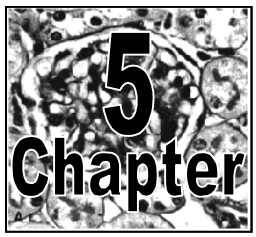

A novel receptor for IgA expressed on human renal mesangial cells[#]

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SUMMARY

Primary IgA-Nephropathy (IgAN) is associated with deposition of IgA in the renal mesangium. It has been demonstrated that IgA can bind to human mesangial cells (MC) in culture and can induce activation of this cell-type leading to increased expression of interleukin-6, tumor necrosis factor α and c-jun. In contrast to earlier reports, we have recently shown that the myeloid receptor for IgA (FcαRI/CD89) is not expressed on human MC and that blocking anti-CD89 antibodies do not inhibit binding of IgA to MC. To further identify alternative IgA receptors on MC, we raised mouse monoclonal antibodies against IgA-binding proteins isolated from neutrophils. We found one monoclonal antibody, 72G12, which reacts with MC by FACS and inhibited the binding of IgA to these cells in vitro. Precipitation experiments of biotin labeled surface proteins from human MC with 72G12 revealed a protein band of 83 kD. Precipitation experiments using human IgA revealed enrichment of a protein with a similar size. Preclearing experiments with human IgA, followed by precipitation with 72G12 resulted in an almost complete reduction of precipitation of the described protein band, indicating that human IgA and 72G12 bind to the same protein. These results strongly suggest that human MC express a novel 83kD IgA-binding protein on their surface which might be critically involved in the inflammatory process initiated often mesangial deposition of IgA in patients with IgAN. FACS analysis showed that this molecule is not exclusive for mesangial cells and found on various cells of the myeloid lineage.

[#] ***Submitted***

INTRODUCTION

Primary IgA-Nephropathy (IgAN) is the most common form of glomerulonephritis in man leading to progressive renal failure in nearly half of the patients. IgAN is often associated with higher serum IgA1 levels and characterized by deposition of mainly IgA1 in the mesangial area. IgA deposition in the mesangium is thought to play a crucial role in the inflammatory process in this disease (1,2). However the mechanism responsible for IgA deposition remains unknown. Increasing evidence on the existence of a mesangial receptor for IgA has resulted from the observation that IgA is able to bind to rat and human mesangial cells (MC) and that bound IgA can be internalized and degraded by these cells (3-7) . Binding of IgA to MC leads to activation of MC and subsequently to increased expression of NF-$_{kappa}$B (8), c-jun (9), interleukin-6 (IL-6) (3,7), IL-8 (8), monocyte chemoattractant protein-1 (MCP-1) (8) and Tumor Necrose Factor-α (TNF-α) (3). These findings strongly indicate the existence of a receptor for IgA on human MC.

In the past it has been suggested that CD89 (FcαR), the known myeloid Fc receptor for IgA, is expressed on human MC and could therefore be responsible for binding of IgA to these cell type (3,5,10). Recently it was shown by different groups, that there is no protein expression of CD89 on human MC neither *in situ* nor *in vitro* (7,9). In addition, no mRNA expression could be demonstrated in cultured human MC. Most importantly, we found that binding of IgA to MC could not be prevented by a blocking ant-CD89 mAb (My43), which is perfectly capable of inhibition of binding of IgA to CD89 expressing cells (7).

The aim of the present study was to identify alternative IgA-binding receptors on human MC. Using a newly raised monoclonal antibody and immunoprecipitation experiments. We show that the IgA-binding moiety on MC is most likely a 83 kD surface receptor with a broad destination on various cells of the myeloid lineage.

MATERIALS AND METHODS

Cell culture and isolation

Primary human MC were cultured from normal donor kidneys and characterized as reported in detail elsewhere (11,12). After outgrowth of the MC, hillocks were lifted off the culture flasks and explanted into 24-wells culture plates (Greiner, Alphen aan de Rijn, The Netherlands) and subcultured in T25 or T75 flasks (Greiner) in 10 % FCS, 100U/l penicillin and 100 μg/ml streptomycin (Life Technologies). For FACS-experiments primary MC-cultures were used between subculture 3 and 10.

The CD89-transfected murine B-cell line IIA1.6 was cultured as described (7,13). Mononuclear cells were isolated from heparinized venous blood of healthy controls by Ficoll-Paque density-gradient centrifugation. The mononuclear cells (lymphocytes/monocytes) were isolated out of the interphase; the PMN's out of the pellet after lysing the erythrocytes. The cells were washed in PBS and immediately used for flow cytometry.

Dendritic cells were generated from human monocytes, by culturing the adherent PBMC fraction for 6 days in granulocyte-monocyte colony-stimulating-factor (GM-CSF, 50 ng/ml) and IL4 (5ng/ml) as described (14).

Isolation of IgA-binding proteins from PMN

Membrane proteins of human PMN's were isolated using freshly isolated cells from buffy coat. The cells were washed three times in ice cold PBS and lysed in 1 ml lysis buffer (20 mM HEPES, 150 mM NaCl, 50 mM NaF, 1 mM Na3So4, 1 mM EGTA, pH 7.5, 1 % Nonidet P-40, 10 mN iodoacetamide, 1mM PMSF, and 1 mg/ml of antipain, chymostatin, leupetin, and pepstain A (Sigma)) for 24 hours at 4 °C. Insoluble material was removed by centrifugation (13000 rpm for 10 minutes). The isolated proteins were used for isolation of IgA-binding proteins by affinity chromatography using human IgA bound to A5-Biogel. Human IgA was isolated from human serum as described previously (15). The proteins bound to the column were eluted using Glycin/HCl and immediately buffered with 1 M TRIS-solution. After concentrating the eluate the IgA-binding proteins were used for immunizing mice.

Generation of monoclonal antibodies against IgA-binding proteins derived from PMN's

To generate monoclonal antibodies female BALB/C mice were immunized with (20 □g/mouse) purified IgA-binding proteins derived from PMN's combined with complete Freund's adjuvant (250 □l/mouse, subcutaneous). After 14 and 30 days the injection was repeated with the isolated protein together with incomplete Freund's adjuvant (both subcutaneous). Finally, the mice were boostered 6 weeks after the first immunization by injecting 20 □g isolated IgA-binding proteins dissolved in PBS directly into the spleen. After 3 days splenocytes were fused with myeloma cells (SP20) using 50% polyethylene glycol. The cell suspension was diluted in RPMI 1640 medium supplemented with 10% FCS, hypoxantine (100 μmol), aminopterin (0.4μM), thymidine (16μM), 100U/l penicillin and 100 μg/ml streptomycin. Cells with positive reactivity were subcloned by limiting dilution. One clone produced anti-IgA binding protein mAb tested by flowcytometry using human PMN's (see below) were expanded. This clone was designated 72G12 and identified as being a mouse IgM using an ELISA system.

Immunohistochemistry

To analyze MC of their reactivity with the new mAb MC's were grown on glass cover slips for 24 hours washed in PBS, air-dried and fixed in acetone for 10 minutes at room temperature. The cover slips were incubated with Ms mAb 72G12. As a negative control the isotype matched anti-CD89 mAb My43 was used. After washing the cover slips were incubated with rabbit anti-mouse-IgM polyclonal Ab. Following incubation with HRP (horseradish peroxidase)-conjugated Swine anti-rabbit polyclonal Ab (all antibodies from Dako, Denmark) the slides were subsequently incubated with tyramide-FITC for 30 minutes at room

temperature. Between the one-hour incubation steps the slides were washed two times with PBS containing 0.05 % Tween-20 and two times with PBS.

FACS (fluorescence-activated cell sorter)-analysis

To evaluate a trypsin sensitivity of the possible new receptor human MC were treated with 0,05 % trypsin/0,02% EDTA (all from Sigma) for 3 minutes at room temperature and assessed for binding of human IgA by FACS (see below). No differences between trypsinized and non-trypsinized human MC-cells were observed. Therefore 0,05 % trypsin/0,02% EDTA was used for the detachment of MC.

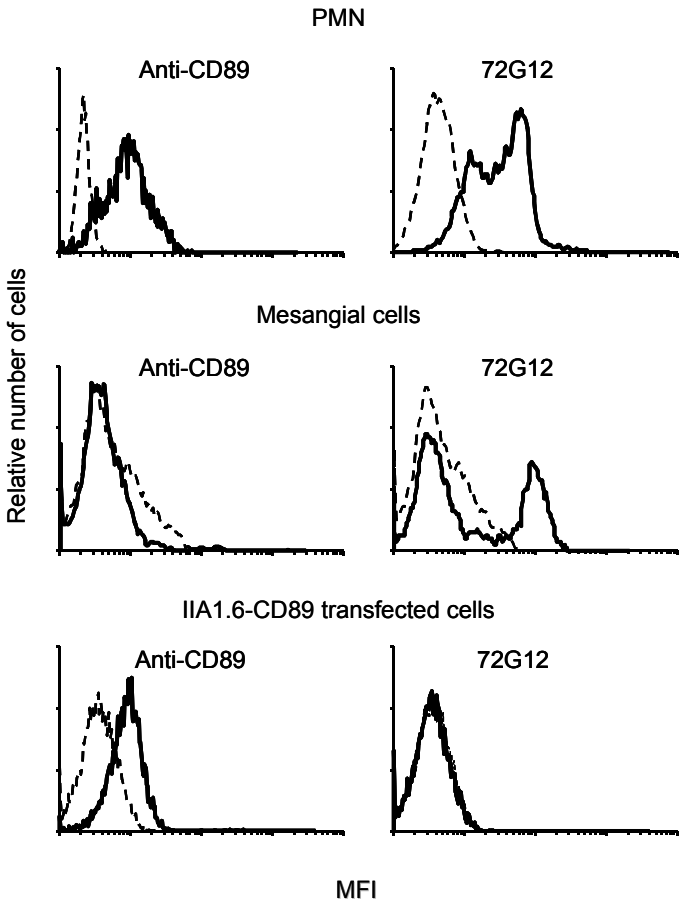

Figure 1: FACS analysis of MCs and IIA1.6 CD89 transfected cells. Top: Human PMN showed positivity after staining with the anti-CD-89-mAb My43 and the new developed mAb 72G12. Middle: Human MC were stained for CD89 (My43, left) and with 72G12 for expression of a new IgA-binding protein (right). Bottom: Also IIA1.6 CD89 transfected cells were stained for CD89 and with mAb 72G12. Broken lines indicate the control staining only using the second antibody.

Cells were washed twice in FACS buffer (PBS/1%BSA/0,1%NaN3) and incubated for 1 hour with mouse mAb 72G12 (culture supernatant diluted 1:2 in FACS buffer). As negative control an isotype matched Ms mAb was used (culture supernatant 1:2 in FACS buffer). Following incubation, cells were washed twice with FACS buffer and incubated for 1 hour with goat anti-mouse Ig-PE. (From Dako, Denmark). All staining procedures were performed at 4°C.

The binding of human IgA to PMN's and MC's was analyzed as previously described (7), in brief: Cells were washed twice with FACS-buffer and incubated for 1 hour with 800, 400 and 200 μg/ml purified IgA. Following incubation, the cells were washed and bound IgA was detected by incubation with mouse monoclonal anti-human IgA antibody (4E8) and subsequently with PE-labeled goat anti-mouse IgG1 polyclonal antiserum.

To demonstrate the specificity of binding of IgA to the new IgA-binding receptor blocking studies were performed in which PMN's and MC were incubated

with human IgA in the presence and absence of the new mAb 72G12. An isotype-matched Ms mAb served as a control.

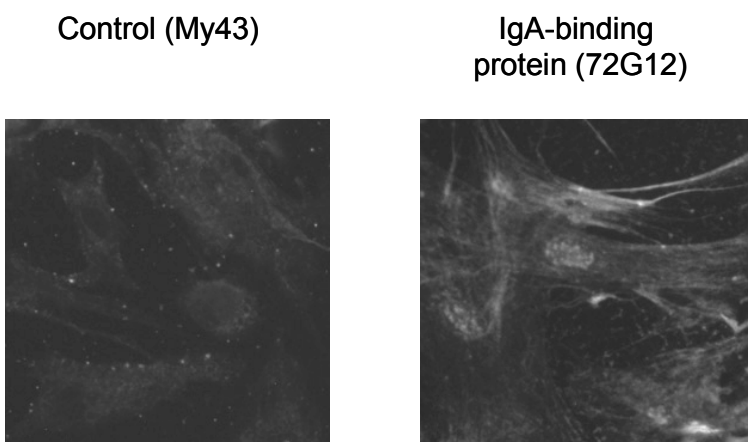

Figure 2: Stainings of MC grown on glass cover slips. Human MC derived from 4 different individuals were stained with 72G12. As an example a staining of one MC is shown. The negative control staining was performed using My43; an isotype matched anti-CD89 mAb

The percentage positive cells were used in all FACS-experiments as a measure for expression of the new IgA-receptor and IgA-binding. All measurements were done with FACScan (Bexton Dickinson, Mountain Vieuw, CA, USA) and data were analyzed using Lysis II software.

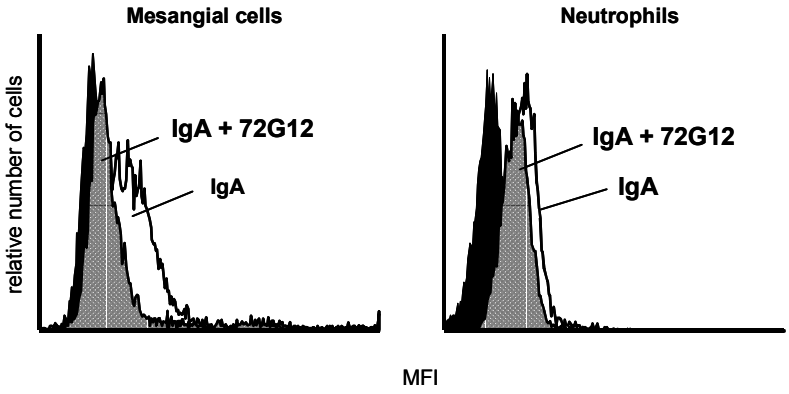

Figure 3: IgA-blocking effect of 72G12 and My43 to human MCs and PMN's. Histograms for binding of IgA to MC (left) and PMN (right) are shown. The empty histogram showed binding of IgA to these cell types. After preincubation of MC with 72G12 binding of IgA was almost completely reduced (gray patterned histogram). In contrast to this binding of IgA to PMN's is only slightly reduced by 72G12 preincubation (gray patterned histogram).

Isolation of NHC-LC Biotin labeled membrane proteins from human MC

MC were trypsinized as described and washed twice with PBS (pH8). After that $10x10^6$ MC were incubated in 500 µl/ml EZ-Link™ Sulfo- NHC-LC Biotin (Pierce Chemical Company, Rockford IL, USA) for one hour at 4 °C, washed 3 times in ice cold PBS and finally lysed in 1 ml lysis buffer (20 mM HEPES, 150 mM NaCl, 50 mM NaF, 1 mM Na3So4, 1 mM EGTA, pH 7.5, 1 % Nonidet P-40, 10 mN iodoacetamide, 1mM PMSF, and 1 mg/ml of antipain, chymostatin, leupetin, and pepstain A (Sigma)) for 24 hours at 4 °C. Insoluble material was removed by centrifugation (13000 rpm for 10 minutes). Lysate was further used for immunoprecipitation experiments (16).

Immunoprecipitation experiments

Precipitation experiments were performed to identify the molecule recognized on human MC by the developed mAb 72G12. Therefore we first preincubated the biotinylated membrane proteins derived from $10x10^6$ human MC with 200 µl bovine serum albumin (BSA)-A5-Biogel for two hours with rotation. Then the lysate was incubated for 18 hours with IgA-A5-Biogel. As a control human serum albumin (HSA) bound to A5-Biogel (200 µl) was used. The pellets were washed extensively with PBS, resuspended in reducing sample buffer (Biolabs, The Netherlands), boiled for about 5 minutes, separated by SDS-PAGE (10% or 7% acrylamide), and transferred to polivinylidine difluoride (PVDF) membranes. Detection of biotinylated proteins bound to IgA or HSA was performed by incubation the PVDF-membrane with streptavidine-horseradish peroxidase and the chemiluminescent method (Chemiluminescent Substrate, Super Signal, Pierce Chemical company, Rockford IL, USA). For detection of chemiluminescence we used a high performance chemiluminescence film (Hyperfilm™ECL™, Life Science, Amersham, England).

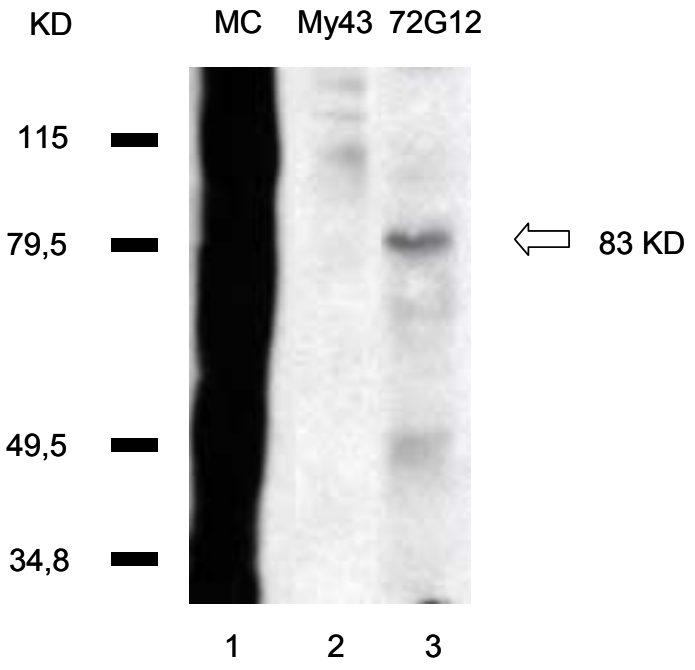

Figure 4: Immunoprecipitation of protein isolated from human MC using 72G12. Precipitation experiments of MC-protein using 72G12 revealed an enriched band at the size of 83 Kd using the chemiluminescence method (lane 3) whereas no comparable enriched protein was found using My43 (lane 2). Starting material is shown in lane 1.

Before precipition of biotinylated membrane proteins with 72G12 we precleared two times with Protein G (20µl). Then the lysate was incubated for 18 hours with 72G12 or an isotype matched irrelevant control Ab followed by incubation with a Rabbit anti-mouse-IgM polyclonal Ab for 18 hours. Finally we precipitated using 20 µl Protein G. The following steps were done as described. Similar experiments were also performed using not biotinylated protein isolates from MC. Those precipitates were also plotted to PVDF-membranes and stained using Coomassie-blue.

To investigate whether IgA and 72G12 recognize the same protein we preclearing lysates with Protein G and BSA A5-Biogel. Next 50% of the material was precleared with IgA-A5-Biogel. Thereafter we did precipitation experiment using 72G12 as described.

RESULTS

Generation of a new antibody against an IgA-binding protein

To identify new IgA-binding receptors, IgA binding-proteins were isolated from human PMN's using an IgA-immunoabsorbance. After immunization one monoclonal antibody, 72G12 (IgM isotype) was identified which not only reacted with PMN's, but also showed a surface staining of human MC (Figure 1). In accordance with the absence of CD89 expression on human MC, the antibody 72G12 did not react with CD89 transfected cells (Figure 1). The 72G12 antibody reacted with a large population of human mesangial cells in culture using FACScan and immunohistochemistry of cells grown on glass cover slips (Figure 2). Since the antibody was raised against fractions of IgA-binding proteins from human PMN, the antibody could potentially recognize a novel IgA-receptor on MC.

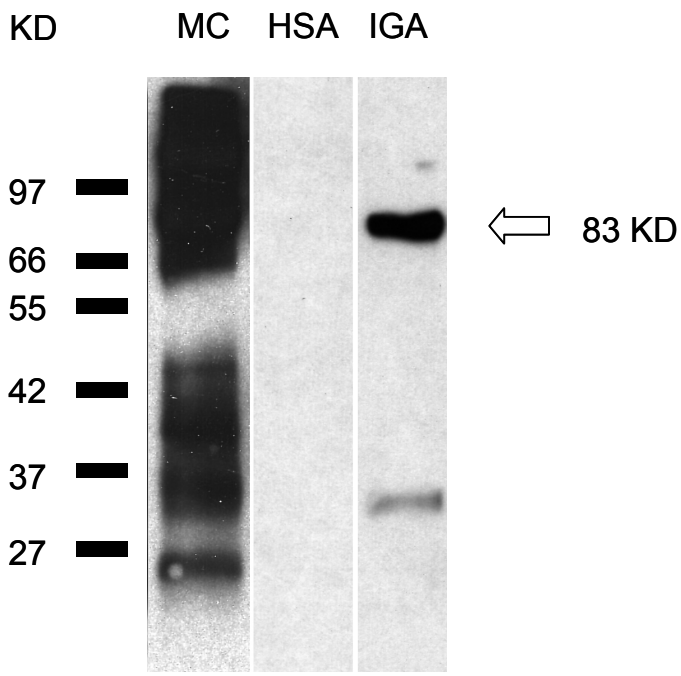

Figure 5: Immunoprecipitation of protein isolated from human MC with IgA Precipitation experiments of MC-protein using HSA revealed no enrichment of a specific protein (HSA-lane). In contrast precipitations using human IgA revealed a strong enhancement of an 83 kD protein (IgA-lane) using the chemiluninescence method as compared to the starting material (MC-lane).

Binding of human IgA to MC and PMN is inhibited by 72G12

To determine whether the binding of IgA to MCs was mediated by the postulated IgA-binding protein, inhibition experiments were performed. Incubation of MC with IgA and subsequent analysis by FACS revealed that all 4 different primary MC-cultures bound human IgA well. Also PMN showed a homogenous binding of IgA to their surface (Figure 3). Preincubation with 72G12 showed that this antibody is able to reduce the binding of IgA to both MC and PMN (Figure 3) effectively whereas an isotype matched control Ab did not. Calculation of the mean fluorescence intensities showed a $76.7 \pm 15.2\%$ reduction on MC and a $25.4 \pm 6\%$ reduction on PMN (mean of 3 experiments) after preincubation with 72G12. IgA-binding is not affected by an isotype-matched antibody. In accordance with our previous results, the blocking anti-CD89 antibody (My43) reduced the binding of IgA to PMN ($54,7 \pm 11,3$ % reduction of the mean fluorescence intensity), whereas no effect is seen on the binding of IgA to MC (not shown).

72G12 and IgA recognize a similar 83 Kd surface protein

Precipitation experiments using the new monoclonal antibody 72G12 and biotinylated membrane proteins from human MC led to the detection of a protein of 83 Kd. Control experiments using monoclonal antibody My 43 did not reveal any enrichment, indicating the specificity of the proteins precipitated by 72G12 (Figure 4). Similar results were obtained using non biotinylated membrane proteins from human MC after staining with Coomassie-blue (not shown).

Incubation of human IgA bound to Biogel-A5 led to precipitation of a protein with the size of about 83 Kd using biotinylated membrane proteins derived from human MC. Control precipitations using HSA-A5-Biogel did not reveal any band indicating the specificity of the precipitated protein to human IgA (Figure 5)
To determine whether the proteins precipitated with human IgA and 72G12 are identical we performed precipitation experiments where we precleared one part of the membrane proteins with human IgA bound to A5-Biogel. After preclearence with human IgA we found an almost complete reduction of the enrichment of the 83 Kd protein after 72G12 precipitation (Figure 6).

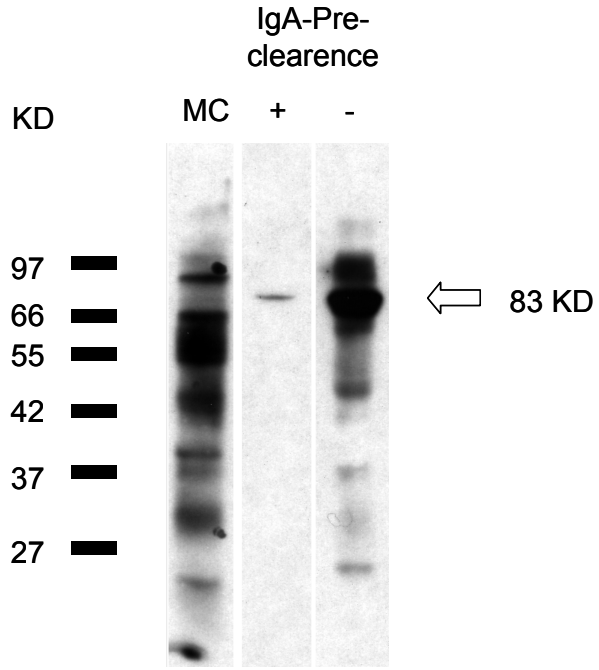

Figure 6: Effect of preclearence with human IgA on immunoprecipitations by 72G12 of proteins isolated from human MC After preclearence using human IgA BiogelA5 of proteins derived from human MC 72G12 precipitated much less protein with the expected size of 83 Kd (lane +). The result of a precipitation without preclearence with human IgA is shown in lane " – ". Lane "MC" shows the starting material.

72G12 is expresses on different cells of the myeloid lineage

Mononuclear cells and PMN derived from human buffy coat were investigated for expression of 72G12 antigen. As expected human PMN's were stained clearly positive using 72G12. Monocytes showed little reactivity, whereas monocyte derived dendritic cells have a higher expression of the IgA-binding moiety. In contrast, lymphocytes failed to be positive for the 72G12 antigen (Figure 7).

DISCUSSION

Although deposition of mainly IgA1 (17) in the mesangium of IgAN patients is well documented the mechanism by which binding and deposition of this type of IgA take place is barely understood. Recent investigations showed that the glycosylation of the hinge region of human IgA1 is different between normals and

patients with IgA-nephropathy (17). These differences may contribute to the increased deposition and may be metabolisation of IgA1 during the course of IgA-Nephropathy.

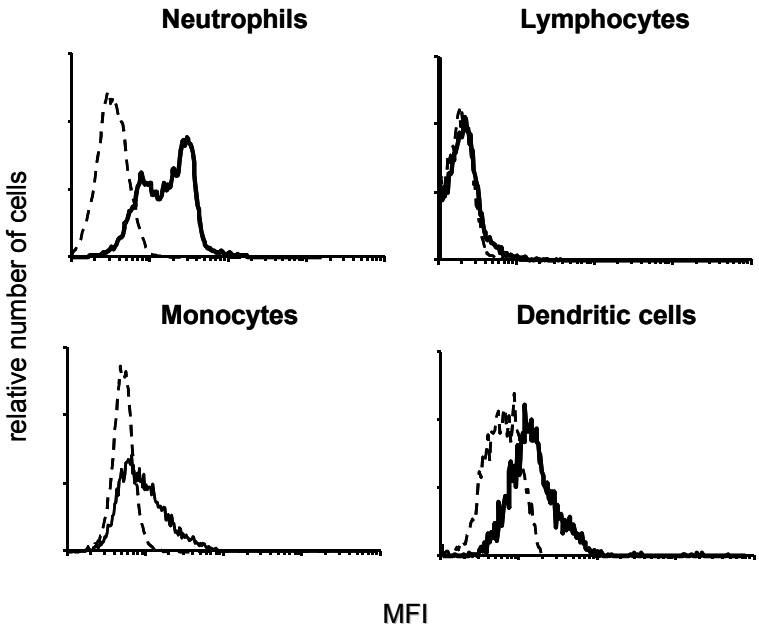

Figure 7: FACS analysis of expression of the new receptor for IgA on white blood cells. At the top a strong reactivity of freshly isolated PMN with 72G12 is shown. Monocytes revealed only a slight expression of the investigated protein whereas dendritic cells showed an increasing expression of the new IgA-binding protein. Lymphocytes were negative for this antigen. Broken lines show the staining of the cells by an isotype matched control antibody, the full lined histograms show the staining with 72G12.

However it is well known that binding of IgA to MC is able to induce activation of these cells which subsequently lead to increased phagocytosis and expression of IL-6 (3,7), IL-8 (8), MCP-1 (8), TNF-α (3), NF-$_{kappa}$B (8) and the protooncogen c-jun (9) suggesting a specific binding of IgA to MC. Recently we and others published data that the known Fc receptor for IgA (CD89) is not involved in the binding of IgA to human MC in vitro and the mesangium of patients with IgA-Nephropathy and healthy controls in situ (7,9).

Because of the failure of CD89 blocking antibodies to reduce binding of IgA to human PMN's completely expression of more than one IgA-binding protein of the surface of this cell type is most likely (7). Therefore we raised new monoclonal Abs against IgA-binding proteins derived from human PMN. One of this Abs showed cross-reactivity with human MC in vitro. In contrast this mAb designated 72G12 did not react with IIA1.6-CD89 transfected cells indicating its inability to bind CD89. Furthermore 72G12 was able to block binding of human IgA to a subpopulation of cultured human MC. As expected binding of IgA to PMN's were partly reduced suggesting that MC bind IgA via a receptor distinct from CD89. We have shown earlier that binding of IgA to PMN was reduced by My43, an IgA-blocking monoclonal antibody against CD89. In contrast to this binding of IgA to MC was not reduced (7).

Precipitation experiments with human IgA and 72G12 using membrane proteins derived from human MC revealed a protein band with the size of 83 Kd. Experiments in which we first incubated membrane proteins from human MC with human IgA and then precipitated with 72G12 showed that IgA is able to reduce the amount of precipitated protein almost completely. These data indicate that MC express a receptor able to bind human IgA distinct from CD89.

Furthermore this new receptor seems to be distinct from an earlier described Fc receptor for IgA expressed on a small fraction of human T-cells (18-20) because the new mAb 72G12 did not react with human lymphocytes.

Also other molecules have been suggested to be responsible for binding of IgA to human MC. Since reliable poly- and monoclonal antibodies against the mannose receptor (175 Kd) have been generated (21,22) and it has been suggested that activated mouse MC express this receptor (23), we investigated its expression on human MC. However we did not find mannose receptor expression neither on resting nor on activated human MC (7).

Asialoglycoprotein (ASGP-R) receptor is known to be expressed on human and rat hepatocytes and involved in the clearance of galactose-terminal glycoproteins for example human IgA1 (24-27). It was reported that ASGP-R is expressed on human and rat MC using RT-PCR, no protein expression was shown (16). However recent investigations were not able to confirm these data mainly basing on results obtained from rat MC (Lai, K., in press). Additionally the expected sizes of the three subunits of the hepatic ASGP-R are much smaller as compared to the size of the suggested novel receptor for IgA. Even more because the failure of PMN's to express ASGP it is not likely that 72G12 reacts with this molecule.

Another candidate for binding of IgA to human MC could be the polymeric immunoglobulin receptor also designated secretory component (SC). Northern blot analysis and immunohistochemical stainings showed mRNA and protein expression of this receptor in various human exocrine tissues but not in the kidney (28). Until now non of the publications revealed expressed of this receptor neither on PMN nor on MC.

Taken together, for the first time we presented data about a novel IgA binding protein expressed on human MC. Furthermore this 83 Kd protein is also expressed on monocytes, dendritic cells and PMN's. Binding studies to the CD89-transfected mouse myeloid cell line IIA1.6 showed that the new receptor is distinct from CD89. A new developed mAb (72G12) directed against this postulated receptor is able to block binding of IgA to MC indicating the major role of this receptor in mediating binding of IgA to this cell type. Further work has to be done to isolate and characterize this new IgA-binding receptor to be able to determine its possible role of this protein in the pathogenesis during human IgA-nephropathy.

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