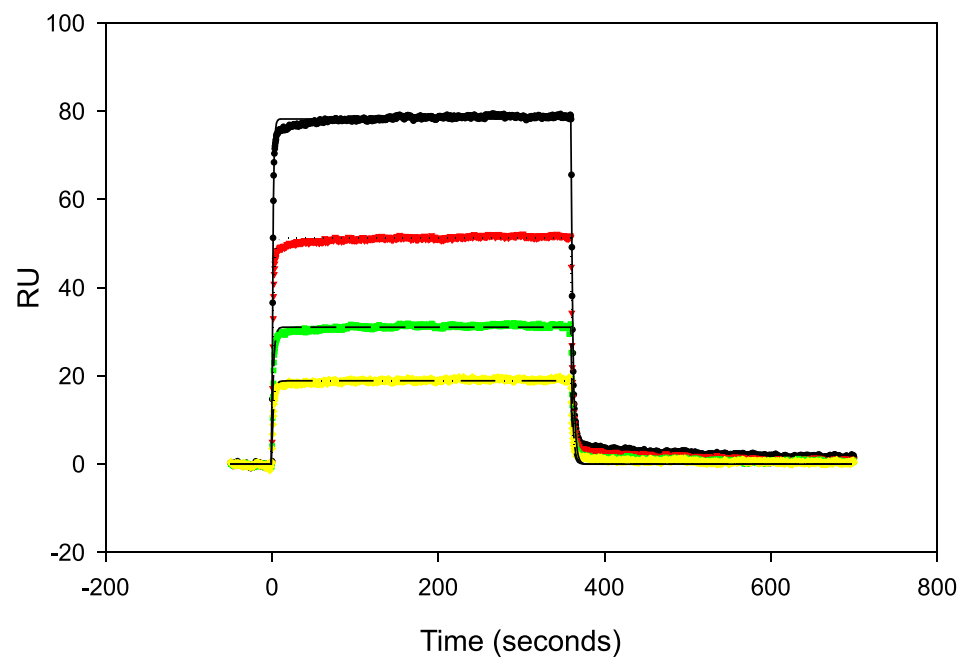
(b) exCD23 binding to C ϵ 2-4



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Figure 4

(a) derCD23 binding C_ε2-4



(b) zCD23 binding C_ε2-4

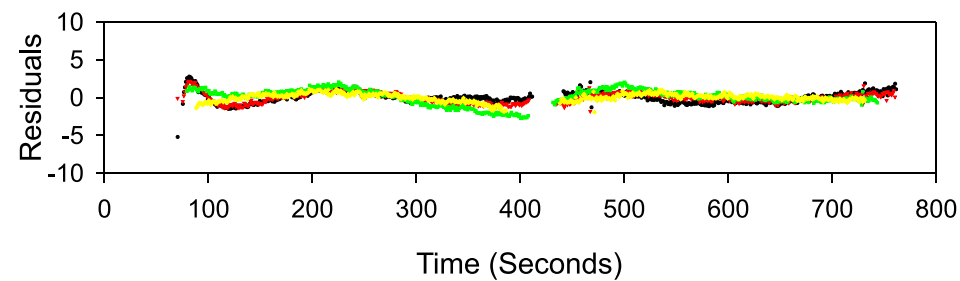
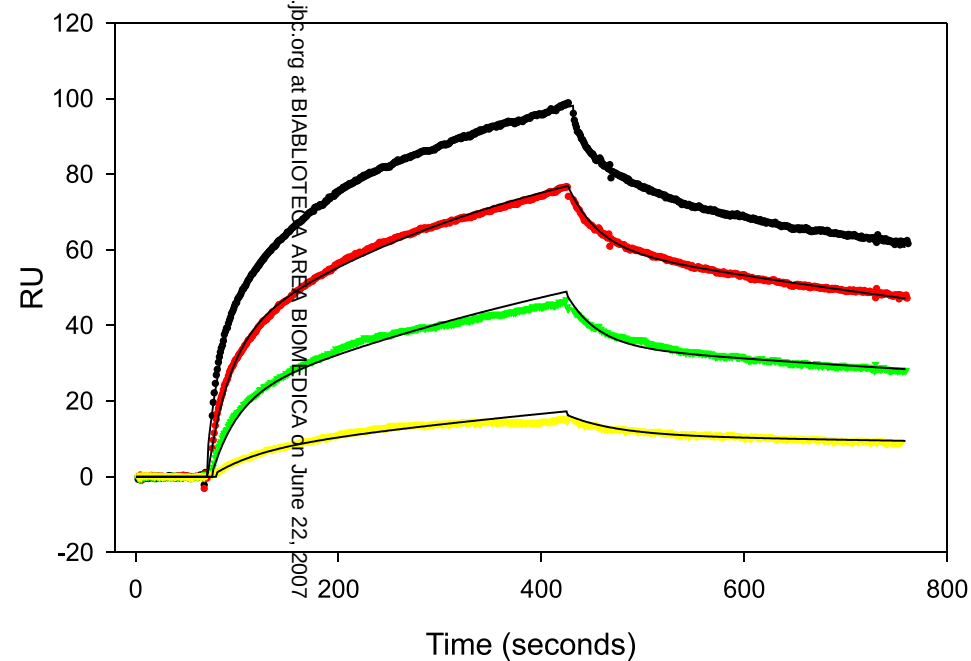


Figure 5

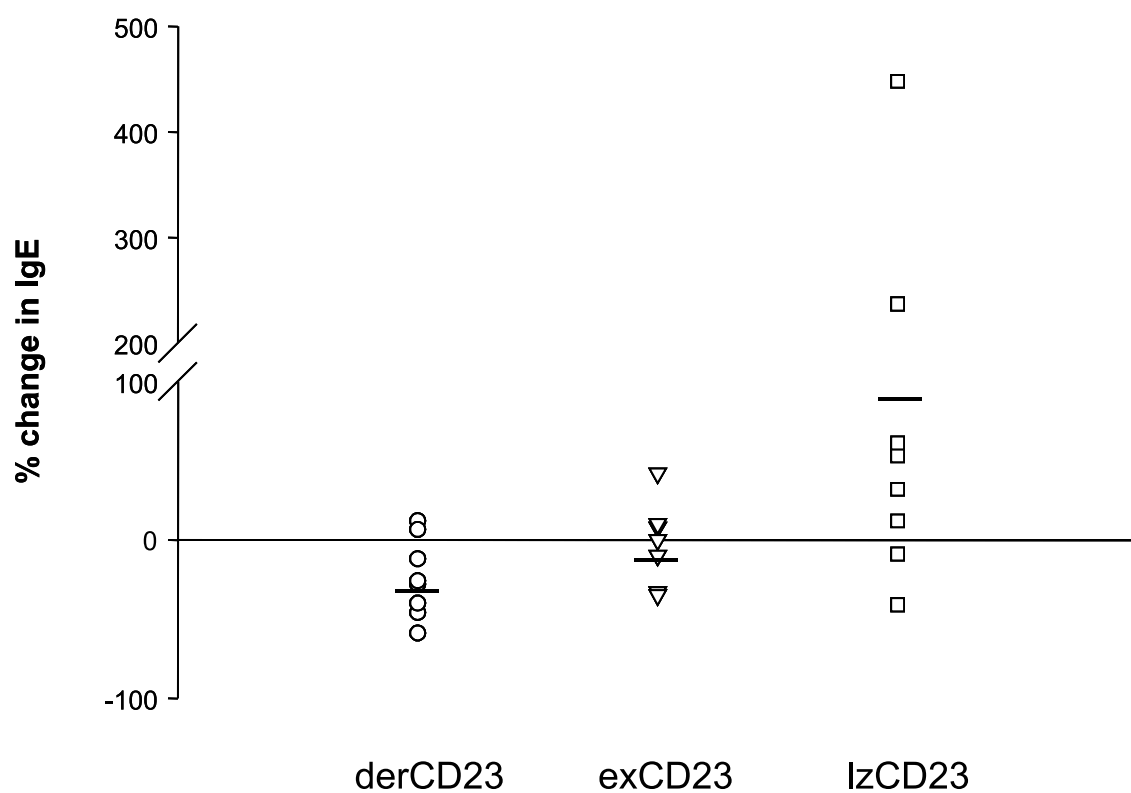
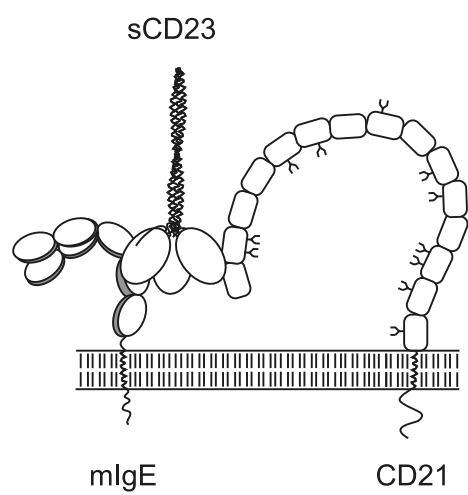
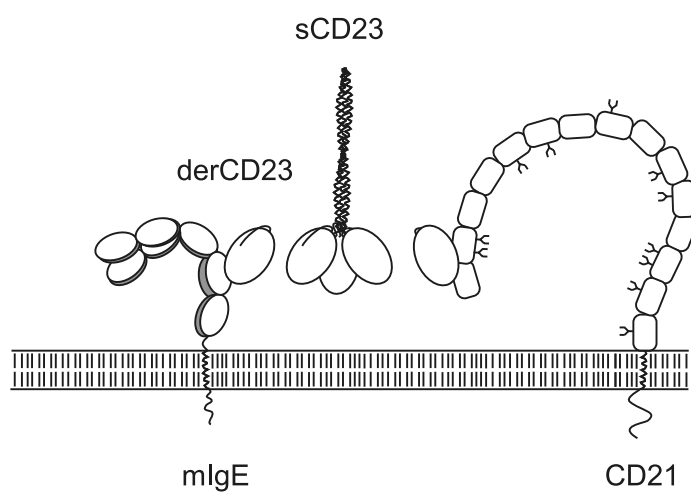


Figure 6

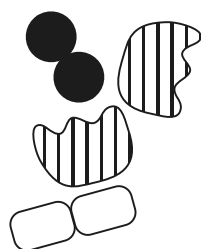
(a)



(b)



(c)



(d)

