Pathogenic role of (S)IgA in IgA nephropathy

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CHAPTER 2

Monomeric and polymeric IgA show a similar association with the myeloid FcαRI/CD89

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Summary
IgA is found in both mucosal secretions and serum and is the dominant immunoglobulin isotype produced in humans. It exists in different molecular forms, namely monomeric IgA, dimeric IgA, polymeric IgA and secretory IgA, all exhibiting interactions with FcaRI/CD89 to some extent. CD89 is an activating, γ-chain associated, Fc receptor for IgA expressed on myeloid cells. Here, we investigated the interaction of monomeric and polymeric IgA purified from human serum with CD89 using surface plasmon resonance. The results demonstrate a similar association for monomeric and polymeric IgA with CD89. In contrast, monomeric IgA dissociated more rapidly from CD89 than polymeric IgA. Removal of N-glycans from mIgA resulted is an increased association with CD89, whereas the dissociation was more rapid, resulting in binding comparable to that of untreated monomeric IgA. We conclude that the initial interaction of monomeric and polymeric IgA with CD89 is similar, whereas monomeric IgA dissociates more rapidly from CD89. In view of the large excess of monomeric IgA in serum, monomeric IgA will compete for CD89 interaction with polymeric IgA, thereby preventing cell activation initiated by receptor aggregation contributing to the anti-inflammatory role of IgA.

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INTRODUCTION

Immunoglobulin A (IgA) is the predominant immunoglobulin isotype, and plays a critical role in protecting the host against environmental pathogens and antigens encountered at mucosal surfaces (1). In secretions, secretory IgA (SIgA) is generated during transcytosis of dimeric IgA (dIgA) by epithelial cells, ultimately leading to its association with the extracellular part of the polymeric Ig receptor (secretory component) (2). In humans, IgA in the circulation primarily consists of monomeric IgA (mIgA), but about 10-20% of the IgA is found in dimeric or polymeric IgA (pIgA) forms, while in rodents IgA is mostly present in a polymeric form (3). Furthermore, IgA consists of two subclasses namely IgA1 and IgA2. IgA1 contains ten potential O-glycosylation sites and two N-glycosylation sites and IgA2 does not contain O-glycosylation sites but contains two or three additional N-glycosylation sites. In vitro deglycosylation of IgA leads to self-aggregation, suggesting that underglycosylation of IgA may contribute to generation of high molecular weight IgA (4).

The transmembrane molecule FcαRI (CD89) has been identified as receptor for the Fc portion of human IgA (5). This receptor is constitutively expressed on polymorphonuclear leukocytes (PMN), monocytes, eosinophils, dendritic cells and a subset of macrophages (5-7), as well as on Kupffer cells in the liver, where it has been suggested to provide a second line of defence (8). CD89 consists of two extracellular Ig-like domains followed by a stretch of hydrophobic amino acids representing the predicted transmembrane domain, with a positively charged arginine which is essential for association of CD89 with the FcR γ-chain homodimeric signalling subunit (9), and a short cytoplasmic tail devoid of recognition signalling motifs. The protein core of CD89 has a predicted molecular mass of 30 kDa with differential glycosylation at six potential N-linked sites, and the probability of additional O-glycosylation contributing to the variable size observed for the mature receptor (55-110 kDa) (10). CD89 has been reported to bind both IgA1 and IgA2 with similar affinity (Ka ~10^6 M-1). The site of interaction between CD89 and IgA was identified in the first extracellular domain of CD89 (11,12) and in the Cα2/Cα3 junction of IgA (13,14).

CD89 participates in different aspects in host defence. CD89 induce phagocytosis of IgA complexed antigens (15), initiates antibody dependent cellular cytotoxity (16) and CD89 is important for the clearance of IgA from the circulation (9). Binding studies with different molecular forms of IgA have shown that pIgA binds stronger to CD89 than mIgA (16-18).

Furthermore, one study suggested that secretory IgA (SIgA) can only interact with CD89 if MAC-1 is present (19). For most of these binding studies total IgA was purified from serum as a source for mIgA followed by production of pIgA by an artificial aggregation process (17). Alternatively, mIgA was generated from SIgA via a chemical treatment (16).

The purpose of present study was to study the interaction of mIgA and pIgA in their physiological conformation with CD89 in a quantitative manner. Therefore, these two biological forms of IgA were directly isolated from serum and their interaction with CD89 was studied by ELISA and surface plasmon resonance. Furthermore, we studied a potential role for IgA glycosylation in the differential interaction of the molecular forms of IgA with CD89. The results indicate that the initial

interaction of mIgA and pIgA with CD89 is comparable, but since mIgA dissociates more rapidly from CD89, the final outcome is that pIgA remains associated with CD89 for an extended time, thereby potentially increasing its signalling potency.

MATERIAL AND METHODS

IgA purification

Serum from 6 individuals was used for IgA purification, according to methods described before (20). In brief, serum was applied to an anti-IgA (HisA 43, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) affinity column. The column was washed with 0.5 x PBS and 0.3 M NaCl, and subsequently IgA was eluted with 0.1 M glycine/ 0.3 M NaCl (pH 2.8). Directly after elution the fractions were neutralized with 1 M Tris pH 8.0. The eluted protein fractions that contained IgA, as assessed by ELISA (21), were pooled and dialysed against PBS containing 2 mM EDTA.

Isolated IgA was size-separated with a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands) into polymeric IgA and monomeric IgA. These pools were analysed for total IgA content using ELISA (21).

Secretory IgA that was used in some experiments was obtained from Sigma.

ELISA

Binding of IgA to Fc-$(CD89)_2$ was analysed by coating ninety-six well Nunc Maxisorb microtitre plates (Gibco/Invitrogen, Carlsbad, CA) with Fc-$(CD89)_2$ (2 µg/ml) in carbonate buffer (pH 9.6) (100 µl/well) overnight at room temperature. After coating, the plates were washed with PBS/ 0.05 % Tween. Plates were incubated with different concentrations of IgA in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgA was detected using mouse anti-human IgA (4E8), followed by HRP-conjugated goat anti-mouse IgG (Dako, Heverlee, Belgium), both diluted in PBS/ 1 % BSA/ 0.05 % Tween and incubated for 1 hour at 37 ºC. Enzyme activity of HRP was developed using ABTS (2, 2'-azino-bis (3-ethyl benzathioline-6-sulphonic acid)) (Sigma, St. Louis, MO). The O.D. at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, USA).

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis was carried out using a BIAcore instrument (Biacore AB, Uppsala, Sweden). A CM5 sensor chip (BIAcore AB) was coupled with recombinant CD89 (10000 response units (RU)), chimeric Fc-$(CD89)_2$ (10000 RU), and BSA (10000 RU), following manufacturers' instructions. Binding assays were performed at flow rates of 5 µl/min using HBS EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20). A 10 µl aliquot of analytes was injected and subsequently allowed to dissociate for 10 minutes. Subsequently, the surfaces were regenerated with 0.1 M glycine/ 0.3 M NaCl, pH 2.8. Early binding was defined as the response units at 450 seconds, and late binding was defined as the response units at 1100 seconds.

Another CM5 sensor chip was coupled with BSA (200 RU) and chimeric Fc-$(CD89)_2$ (250 RU). Kinetic assays were performed at flow rate of 30 µl/min and 180 µl IgA was injected. Data collected for each experiment were analyzed in a bivalent model using biaevaluation software (mIgA 180 kDa and pIgA 360 kDa).

Glycosidase treatment of IgA

Monomeric IgA (800 µg) was first digested with N-glycosidase F (PNGase F), cloned from *flavobacterium meningosepticum* and expressed in *E. coli* (6 U/ 200 µg IgA, Roche) in 0.02 M sodium phosphate buffer pH 7.2 with 2 mM EDTA. The first sample, to be used as control, was IgA with incubation buffer (mock IgA); the second sample was IgA with enzymes (treated IgA). After 72 hours of incubation at 37ºC this IgA was further digested with Neuraminidase from *Arthrobacter ureafaciens* (6 mU/200 µg IgA, Roche, Mannheim, Germany),β (1-3,4,6) galactosidase isolated from a cloned gene expressed in *E. coli* (7 mU/ 200 µg IgA, Prozyme), or combinations of the enzymes. This deglycosylation step was performed in 50 mM sodium acetate pH 5.6 for 72 hours at 37ºC. After treatment, samples (10 µg) were analysed by loading on a 10 % reduced SDS-PAGE gel and Coomassie stained.

HAA ELISA

To confirm deglycosylation, the deglycosylated IgA was assessed for binding to biotinylated *Helix Aspersa* (HAA, Sigma) lectin, known to recognize terminal GalNAc, was performed. Ninety-six well NUNC Maxisorp microtitre plates were coated with 2 µg/ml IgA in carbonate buffer (pH 9.6) (100 µl/well), overnight at room temperature. After washing with PBS/ 0.05 % Tween and blocking for one hour 37 ºC with PBS/ 1 % BSA, the plate was incubated with 5 µg/ml biotinylated HAA in PBS/ 1 % BSA/ 0.05 % Tween. Binding of HAA to IgA was detected with HRP-conjugated streptavidin (Zymed). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. was measured at 415 nm.

Western blot analysis

IgA preparations (3 µg) were subjected to 10 % SDS-PAGE under reducing conditions, followed by semi-dry blotting to PVDF (immobilin-P, Millipore, Bedford, MA). Blots were blocked for 2 hours in PBS/ 0.1 % Tween/ 5 % BSA. Blots were subsequently incubated with 2 µg/ml biotinylated HAA in PBS/ 0.1 % Tween/ 2.5 % BSA overnight at 4ºC. After washing with PBS/ 0.1 % Tween, blots were incubated with HRP-conjugated streptavidin (Zymed) for 2 hours at room temperature. After extensive washing bands were visualized with Supersignal (Pierce Chemical Co., Rockford, IL) and exposure to HyperfilmTM films (Amersham Pharmacia).

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed rank test. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Dose dependent binding of IgA to Fc-$(CD89)_2$ in ELISA

To investigate the interaction of different molecular forms of IgA with CD89, IgA was purified from serum using a monoclonal anti-IgA affinity column and the eluate was size fractionated by gel filtration (Figure 1A). Pools containing mIgA or pIgA were obtained and assessed for the binding to Fc-$(CD89)_2$ in ELISA (Figure 1B). The binding of IgA was dose-dependent over a wide range of concentrations. Binding of pIgA to CD89 was much stronger than that of mIgA, the latter requiring a

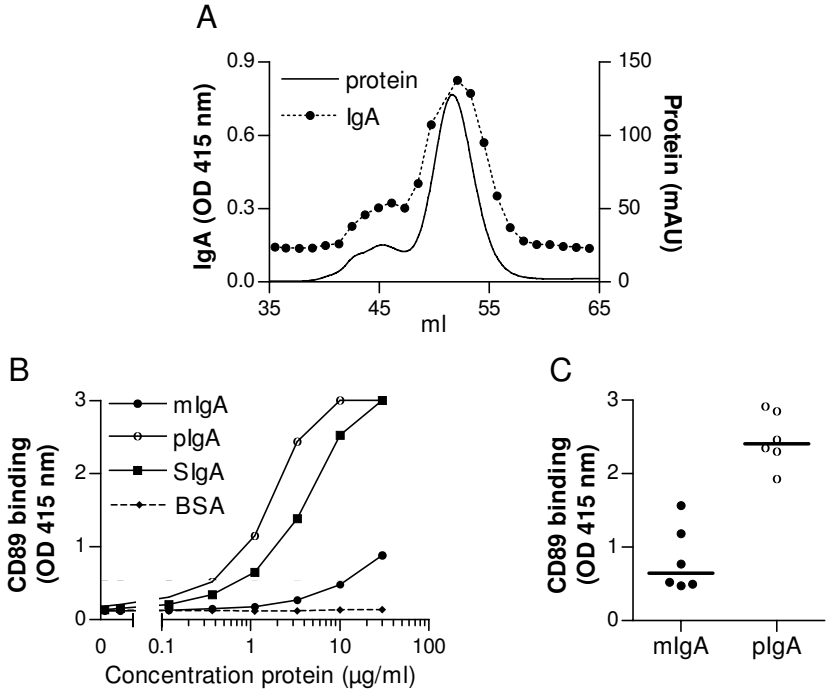

Figure 1: Binding of pIgA and SIgA to CD89 in ELISA is stronger than that of mIgA.

A) IgA was affinity purified and size-fractionated on a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA by ELISA. B) Isolated IgA was pooled in pIgA fraction (39-47.5ml) and mIgA fraction (47.5-57ml). Binding of mIgA, pIgA and SIgA to immobilized Fc-$(CD89)_2$ was measured in ELISA. C) From 6 individuals, IgA was purified and size fractionated as above. The binding of IgA (10 µg/ml) to Fc-(CD89)2 was measured in ELISA.

50-fold higher concentration to reach the same level of binding. We have reported earlier that in serum low concentrations of SIgA are present (22). Therefore we also tested the binding of SIgA to CD89. The binding of SIgA to CD89 was similar to that of pIgA, whereas it was much stronger than the binding of mIgA.

Using the same procedure, mIgA and pIgA were isolated from serum of 6 different individuals and tested for binding to Fc-$(CD89)_2$ in ELISA at a fixed concentration (Figure 1C). In all cases, pIgA bound stronger to Fc-$(CD89)_2$ compared to mIgA.

Difference in IgA binding is determined by dissociation from immobilized CD89 and not by association

The interaction of mIgA or pIgA with CD89 was evaluated using a biosensor. Two channels of a CM-5 sensor chip were coupled with recombinant CD89 and chimeric Fc-$(CD89)_2$. A third control channel was coupled with BSA. Monomeric IgA and polymeric IgA exhibited strong binding to both CD89 (not shown) and Fc-$(CD89)_2$

(Figure 2). The association of pIgA and mIgA to Fc-$(CD89)_2$, was similar while the dissociation was more rapid for mIgA compared to pIgA which led to a higher late binding level for pIgA (Figure 2A). SIgA binding was assessed as well and it was found that the association of SIgA to CD89 was lower compared with mIgA and pIgA. The binding of SIgA remained stable, comparable to that of pIgA (Figure 2A).

Similar results were obtained from IgA preparations from 6 different individuals (Figure 2B). The profiles of the binding of IgA isolated from 6 different individuals to Fc-$(CD89)_2$ were also analysed for the early (450 s) and late binding (1100 s). Monomeric and polymeric IgA, applied at identical concentrations, showed similar early binding to Fc-(CD89)2 (Figure 2B, C). The late binding assessed by SPR, in agreement with the measurements in ELISA, showed a stronger binding of pIgA to CD89 compared to mIgA (Figure 2B, C).

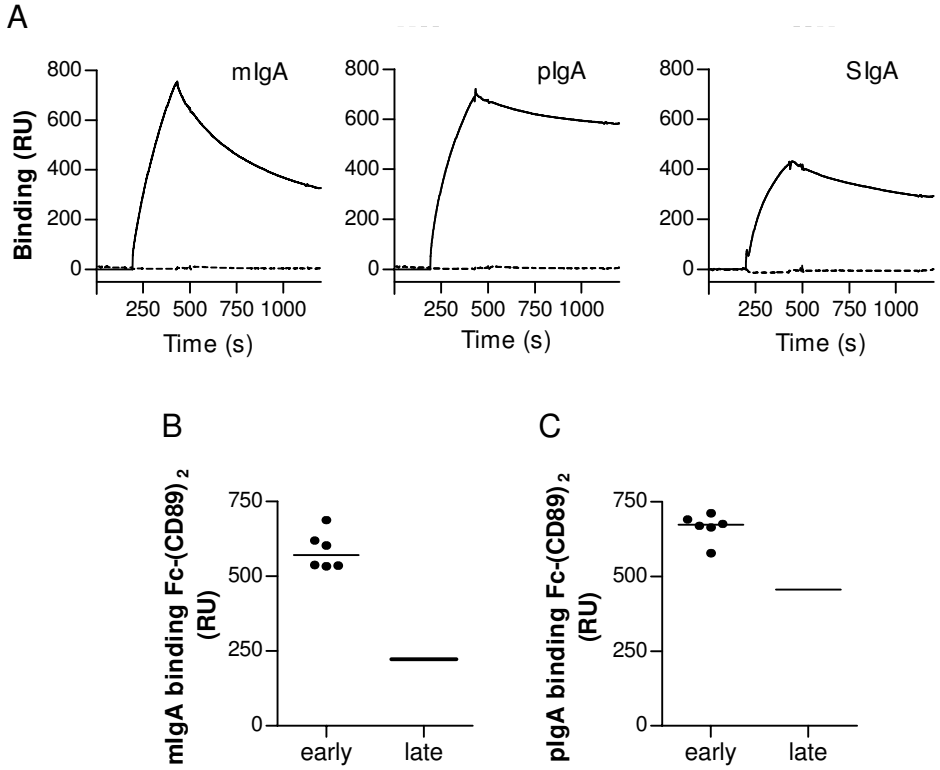

Figure 2: Real time binding of IgA to CD89.

A) Binding profile of mIgA (200 µg/ml), pIgA (200 µg/ml), isolated from serum, and SIgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. From 6 individuals, IgA was purified and size fractionated as above. The binding of mIgA B); (200 µg/ml) and pIgA C); (200 µg/ml) to Fc-$(CD89)_2$ was measured with SPR at two different time points (early (450 s) and late (1100 s) binding). Results represent response units of binding to Fc-$(CD89)_2$ after subtraction of response units obtained with BSA.

To determine the binding constants of the interaction of IgA with CD89, another CM-5 sensor chip was coupled with a low concentration of Fc-$(CD89)_2$ and BSA, and the binding of different concentrations of mIgA and pIgA was measured. To investigate the dissociation constants (kd) for mIgA and pIgA we made use of a bivalent model with a molecular mass of 180 kDa for mIgA and 360 kDa for pIgA. As presented above the dissociation of mIgA was more rapid compared with pIgA. Kinetic analysis showed a lower affinity for mIgA (kd = $3.9x10^{-4}$) compared to pIgA (kd = $2.6x10^{-4}$). These dissociation constants explain why pIgA ultimately remains associated with CD89 for a longer period, and also provide a rationale for the higher binding of pIgA as compared to mIgA to CD89 in ELISA.

Binding of deglycosylated IgA to CD89 is not changed while association is increased

Results presented above demonstrate that the interaction between pIgA and CD89 is more stable than the interaction between mIgA and CD89. Since previously published results suggest that underglycosylation promotes the production of polymeric IgA (4), and since the glycosylation of IgA has been implicated in its interaction with CD89 (23), we postulated that the glycosylation differences between monomeric and polymeric IgA could account for their differential interaction with CD89. Furthermore, underglycosylated IgA has been shown in serum of IgA nephropathy patients (24,25). This patient-derived IgA shows enhanced exposure of terminal GalNAc on O-linked glycans, which can be recognized by a GalNAc-specific lectin, *Helix Aspersa* (HAA). Previously, it has been reported that HAA might specifically bind to high molecular weight proteins in human serum (26), but interaction with pIgA has not been studied. The binding of HAA lectin to immobilized highly purified monomeric and polymeric IgA was studied by ELISA. We observed a dose-dependent binding of both mIgA and pIgA with HAA, with mIgA exhibiting significantly less binding to HAA as compared to pIgA (Figure 3A). These results suggest that pIgA, compared with mIgA, has smaller O-linked glycans with increased exposure of GalNAc.

To investigate whether exposure of terminal O-linked GalNAc might lead to an increased interaction of IgA with CD89, we treated mIgA with neuraminidase and galactosidase. Furthermore, to evaluate a possible role of N-glycans in the binding of IgA to CD89, mIgA was treated with PNGase F to remove N-linked sugars. These enzymatic treatments resulted in a clearly reduced MW of the IgA heavy chain as shown by SDS-PAGE, without affecting the light chain which is known to be non-glycosylated (Figure 3B). Increased exposure of terminal GalNAc after treatment of mIgA with neuraminidase and galactosidase was confirmed by a lectin-blot using biotinylated HAA (Figure 3B, C) and by using an ELISA system (Figure 3D). As expected, removal of N-glycans with PNGase F did not result in increased exposure of GalNAc (Figure 3B-D). Interestingly we observed that treatment of mIgA with neuraminidase alone already increased HAA reactivity, suggesting the presence of undergalactosylated O-linked glycans on mIgA.

To investigate whether exposure of terminal GalNAc or removal of N-glycans might enhance the interaction between IgA and CD89, we tested these well charac-

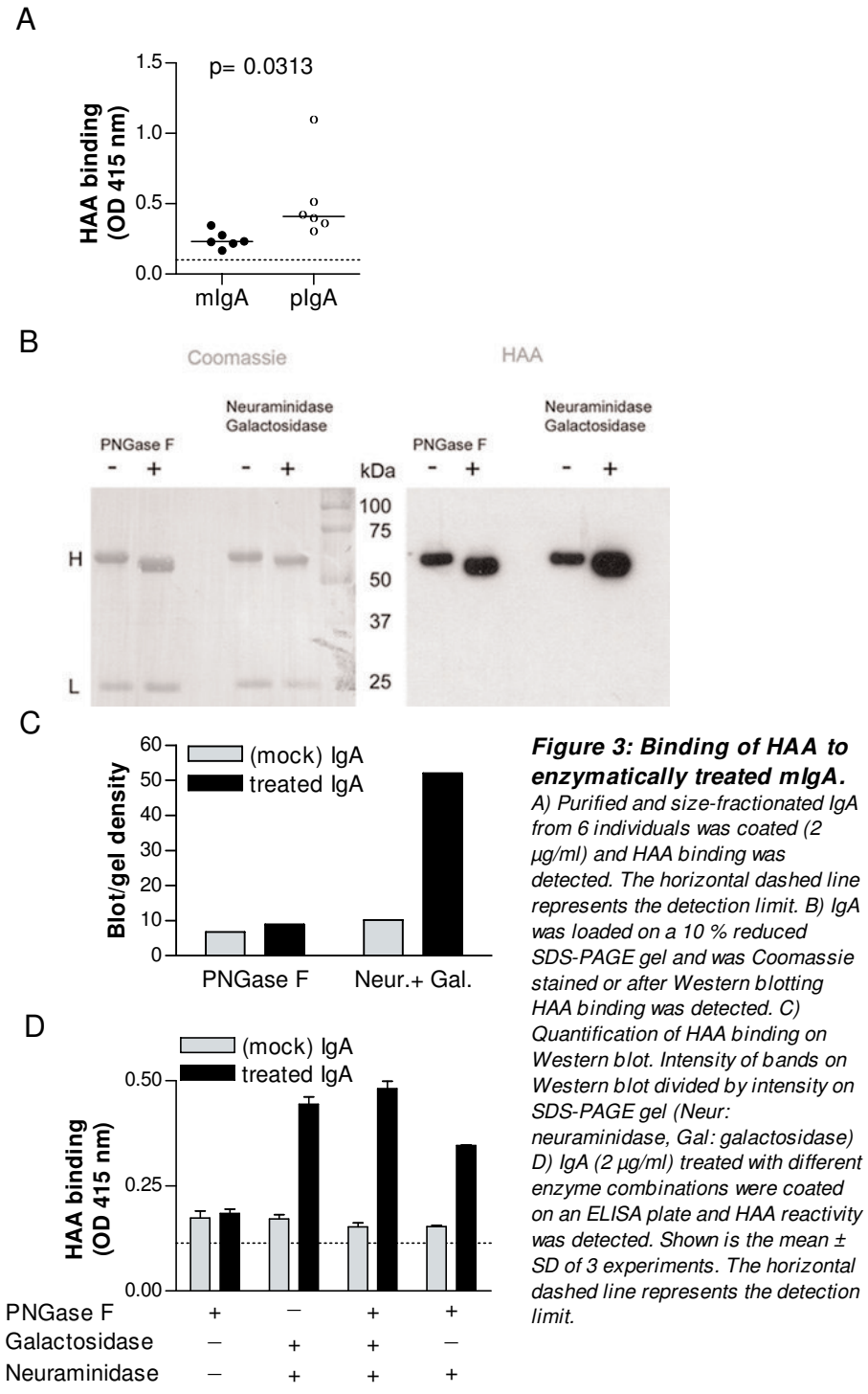

Figure 3: Binding of HAA to enzymatically treated mIgA.
A) Purified and size-fractionated IgA from 6 individuals was coated (2 µg/ml) and HAA binding was detected. The horizontal dashed line represents the detection limit. B) IgA was loaded on a 10 % reduced SDS-PAGE gel and was Coomassie stained or after Western blotting HAA binding was detected. C) Quantification of HAA binding on Western blot. Intensity of bands on Western blot divided by intensity on SDS-PAGE gel (Neur: neuraminidase, Gal: galactosidase) D) IgA (2 µg/ml) treated with different enzyme combinations were coated on an ELISA plate and HAA reactivity was detected. Shown is the mean ± SD of 3 experiments. The horizontal dashed line represents the detection limit.

terized deglycosylated IgA preparations for binding to Fc-$(CD89)_2$ using ELISA and SPR. Treatment of mIgA with combinations of neuraminidase, galactosidase and/or PNGase F did not change the binding of IgA to CD89 in ELISA (Figure 4A) and this was confirmed by showing a similar level of binding to CD89 in the late phase of SPR (Figure 4B, C). However, the early binding of mIgA after removing the N-glycans was increased compared to mock treated IgA or IgA treated with other enzymes (Figure 4B, D). These results suggest that N-glycans negatively affects the interaction of IgA with CD89.

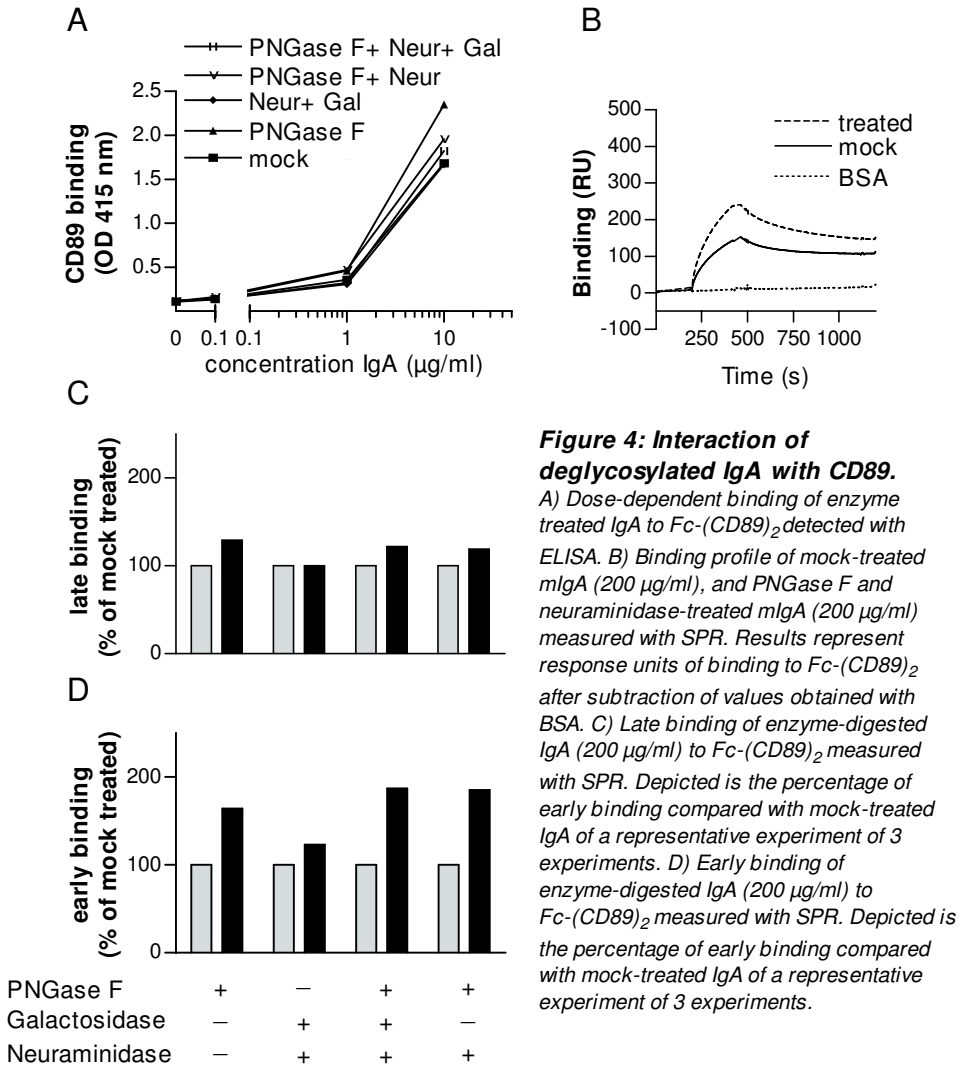

Figure 4: Interaction of deglycosylated IgA with CD89.
A) Dose-dependent binding of enzyme treated IgA to Fc-$(CD89)_2$ detected with ELISA. B) Binding profile of mock-treated mIgA (200 µg/ml), and PNGase F and neuraminidase-treated mIgA (200 µg/ml) measured with SPR. Results represent response units of binding to Fc-$(CD89)_2$ after subtraction of values obtained with BSA. C) Late binding of enzyme-digested IgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. Depicted is the percentage of early binding compared with mock-treated IgA of a representative experiment of 3 experiments. D) Early binding of enzyme-digested IgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. Depicted is the percentage of early binding compared with mock-treated IgA of a representative experiment of 3 experiments.

DISCUSSION

The present study provides evidence that the initial binding of mIgA and pIgA to CD89 by SPR were similar. However the dissociation of mIgA is more rapid than that of pIgA leading to a more sustained binding of the latter to CD89. Furthermore we show that SIgA is able to bind to CD89 without the presence of accessory molecules. It seems that N-glycans are important for the initial interaction of mIgA to CD89 because removal of the N-glycans by enzymatic treatment of mIgA resulted in enhanced association of IgA with CD89, whereas the final binding level remained the same as that for mock treated IgA. Taken together, these data provide more insight in the interaction of different molecular forms of IgA with CD89.

Binding studies with IgA and CD89 using mutagenesis and chemical modification showed that R82 and H85 in the FG loop of the first domain of CD89 are essential for the binding activity of CD89 (11). In a model (27), based on the KIR structure, it was suggested that the ligand binding site of CD89 is located in the first domain and not in the second domain, as described for other Fc receptors. The crystal structure of the IgA/CD89 recently confirmed these findings (28). The binding site for CD89 on IgA seems to overlap with that of bacterial IgA-binding proteins, suggesting involvement of the Cα2/Cα3 interdomain regions (29). Furthermore, using recombinant IgA molecules it has been shown that the presence of certain regions in both the Cα2 and Cα3 domains of IgA are required for efficient interaction with CD89 and for triggering of this receptor (14). Such a model (27) is consistent with a study that revealed a 2:1 stoichiometry for the CD89-IgAFc interaction (30,31). Furthermore, the crystal structure of the IgA1Fc-CD89 complex also showed that IgA could be in complex with two CD89 molecules (30). Our data indicate that mIgA molecules are able to interact similar with CD89 as pIgA, in contradiction with earlier suggestions (16,17).

In the circulation a large excess of mIgA is present and therefore CD89 might be permanently occupied with mIgA, thereby competing for CD89 binding by pIgA and prevents cell activation. The interaction of mIgA with CD89 might also be sufficient for internalization of mIgA via CD89, thereby creating intracellular CD89-IgA complexes which then can be secreted in the circulation (20).

In IgA nephropathy (IgAN), the most common primary glomerulonephritis with IgA deposits in the glomeruli, an altered O-glycosylation of the IgA1 molecule is described (24,32-34). Patient IgA1 contains smaller O-linked glycans with more terminal GalNAc and this could play a role in the deposition of IgA1 in the mesangial area of these patients (24,25,35). IgA treated with neuraminidase and galactosidase resembles the underglycosylated IgA as described in IgA nephropathy (24). In our studies we did not find a difference in binding of IgA treated with neuraminidase and galactosidase to CD89 as compared to control IgA. However after treatment of IgA with PNGase F, to remove the N-glycans, the initial interaction of mIgA with CD89 was enhanced, suggesting an important role for the N-glycans on IgA in the initial interaction with CD89. Several different N-glycans are described to be present on IgA, among which high mannose type N-linked glycan chains (36). These high mannose N-glycans may be likely candidates for the interaction of IgA with MBL (37). Recent studies have suggested the presence of MBL in association with IgA in the

mesangial area of patients with IgAN (38,39).

In literature it is still controversial whether N-glycans present on IgA do influence the interaction with CD89. One study showed that IgA with a mutation in ASN-263 in IgA, which lacks the Cα2 N-glycan, led to loss of binding to CD89 (23). However, another study showed a normal binding of the IgA1 mutant N263A to CD89 (32). In the first study mutant IgA was expressed in insect cells. This could lead to differences in the glycan structure (23). In the present study we show that deglycosylation of IgA could lead to an increase in early binding and therefore more interaction in the circulation, however this will not lead to an increase in late binding, which is in agreement with the data observed with mutant N263A. In the crystal structure of the IgA-CD89 complex (28), the observed N-glycans on IgA approach within 8 Å, but do not directly contact, the receptor (28). In CD89, a mutation in an amino acid that is not directly in contact with IgA resulted in an 11-fold loss of affinity (28). Because of the presence of the N-glycans on IgA close to these binding sites it could be that the N-glycans hamper the IgA/ CD89 interaction, but more research is needed to confirm this.

In summary we have shown that mIgA is able to interact with CD89 in a similar way as pIgA but that the interaction is more stable for pIgA than for mIgA. Furthermore we show that the N-glycans of IgA are important for the initial interaction with CD89. This binding of mIgA to CD89 could explain the observed presence of IgA-CD89 complexes in serum.

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