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A pathogenic role for secretory IgA in IgA nephropathy

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IgA nephropathy (IgAN) is characterized by deposits of IgA in the renal mesangium. It is thought that deposits of IgA mainly involve high molecular weight (HMW) IgA1. However, there is limited information on the exact composition of HMW IgA in these deposits. In this study, we investigated the presence of secretory IgA (SIgA) in human serum and in the glomerular deposits of a patient with IgAN. Furthermore, we analyzed the interaction of SIgA with mesangial cells. With enzyme-linked immunosorbent assay, SIgA concentrations in the serum of IgAN patients and healthy controls were measured. Both patients and controls had circulating SIgA that was restricted to the HMW fractions. Patients tended to have higher levels of SIgA, but this difference was not significant. However, in patients with IgAN, high serum SIgA concentrations were associated with hematuria. Binding of size-fractionated purified serum IgA and SIgA to mesangial cells was investigated with flow cytometry. These studies showed stronger binding of SIgA to primary mesangial cells compared to binding of serum IgA. Importantly, after isolation and elution of glomeruli from a nephrectomized transplanted kidney from a patient with recurrent IgAN, we demonstrated a 120-fold accumulation of SIgA compared to IgA1 in the eluate. In conclusion, we have demonstrated that SIgA strongly binds to human mesangial cells, and is present in significant amounts in serum. Furthermore, we showed that SIgA is accumulated in the glomeruli of an IgAN patient. These data suggest an important role for SIgA in the pathogenesis of IgAN.

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Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease shows a spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA in the glomerular mesangium.[1–3] It is generally thought that deposits of IgA mainly involve IgA1 and, for a large part, consist of high molecular weight (HMW) IgA.[4] The composition of HMW forms of IgA in the serum is diverse and may include dimeric IgA, CD89/IgA complexes, IgA immune complexes and IgA–fibronectin complexes.[5–9]

Several reports have shown that the glycosylation of IgA1 in patients is different from that in controls. Patient IgA1 contains more terminal GalNAc, and this could play a role in the deposition of IgA1 in the mesangium.[10–12] IgA from the glomeruli of IgAN patients consists at least partly of HMW IgA[13] and is under-O-glycosylated.[10,14] In these studies, no data was presented concerning the presence of secretory IgA (SIgA). When deposited in the kidney, the IgA1-containing complexes are linked to inflammation. Stimulation of mesangial cells with HMW IgA leads to enhanced production of cytokines and chemokines, including interleukin (IL)-6, transforming growth factor-β, tumor necrosis factor-α, monocyte chemoattractant protein-1, IL-8, and migration inhibitory factor.[15–18] These cytokines and chemokines might play a role in the development and progression of renal injury in IgAN patients.

SIgA is the dominant immunoglobulin in external mucosal secretions like oral, respiratory and intestinal cavities, and is often characterized as a component of the immune systems' 'first-line defense' against pathogenic microorganisms.[19] The SIgA molecule is composed of two IgA monomers, linked by a junction peptide called J chain, and the secretory component (SC) that wraps around the dimer.[20] Next to its presence in mucosal secretions, small amounts of SIgA can also be found in human serum.[21,22] Moreover, increased serum levels of SIgA have been reported in various diseases,[23–25] indicating that SIgA may be a marker of clinical interest. A previous study has suggested that the serum concentrations of SIgA are not different in IgAN patients compared to healthy controls.[23] The physiological roles of serum IgA and SIgA are quite different, and the presence of the highly glycosylated SC can have major effects

on the biological functions of SIgA.[26,27] In the literature, it is still controversial if SIgA is able to bind to mesangial cells.[28,29] One study showed that SIgA is able to bind to mesangial cells;[28] however, this could not be confirmed in another study.[29]

In the present study, we investigated the presence of SIgA in the sera of IgAN patients and healthy controls, and examined the binding of different molecular forms of IgA to human mesangial cells with special interest for SIgA. Finally, we investigated the presence of SIgA in the glomerular eluate of an IgAN patient.

RESULTS

Specific detection of SIgA in human serum

An enzyme-linked immunosorbent assay (ELISA) system was developed to specifically measure the amounts of SIgA in the serum. In this system, anti-SC antibodies (Abs) were coated, samples were applied and SIgA was detected with anti-IgA Abs. Purified SIgA was readily detected by this ELISA with a detection limit of 100 ng/ml (Figure 1a). In contrast, purified monomeric serum IgA is not recognized in the ELISA, even when applied at high concentrations (Figure 1a). In accordance with previous publications,[23] a specific signal for SIgA could be detected in the serum of healthy individuals (Figure 1b). The specificity of this assay is especially dependent on the specificity of the anti-SC Ab. Using Western blot, we showed that this monoclonal Ab only recognized the 75 kDa SC (Figure 2a). Furthermore, comparison of the NI194-4 Ab with another anti-SC Ab 3F8[30] showed specificity for SIgA, both in a direct ELISA (Figure 2b) and in a sandwich ELISA (Figure 2c).

To determine the molecular size of SIgA in the serum, IgA was isolated from the serum using affinity chromatography. Size fractionation revealed that SIgA was specifically present in the HMW fractions (Figure 3a). Using the same procedure, IgA was isolated from eight healthy controls followed by gel filtration. Pools containing polymeric serum IgA (pIgA) and monomeric serum IgA (mIgA) were obtained and assessed for the amount of SIgA (Figure 3b). In all cases, SIgA was demonstrated exclusively in the pIgA pool.

Next, we assessed concentrations of SIgA in the serum of 47 IgAN patients and 19 healthy controls (Figure 4a). Both in controls and in patients, significant serum concentrations of SIgA were detected. There was no significant difference ($P = 0.159$) in the SIgA concentrations in the serum of patients ($3.3 \pm 3.0\ \mu$g/ml) compared to that of controls ($2.2 \pm 1.2\ \mu$g/ml). After comparison of the SIgA concentration in the serum of IgAN patients with different clinical parameters, there were no correlations found. However, concentrations of SIgA in IgAN patients were more heterogeneous than in controls. Therefore, we divided the IgAN group into patients with elevated levels of SIgA ($\geqslant 5\ \mu$g/ml, mean $\pm$ 2 s.d. of control sera) and patients with normal levels ($< 5\ \mu$g/ml). There were no significant correla-

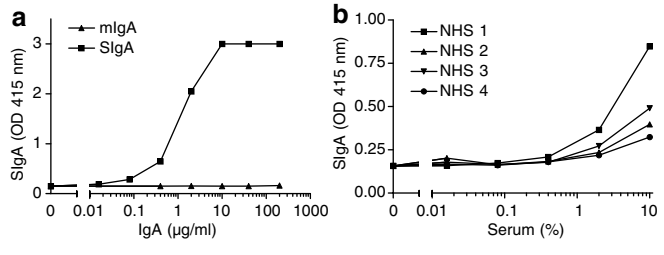

Figure 1 | Specific detection of SIgA in human serum. (**a**) Purified SIgA and monomeric serum IgA were measured in a sandwich ELISA, as detailed in the Materials and Methods. (**b**) Measurement of SIgA in serum from healthy individuals in different serum dilutions. NHS, normal human serum.

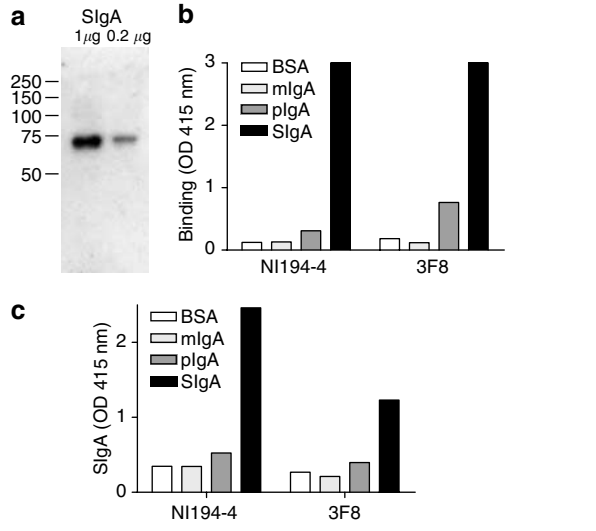

Figure 2 | Specific detection of SIgA and secretory component. (**a**) Detection of secretory component in SIgA, 1 and 0.2 μg SIgA were loaded on 10% SDS-PAGE gel under reducing conditions after blotting secretory component was detected with 2 μg/ml NI194-4. (**b**) Detection of secretory component with NI194-4 and 3F8 (2 μg/ml) in different molecular forms of IgA (2 μg/ml) coated on 96- well Nunc maxisorp microtiter plate. (**c**) Different forms of IgA measured with sandwich ELISA using two different coating antibodies (NI194-4 and 3F8).

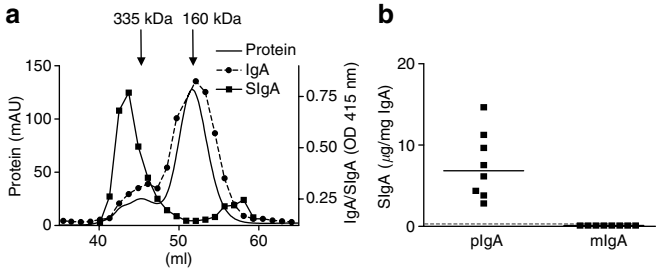

Figure 3 | SIgA is present in high molecular weight fractions of serum IgA. (**a**) IgA was affinity purified and size fractionated on a HiLoad™ 16/60 HR200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA and SIgA by ELISA. (**b**) From eight healthy controls, IgA was purified and size fractionated as above. IgA was pooled in pIgA fraction (39–47.5 ml) and mIgA fraction (47.5–57 ml). Both IgA and SIgA concentrations were determined, and depicted is the amount of SIgA corrected for the amount of total IgA. The horizontal dashed line represents the detection limit. The horizontal solid lines indicate the median.

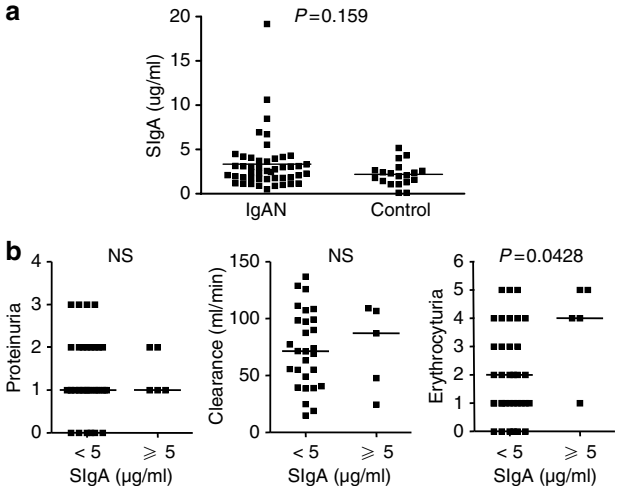

Figure 4 | Measurement of SIgA in the serum of IgAN patients and controls and relation to clinical parameters. (**a**) The concentrations of SIgA were determined in serum from patients ($n = 47$) and controls ($n = 19$). The horizontal dashed line represents the detection limit and the horizontal solid lines indicate the median. (**b**) Based on SIgA concentrations, IgAN patients were divided in two groups: normal SIgA levels ($< 5\ \mu$g/ml; mean + 2 s.d. of control sera) and increased SIgA levels ($\geqslant 5\ \mu$g/ml). These groups were analyzed for proteinuria, creatinine clearance (700*: 700 multiply with creatinine in urine (mmol/24 h)/creatinine in serum (μmol/l)) and erythrocyturia at the time of sampling. The horizontal solid lines indicate the median. Statistics were performed using the Mann–Whitney test (NS = not significant).

tions between the degree of proteinuria or creatinine clearance and the serum concentration of SIgA (Figure 4b). However, there was significantly more pronounced hematuria in the group with higher levels of SIgA ($P = 0.04$) (Figure 4b).

SIgA binds to mesangial cells and induces cell activation

After demonstrating the presence of SIgA in the circulation, we investigated its capacity to interact with mesangial cells. After incubation of the mesangial cell line adult mesangial cells (AMC)11 or normal human mesangial cells (NHMC) with 200 μg/ml of different molecular forms of IgA, the binding was examined by fluorescence-activated cell sorting (FACS) analysis. Binding of mIgA to NHMC is very low (Figure 5a). In contrast, the polymeric form of serum IgA showed a clear binding to mesangial cells. However, the best binding was observed with similar concentrations of SIgA, which occurred in a dose-dependent manner, present over a wide range of concentrations (Figure 5b). Although the mean fluorescence intensity of IgA binding to NHMC, compared with AMC11, was overall higher for all forms of IgA, the relative differences between the different forms of IgA were the same.

The different molecular forms of IgA were compared for their capacity to induce IL-6 production by mesangial cells. Stimulation of NHMC for 72 h with IgA resulted in an increased IL-6 production. This increase was most prominent following stimulation with SIgA (Figure 6a). The induction of IL-6 production by mesangial cells was dose dependent: a 17-fold increase was observed compared to the negative control upon stimulation with 200 μg/ml SIgA (Figure 6b).

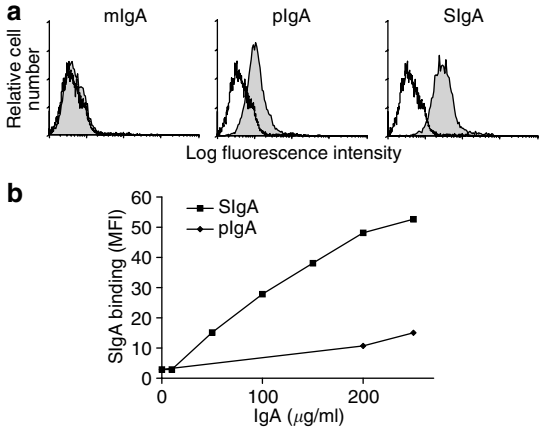

Figure 5 | Binding of SIgA to mesangial cells. (**a**) Normal human mesangial cells (NHMC) were incubated with different molecular forms of IgA (200 μg/ml) and assessed for IgA binding by flow cytometry. Filled histograms represent the binding of IgA and the open histograms represent the control staining with secondary antibodies. (**b**) FACS analysis with AMC11 was performed using increasing doses of SIgA (Sigma) and pIgA (isolated from a healthy individual). Depicted is the mean fluorescence intensity (MFI).

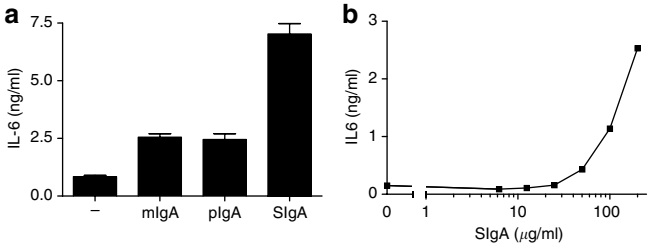

Figure 6 | SIgA increase IL-6 production by mesangial cells. (**a**) Normal human mesangial cells (NHMC) (25×10^3 cells/well) were stimulated with different molecular forms of IgA (200 μg/ml). After 72 h, supernatants were harvested and tested for IL-6 using ELISA. Depicted is the mean ± s.d. (**b**) NHMC was stimulated with different concentrations of SIgA (Sigma) as described above and IL-6 production was assessed.

Mesangial cells do not express the mannose receptor (CD206) or MAC-1 (CD11b/CD18)

Recently, we showed that dendritic cells (DCs) are able to bind SIgA via the mannose receptor (CD206).[31] Therefore, we investigated the presence of the mannose receptor as a potential SIgA receptor on mesangium cells. However, using both FACS analysis (Figure 7a) and reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 7b), we were not able to demonstrate the presence of the mannose receptor on mesangial cells. In both cases, DCs served as a positive control. Similarly, we were not able to demonstrate the presence of CD11b/CD18 (Figure 7c), recently identified as a coreceptor for SIgA binding.[32]

Binding of SIgA to mesangial cells is not inhibited by SC, EDTA and calcium

To investigate in more detail the mechanism of binding of SIgA to mesangial cells, the cells were preincubated with free

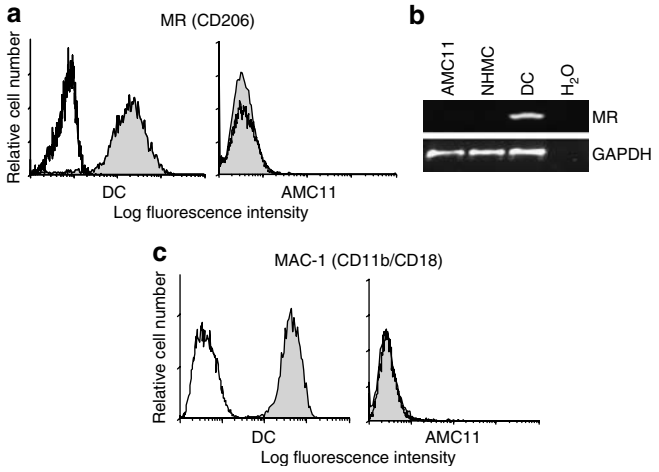

Figure 7 | Mannose receptor and MAC-1 are not present on mesangial cells. (**a**) FACS analysis of mannose receptor on DC and AMC11. Filled histograms represent expression of mannose receptor; open histograms represent the control staining with secondary antibodies. (**b**) Mannose receptor mRNA expression was analyzed by RT-PCR as described in the Materials and Methods (**c**) Presence of MAC-1 on mesangial cells and DC was tested with FACS analysis. Filled histograms represent expression of MAC-1. Open histograms represent the control staining with secondary antibodies.

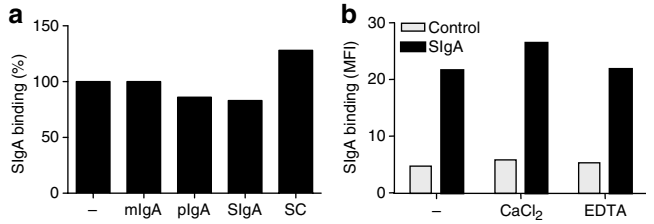

Figure 8 | Binding of SIgA to mesangial cells is not affected by free secretory component or calcium but is affected by IgA. (**a**) Mesangial cells were preincubated with mIgA, pIgA, SIgA (400 μg/ml) or free secretory component (SC) (100 μg/ml). After 1 h, SIgA (200 μg/ml) was added and the binding of SIgA to mesangial cells was examined with flow cytometry. Depicted is the percentage of SIgA binding of a representative experiment of two experiments. (**b**) Cells were incubated with SIgA (200 μg/ml) in the presence or absence of EDTA (10 mM) or calcium (10 mM) followed by detection of SIgA binding as described in the Materials and Methods.

SC. After preincubation with SC, the binding of SIgA to mesangial cells was not affected (Figure 8a). Binding of SIgA was slightly inhibited with SIgA and pIgA but not with mIgA (Figure 8a), suggesting interaction with the IgA part of the molecule.

To investigate the potential contribution of C-type lectins, a family of cell surface molecules including the mannose receptor, the role of calcium in SIgA binding to human mesangial cells was examined. However, neither the addition of extra calcium nor the removal of calcium using ethylenediaminetetraacetic acid (EDTA) showed a significant effect on the binding of SIgA to mesangial cells (Figure 8b).

Table 1 | Immunoglobulin concentrations in serum and glomerular eluate of IgAN patient

	IgA1	IgA2	SIgA	IgM	IgG
Serum (mg/ml)	5.9	0.59	0.016	0.74	10.0
Glomerular eluate (μg/ml)	6.1	0.65	2.0	2.2	6.8

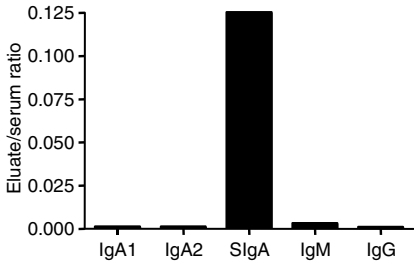

Figure 9 | SIgA is accumulated in the glomerular eluate of an IgAN kidney. To make an estimate for the specific accumulation in the glomerular deposit, immunoglobulin concentrations in the eluate and serum were compared. Depicted is the ratio of concentration of different immunoglobulins in the eluate and the serum.

SIgA is present in the glomerular eluate from a kidney of an IgAN patient

To determine the potential role of SIgA in the pathogenesis of IgAN, we had the unique opportunity to analyze the glomerular eluate of a nephrectomized specimen derived from a patient with recurrent IgAN. After elution, concentrations of specific immunoglobulin isotypes were determined. In the glomerular eluate, all immunoglobulin classes measured were detectable (Table 1), including SIgA in a concentration of 2 μg/ml. To exclude that the immunoglobulins in the eluate were the result of aspecific trapping from the circulation, serum immunoglobulin levels were determined in the serum of this patient at the time of nephrectomy (Table 1). The ratio of the immunoglobulin concentrations in the eluate and the serum can be used as a measure of the specific accumulation in the glomerular deposit (Figure 9). In this analysis, we observed a ratio for SIgA, which was 120-fold higher that the ratio for IgA1.

DISCUSSION

This is the first study to support a role for SIgA in the pathogenesis of IgAN. We show that SIgA is present in low concentrations in the serum of healthy individuals as well as in IgAN patients. In patients with higher SIgA serum concentrations, hematuria is more pronounced. Furthermore, we show that SIgA exhibits the strongest binding to mesangial cells than serum IgA. Finally, in the eluate of glomeruli from a kidney of an IgAN patient, a strong accumulation of SIgA was detected. Taken together, these data suggest an important role for SIgA in the pathogenesis of IgAN.

The high incidence of IgAN recurrence after renal transplantation, and the disappearance of deposits of IgA from accidentally transplanted kidneys clearly suggests that intrinsic alterations and/or structural characteristics of IgA

contribute to the process of deposition.[33,34] The predominance of IgA1 deposits and the specific hinge region of IgA1 with potential O-linked glycosylation sites have initiated a directed search for alterations in glycosylation. Indeed, both in the serum, but more importantly, in the eluate of renal deposits,[14] a specific reduction of O-linked galactosylation has been observed.[10,12,14,35] Furthermore, with size fractionation of eluted proteins from kidney sections, it was shown that deposited IgA was mostly HMW of nature.[13] In addition, based on different staining methods, it has been proposed that a large part of the deposited IgA is of HMW of nature.[36–38]

The results from all these methods provide indirect indications for the composition of the IgA1 deposits. We now show by eluting glomeruli that glomeruli display a strong and specific accumulation of SIgA compared to other serum immunoglobulins. However, this technique can only be applied in limited cases of situations. We have tried to demonstrate the presence of SIgA deposits using traditional immunofluorescence on cryosections. Until now, we were not able to show SIgA in renal sections, not even in cryosections of the kidney used for our elution study. This might be due to inappropriate reagents or conformational changes of the deposited SIgA, thereby masking the SC epitope. Therefore, it will be necessary to generate other reagents for the detection of deposited SIgA and a more thorough analysis of renal biopsies.

As described above, it is assumed that glycosylation of IgA is an important factor in IgAN. Previous studies have shown the role of the glycosylation of IgA on the activation of mesangial cells,[39,40] which could be partially explained by altered interaction with mesangial cells.[41] The glycosylation of SIgA is different compared to that of serum IgA in several aspects. First, SIgA is a tetramolecular complex consisting of two IgA molecules, a J chain and the SC wrapped around the H chain. Modelling of SIgA suggests that the N-glycans on the heavy chain can be masked by the SC.[42] This may also result in a different exposure of the O-glycans. Moreover, specific analysis of the glycosylation of the IgA heavy chain present in SIgA demonstrated different N-glycan structures compared to that of serum IgA, with terminal GlcNAc residues on the majority of the N-glycans.[42] The O-glycans on the hinge region of the heavy chain of SIgA1 presented a wide range of glycan structures, of which the major part is now characterized.[42] Finally, the SC itself is also heavily glycosylated. However, we were not able to inhibit the binding of SIgA to mesangial cells with SC, suggesting that the SC part is not important for interaction with mesangial cells. It will be a major challenge to isolate SIgA from the serum of IgAN patients and to determine specific alterations in glycosylation.

Having shown that SIgA strongly binds to mesangial cells, an important question is which receptor is involved in this binding and whether this receptor is different from that of serum IgA. Several IgA receptors have been described in the literature. The best-known receptors, the polymeric Ig receptor (pIgR), the asialoglycoprotein receptor and CD89, have already been described to be absent on mesangial cells.[28,43,44] The transferrin receptor is described as an IgA receptor present on mesangial cells, but it has been reported that this receptor is not able to bind SIgA.[29] We have previously reported the binding of SIgA to the mannose receptor on DCs,[31] but we were not able to demonstrate the presence of the mannose receptor on mesangial cells. Similarly, we were not able to demonstrate the presence of CD11b/CD18 (MAC-1), a coreceptor for CD89 specifically involved in recognizing SIgA or free SC.[32] Therefore, until now, we have no indication for the mesangial IgA receptor involved in SIgA binding. Our inhibition experiments suggest that IgA rather than SC is recognized and that no C-type lectin is involved. Importantly, we found that the putative receptor is able to transmit proinflammatory signals, since SIgA induced a strong dose-dependent increase in IL-6 production by mesangial cells. This seems in contrast with the proposed anti-inflammatory role of SIgA.[45]

Generation of SIgA, that is, production of dimeric IgA (dIgA) followed by transcytosis using the pIgR, is a specific process taking place at mucosal surfaces.[46] Interestingly, IgAN patients often present macroscopic hematuria following upper respiratory tract infections. Mucosal challenge also leads to an increased production of IgA in the systemic compartment, probably based on the migration of B cells (the mucosa–bone marrow axis).[47] This mucosa–bone marrow traffic has been confirmed by challenging healthy individuals intranasally with the neoantigen cholera toxin subunit B (CTB).[48] In patients with IgAN, we observed a reduced mucosal IgA response to mucosal immunization with CTB.[48] At present, it is not clear whether a mucosal challenge also regulates levels of circulating SIgA. Still, our finding of glomerular accumulation of SIgA provides a link between the mucosal immune system and renal deposits.

In summary, we have shown that SIgA is able to bind to mesangial cells in a dose-dependent manner and that this binding is calcium independent and cannot be inhibited with free SC. Furthermore, low concentrations of SIgA can be detected in serum. Further research is needed to determine to which receptor SIgA can bind to and what the mechanism of cell activation induced by SIgA. SIgA is strongly accumulated in the glomeruli of a kidney from an IgAN patient. Therefore, we conclude that our data support a role for SIgA in the pathogenesis of IgAN, and further research to define such a pathogenic role is warranted.

MATERIALS AND METHODS

Human subjects

In this study, we included 19 healthy volunteers and 47 patients with primary IgAN.[49] The latter were defined by mesangial deposits of IgA. None of these patients had clinical or laboratory evidence of Henoch Schoenlein purpura, systemic lupus erythematosus, liver disease, or received immunosuppressive therapy. Patients were included in the study between October 1998 and February 1999. Blood and urine samples were collected, clinical characteristics and

Table 2 | Clinical characteristics of the patients with IgAN at the point of serum SIgA measurement

	Number of patients	Median	Range
Male/female	38/9		
ACE-inhibitor/AII antagonist	24/3		
Age (years)		47	19–69
Systolic blood pressure (mmHg)		130	100–160
Diastolic blood pressure (mmHg)		80	55–100
Proteinuria (g/day)		0.6	0.1–5.2
Glomerular filtration rate (ml/min)		71	15–137

laboratory data of the patient group were obtained (Table 2) retrospectively (observation time 5.9 ± 0.5 years) and prospectively (follow-up time 3.7 ± 0.2 years).

Renal cortex was obtained from a transplant nephrectomy from a male patient (1977), with biopsy-proven recurrent IgAN. He was diagnosed with macroscopic hematuria (serum creatinine 146 μmol/l) in 1993. The renal biopsy of 1996 showed characteristic features of progressive IgAN, and dialysis was started. In April 2002, he received a cadaveric renal transplantation. Three months later, a decline in renal function was observed and a renal biopsy showed the presence of interstitial nephritis and recurrent IgAN. In a biopsy taken 6 months following transplantation, characteristics of the interstitial nephritis disappeared, but IgA was still present. Owing to decreased patient compliance, there were two episodes of acute rejection in 2003 and 2004, which eventually led to graft loss. Informed consent was obtained from all subjects.

IgA purification

Serum from healthy controls was used for IgA purification, according to methods described earlier.[9] 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. IgA was eluted with 0.1 M glycine/0.3 M NaCl (pH 2.8). The eluted protein fractions containing IgA, as assessed by ELISA,[50] were pooled and dialyzed. The IgA was size separated with a HiLoad™ 16/60 HR200 Superdex prep grade gelfiltration column (120 ml; Amersham Pharmacia, Roosendaal, The Netherlands) into pIgA and mIgA.

Cell culture

NHMC (Cambrex, NJ, USA) were expanded according to the protocol provided by the manufacturer. Experiments with NHMC were performed in RPMI with 10% fetal calf serum (FCS), 1% nonessential amino acids, 0.5% transferrin/insulin/selenium, 1% sodium pyruvate, and 1% L-glutamine (all purchased at Gibco/life Technologies, Paisley, Scotland). AMC11, a spontaneously growing adult human mesangial cell line (kindly provided by Professor Holthofer, Helsinki, Poland), was cultured in Dulbecco's modified Eagle's medium with 10% FCS. Cells were cultured in culture flasks (Greiner, Frickenhausen, Germany) at 37°C in a humidified incubator with 5% CO_2/95% air. For passage, the cells were harvested by trypsinization (0.02% (w/v) EDTA/0.05% (w/v) trypsin in phosphate-buffered saline (PBS) from Sigma (St Louis, MO, USA)).

Monocyte-derived DCs were generated as described earlier.[51]

Flow cytometry

Cells were harvested, washed with FACS buffer ($0.5 \times$ PBS containing 1% bovine serum albumin (BSA)/2.8% glucose/0.01% NaN_3) and incubated with mIgA and pIgA, and SIgA (Sigma). Following incubation for 1 h at 4°C, cells were washed and incubated for 1 h at 4°C with monoclonal anti-IgA Ab 4E8 (IgG1).[50] IgA binding was visualized using phycoerythrin (PE)-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL, USA) and assessed for fluorescence intensity by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences, San Jose, CA, USA). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

For analyzing the presence of the mannose receptor, anti-mannose receptor Ab (D547.3; kindly provided by F Koning, Leiden University Medical Center, Leiden, The Netherlands) was used followed by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark). For the detection of the two chains of MAC-1, anti-human CD11b (leu-15-PE; BD Biosciences) and anti-CD18 (IB4; ATCC, Manassas, VA, USA) were used.

For inhibition of the binding of SIgA to mesangial cells, the cells were preincubated with purified free SC (100 μg/ml),[52] 10 mM EDTA or 10 mM $CaCl_2$ for 1 h at 4°C. Subsequently, without washing, SIgA was added and this binding was visualized as described above. Inhibition of binding of SIgA was also detected with a preincubation of IgA for 1 h at 4°C, and subsequently, Alexa-conjugated SIgA was added. After 1 h, the binding of Alexa-conjugated SIgA was measured.

Cytokine analysis

Production of IL-6 was measured in supernatants of mesangial cells after 72 h stimulation. Prior to stimulation, cells were transferred to 48-well plates (Costar, Corning, NY, USA) at a density of 25×10^3 cells/well and cultured overnight in culture medium with 0.5% serum. Cells were stimulated with IL-1α, serum IgA, or SIgA. The concentration of IL-6 in culture supernatants was measured by specific ELISA as described previously.[53]

RNA extraction and RT-PCR

Total RNA was extracted from mesangial cells using RNeasy mini kit (Qiagen, Valencia, CA, USA). Optical density (OD)260/280 ratio was measured to determine the quantity and purity of RNA preparations. Fixed amounts of total cellular RNA (1 μg) were reverse transcribed into cDNA by oligo(dT) priming, using Moloney-murine leukemia virus reverse transcriptase (Gibco/Life Technologies, Breda, The Netherlands). PCR to detect the human mannose receptor was performed with specific primers (sense 5′-TTG AGT GGA GTG ATG GGA CC-3′; antisense 5′-TTT CTG GAC CTT GGC TTC GT-3′) using AmpliTaq DNA polymerase (Applied Biosystems, Roche, Mannheim, Germany). The PCR reaction was performed under standard conditions.[54] The cDNA samples were also subjected to PCR for glyceraldehyde-3-phosphate dehydrogenase as an internal control[54] PCR products were resolved on 1% agarose gels and bands were visualized by ethidium bromide staining.

Preparation of glomerular eluate

Glomeruli were isolated from a nephrectomized kidney from a transplanted IgAN patient with recurrent disease. For this purpose, the renal cortex was separated from the medulla. After slicing the cortex into little pieces, the glomeruli were collected on a 150-mesh sieve and stored at −70°C with protease inhibitors (Complete, Mini, Roche). The glomeruli were washed with PBS and the final pellet was resuspended in 5 ml of elution buffer (2 M potassium thiocyanate in 0.01 M phosphate buffer, pH 7.6) as described earlier.[55] After stirring at room temperature (RT) for 60 min, the

suspension was centrifuged at 8000 *g* for 15 min at 4°C in a high-speed centrifuge (Beckman, Avanti J25-1). The supernatant was collected and dialyzed overnight against PBS. The precipitate that was formed during dialysis was removed by centrifugation for 15 min at 17 000 *g* and was negative for immunoglobulins. The remaining supernatant was concentrated to one-third of the original volume.

ELISA for human SIgA, IgA1, IgA2, IgG and IgM

To test the specificity of the Abs used for the SIgA ELISA, 96-well Nunc Maxisorb microtiter plate (Gibco/Invitrogen, Carlsbad, CA, USA) was coated with 2 μg/ml IgA and BSA in carbonate buffer (pH 9.6) overnight at RT. After washing, the plate was incubated with monoclonal Abs (2 μg/ml) specific for SC: NI194-4 (IgG1-k; Nordic Immunology, Tilburg, The Netherlands)[56] or 3F8 (kindly provided by Dr RM Goldblum, University of Texas Medical Branch, Galveston, TX, USA)[30] in PBS/1% BSA/0.05% Tween for 1 h at 37°C. Bound Ab was detected with goat anti-mouse Ig horseradish peroxidase (HRP) (Dako). Enzyme activity of HRP was developed using 2,2′-azino-di(3-thylbenstialozone (ABTS) (Sigma). The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, CA, USA).

In order to quantify SIgA levels in isolated IgA and serum, a sandwich ELISA specific for SIgA was developed. Plates were coated with 2 μg/ml monoclonal Ab specific for SC (NI194-4; 3F8) in carbonate buffer (pH 9.6) (100 μl/well) overnight at RT. Subsequently, the plates were washed with PBS/0.05% Tween. Plates were incubated with IgA or with serum from patients and controls in PBS/1% BSA/0.05% Tween for 1 h at 37°C. After washing, bound IgA was detected using mouse anti-human IgA (4E8) conjugated to digoxigenin (Dig), followed by $F(Ab)_2$ anti-Dig Abs conjugated to HRP (Roche). Enzyme activity of HRP was developed using ABTS (Sigma). The OD at 415 nm was measured. A calibration line was produced using purified SIgA (Sigma).

Concentrations of IgA1, IgA2, IgG, and IgM in sera and glomerular eluate were determined using specific ELISAs.[57]

Western blot analysis

IgA preparations were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, followed by semidry blotting to polyvinylidine difluoride (immobilin-P, Millipore, Bedford, MA, USA). Blots were blocked for 2 h at RT in TBS/0.1% Tween/5% skim milk powder (Fluka, Buchs, Switzerland). Blots were subsequently incubated with 2 μg/ml monoclonal Ab specific for SC (NI194-4) in TBS/0.1% Tween/2.5% skim milk powder overnight at 4°C. After washing with TBS/0.1% Tween, blots were incubated with HRP-conjugated goat anti-mouse immunoglobulin (Dako) for 2 h at RT. After washing, bands were visualized with Supersignal (Pierce Chemical Co., Rockford, IL, USA) and exposure to Hyperfilm™ films (Amersham Pharmacia).

Statistical analysis

Statistical analysis was performed using the Mann–Whitney test. Differences were considered statistically significant when *P*-values are less than 0.05.

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