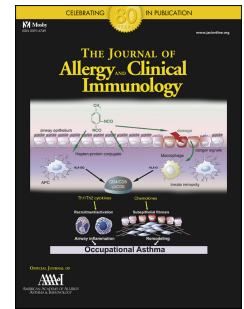


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Critical and direct involvement of the CD23 stalk region in IgE binding

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ABSTRACT

BACKGROUND: The low-affinity receptor for IgE, FcεRII (CD23), contributes to allergic inflammation by allergen presentation to T cells, regulation of IgE responses and enhancing trans-epithelial allergen migration.

OBJECTIVE: To investigate the interaction between CD23, chimeric monoclonal human IgE and the corresponding birch pollen allergen, Bet v 1 at a molecular level.

METHODS: We expressed four CD23 variants. One variant comprised the full extracellular portion of CD23 including stalk and head domain, one was identical with the first except of an amino acid exchange in the stalk region abolishing the N-linked glycosylation site, and two variants representing the head domain, one complete and one truncated. The four CD23 variants were purified as monomeric and structurally folded proteins as demonstrated by gel filtration and circular dichroism. Using a human monoclonal IgE antibody, the corresponding allergen Bet v 1 and a panel of antibodies specific for peptides spanning the CD23 surface, binding as well as inhibition assays and negative stain electron microscopy were performed.

RESULTS: A hitherto unknown IgE binding site was mapped on the stalk region of CD23 and the non-N-glycosylated monomeric version of CD23 was superior in IgE binding compared to glycosylated CD23. Furthermore, we demonstrated that a therapeutic anti-IgE antibody, omalizumab, which inhibits IgE binding to FcεRI, also inhibited IgE binding to CD23.

CONCLUSION: Our results provide a new model for the CD23-IgE interaction. We show that the stalk region of CD23 is crucially involved in IgE binding and that the interaction can be blocked by the therapeutic anti-IgE antibody omalizumab.

CLINICAL IMPLICATION: The new interaction model of CD23 and IgE and its inhibition by omalizumab may open new opportunities for allergy treatment.

CAPSULE SUMMARY.

We provide a new model of interaction between CD23 and IgE by showing a direct involvement of the CD23 stalk region in IgE binding. Furthermore, we demonstrate that a therapeutic anti-IgE antibody, omalizumab, blocks the binding of IgE to CD23.

KEY WORDS:

CD23, allergy, IgE, low-affinity IgE receptor, B cell, allergen

ABBREVIATIONS

A 405nm: adsorption at 405 nm

Bet v 1: major birch pollen allergen

CD23/ FcεRII: cluster of differentiation 23, low-affinity IgE receptor

Cε3-Cε4: IgE constant domains 3-4

FcεRI: high-affinity IgE receptor

HRP: horse radish peroxidase

IgE: Immunoglobulin E

IgG: Immunoglobulin G

N63: asparagin 63

N-linked glycosylation: asparagin-linked glycosylation

NMR: nuclear magnetic resonance

Q63: glutamine 63

INTRODUCTION

Immunoglobulin E (IgE)-associated allergy is the most frequent immunologically-mediated hypersensitivity disease affecting more than 25% of the population worldwide¹. IgE is the least abundant immunoglobulin class and therefore exerts its pathological effects mainly via the interaction with two cellular receptors, the high-affinity receptor FcεRI and the low-affinity receptor CD23 (FcεRII)². The molecular and structural interaction between IgE and FcεRI has been investigated in great detail³ and represents an important target for therapeutic strategies for allergy⁴⁻⁶.

The interaction between IgE and the low-affinity receptor, CD23, is less well understood than that between IgE and the high-affinity receptor FcεRI, although CD23 plays several important roles in allergic inflammation. CD23 is a key molecule in IgE-facilitated allergen presentation and subsequent activation of allergen-specific T cells⁷, a mechanism that can be blocked by allergen-specific IgG after specific immunotherapy⁸. Furthermore, CD23 was suggested to be important in the regulation of IgE production⁹ and there is growing evidence that CD23 mediates transport of allergens through respiratory and gut epithelial barriers^{10,11}.

So far, only parts of the three-dimensional structure (i.e., parts of the head domain) of CD23 have been analysed by crystallography¹² and NMR analysis¹³ and results were not in full agreement. By site-directed mutagenesis of the head domain and subsequent expression of the mutants in cells, evidence for an involvement of the head domain in IgE binding was provided¹⁴. These findings were supported by structural studies which have analysed the interaction between an incomplete recombinant CD23 head domain expressed in *Escherichia coli* and a recombinant Cε3-Cε4-comprising sub-fragment of the Fc part of human IgE¹⁵. One study observed enhanced IgE binding of CD23 variants containing the stalk region as compared to

CD23 variants representing only the head domain¹⁶ and another study observed enhanced IgE binding of a CD23 variant containing a single amino acid exchange in the stalk region¹⁷. Both attributed the enhanced IgE binding to the fact that the stalk region may contribute to IgE binding through accurate formation of CD23 trimers, since the current assumption is that CD23 occurs in trimeric form on the cell surface.

In this study we expressed four monomeric, folded CD23 variants in insect cells, one comprising the full extracellular portion of CD23 including stalk and head domain, one identical with the first apart from a single amino acid exchange in the stalk region abolishing the only N-linked glycosylation site of CD23, a third representing the full head domain and a fourth consisting of a truncated head domain. We were able to directly map hitherto unknown IgE binding sites on the stalk region of CD23 and could show that a non-N-glycosylated monomeric version of CD23 bound IgE much stronger than glycosylated CD23. Furthermore, we demonstrate that omalizumab, a therapeutic antibody that inhibits IgE binding to FcεRI, also inhibits IgE binding to CD23.

METHODS

Construction and recombinant expression of CD23 derivatives in SF9 insect cells. Four constructs of the human low-affinity IgE receptor FcεRII /CD23 (NCBI Reference Sequence: NP_001993.2) were expressed in insect cells based on synthetic genes. CD23A represented the full extracellular CD23 protein starting from aspartic acid 48 (D48) to the end of the protein (i.e., S321). CD23B was identical to CD23A but asparagine 63 (N63) was substituted by a glutamine residue (N63→Q63). CD23C represented only the head part of the protein (i.e., from methionine 150 to serine 321), and CD23D consisted of a small head part starting at serine 156 (S156) and ended at glutamic acid 298 (E298). Further description of the expression of the CD23 derivatives can be found in the online repository.

Purification, biochemical and biophysical characterization of recombinant CD23 constructs. A detailed description can be found in the online repository.

Synthesis of CD23 peptides and generation of CD23 peptide-specific antibodies. See online repository.

Expression and purification of human Bet v 1-specific monoclonal IgE. Expression and purification of the IgE antibody is described in the online repository.

Binding experiments with recombinant CD23 derivatives and statistical analysis. Binding of antibodies specific for CD23 peptides and of a monoclonal anti-

CD23 antibody (BD Biosciences, Isotype M-L233) to the CD23 derivatives was tested by ELISA. A detailed description of the binding experiments can be found in the online repository.

Statistical analysis of the ELISA results was done by calculating student's t-test for independent means with the programme IBM SPSS Statistics 23.0. Coefficients were considered significant at a p-value < 0.05. Significant p-values below 0.05 (two-tailed test) are marked with * in figures. P-values below 0.01 (two-tailed tests) are marked with **.

Binding and inhibition experiments in a cell-based model. See online repository.

Negative stain electron microscopy. A description of the negative stain electron microscopy methods used can be found in the online repository.

CD23 structural calculations and predictions. Relative surface exposures were calculated using the MSMS package¹⁸ in the USCF Chimera package¹⁹ and using Gly-X-Gly tripeptides²⁰ as reference states. Graphical depiction of the CD23 head was rendered with PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.). The structural basis for the calculation and depiction was the PDB model 2H2T¹². The secondary structure prediction was performed using the Psipred server^{21,22}.

RESULTS

Expression and purification of folded and monomeric CD23 derivatives

Four different CD23 constructs spanning different regions of CD23 were expressed in SF9 insect cells (Fig 1). Two constructs (i.e., CD23A, CD23B) represented the complete stalk and head region of CD23 and differed by a single amino acid exchange (i.e., N63 to Q63) which eliminated the only N-linked glycosylation site of CD23 (Fig 1, A and C). The other two constructs represented the head domain of CD23 for which the three-dimensional structures alone (CD23C, CD23D) and in complex with IgE (CD23D) have been reported (Fig 1, A, B and C)^{12,13,15}. CD23D, for which the structure in complex with IgE has been reported, comprises amino acids 156-298 and lacks 6 amino acids at the N-terminal end compared to CD23C, as well as 23 amino acids at the C-terminal end (Fig 1, A, B and C). The four recombinant CD23 derivatives were expressed with a C-terminal hexahistidine tag in baculovirus-infected insect cells as soluble proteins and could be detected with an anti-his-tag antibody in supernatants from infected cells (Fig E1A, Online Repository). The molecular masses of the expressed and purified CD23 derivatives observed in SDS-PAGE and after blotting (Fig 2A; Fig E1A and E1B, Online Repository) corresponded to those determined by mass spectrometry (MALDI) (i.e., CD23A: 33.7 kDa; CD23B: 33.4 kDa; CD23C: 24.7 kDa; CD23D: 16.8 kDa (data not shown)). Only CD23A and CD23B appeared larger (i.e., approximately 37 kDa) in SDS PAGE, which has been reported earlier²³ (Fig E1B, Online Repository). Compared to a glycosylated control protein containing 9 N-linked glycosylation sites (i.e., horseradish peroxidase), the CD23 derivatives showed weak reactivity when stained for carbohydrates with CD23A giving the strongest staining of the four constructs indicating that the mutation of N63 to Q63 has successfully removed the carbohydrates attached to this site. No

carbohydrate staining was observed for a non-glycosylated control protein (i.e., soybean trypsin inhibitor) (lane N, Fig E1C, Online Repository).

All four recombinant CD23 derivatives could be purified (Fig 2A) and, when studied by size exclusion chromatography, were found to occur in a strictly monomeric form (Fig 2B). Circular dichroism (CD) analyses revealed that all four proteins were folded (Fig 2C) and consisted of a mixture of alpha helices (CD23A: 17%, CD23B: 19%, CD23C: 12%, CD23D: 12%), β -sheets (CD23A: 28%, CD23B: 27%, CD23C: 33%, CD23D: 34%), turns (CD23A: 24%, CD23B: 24%, CD23C: 25%, CD23D: 23%) and unordered parts (CD23A, B, D: 31%, CD23C: 30%) (data not shown). We thus were able to assemble a set of four folded and monomeric CD23 derivatives representing different parts of CD23, with and without the N-linked glycosylation site for functional assays.

CD23 derivatives containing the stalk region and in particular the non-glycosylated CD23B variant show superior binding to IgE

In a first set of experiments we investigated the binding of IgE and of IgE-allergen immune complexes to the CD23 derivatives. For this purpose, the CD23 variants were immobilized to ELISA plates and incubated with a monoclonal human IgE antibody specific for the major birch pollen allergen Bet v 1 alone, or in complex with Bet v 1 (Fig 3). When IgE (Fig 3A, left panel) or IgE in complex with Bet v 1 (Fig 3A, right panel) bound to the CD23 derivatives was detected with anti-human IgE antibodies, we observed that the CD23 derivatives containing the stalk region (i.e., CD23A, CD23B) bound IgE stronger than the CD23 derivatives without stalk region (i.e., CD23C, CD23D). Interestingly, the strongest IgE binding was obtained for

CD23B which had the amino acid exchange in the N-linked glycosylation site (Fig 3A). A similar result was obtained when the bound IgE-Bet v 1 complexes were detected with antibodies specific for the allergen. As exemplified for CD23A and CD23C, the CD23 construct containing the stalk region bound the IgE-allergen complexes much better than the construct containing only the head domain (Fig 3B). The latter experiment also confirmed the correct formation of the IgE-allergen complexes and their binding to the CD23 derivatives because the complex was detected by anti-allergen-specific antibodies.

The specificity of IgE binding to the CD23 derivatives was further investigated using a monoclonal anti-CD23 antibody which inhibits IgE binding to CD23. We found that this antibody, but not an isotype-matched control antibody, inhibited IgE binding to CD23A and CD23B (Fig 3C). Next, we investigated if omalizumab, a registered therapeutic anti-human IgE antibody is able to block IgE binding to CD23. Using the four recombinant CD23 constructs, we were indeed able to show that pre-incubation of IgE with omalizumab inhibited IgE binding to CD23 as compared to an isotype-matched control antibody (Fig 3D).

Mapping of direct IgE binding sites in the CD23 stalk region with peptide-specific antibodies

In order to investigate the involvement of different portions of CD23 in IgE binding we produced a set of synthetic CD23 peptides (Repository Table E1) which are derived from different regions in the CD23 molecule. The peptides in the CD23 sequence (Fig 1A) were located in the trans-membrane region (i.e., peptide 2), in the stalk region (i.e., peptides 1 and 4), comprised several portions of the head domain (i.e., peptides

5, 6 and 7) and included the C-terminus of CD23 (i.e., peptide 3). Three shorter versions of peptide 6 (peptides P6a, P6b, P6c; Repository Table E1) were synthesized for direct binding experiments with anti-CD23 antibodies. Peptides (peptides 1-6) were then used to immunize rabbits to generate anti-sera that should be capable of targeting the corresponding sequences in the intact, folded CD23 molecule. In a first set of experiments the resultant antibodies were used as probes to study surface exposure of certain portions of CD23. When we tested the anti-peptide antibodies for reactivity with the folded CD23 derivatives, we found that antibodies raised against peptide 2, primarily including the trans-membrane domain, did not bind to the four constructs because the peptide was either not present (i.e., CD23C and CD23D) or was only partially represented (i.e., CD23A and CD23B) in the CD23 derivatives (Fig E2 A-D, Online Repository). Antibodies raised against peptides 1 and 4 reacted specifically with the constructs containing the stalk region (i.e., CD23A, CD23B) but not with the derivatives lacking the stalk region (i.e., CD23C, D) (Fig E2 A-D, Online Repository). Interestingly, only antibodies raised against peptides 6 and 7 from the head domain, but not peptide 5-specific antibodies, reacted with the CD23 constructs. Since peptide 5-specific antibodies reacted with the isolated peptide 5, lack of reactivity is not due to the failure of obtaining peptide-specific antibodies (data not shown). Anti-peptide 3 antibodies reacted specifically with each of the constructs containing the CD23 C-terminal end (i.e., CD23A, B, C) but not with the construct lacking the C-terminal end (CD23D) (Fig E2 A-D, Online Repository). All constructs were recognized by the monoclonal anti-CD23 antibody which inhibited IgE binding to CD23 (positive control) (Fig E2 A-D, Online Repository).

Next, we investigated if the monoclonal anti-CD23 antibody that inhibited IgE binding to CD23 reacts with the synthetic CD23 derived peptides (Repository Table E1) and

found that this antibody only reacted with folded CD23 but not with any of the peptides (Fig 4A). We then tested whether the peptide-specific antibodies inhibit binding of the monoclonal anti-CD23 antibody. We observed a weak inhibition with anti-peptide 6 antibodies as well as with a mix of all anti-peptide antisera or other combinations containing anti-peptide-6 (i.e. anti-peptide 1,4 and 6 antibodies or anti-peptide 1 and 6 antibodies in combination) (Fig 4B). These experiments suggest that the anti-CD23 monoclonal antibody recognizes an epitope close to the peptide 6 amino acid sequence. Furthermore, it likely binds with higher affinity to CD23 than the peptide-specific antibodies, since the inhibition by anti-peptide 6 antibodies was weak (i.e., 18.5% inhibition of binding, data not shown).

We then used the peptide-specific antibodies to block the binding of IgE to CD23 (Fig 4, C and D). We found that the antibodies against peptides 1 and 4 from the stalk region inhibited IgE binding to the CD23A and CD23B variant (Fig 4, C and D). Inhibitions of IgE binding by antibodies specific for peptide 1 were 29% for CD23A and 39% for CD23B. Peptide 4 antibodies were able to inhibit IgE binding to CD23A by 20% and for CD23B, 31% (data not shown). Likewise, antibodies specific for peptide 6 from the head domain partially inhibited IgE binding to both CD23 variants (i.e., CD23A: 32%, CD23B: 44%, data not shown) whereas the other peptide antibodies had no effect on IgE binding. Inhibition of IgE binding to CD23A and CD23B was also observed with combinations of anti-peptide 1, 4 and 6 (CD23A: 25%, CD23B: 55%, data not shown), and was even more pronounced when a combination of anti-peptide 1 and 6 antibodies was used (CD23A: 43%, CD23B: 58%, data not shown) (Fig 4, C and D). These experiments clearly show that the binding of IgE to monomeric CD23 can be inhibited with antibodies directed against two distinct peptides in the CD23 stalk region, which demonstrate a direct involvement of the stalk region in IgE binding. Our experiments also confirm that

amino acids in the peptide 6-defined region on the head domain are involved in IgE binding (Fig 1A). Interestingly, anti-peptide 7 antibodies did not inhibit IgE binding to CD23 although this peptide contained amino acids which were reported to be directly involved in IgE binding (Fig 1A)^{13,15}. The lack of inhibition of IgE binding by anti-peptide 5 antibodies can be explained by the fact that these antibodies did not react with folded CD23.

Results obtained for the protein interactions could be confirmed in cellular experiments. Antibodies specific for peptides 4 and 6 inhibited IgE binding to B cells expressing CD23 (Fig 4E). A weak inhibition was also obtained with anti-peptide 1 antibodies, but not with anti-peptide 7 antibodies (Fig 4E).

Visualization of the tri-molecular complex consisting of CD23A, IgE and allergen by negative stain electron microscopy

Negative stain electron microscopy allows to directly visualize the interaction of molecules^{24, 25}. It has been used successfully to visualize human IgE alone and in complex with FcεRI and an anti-human IgE antibody^{26, 27}. We used CD23A together with a human monoclonal IgE antibody specific for the major birch pollen allergen, Bet v 1 and the Bet v 1 allergen to visualize complexes of the three molecules (Fig 5). The challenge regarding the visualization of the interaction of CD23 with IgE and the Bet v 1 allergen is that CD23 (33.7 kDa) and Bet v 1 (17 kDa) are very small molecules compared to IgE (190 kDa). We therefore used an anti-His Fab which has a size and shape (i.e., donut-like shape) distinct from IgE to investigate the tri-molecular interaction. In Figure 5A, monoclonal, Bet v 1 specific IgE alone is shown. The three domains (Fc arm and 2 Fab arms) of the molecules can clearly be seen,

however, differentiation of Fc from Fab is not possible. Figure 5B shows the donut-shaped anti-His Fab fragment by itself. In Figure 5C we added the anti-His Fab fragment to the IgE preparation. The Fab fragment is not able to bind to the IgE and is therefore separate from IgE. However, when CD23A was added to the preparation, complexes consisting of one IgE molecule and one CD23 molecule, indicated by the anti-His-Fab, became visible (Fig 5D). These complexes were a minority in the preparation, however, they were only seen when CD23 was added compared to controls. A possible explanation for the relatively small number of complexes observed in the mixes could be weak affinity of the IgE-CD23 complex. We then additionally added Bet v 1 allergen to the anti-His-Fab-CD23-IgE complex and observed an increase of the size of the previously observed complexes (Fig 5E). Interpretation of the samples was challenging, since several possible complexes exist in one sample. In addition to the anti-His Fab-CD23-IgE-Bet v 1 complex, IgE-Bet v 1, IgE-CD23-Bet v 1, anti-His Fab-CD23-IgE, anti-His Fab-CD23, CD23-IgE complexes are also possible as well as each of the added molecules by themselves. However, again, even though they were a minority, large anti-His Fab-CD23-IgE-Bet v 1 complexes could be observed repeatedly and only in CD23-containing preparations as compared to controls. Their sizes and shapes are best explained by a 1:1 interaction between CD23 and IgE indicating that CD23 interacts as a monomer with IgE in this experimental set-up.

DISCUSSION

The IgE-CD23 interaction has regained considerable attention because it contributes to allergic inflammation through IgE-facilitated allergen presentation to T cells and enhancement of trans-epithelial allergen migration^{7, 10, 11}. Our analysis of the interaction between IgE and CD23 has revealed several novel findings which may change current concepts of the interaction of these molecules and in addition may be of relevance for therapeutic approaches for IgE-associated allergy. We expressed and purified a set of folded and monomeric CD23 protein derivatives differing in size and N-linked glycosylation to conduct a series of functional assays investigating the binding of the tri-molecular complex between CD23, IgE and a corresponding allergen. Direct IgE binding experiments showed that monomeric CD23 containing the stalk region exhibited superior IgE binding compared to CD23 derivatives without stalk region which indicated a direct involvement of the stalk region in IgE binding. This finding was unexpected because so far it has been thought that the CD23 stalk region is important for the trimerization of CD23 and that this trimeric state indirectly enhances IgE binding to CD23^{16, 28}. However, our data demonstrate that CD23 derivatives containing the stalk region are monomeric and can bind IgE. According to preliminary plasmon surface resonance (Biacore) experiments the affinity of the monomeric protein to IgE was similar to that described in earlier studies¹³. Further support for a 1:1 binding of monomeric CD23 to IgE and IgE-allergen complexes came from negative stain electron microscopy experiments.

Another interesting observation was that a single amino acid exchange in the only N-linked glycosylation site of CD23 strongly enhanced IgE binding. In fact, it has been reported that single nucleotide polymorphisms in this region were related to the development of asthma and it has been observed that cells expressing CD23

variants containing an amino acid exchange close to but not within the N-linked glycosylation site (i.e., R62-W62) showed better IgE binding¹⁷. The authors who made this observation considered that this exchange might have affected the trimerization of CD23 on the cell surface and thus might have caused increased IgE binding. However, our experiments demonstrate that the exchange directly enhanced the binding of monomeric CD23 to IgE. The enhancement of IgE binding by the mutation in the glycosylation site may be due to the removal of sugar residues hindering IgE binding. Alternatively it is possible that mutations of certain critical amino acids may improve the affinity of CD23 for IgE. It is thus quite possible that the binding of IgE to CD23 may be regulated in cells through point mutations through varying glycosylation and/or altered affinity. The reported nucleotide polymorphisms in the DNA encoding the stalk region may hence directly affect the affinity for IgE binding and thus result in different susceptibilities to asthma. According to this model, atopic patients expressing less glycosylated CD23 may bind IgE better via CD23 and, consequently, exhibit more severe forms of allergic inflammation since CD23 is important for IgE-facilitated allergen presentation leading to activation of allergen-specific T cell and cytokine responses.

We therefore tested our hypothesis of a critical and direct stalk involvement of CD23 in binding its ligand IgE by inhibition experiments. Using peptide-specific antibodies, binding sites previously identified by structural studies could be confirmed. For example, site-directed mutagenesis in the peptide 6 region in the head domain of CD23 was able to affect IgE binding to the receptor molecule¹⁴. However, we did not observe a blocking effect by anti-peptide 7 antibodies, even though certain amino acids contained in peptide 7 were described to be directly involved in IgE binding^{13, 15}. Peptide 5 specific antibodies did not bind to the CD23 constructs. Since these antibodies reacted with the isolated peptide 5, lack of reactivity is not due to the

failure of obtaining peptide-specific antibodies but could be accounted to a lack of surface exposure of the amino acids contained in peptide 5 on the folded CD23 molecules. In fact, according to the three-dimensional structure determined for the head domain¹² several amino acids included in peptide 5 are not exposed on the surface of the CD23 molecule. Unlike in peptides 6 and 7, no exposed segment comprising several contiguous amino acids can be found in peptide 5 (Fig 1A). We also noted that certain amino acids throughout the CD23 head domain (e.g., W184, L198, I221, G222, A279), which have been described as contact residues for the IgE interaction^{13, 15} were not found to be surface exposed in the three-dimensional structure determined for the head domain¹².

Importantly, antibodies against peptides 1 and 4 which are both located in the stalk region strongly inhibited IgE binding to CD23 in protein interaction assays and using cultured cells expressing CD23. Since the CD23 molecules used in the molecular inhibition assays represented monomeric proteins, an indirect involvement of the stalk region by formation of CD23 oligomers with enhanced IgE binding can be excluded. These experiments thus unambiguously mapped IgE binding sites on the CD23 stalk region.

Taken together, our experimental data with blocking antibodies identified antibodies against two distinct regions in the stalk domain of CD23 which can shield CD23 from IgE binding. It is however known that cleavage of the head domain from CD23-expressing cells abolishes IgE binding²⁹ and one must therefore assume that the isolated stalk alone does not bind IgE but rather cooperates with the head in IgE binding. Nevertheless, our observations point to a direct and critical involvement of the CD23 stalk domain in IgE binding and provide a novel view regarding the molecular mode of binding of IgE to CD23.

Using the molecular model of the CD23-IgE interaction we made another observation of high clinical relevance. We found that a therapeutic anti-human IgE antibody, omalizumab, was also able to inhibit the binding of IgE to CD23. In fact, it has been hypothesized previously that omalizumab which inhibits binding of IgE to FcεRI may also affect IgE binding to CD23 and thus may offer additional benefit to patients through an effect that goes beyond the inhibition of allergen-induced mast cell and basophil degranulation such as IgE-facilitated allergen presentation^{6, 7, 30}. Recently, the three-dimensional structure of the omalizumab Fab has been determined by x-ray crystallography³¹. Based on *in silico* docking experiments it has been proposed that omalizumab may indeed inhibit IgE binding to CD23³², but so far no definitive experiments have been performed to investigate this possibility. Our experiments now unequivocally demonstrate for the first time in a defined molecular experimental set-up that the therapeutic anti-human IgE antibody omalizumab inhibits IgE binding to CD23 and thus has the potential to inhibit also CD23-mediated allergic inflammation.

In summary our studies provide a new molecular model for the CD23-IgE-allergen interaction and conceivably may open new doors for the treatment of IgE-associated allergy based on the interference of this interaction.

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FIGURE LEGENDS

FIG 1. Overview of CD23 constructs. **A**, Amino acid sequence of CD23. Indicated are constructs CD23A, B, C and D, peptides (P1-P7) used to raise specific antisera (brown bars), N-linked glycosylation sites (red letters), secondary structure and surface exposure (blue bars) of amino acids as derived from the crystal structure PDB:2H2T (Wurzburg *et al.* 2006¹²). A region where no data is available (R253-E257) is indicated by a grey bar. Amino acids involved in IgE binding according to Hibbert *et al.* and Dhaliwal *et al.* are printed in green and blue, respectively^{13, 15}. Residues involved in IgE binding according to both publications are printed in orange. Cysteine residues involved in disulfide bond formation are numbered. Domains of CD23 are shaded (intracellular domain: red, trans-membrane domain: yellow, stalk domain: green, head domain: purple) **B**, Schematic representation of the known (Wurzburg *et al.* 2006¹²) and predicted three-dimensional structure of CD23. CD23 peptides are indicated. Data for region R253-E257, as well as for C-terminal end is not available. **C**, Sketch of the recombinant CD23 constructs (CD23A, B, C, D) as cloned into plasmid pTM1 with a C-terminal hexa-histidine tag (6xHis) and an N-terminal melittin-signal sequence. Amino acids at the N- and C-terminal ends of CD23 and one mutated in CD23B are indicated in black.

FIG 2. Biochemical, biophysical and immunological characterization of recombinant CD23 constructs. **A**, Coomassie-stained gel containing aliquots of purified CD23 constructs. **B**, Gel filtration profile of the four CD23 constructs. Adsorptions at 210 nm and 280 nm (y-axes: milliabsorbance units, mAU) recorded during elution (x-axes:

elution volumes in ml and corresponding protein size ranges in kDa) are shown. **C**, Circular dichroism analysis of the four CD23 constructs. An overlay of the four CD spectra of constructs CD23 A-D is shown. CD23A: red, CD23B: pink, CD23C: blue, CD23D: yellow. X-axis: wavelength [nm], y-axis: $[\theta]_{mr}$ (mean-residue molar ellipticity).

FIG 3. Binding of IgE to CD23. **A**, Varying binding of IgE to the CD23 constructs. ELISA plates were coated with recombinant CD23 constructs A-D or a control protein and incubated with either IgE alone (IgE) or IgE-Bet v 1 complexes (IgE+Bet v 1). Absorbance values (A 405 nm) corresponding to bound IgE are indicated on the y-axis. **B**, Binding of the IgE-Bet v 1 complexes to CD23A and CD23C. For control purposes, IgE was omitted (Bet v 1 only) or rabbit antibodies without specificity for Bet v 1 (Control Ab) were used. Absorbance values (A 405 nm) corresponding to bound Bet v 1 detected with rabbit anti-Bet v 1 antibodies are indicated on the y-axis. Error bars show standard deviations of triplicates.

Binding of monoclonal IgE to recombinant CD23 constructs can be blocked with **C**, α -CD23 or **D**, omalizumab. **C**, ELISA plate bound CD23A and B were pre-incubated with α -CD23 (black bars) or with an isotype control (white bars) and incubated with IgE. In **D**, IgE was pre-incubated with omalizumab (black bars) or with an isotype control (white bars) before incubation with CD23 constructs (x-axis: CD23A, B, C, D). Absorbance values (A 405 nm) corresponding to bound IgE are indicated on the y-axis. Error bars show standard deviations of triplicates.

FIG 4. Blocking ability of CD23 peptide-specific antibodies. **A**, Ability of monoclonal anti-CD23 to bind to different anti-CD23 peptides. CD23A was used as positive

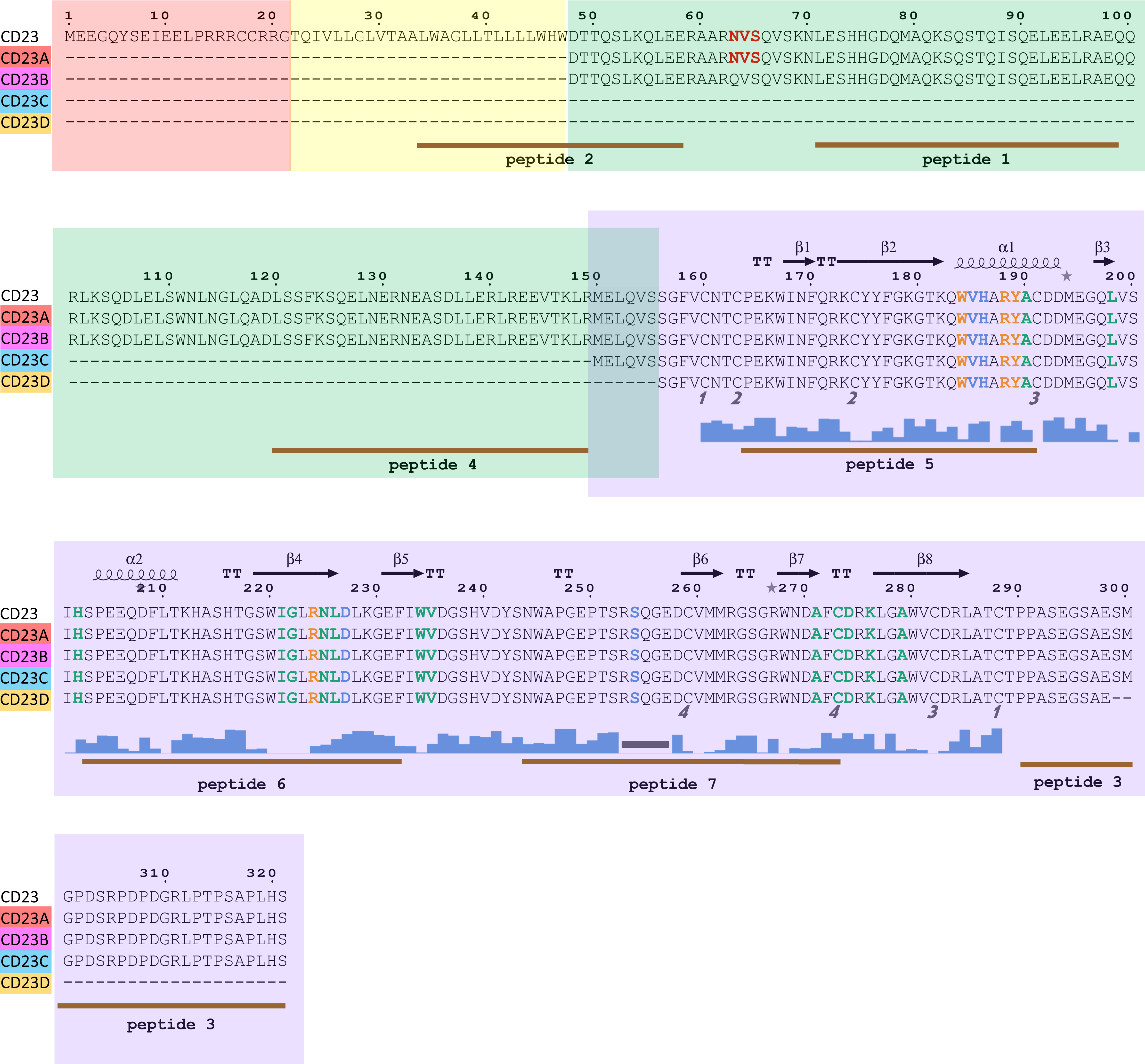
control. **B**, Influence of anti-CD23 peptide antibodies on the binding of monoclonal anti-CD23. CD23A was pre-incubated with anti-peptide antibodies or the corresponding pre-immune sera (x-axis) and then incubated with the anti-CD23 antibody. Absorbance values (A 405 nm: y-axes) correspond to bound anti-CD23 antibody. Error bars show standard error of at least triplicates. The experiments were repeated 3 times with similar results. Influence of anti-CD23 peptide antibodies on the binding of IgE to **C**, CD23A and **D**, CD23B. CD23 constructs were pre-incubated with anti-peptide antibodies or the corresponding pre-immune sera (x-axes) and then incubated with IgE. Absorbance values (A 405 nm: y-axes) correspond to bound IgE. Error bars show standard deviations of triplicates.

E, Influence of antibodies on IgE binding to CD23 expressing B cells. Shown is the percentage blocking of IgE binding (y-axis) to CD23-expressing B cells by a monoclonal anti-CD23 antibody and by antibodies specific for CD23 peptides (x-axis) as determined by flow cytometry. Error bars show standard deviations of triplicates. The experiment was performed twice with similar results.

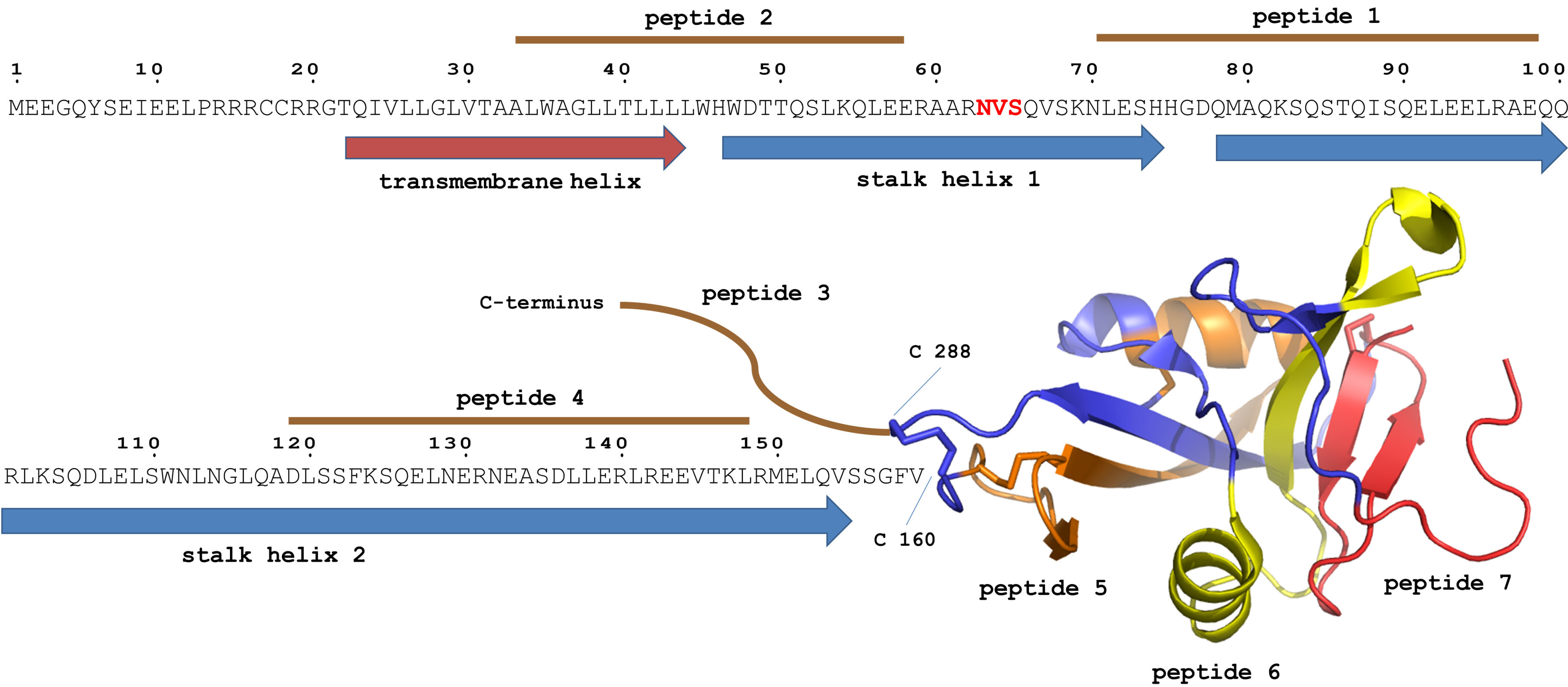
FIG 5. Negative stain electron microscopy of recombinant CD23 in complex with IgE and allergen. Shown are three representative negative stain EM images and interpretive sketches of **A**, IgE alone, **B**, an anti-His IgG Fab fragment, **C**, a mix of IgE plus anti-His IgG Fab fragment, **D**, IgE-CD23 complexes marked with an anti-His IgG Fab fragment and **E**, IgE-CD23-allergen complexes marked with an anti-His IgG Fab fragment. The black bars represent 25 nm.

FIG 1.

A



B



C

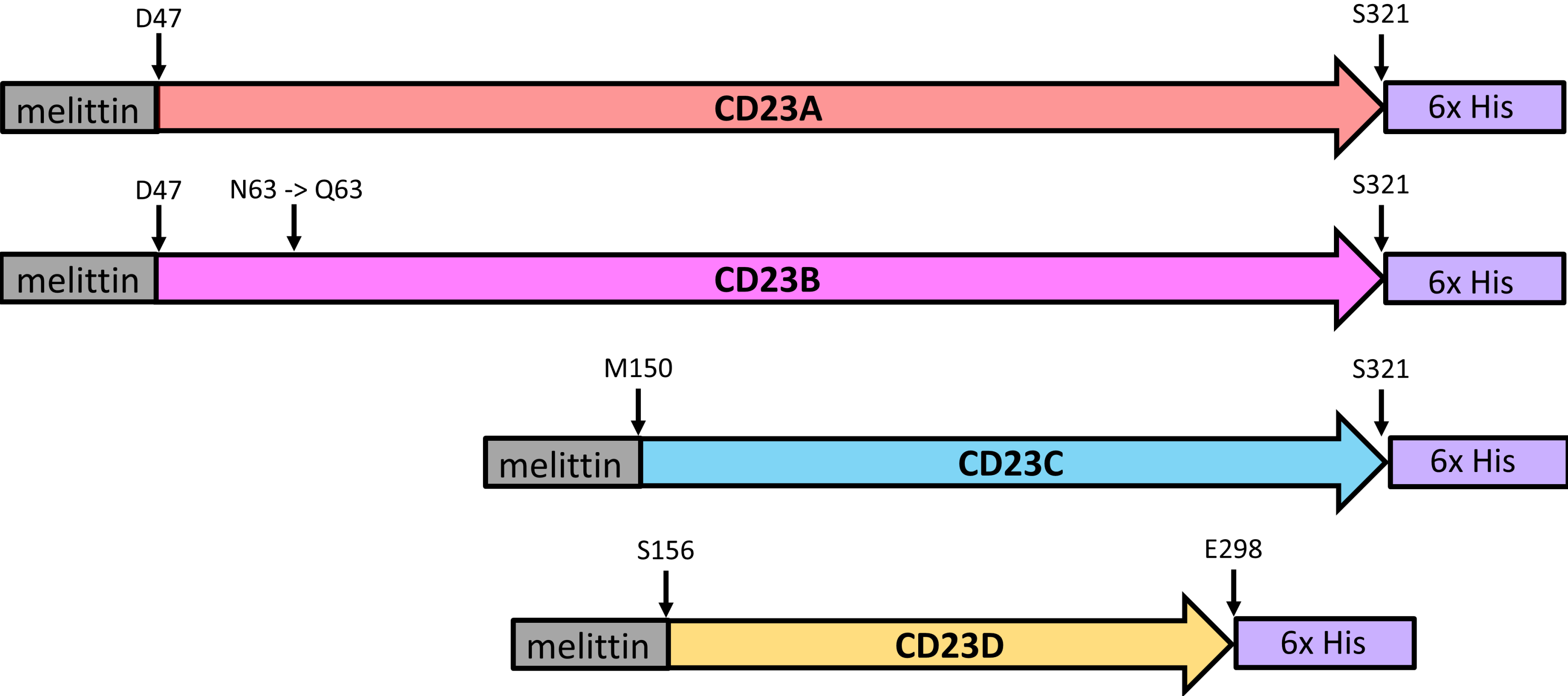
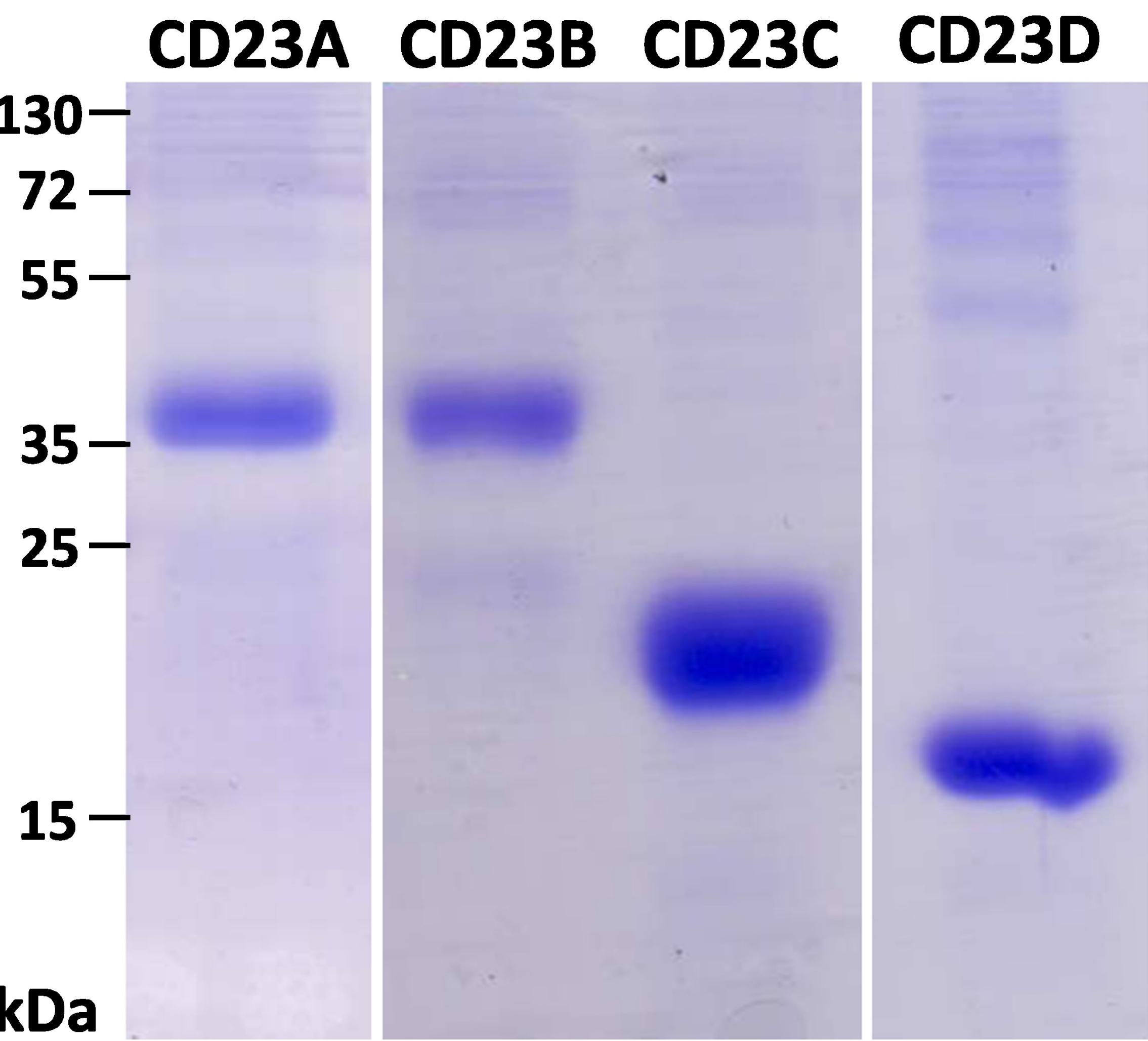
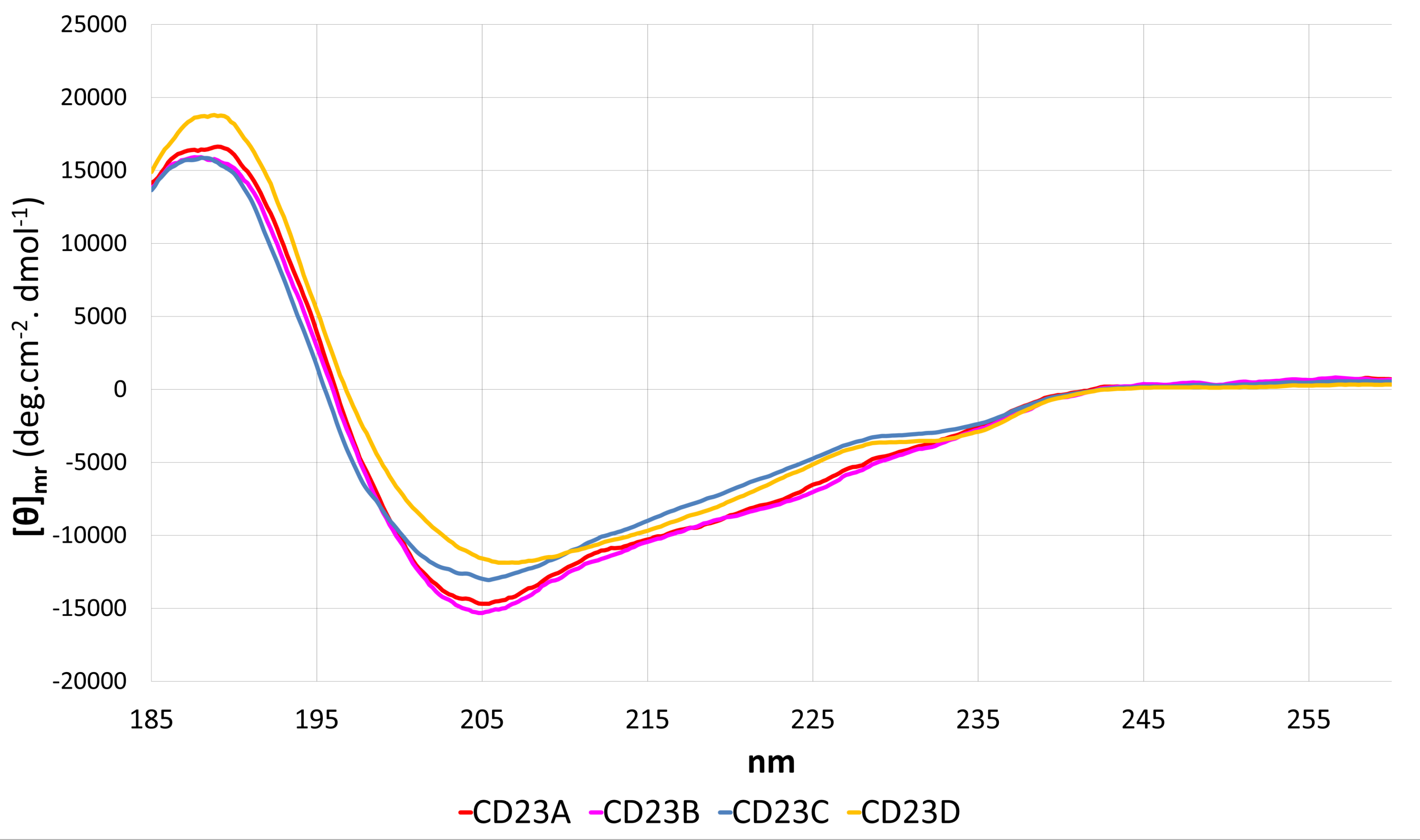


FIG 2.

A



C



B

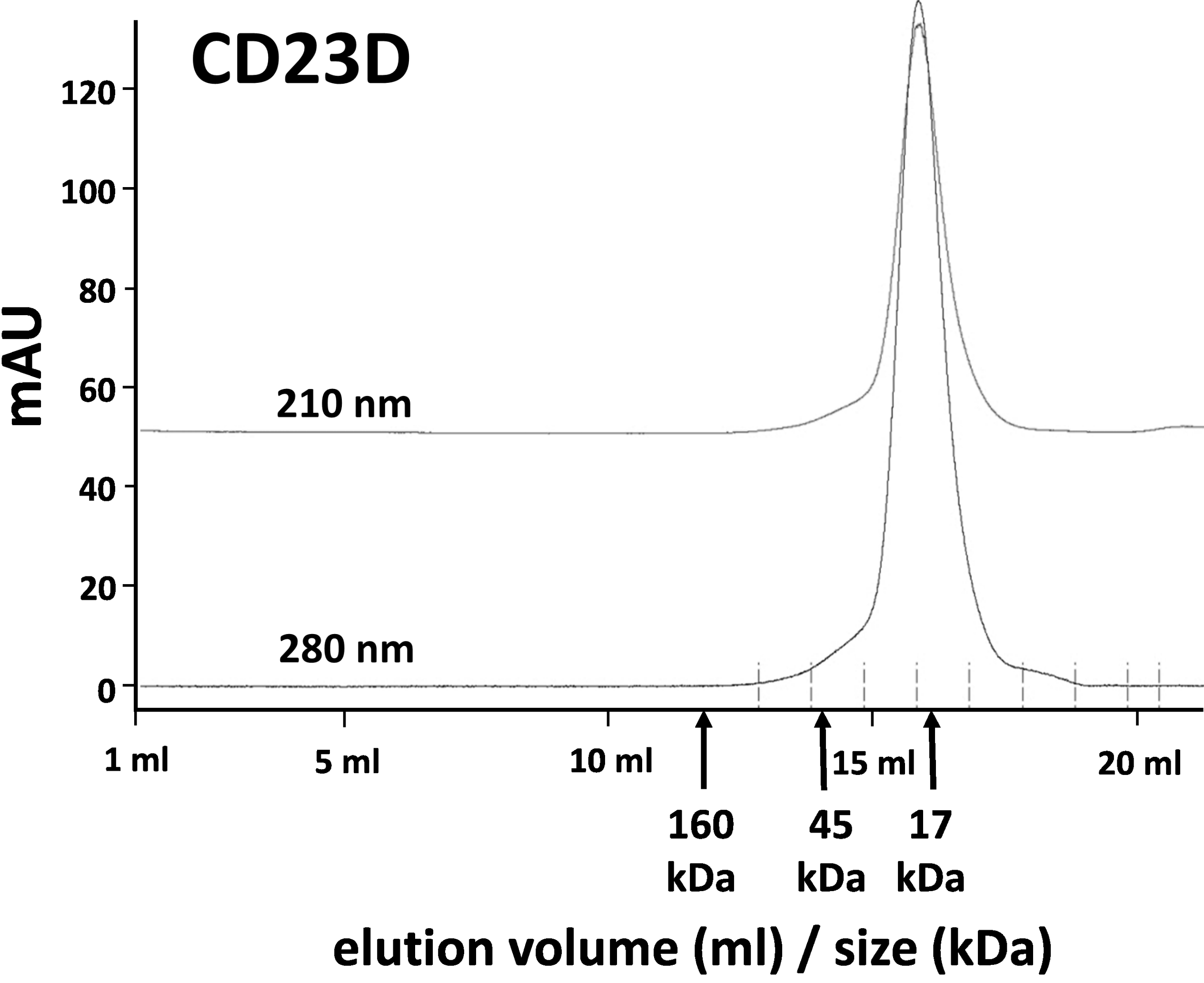
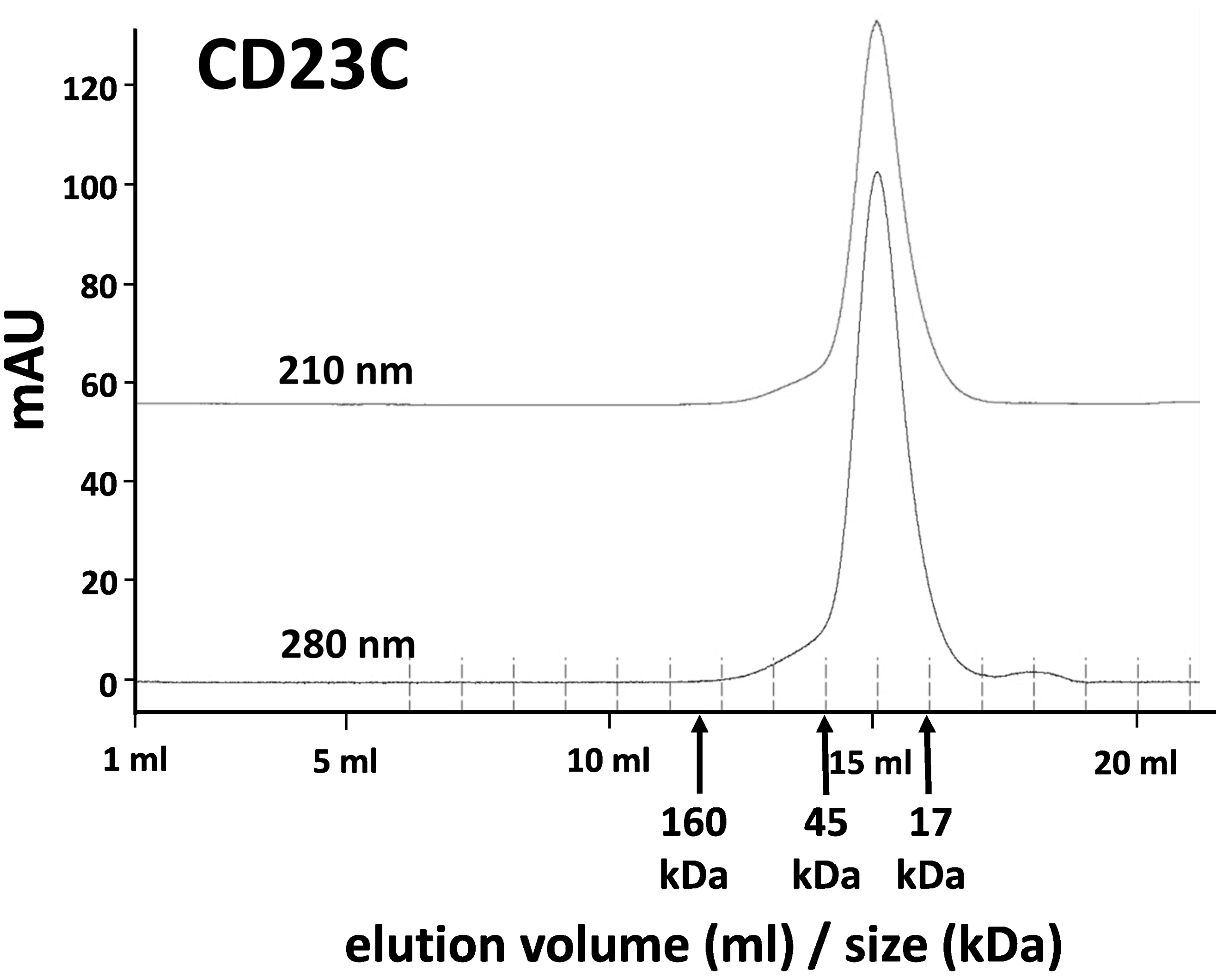
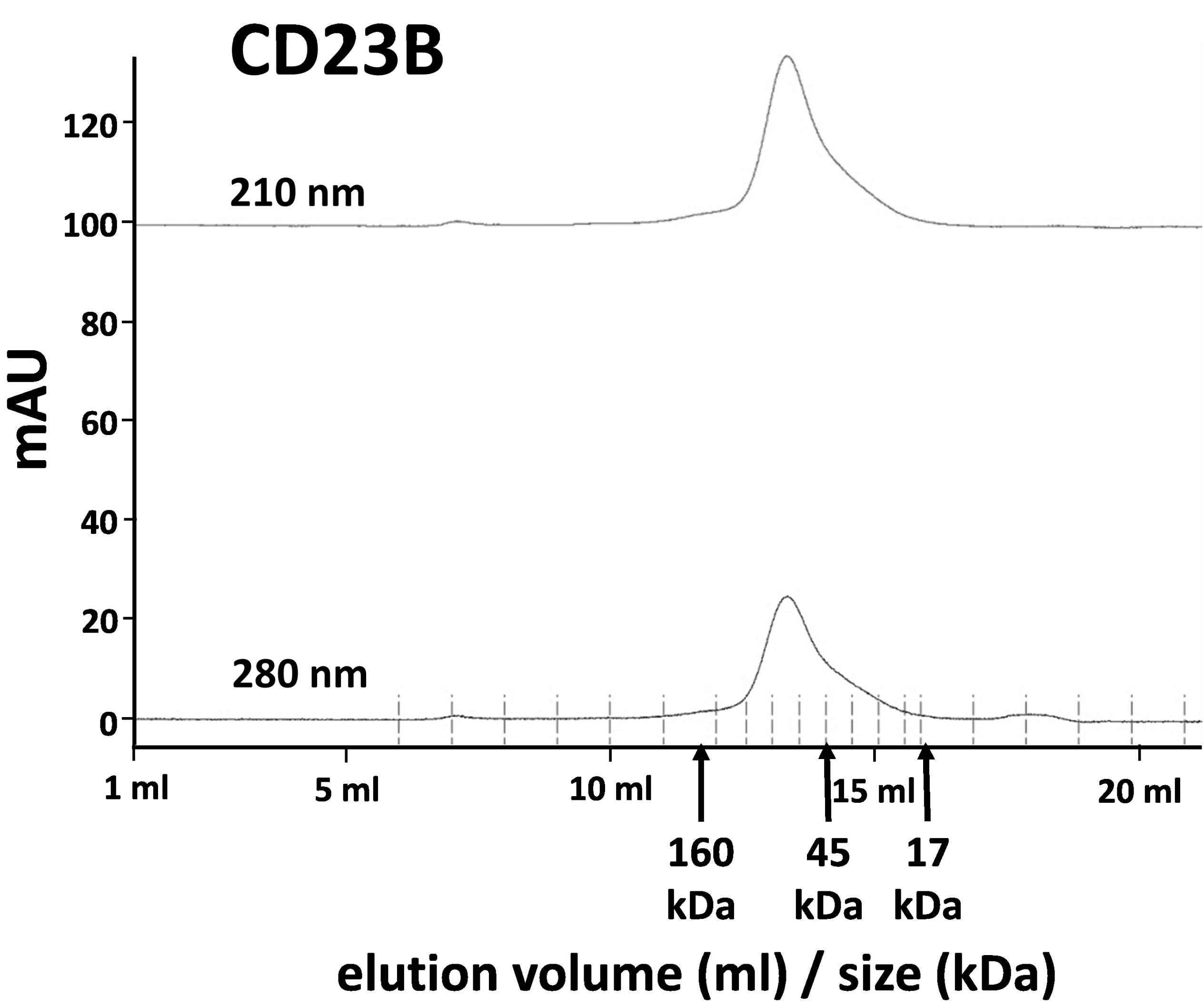
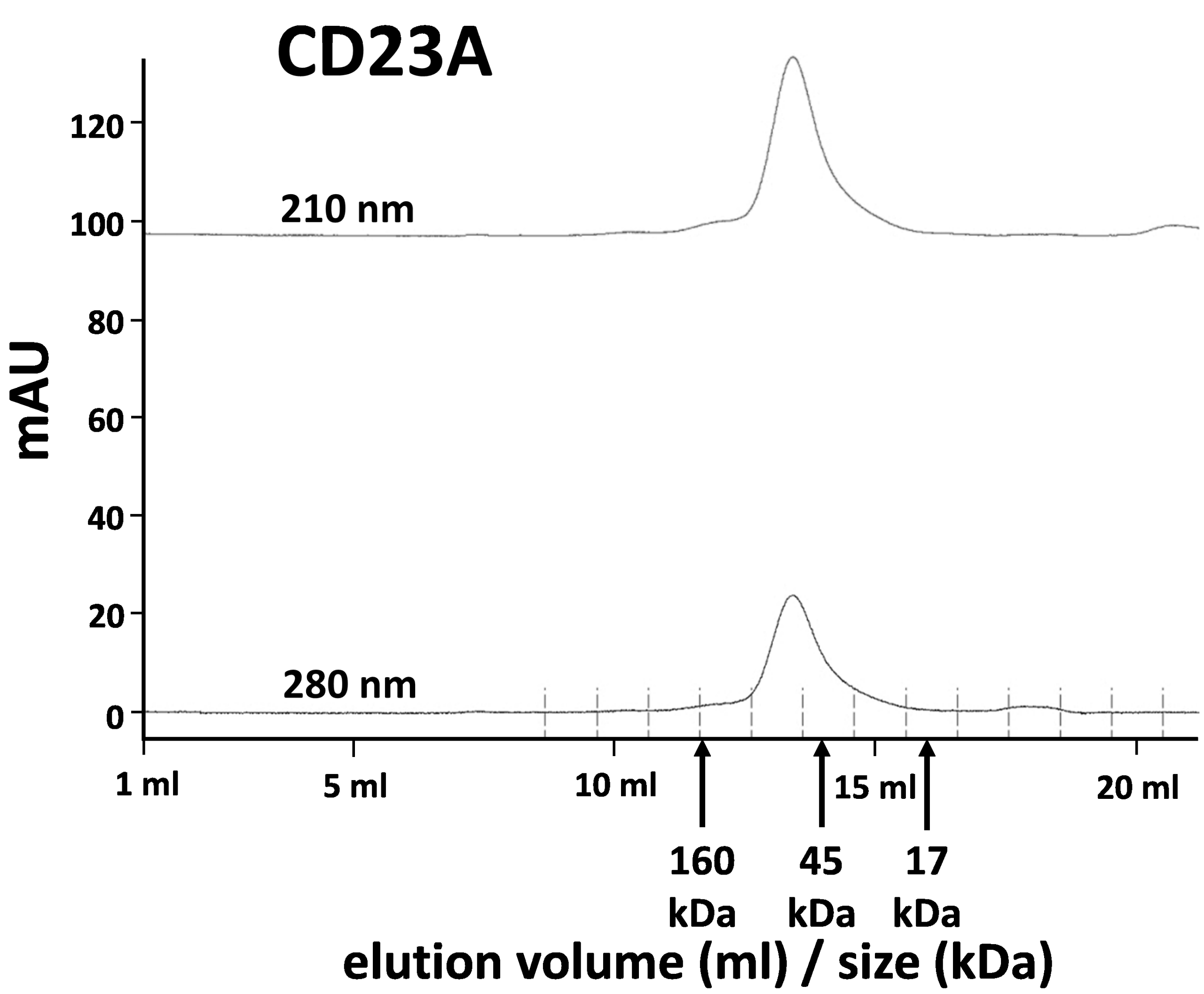


FIG 3.

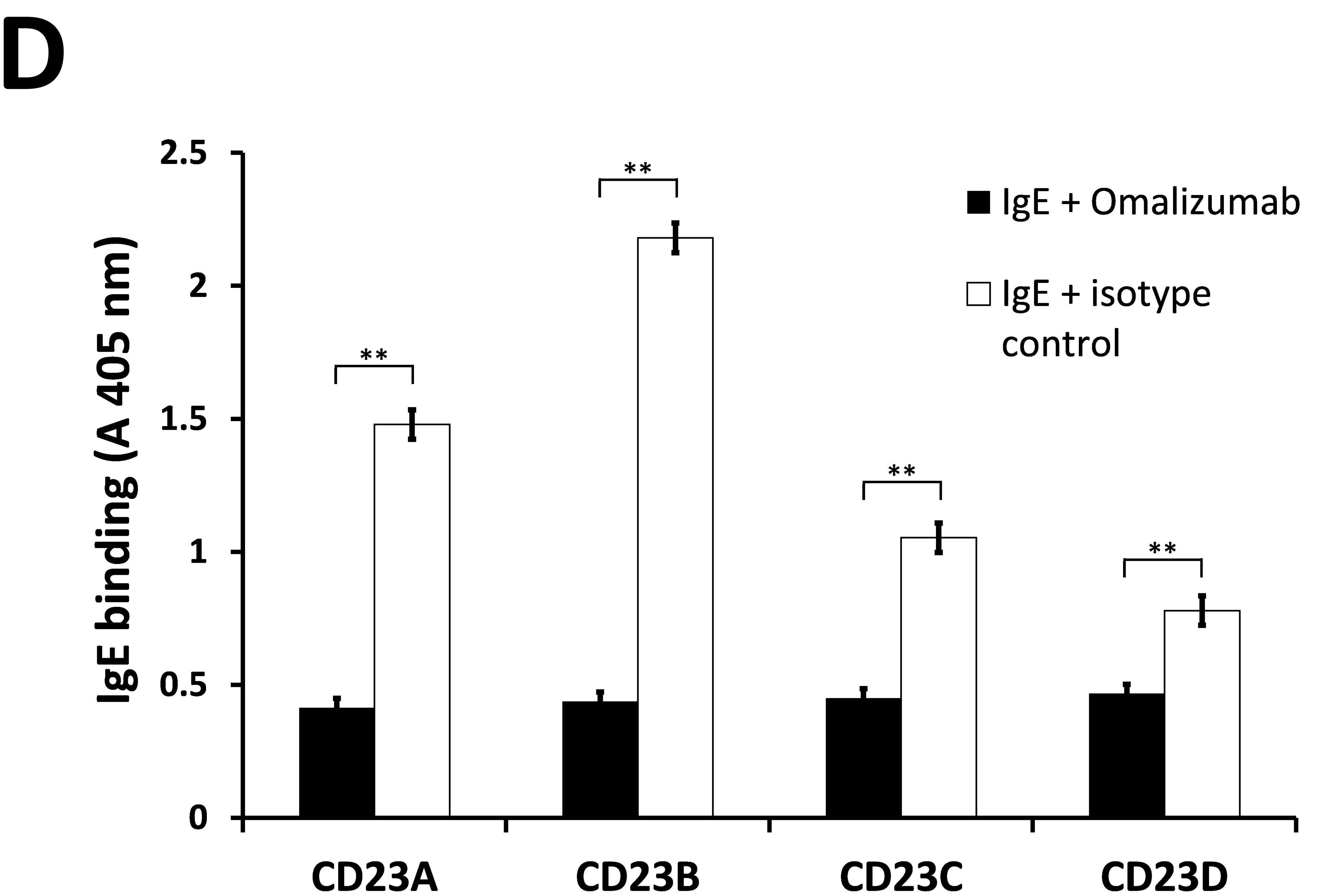
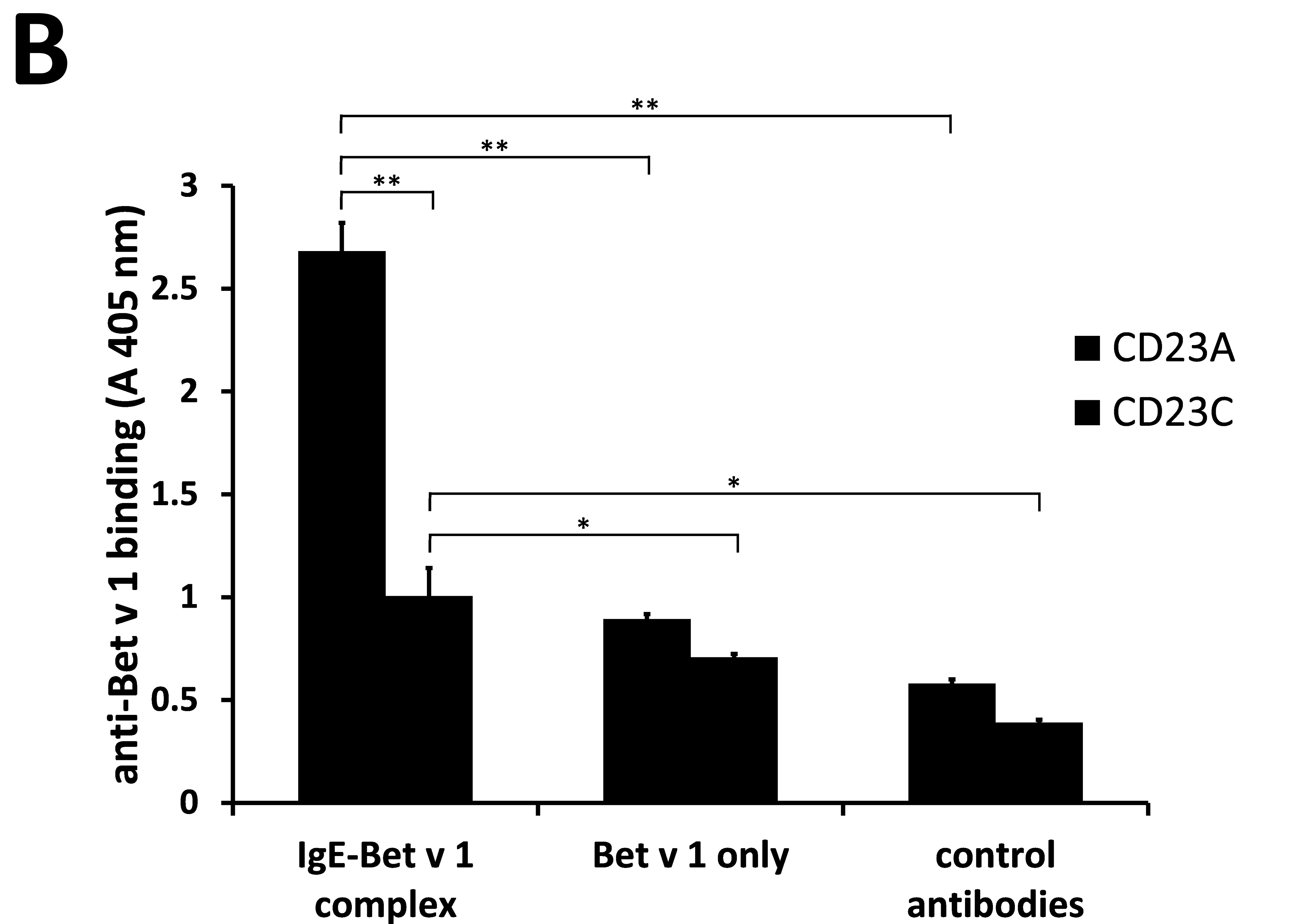
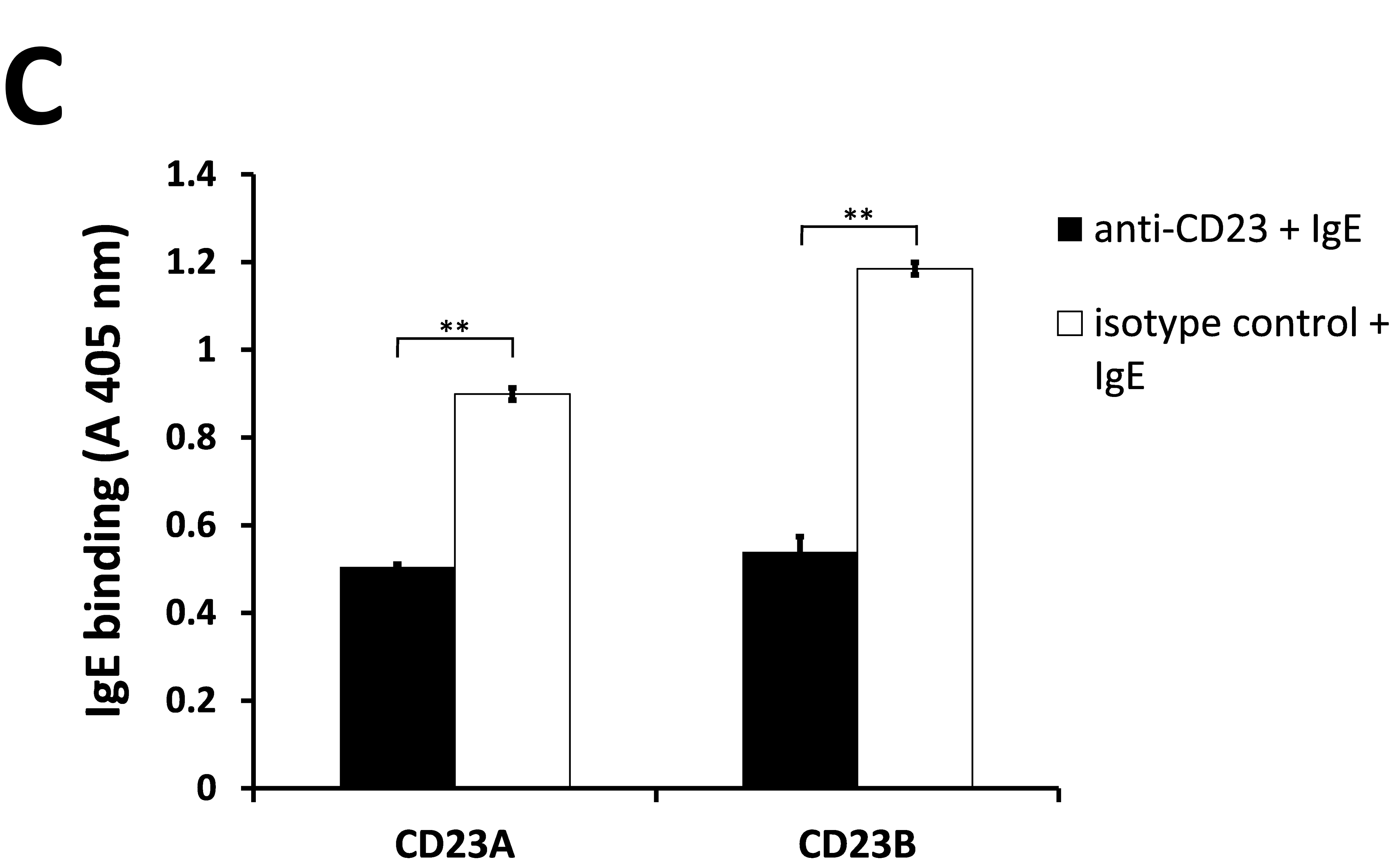
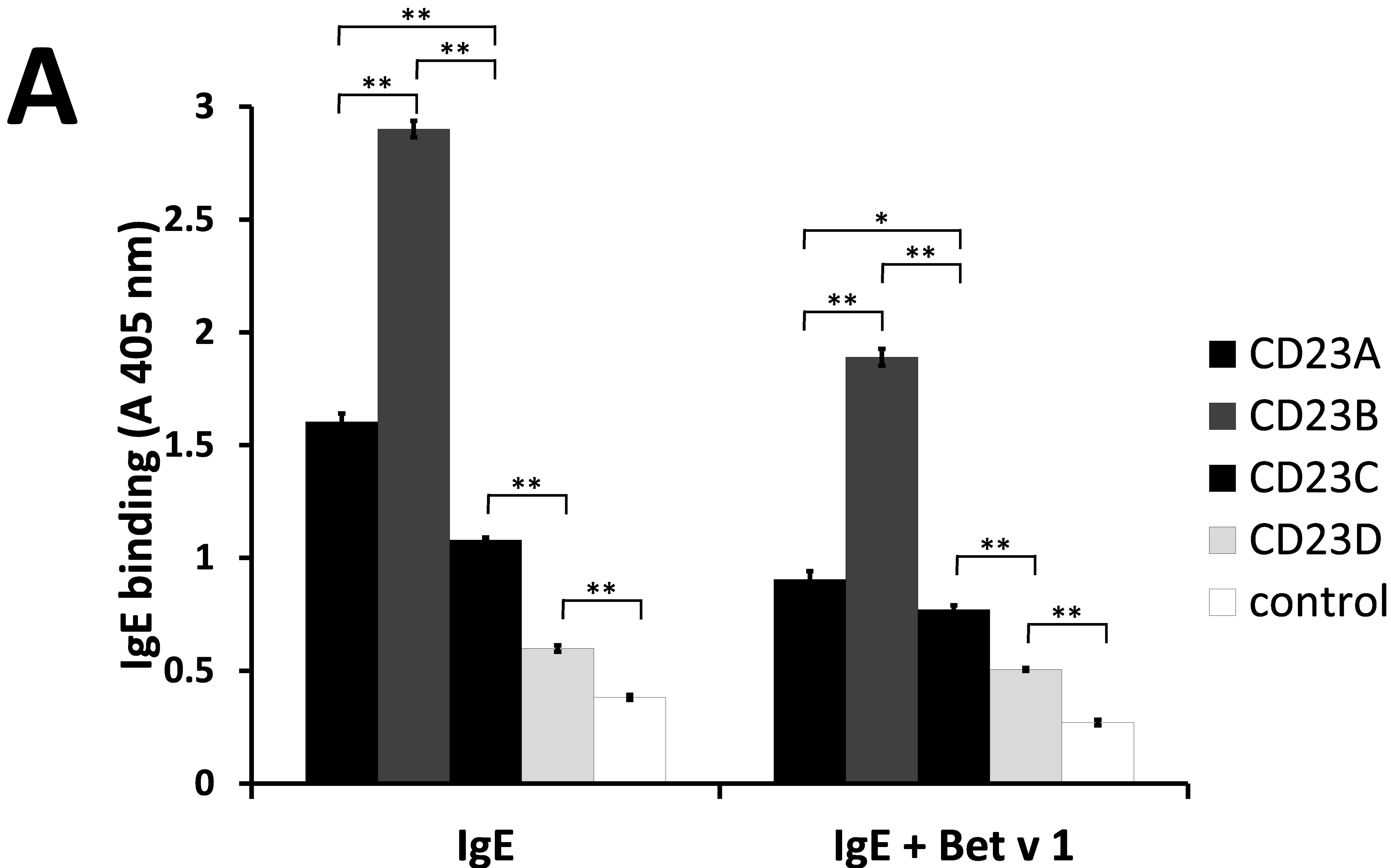
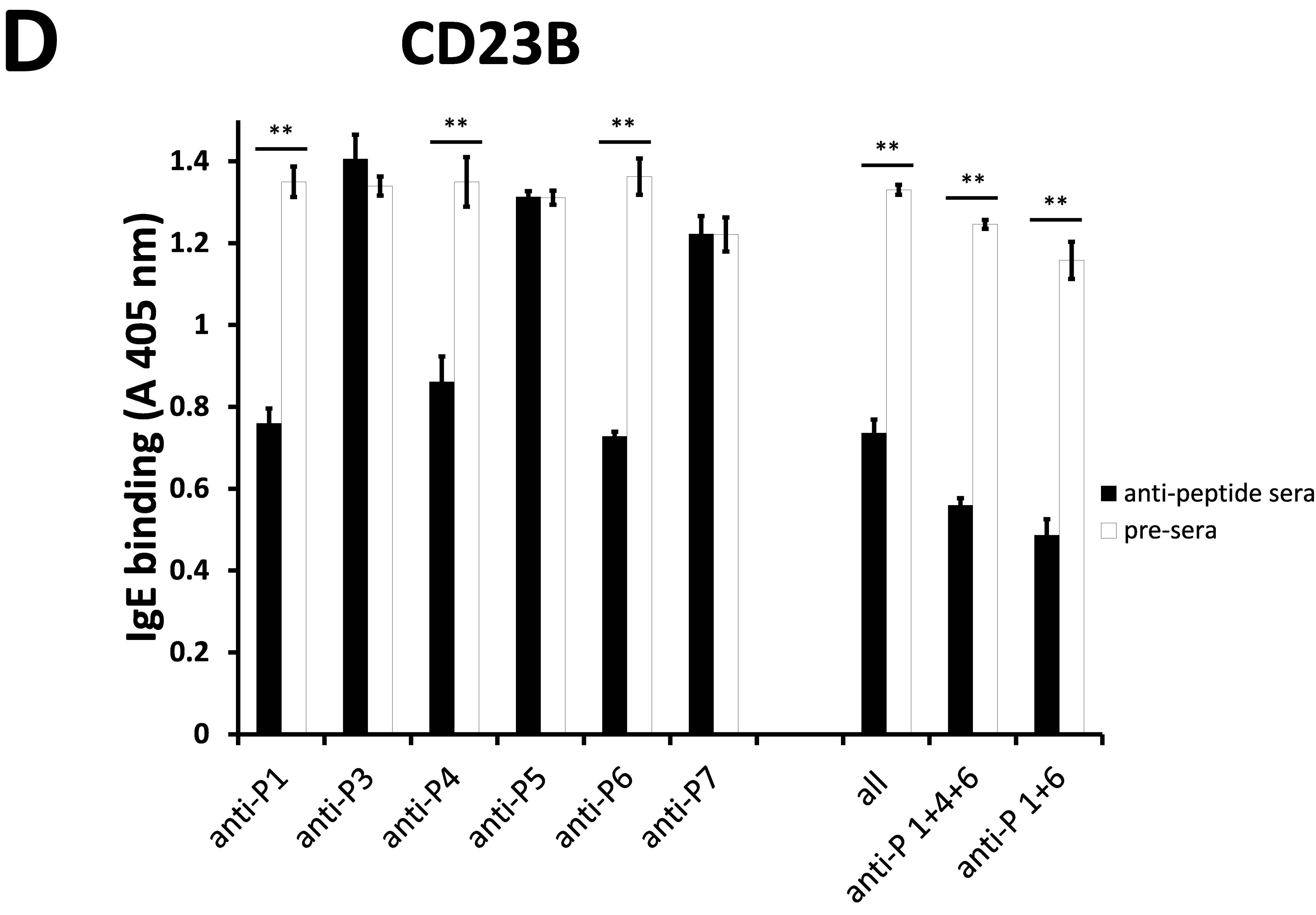
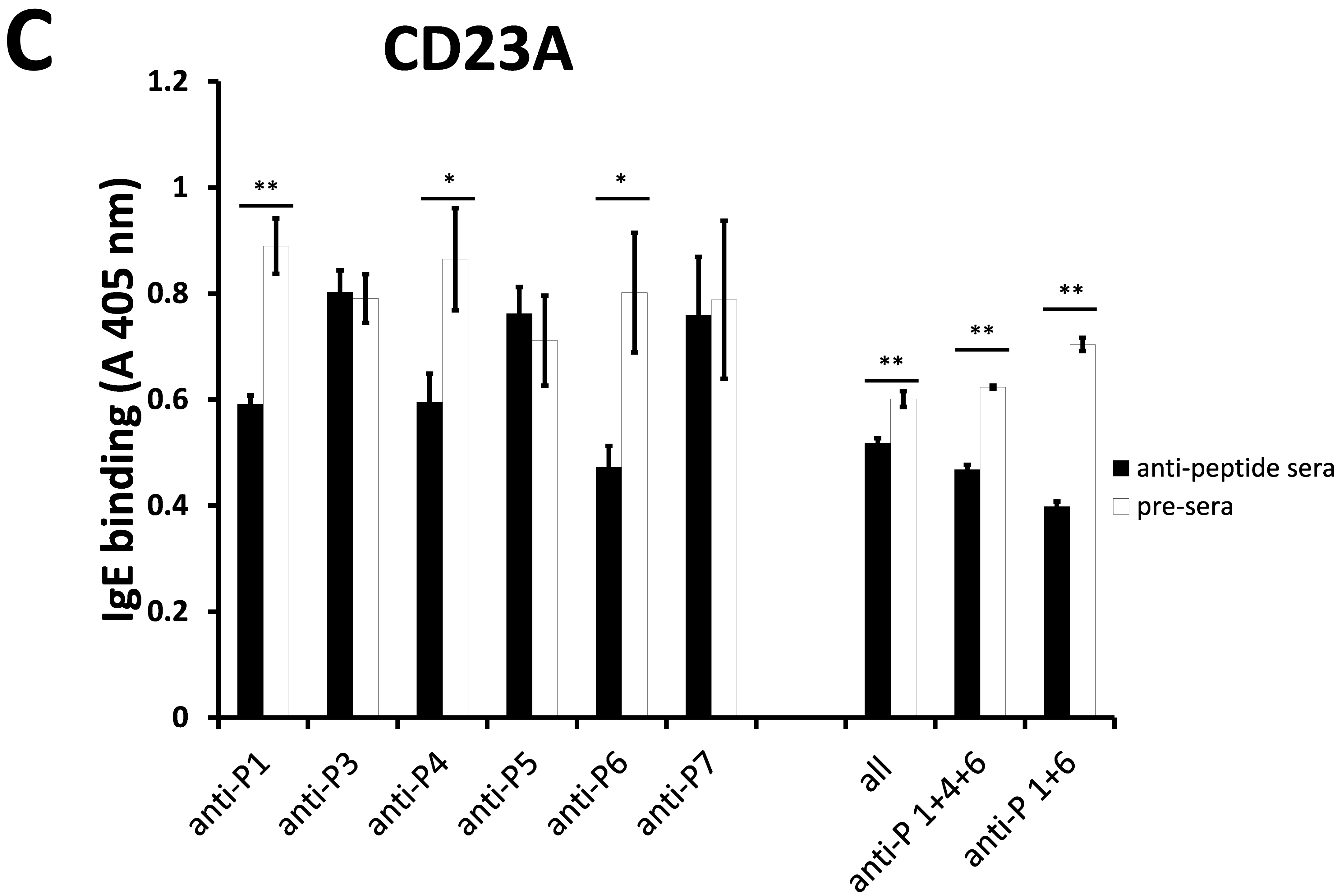
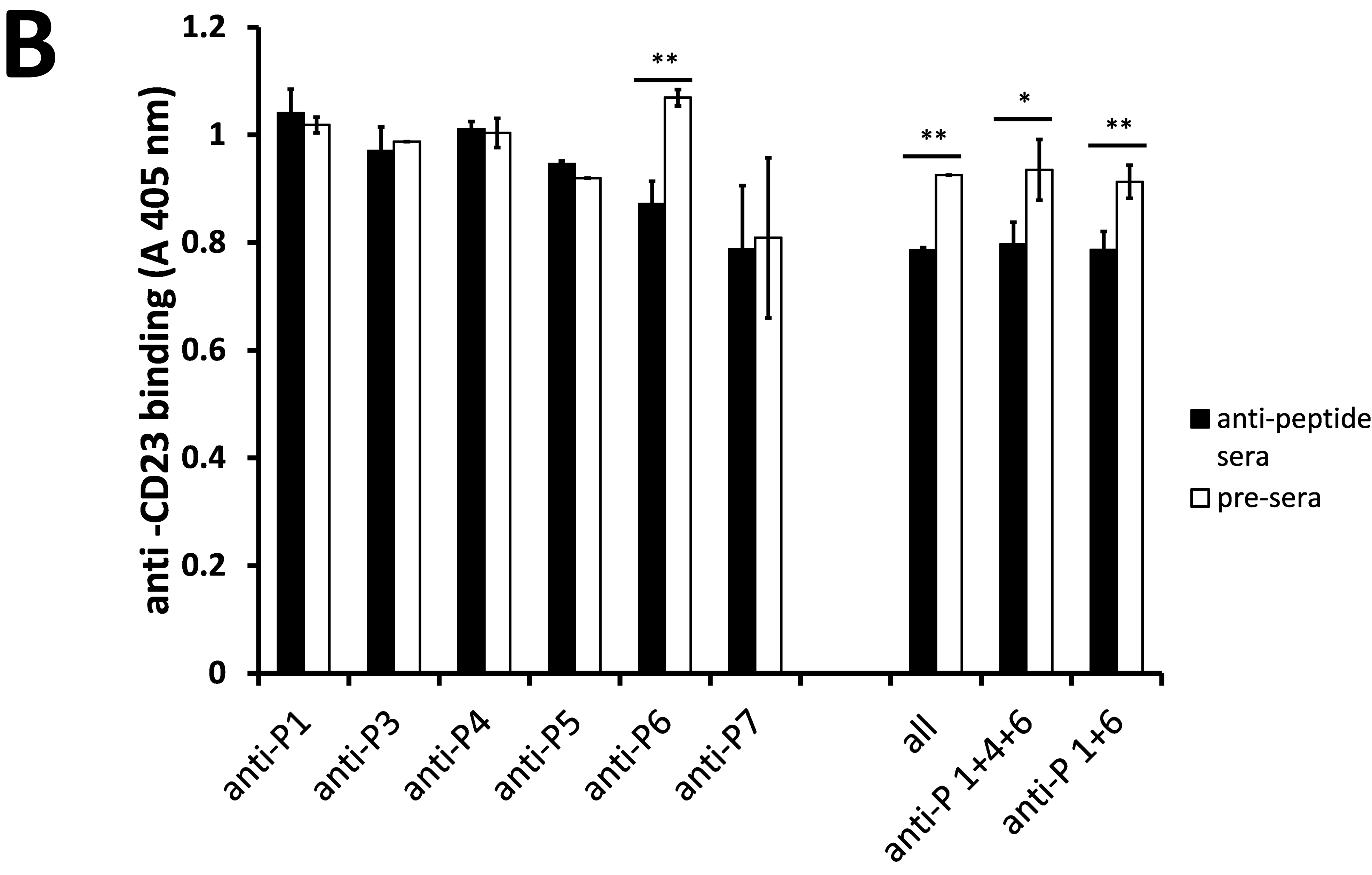
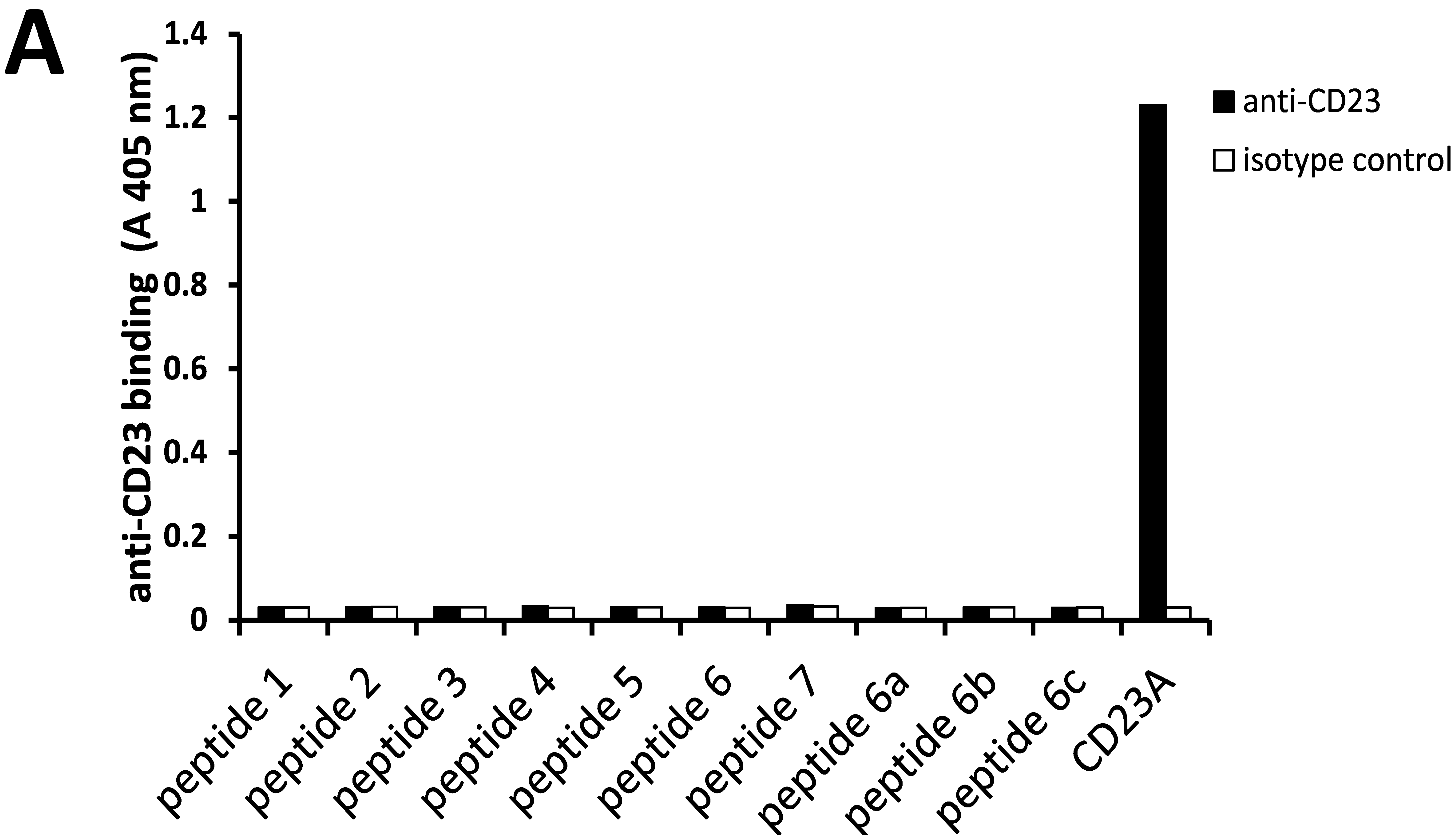


FIG 4.



E

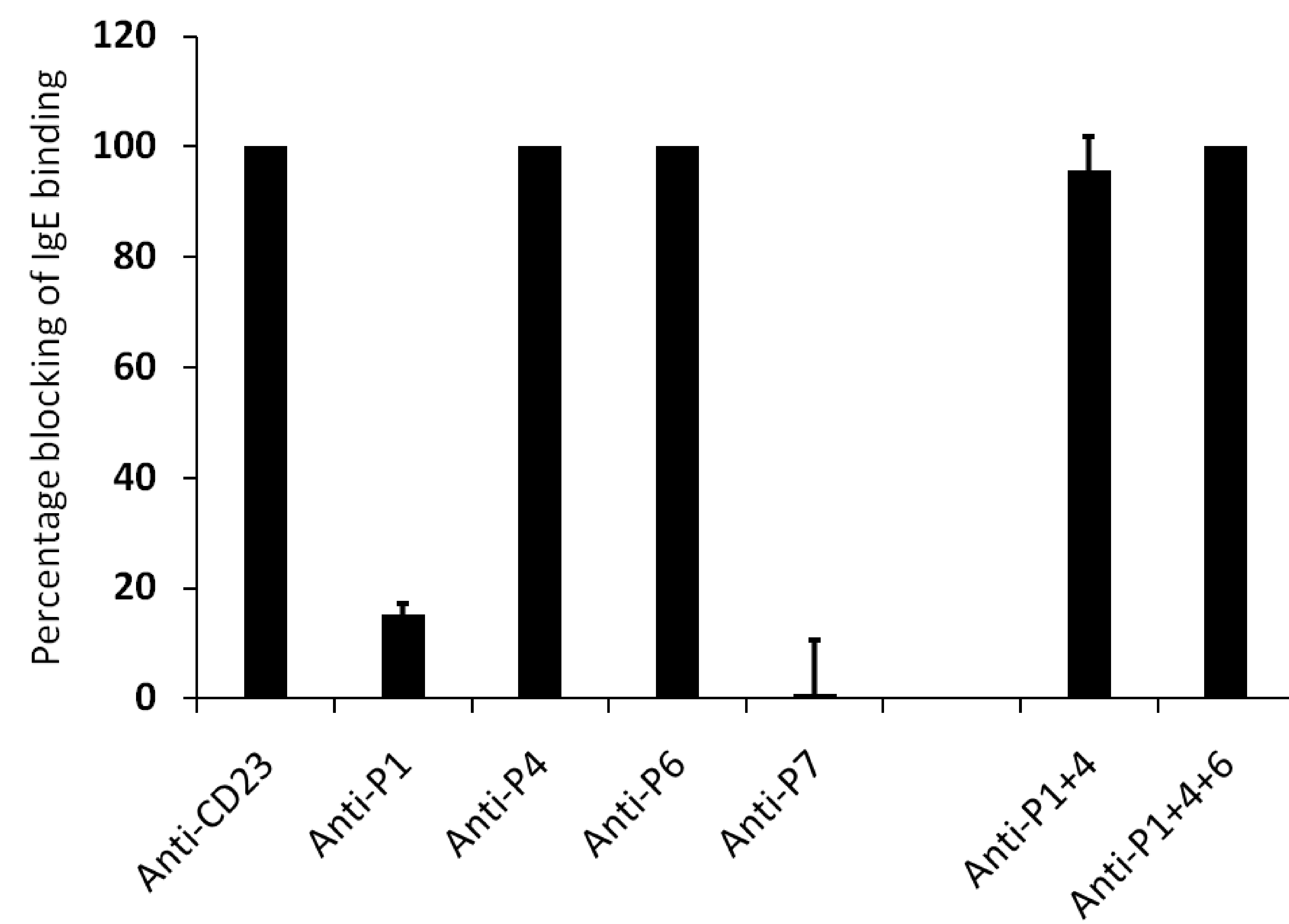
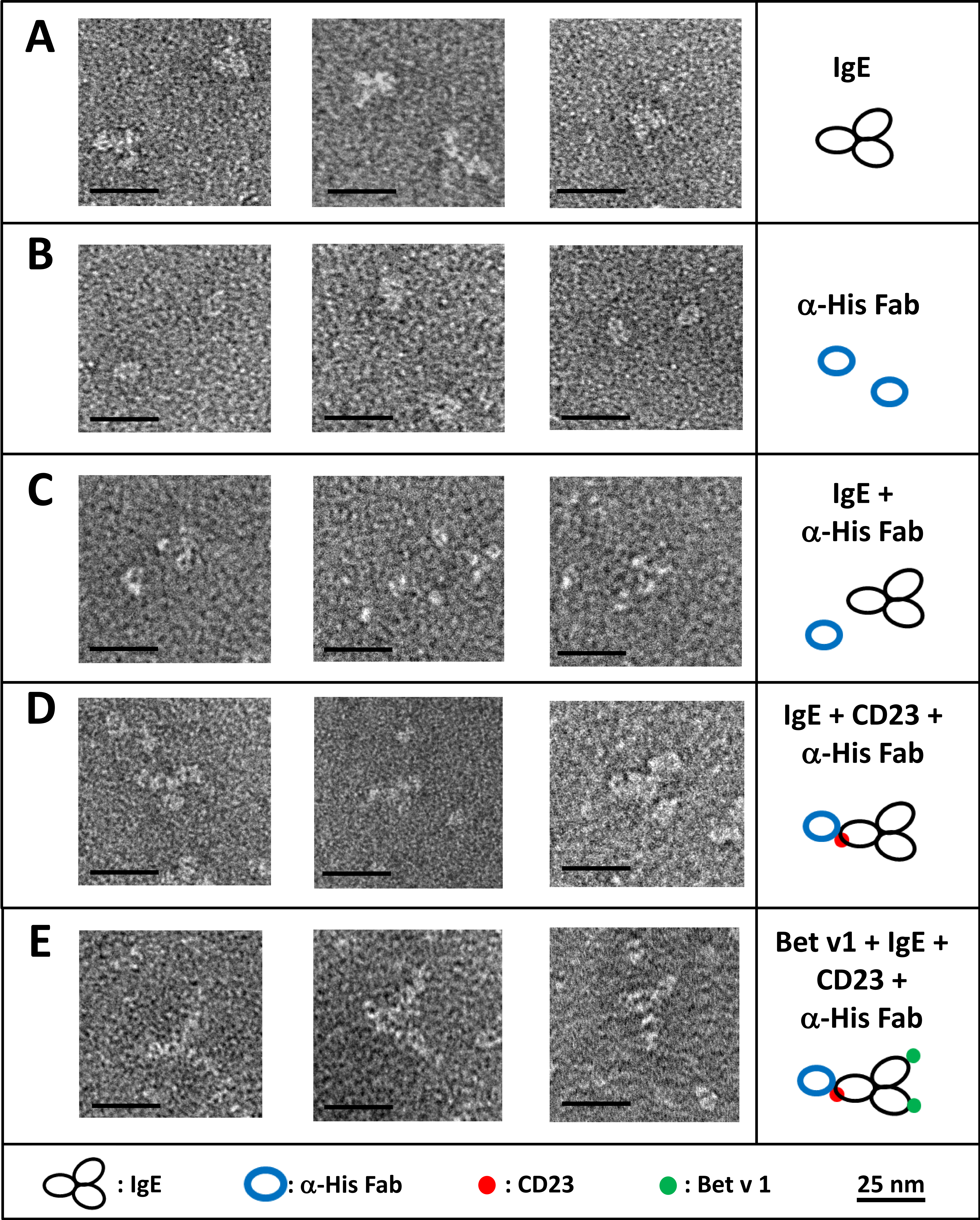


FIG 5.



Critical and direct involvement of the CD23 stalk region in IgE binding

ONLINE REPOSITORY

METHODS:

Construction and recombinant expression of CD23 derivatives in SF9 insect cells. Each of the four constructs contained a C-terminal 6x histidine tag to facilitate purification. Synthetic genes coding for the constructs were codon optimized for insect cell expression (GeneScript, NJ, USA) and were cloned into the polyhedrin promoter-harboured expression vector pTM1^{E1} via *Bam*HI and *Sma*I sites. DNA sequences and their correct insertion were verified by double-strand sequencing using the primers CGCCCAGGACTCTAGCTATAG (forward) and TGATGATGATGATGATGG (reverse). The melittin sequence of the pTM1 vector ensures export of the protein into the culture supernatant whereby the export sequence is cleaved off, leaving two additional amino acids (aspartic acid and proline) at the N-terminal end of each protein. For generation of recombinant baculovirus harbouring CD23 cDNA and the subsequent expression of the CD23 proteins in SF9 insect cells, the Bac-to-Bac TOPO expression system (Life Technologies, Carlsbad, USA) was used according to the manufacturer's protocol^{E2}. The time course of expression was monitored in culture supernatants by Western blotting. For this purpose, aliquots of the culture supernatants containing CD23 proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Whatman, Little Chalfont, UK). Membranes were

blocked with buffer A (Tris-buffered saline containing 0.05% Tween 20) containing 5% w/v milk powder (AppliChem, Darmstadt, Germany) for 1 hour at room temperature, washed 3 times for 10 minutes with buffer A and incubated with a 1:4000 in buffer A diluted horseradish-peroxidase-labelled anti-His-tag antibody (Thermo Scientific, Waltham, MA) for 1 hour at room temperature. Bound antibodies were visualized by chemoluminescence using an ECL-detection kit (BioRad, Hercules, CA, USA) and exposure of blots to x-ray films (Kodak, Rochester, NY).

Purification, biochemical and biophysical characterization of recombinant CD23 constructs. Protein-containing culture supernatants were concentrated using an Amicon concentration cell (Millipore, Darmstadt, Germany) and recombinant proteins were bound via the His-tag to a Co^{2+} -containing column (ClonTech, Mountain View, CA, USA), eluted with 150 mM imidazole and dialysed against 10 mM Tris pH 8 containing 100 mM NaCl. Protein aliquots were stored at -80°C . The purity and identity of the recombinant proteins was checked by SDS-PAGE followed by Coomassie Blue staining and by immunoblotting using the anti-His tag antibody or rabbit anti-peptide 6 antibodies (1:10 000) (Repository Table E1). Bound rabbit antibodies were detected with HRP-labelled goat-anti-rabbit antibodies (GE Healthcare, Chalfont St Giles, UK) diluted 1:50 000 in buffer) and ECL detection. Glycoprotein staining of an SDS-PAGE gel containing CD23 derivatives was performed with the Pierce glycoprotein staining kit (Pierce, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

Purified CD23 proteins in 10 mM sodium phosphate pH 7 were used to acquire mass spectra by MALDI-TOF (Microflex, Bruker, Billerica, MA, USA). To this end, the samples were mixed at a 1:1 ratio with matrix (sinapinic acid dissolved in 60%

acetonitrile, 0.1% trifluoroacetic acid), spotted onto the target and air-dried^{E2}. Folding of the recombinant proteins was analysed by CD spectroscopy on a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 1-mm path length cell at room temperature and a wavelength range from 185 to 260 nm at a resolution of 1-nm, at 20nm/min scanning speed and 1-s response time^{E3}. Data of three measurements were averaged. The final spectra were baseline-corrected and results were expressed as the mean-residue molar ellipticity ($[\theta]_{mr}$) at a given wavelength. The secondary structure content of the CD23 derivatives was calculated using the secondary structure estimation program CDSSTR^{E4} and the reference set 4^{E5}.

Recombinant CD23 constructs were checked for possible aggregation by gel filtration/size exclusion chromatography^{E3}. Aliquots of 1 ml containing the different CD23 constructs (250-600 µg/ml in buffer B: 20 mM Tris buffer pH 7.4, 150 mM NaCl) were loaded on a Superdex 200 10/300 GL column (GE Healthcare, Uppsala, Sweden) for gel filtration. For gel filtration, buffer B was used at a flow rate of 0.5 ml/min at 4°C. The absorbance was monitored at 210 and 280 nm. Gel filtration standard proteins (BioRad: thyroglobulin, 670 kDa; bovine gamma globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; vitamin B12, 1.35 kDa) were run under similar conditions to determine the constructs' size.

Synthesis of CD23 peptides and generation of CD23 peptide-specific antibodies. CD23 peptides (Repository Table E1) were synthesized by Fmoc (9 fluorenyl methoxy carbonyl)-strategy with HBTU [(2-(1H-benzotriazol-1-yl)1,1,3,3 tetramethyluronium hexafluorophosphat] activation (0.1 mmol small-scale cycles) on an Applied Biosystems (Foster City, CA) peptide synthesizer Model 433A as described^{E6}. Cysteine residues were added to peptides P1, P2, P3, P4 and P6 as

indicated in Table E1, whereas P5 and P7 contained cysteine residues. The short peptides P6a, b and c were made without modification. Peptides were purified to >90% purity by preparative HPLC and their identity was confirmed by mass spectrometry (piChem, Graz, Austria). Antibodies against KLH-coupled peptides (P1, P2, P3, P4, P7) (Maleimide-activated KLH conjugation kit, Sigma-Aldrich, St. Louis, MO) or Blue Carrier Protein-coupled peptides (P5, P6) (Imject EDC Blue Carrier Protein Spin kit, Thermo-Scientific) were raised in rabbits (Charles River, Kisslegg Germany)^{E6}.

Expression and purification of human Bet v 1-specific monoclonal IgE. Human monoclonal IgE specific for the major birch pollen allergen Bet v 1 was expressed as described^{E7}. High yield IgE production was obtained in a bio-reactor (CELLine CL 350, Sartorius, Göttingen, Germany) with DMEM high glucose media (Life Technologies) supplied with 5% ultra-low IgG FBS (Life Technologies). Bet v 1-specific IgE was purified by affinity chromatography using a monoclonal anti-IgE antibody^{E8}.

Binding experiments with recombinant CD23 derivatives and statistical analysis. ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with recombinant CD23 proteins dissolved in coating buffer (100 mM sodium carbonate-bicarbonate pH 9.3, c= 5 µg/ml; 50 µl/well). Plates were then washed with buffer C (PBS, 0.02% v/v Tween 20) and reacted with either the peptide-specific rabbit antisera and the corresponding pre-immune sera as control (diluted 1:1000), or with the anti-CD23 antibody (1:1000) and mouse IgG₁ as control overnight at 4°C. Plates were again washed with buffer C. Bound rabbit antibodies were detected with HRP-

labelled anti-rabbit IgG antibodies (GE Healthcare) diluted 1:5000 and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as substrate. Bound mouse antibodies were detected with an HRP-labelled anti-mouse IgG-ECL antibody (GE Healthcare), diluted 1:5000 and ABTS instead. Results represent means of triplicate determinations $\pm$ SD after subtraction of background as determined with the pre-immune sera and the isotype control, respectively.

Binding of human IgE and IgE-allergen immune complexes to CD23 derivatives was also investigated by ELISA experiments. Plates were coated with equimolar amounts of the recombinant CD23 proteins dissolved in coating buffer (100 mM sodium carbonate-bicarbonate pH 9.3, c=200 nM; 50 μ l/well). Plates were then washed with buffer C (PBS, 0.02% v/v Tween 20). Bet v 1-specific IgE (5 μ g/ml in buffer C) alone or pre-incubated with rBet v 1 (5 μ g/ml in buffer C) (Biomay, Vienna, Austria) for 1 hour at 37°C was then added and incubated overnight at 4°C. After washing with buffer C, bound Bet v 1-specific IgE and IgE-allergen complexes were detected with biotin-labelled anti-human IgE antibodies (KPL, Maryland, USA) at a dilution of 1:2500 in buffer C and incubated overnight at 4°C. After a further washing step, Avidin-alkaline phosphatase (Sigma-Aldrich, Saint Louis, Missouri, USA) was added at a concentration of 1:5000 for 1 hour at RT. Plates were washed with buffer C, 4-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, Saint Louis, Missouri, USA) was added as a substrate and the colour reaction was read at 405 nm with an ELISA reader (Victor3, PerkinElmer, Waltham, Massachusetts, USA). Mean values of triplicate determinations $\pm$ SD were calculated. In another set of experiments, bound IgE-Bet v 1 immune complexes were detected with rabbit anti-Bet v 1 antibodies (1:1000)^{E9} followed by HRP-labelled anti-rabbit IgG antibodies (GE Healthcare) diluted 1:5000 and ABTS as substrate. For control purposes, experiments were

performed only with Bet v 1 without IgE and by using control rabbit antibodies without specificity for Bet v 1 instead of the rabbit anti-Bet v 1 antibodies.

The specificity of IgE binding to the CD23 derivatives was tested in a series of ELISA inhibition experiments. First, inhibition of IgE binding was investigated by pre-incubating plate-bound CD23 derivatives with the α -CD23 antibody (BD Biosciences, Isotype M-L233) at a concentration of 5 μ g/ml or mouse IgG₁ as a negative control for 2 hours at room temperature before IgE was added. In another set of experiments, IgE was pre-incubated with 25 μ g/ml of omalizumab (Xolair, Novartis, Basel, Switzerland) or an isotype-matched human IgG₁ (Sigma-Aldrich) at 37° C for 1 hour before addition to plate-bound CD23 derivatives. Third, the ability of the anti-CD23 peptide antibodies to inhibit IgE binding to CD23 derivatives was investigated. For this purpose, plate-bound CD23 derivatives were incubated with the 1:50 diluted peptide-specific antibodies or the corresponding 1:50 diluted pre-immune Ig for 2 hours at RT, washed and then incubated with IgE.

We also tested if the monoclonal anti-CD23 antibody reacts with the synthetic CD23 peptides. Peptides were coated to ELISA plates (5 μ g/ml) and reacted with the 1:1000 diluted antibody or isotype control. Further, it was studied if the peptide-specific antibodies can inhibit binding of the monoclonal antibody to CD23A. For this purpose, ELISA plate-coated CD23A was pre-incubated with the peptide-specific antibodies (1:50) or the corresponding pre-Ig and then binding of the monoclonal antibody was assessed.

Binding and inhibition experiments in a cell-based model. EBV-transformed B cells (2×10^5 per condition) expressing high levels of CD23^{E10} were blocked with PBS

containing 1% BSA (PAA, Pasching, Austria) and 10% goat serum (Sigma Aldrich, St. Louis, Missouri, USA) for 20 minutes on ice. Then cells were washed (PBS with 1% w/v BSA) and incubated with peptide-specific rabbit anti-sera or the respective pre-immune serum as a control in a dilution 1 in 2 for 20 minutes on ice. For control purposes, cells were incubated with an anti-CD23 antibody (clone M-L233, BD Biosciences, San Jose, California, USA) or the respective isotype control. After washing (PBS, 1% w/v BSA), cells were re-suspended in PBS, 1% w/v BSA and complexes of monoclonal anti-Bet v 1 IgE (5 µg/ml) and major birch pollen allergen Bet v 1 trimer^{E3} (5 µg/ml) were added. IgE and Bet v 1 trimer were pre-incubated for 1 hour at 37°C to ensure complex formation. After a nother washing step, cells were incubated with FITC-labelled anti-IgE (KPL, Maryland, USA) and fixable viability dye eFluor® 450 (eBioscience, Inc., San Diego, California, USA) for 20 min on ice. Expression of CD23 was confirmed in each experiment by staining one extra sample with a PE-labelled anti-CD23 antibody (clone EBVCS2, eBioscience) or the respective isotype control as well as viability dye. All experiments were performed in duplicates. Flow cytometry was performed using a FACS Canto II (BD Biosciences, San Jose, California, USA). Thirty thousand events were acquired per sample and analysed with Flowjo Software (Treestar Inc., Ashland, Oregon, USA). Mean fluorescence intensity (MFI) was calculated with Flowjo Software and MFI of cells stained with FITC-labelled anti-IgE in absence of monoclonal anti-Bet v 1 IgE were subtracted as background. The percentage blocking was calculated according to the following formula: $100 - (100 / \text{MFI pre-serum minus background} \times \text{MFI anti-peptide serum minus background})$.

Negative stain electron microscopy. Negative stain electron microscopy was performed with purified Bet v 1-specific IgE^{E7, E8}, the purified CD23 constructs and rBet v 1. Furthermore, a His-tag specific Fab fragment was generated to indicate the His-tagged CD23 constructs. For this purpose, a mouse IgG₁ anti-His specific antibody (37-2900, Life Technologies) was incubated in digestion buffer (100 mM Tris pH 8, 2 mM EDTA, 25 mM 2-mercaptoethanol) for 2.5 hours at room temperature. Subsequently, the antibody was incubated with a papain resin (Pierce, Rockford, IL) over night at 37°C and Fab fragments were purified by depleting full antibodies and Fc fragments with protein A (Pierce). Negative stain of the samples was done as described^{E11}. For this purpose, CD23A was buffered in 10 mM Tris pH 8, 100 mM NaCl and 2mM CaCl₂. The different molecules (i.e. CD23A, IgE, anti-His Fab, Bet v 1) were then complexed in borate-buffered saline (10 mM sodium borate, 150 mM NaCl, pH 8.2) at 4°C overnight (CD23A: 2 µg/ml; IgE: 3.5 µg/ml; anti-His Fab: 2 µg/ml; Bet v 1: 2 µg/ml). Samples were floated onto a 2 nm carbon layer, stained with 2% uranyl formate (Polysciences Europe, Eppelheim, Germany) and transferred to a T 600-Cu grid (Ted Pella, Redding, CA). Samples were then viewed under an electron microscope (EM-900 TEM, Carl Zeiss, Oberkochen, Germany).

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FIGURE LEGENDS

TABLE E1. Characteristics of synthetic CD23 peptides. Cysteine residues added for carrier coupling are printed in bold letters.

FIG E1. Expression in insect cells and purification. **A**, Time course of expression (x-axes) of CD23 constructs as detected in nitrocellulose-blotted insect cell supernatants with an anti-His-tag antibody. Molecular weights are indicated on the y-axes. **B**, Coomassie- and **C**, glycan staining of purified constructs (CD23A, B, C, D) as well as of a non-glycosylated (N) and glycosylated (P) control protein.

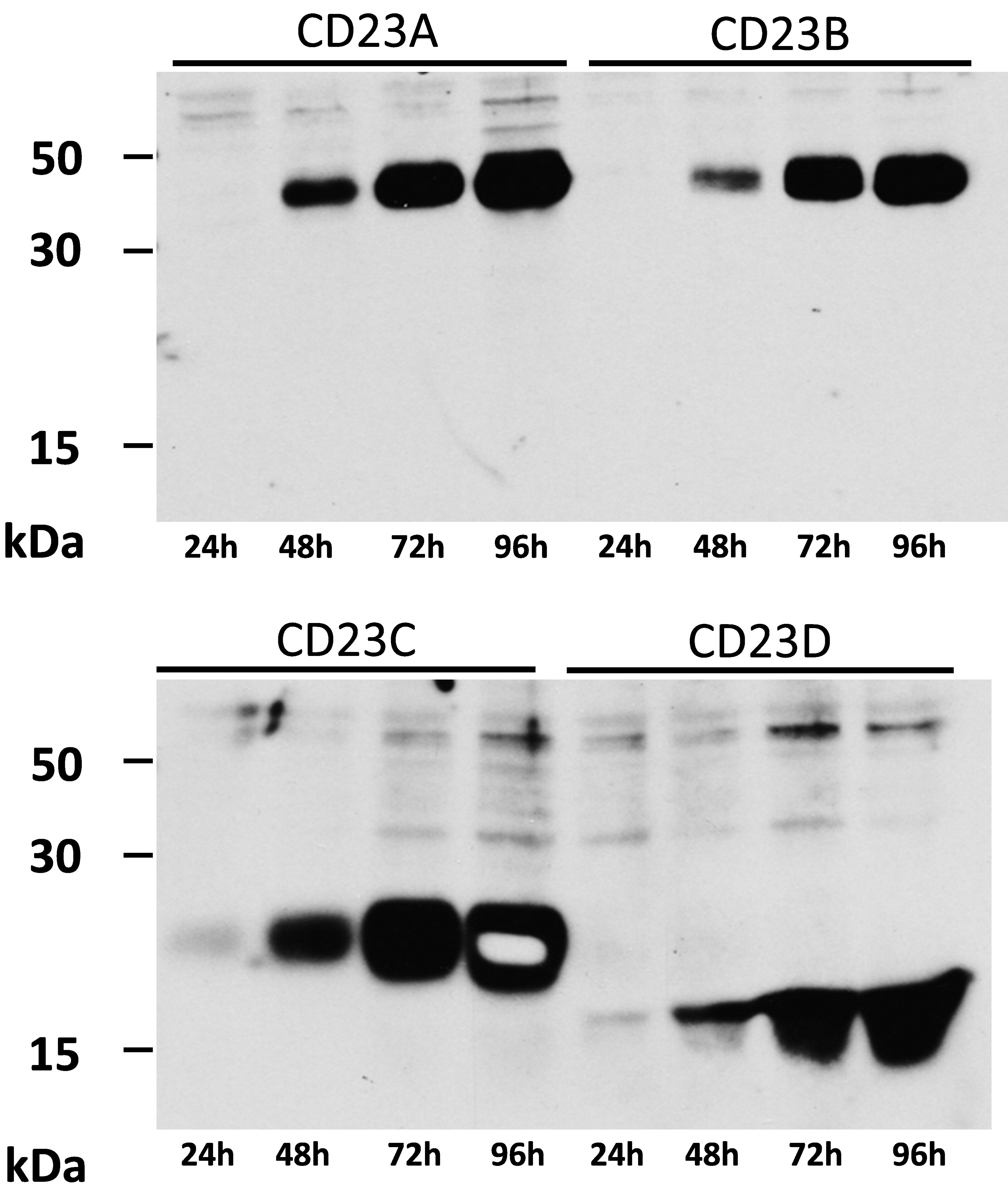
FIG E2. Binding of rabbit antisera specific for CD23 peptides to recombinant CD23 constructs. **A**, CD23A, **B**, CD23B, **C**, CD23C, and **D**, CD23D. CD23 constructs were incubated with anti-CD23 antibodies with different specificities (x-axis; rabbit anti-CD23 peptide antibodies: anti-P1, anti-P2, anti-P3, anti-P4, anti-P5, anti-P6, anti-P7; monoclonal anti-CD23 antibody: anti-CD23). Absorbance values (A 405 nm: y-axes) correspond to bound antibodies. Error bars show standard deviations of triplicates.

Table E1: Characteristics of synthetic CD23 peptides.

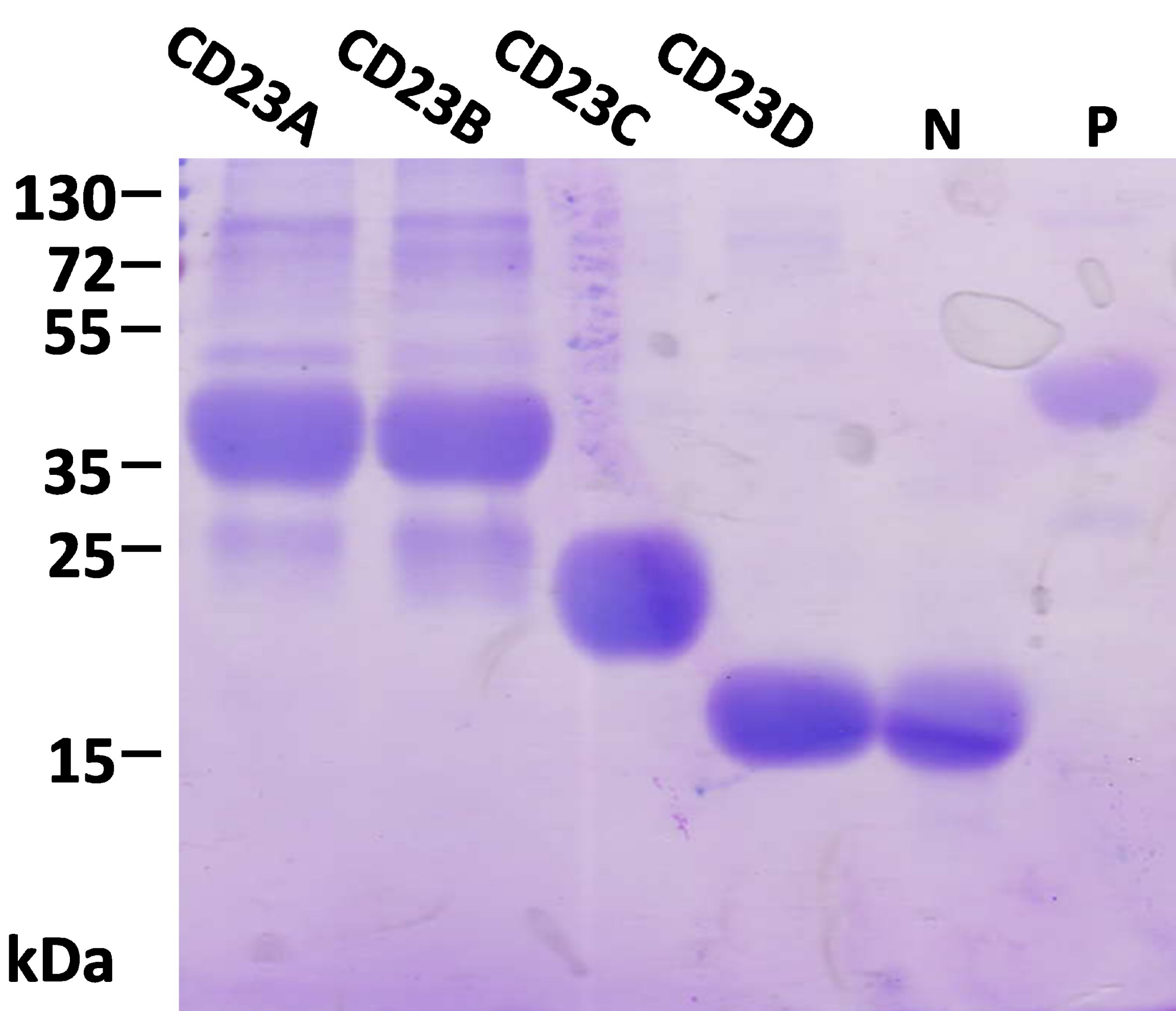
CD23 Peptides	Sequence	theoretical isoelectric point	amino acid residues	molecular weight (Da)
P1	LESHHGDQMAQKSQSTQISQEEELRAEQC	4.68	30	3441.7
P2	CLWAGLLTLLLWHWDTTQSLKQLEE	4.65	26	3111.7
P3	CPASEGSAESMGPDSRPDPDGR LPTPSAPLHS	4.43	32	3219.5
P4	CLSSFKSQELNERNEASDLLERLREEVTKLR	4.19	31	3694.1
P5	PEKWINFQRKCYFYGKGTKQWVHARYAC	9.71	28	3509.1
P6	CSPEEQDFLT KHASHTGSWIGLRNLDLKGEF	5.39	31	3516.9
P6a	SPEEQDFLT KHASHT	5.26	15	1726.8
P6b	GSWIGLRNLDLKGEF	6.07	15	1705.0
P6c	LTKHASHTGSWIGLRN	11	16	1778.0
P7	NWAPGEPTSTSQGEDCVMMRGSGRWND AFC	4.32	30	3345.7

FIG E1.

A



B



C

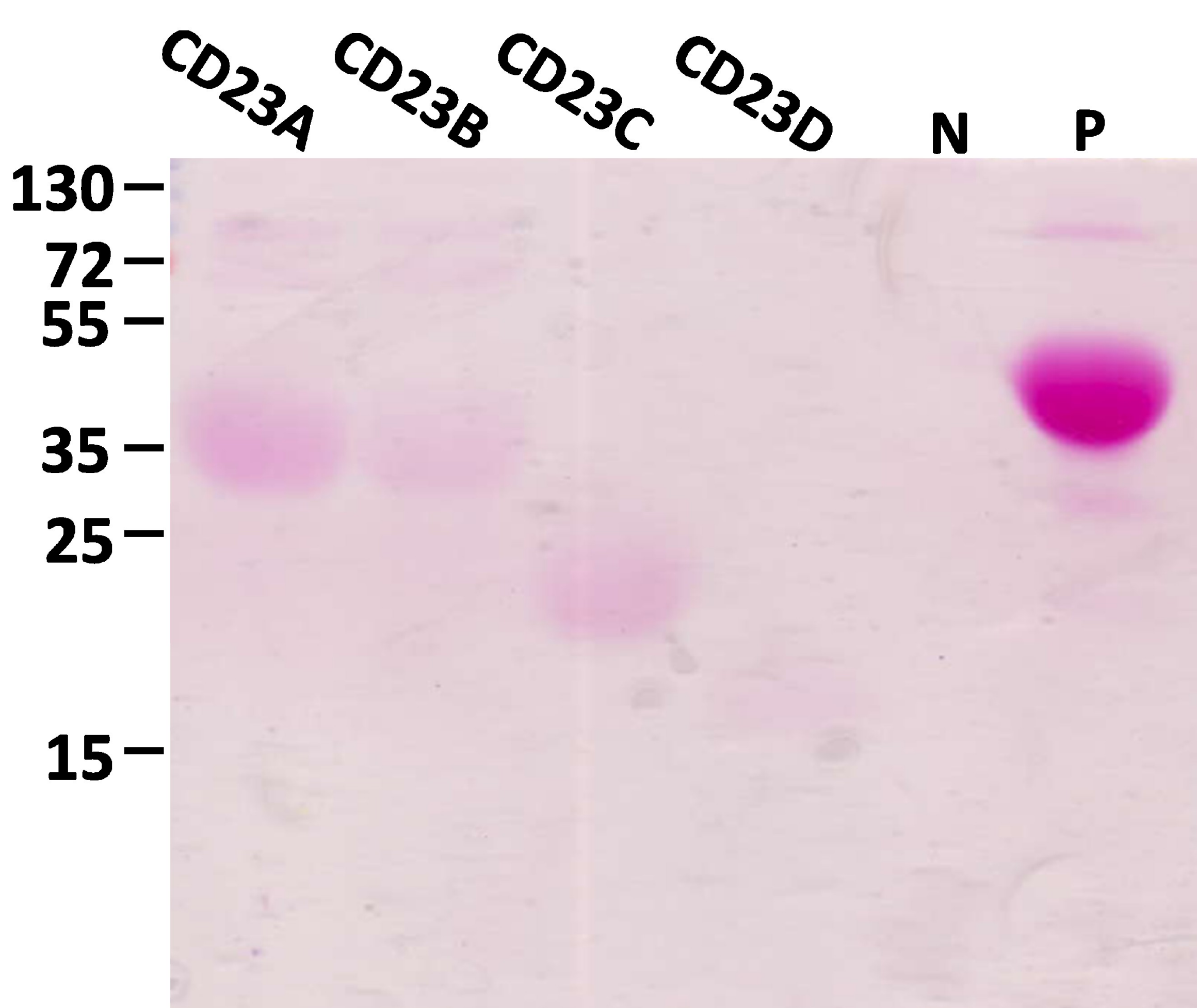
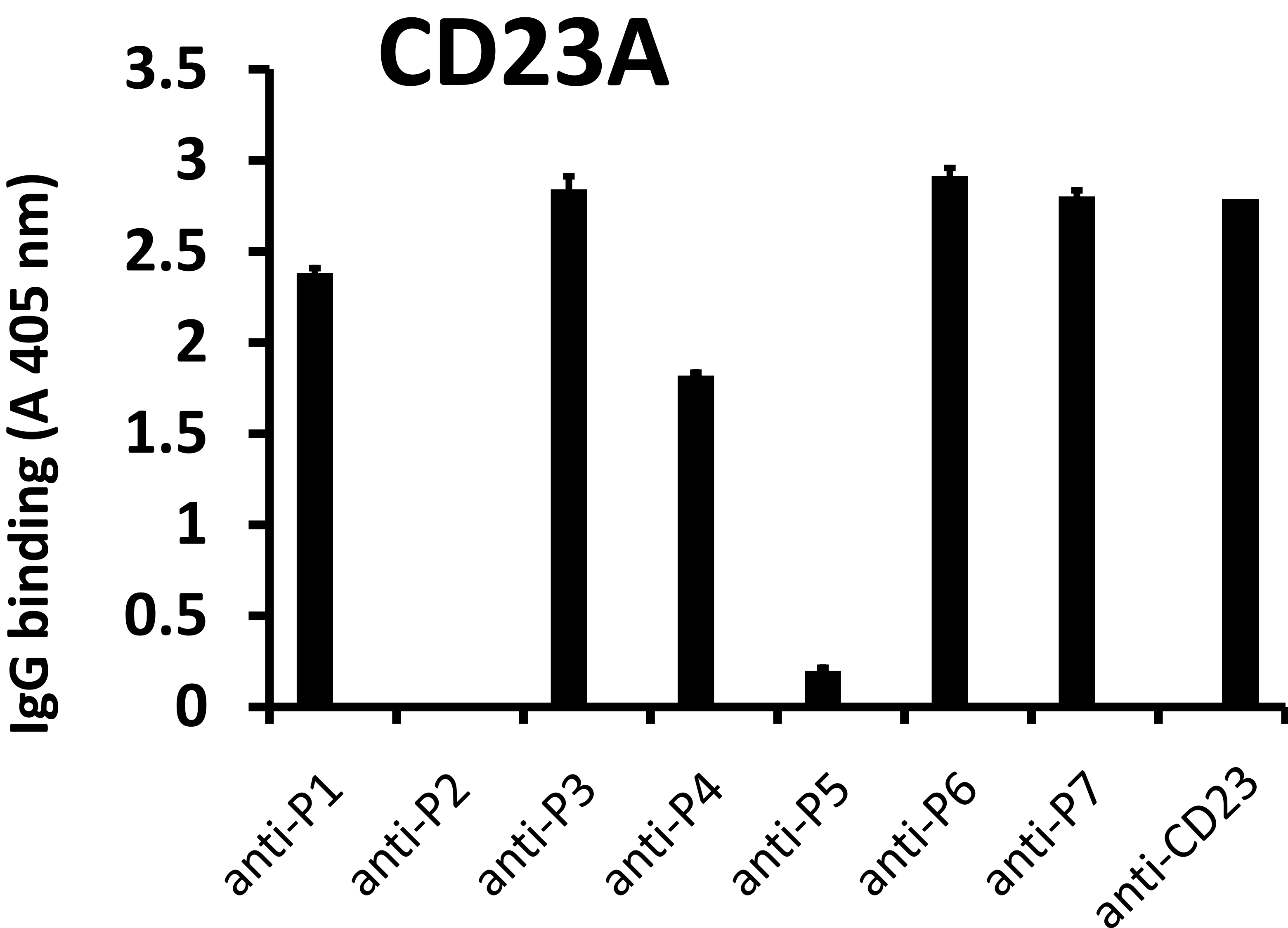
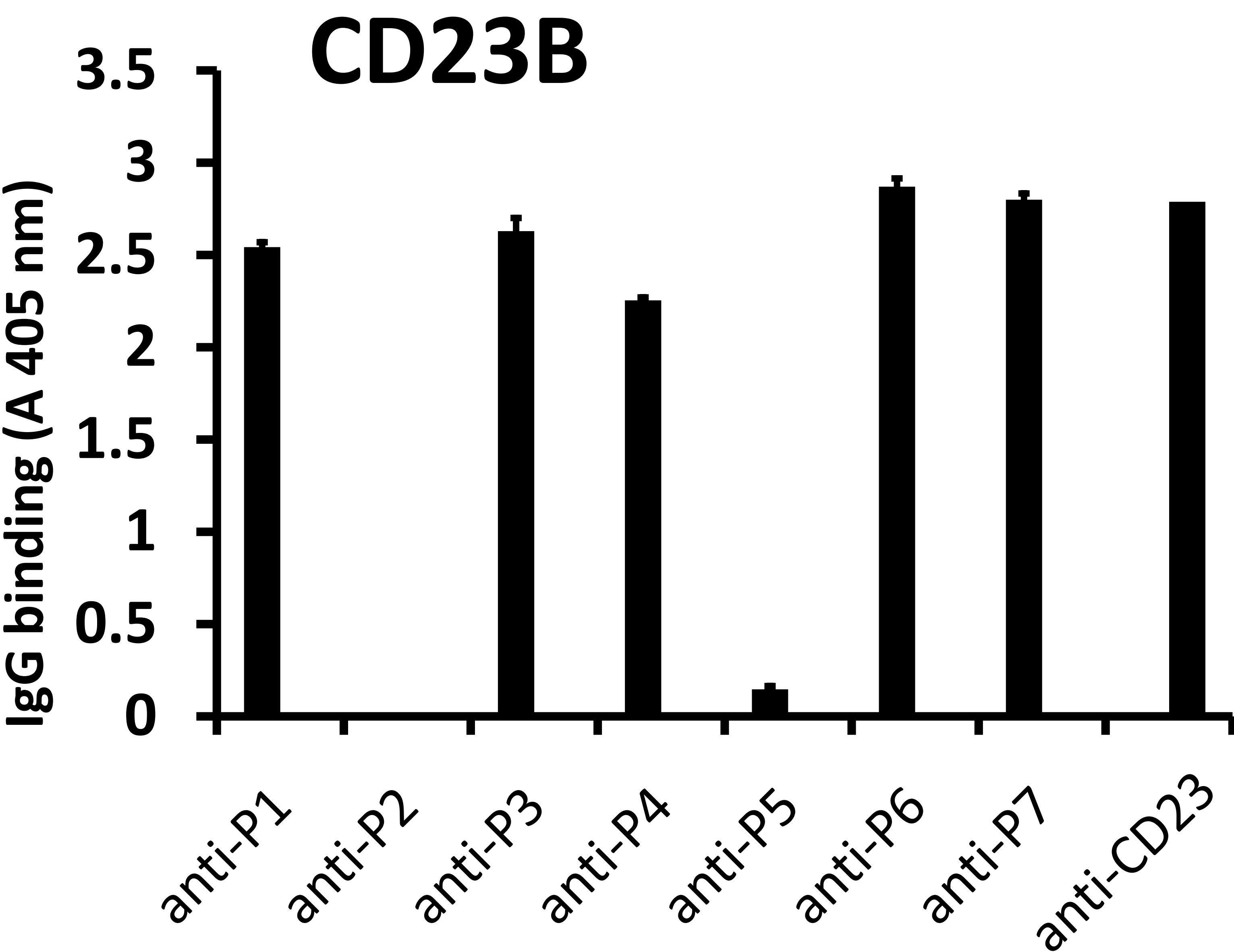


FIG E2.

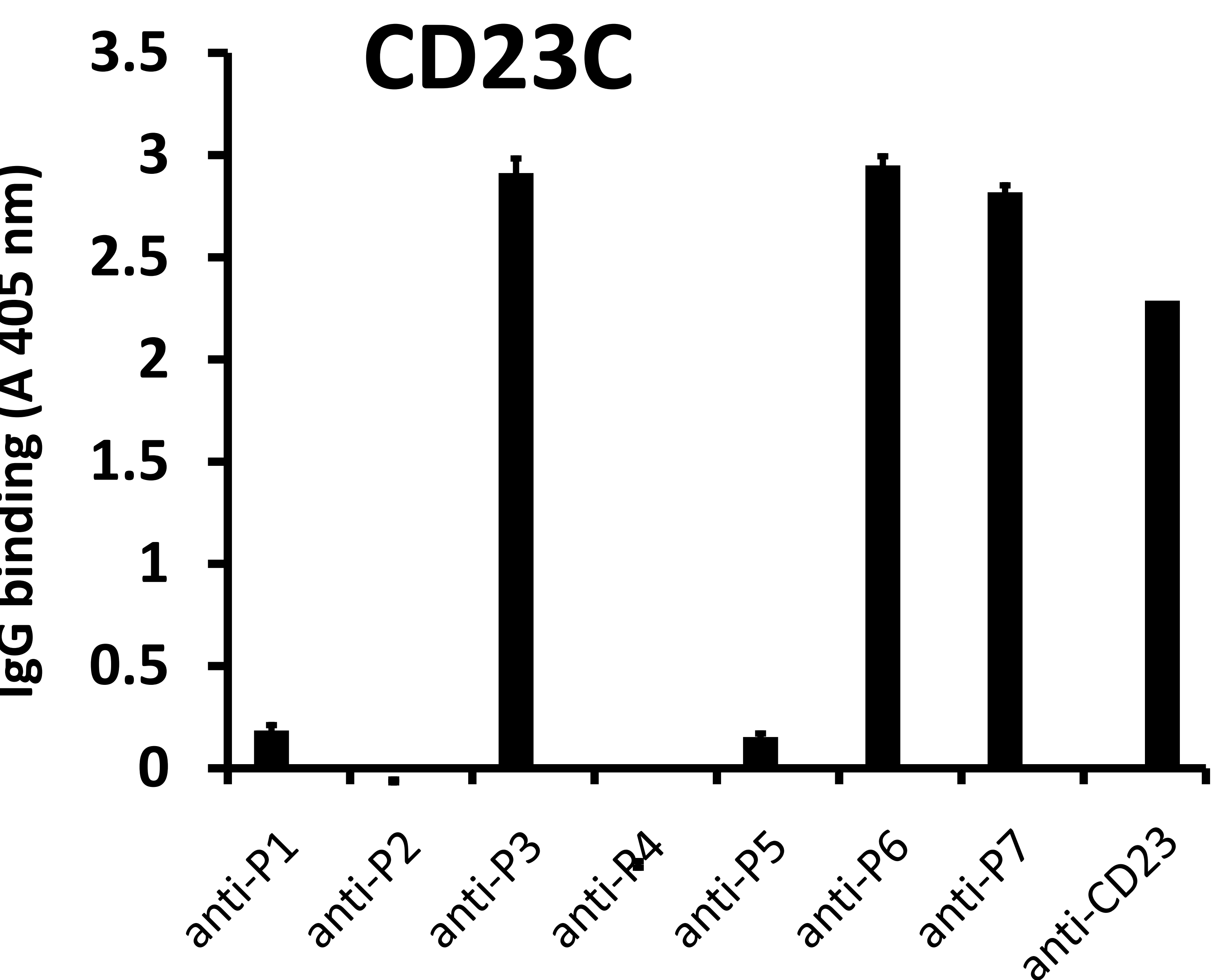
A



B



C



D

