Pathogenetic Aspects of IgA-nephropathy

Ralf Westerhuis

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Ralf Westerhuis

1. Neutrophil granulocytes and monocytes/macrophages play distinctive roles during anti-Thy-1.1 antibody induced mesangial cell proliferative glomerulonephritis in rats (dit proefschrift).

2. Binding of IgA to human MC occurs via an IgA receptor distinct from CD89 (dit proefschrift).

3. IL-6 is not an important mediator of mesangial cell proliferation and matrix overproduction in vivo (dit proefschrift).

4. The myeloid FcRI/CD89 is released as a 30-kDa soluble molecule. The release of sCD89, which can bind IgA, is induced upon activation of myeloid cells by the receptors' own ligand (dit proefschrift).

5. Increased prevalence of the CCR5wt/delta 32 genotype in a high-risk HIV-seronegative cohort in West Africa results in reduced susceptibility to HIV-1 infection among heterozygous individuals.
   Kokkotou E et al, *J Hum Virol* 1998 Nov-Dec;1(7):469-74

6. Seasonal climate forecasts, predicting the likelihood of weather patterns several months in advance, provide helpful early indicators of epidemic risk, particularly for malaria.
   Kovats RS, *Bull World Health Organ* 2000;78(9):1127-35

7. High-dose riboflavin has been shown to be effective in migraine prophylaxis with high efficacy, excellent tolerability, and low cost.
   Schoenen J et al, *Neurology* 1998 Feb;50(2):466-70

8. Blood donated by symptom-free variant Creutzfeldt-Jakob disease (vCJD)-infected human beings may represent a risk to spread this disease because it has been shown that bovine spongiform encephalopathy was transmitted by transfusion with whole blood taken from a sheep during the symptom-free phase of an experimental BSE infection.
   Houston F et al, *Lancet* 2000 Sep 16;356(9234):999-1000

9. Opportunism is the art to sail with the wind produced by others.
   Allessandro Manzoni

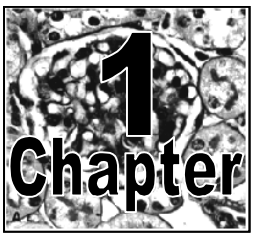
10. If you want to become a real scientist, half an hour a day think the opposite of what your colleagues think.
    Albert Einstein

CONTENTS

ABBREVIATIONS

aa	amino acid
ADCC	Antibody-Dependent Cellular Cytotoxicity
aIgA	heat aggregated IgA
Ag	Antigen
ASPG-R	Asialo-Glyco-Protein Receptor
bp	base pare
bFGF	basic fibroblast growth factor
cDNA	complementary DNA
C3	Complement component 3
C6	Complement component 6
dIgA	dimeric IgA
EC	Extra Cellular
EDTA	Ethylene-Diamine-Tetra-acetic-Acid
FACS	Fluorescence Activated Cell Sorter
Fab'	Fragment antigen binding
Fc	crystallizable Fragment
FCS	Fetal Calf Serum
FcR	Fc Receptor
FcαR	IgA Fc Receptor
FcγR	IgG Fc Receptor
FcεR	IgE Fc Receptor
FITC	fibrinogen-fluorescein isothiocyanate
IC	Immune Complex
IgA	Immunoglobuline A
IgAN	IgA Nephropathy
IgG	Immunoglobuline G
IgM	Immunoglobuline M
IL	Interleukine
ILT	Ig-Like Transcript receptor
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
kD	kilo Dalton
KIR	Killer cell Inhibitory Receptor
LAIR	Leukocyte-Associated Immunoglobuline-like Receptor
LIR	Leukocyte Ig-like Receptor
mAb	monoclonal Antibody
MC	Mesangial Cell
MCP-1	Monocyte Chemoattractant Peptide-1
MFI	Mean Fluorescence Intensity
mIgA	monomeric IgA
MIR	Monocyte/Macrophage Ig-like Receptor
Ms	Mouse
PBS	Phosphate Buffered Saline
PCNA	proliferating cell nuclear antigen
pIgA	polymeric IgA
PIR	Paired Ig-like Receptor
PMN	Poly-Morpho-Nuclear cell
Rb	Rabbit
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor

GENERAL INTRODUCTION



I. CLINICAL FEATURES OF HUMAN IGA-NEPHROPATHY

Immunoglobulin A nephropathy (IgAN) is the most common type of glomerulonephritis in large parts of the world. This glomerular disease leads to end stage renal failure (1,2) in approximately 30 – 50 % of the patients (3,4). About 7 to 10% of the patients with renal replacement therapy have IgAN (5). IgAN is a disease of young adults, males are more frequently affected than females. The clinical presentations of this type of glomerulonephritis show a large variability. Forty percent of the patients have recurrent episodes of macroscopic hematuria associated most commonly with infections of the upper respiratory tract (6) but also other infections have been shown to be associated with increasing disease activity (7-10). In contrast to children adults show no spontaneous remission of the disease (4,11,12). During the course of the disease macroscopic hematuria is less frequent, however long term prognosis of the disease with infection-related macroscopic hematuria is better (12-14). In most of the patients with IgAN the disease has an indolent course and is manifested by microscopic hematuria, intermittent or persistent. In the course of IgAN first mild proteinuria occurs which increases during the timecourse and is frequently associated with rising hypertension (15,16). The degree of hypertension and proteinuria are predictive for progression of the decline of renal function.

II. HISTOPATHOLOGICAL FEATURES

During the initial phase of IgAN MC proliferation is a characteristic finding. This acute phase is accompanied by infiltration of few monocytes, however extra and intracapillary inflammatory processes are severe enough and lead to crescent formation (17). Crescents are frequently found during relapses with macroscopic hematuria. More monocytes and T-cells were found in biopsies of activated disease (17) as compared to those without signs of disease activity.

Immunohistologically IgA-Nephropathy is characterized by deposition of IgA in the renal mesangium (18,19). The IgA deposited in the renal mesangium is mainly of the IgA1 subclass (20-25). Other immunoglobulins are frequently codeposited in the glomeruli in IgAN showing more frequently IgG (45-83%) as compared to IgM (9-66%)(24). In these cases the course of the disease is more severe (26,27).

The deposited IgA has been suggested to be mainly of polymeric origin. There is no direct evidence of the polymeric nature of deposited IgA but the presence of J-chain and the ability of secretory component to bind to glomerular areas with IgA-deposition points out that it is most likely IgA of polymeric origin

(28). Furthermore elution studies using glomeruli of patients with IgAN seem to detect a substantial fraction of polymeric IgA (28,29).

In patients with IgAN deposits of C3 have been described at similar glomerular locations as IgA in up to 90% of the patients (21,30-33). The codepostion of factor H and properdin in the absence of complement components of the classical pathway indicates the activation of complement during IgAN by the alternative pathway (32). Detection of the terminal membrane attack complex (MAC) in the glomerulus during IgAN is correlated with progressive renal failure.

III. IGA RELATED ACTIVATION OF MC

Various investigators have pointed out that IgA binds to rat mesangial cells (MC) (34,35). It was been shown that binding of IgA to MC leads to uptake and degradation of IgA and that IgA induces release of different cytokines (36,34). Previous results have demonstrated that IgA binds to human MC (36-38), however no specific receptor could be identified responsible for binding and activating the cells. Binding studies with human monoclonal IgA1 have shown linear binding curves indicating a single population of an IgA-binding protein on human MC with a Ka of $3.2x10^7$ M^{-1}. Quiescent MC bear approximately $2.55x10^5$ sites/cell, and after stimulation of these cells with different recombinant cytokines (Interleukin-6, Tumor Necrosis Factor-α, Interferon-α) binding-sites increased whereas binding curves were not changed (39). Diven and coworkers demonstrated in their study that IgA1, isolated from healthy individuals (polyclonal), binds dose-dependently and in a saturable manner to human MC. They found $1.2x10^6$ binding-sites/cell and an affinity constant for IgA1(Ka) of $2.3x10^6$ M^{-1} and a dissociation constant (Kd) of $4.4x10^7$ M^{-1}. Binding of dimeric and polymeric IgA led dose-dependently to enhanced expression of the protooncogene c-jun (40) which has been shown to be linked to cell proliferation and matrix synthesis (41).

Another study demonstrated cytosolic calcium release after stimulation of MC with aggregated monoclonal IgA in a dose-dependent and transient manner. This initial induction of calcium rise was due to calcium mobilisation from inositol trisphosphate (IP_3)-sensitive intracellular stores. Protein tyrosin kinase inhibitors abolished calcium mobilization from these stores (42).

Furthermore IgA immunecomplexes or aggregates of soluble IgA and IgG activate human MC to produce IL-6 and TNFα (34). Also rat MC were stimulated to produce increasing amounts of IL-6 using dimeric and polymeric IgA. IL-6 has been reported to induce matrix protein transcription and autocrine growth in MC in vitro (43,44). In vivo glomerular IL-6 overexpression was detected in human

glomerulonephritis types characterized by MC hypercellularity such as IgAN and some types of lupus nephritis (45-49).

Binding of monoclonal aggregated IgA to MC in vitro furthermore leads to enhanced expression of the nuclear factor-kappa B (NF-kappa B) (50). This increased expression of NF-κB induces e.g. expression of monocyte chemo-attractant protein-1 (MCP-1), IL-8 and interferon-inducible protein 10. Preincubation of MC with Fc-fragments of IgA led to a complete blocking of this effect (49,50).

Enhanced production of the chemoattractant IL-8 (51) may lead to accumulation of polymorphonuclear cells (PMN) in biopsies of patients with IgAN. Evenmore increased numbers of IL-8 and TNFα-positive cells correlate with the amount of proteinuria and the number of IL1α, TNFα and IL-6 positive cells are associated with hypercellularity of the renal mesangium (44,49,52,53).

IV. IGA-RECEPTORS AND IGAN

Because of increasing evidence of receptor mediated activation of mesangial cells it seems more and more likely that binding of IgA or IgA-immunecomplexes is mediated via an receptor on the surface of human renal MC. Therefore the known receptors, especially the Fc receptor for IgA (CD89, FcαRI) will be described in the following paragraph in more detail.

IV.1 FcαRI/CD89

The DNA sequence of a receptor for the Fc-tail of IgA expressed on myeloid cells was published earlier (54). The 1.6 cDNA clone was isolated from PMA-stimulated U937 cells, using a known monoclonal antibody specific for CD89 (My43) (55). The gene of CD89 consists of 5 exons and is located on chromosome 19, at position 19Q13.4 (56), which is different from all other Fc receptors. They are located on chromosome 1.

The protein backbone of CD89 consists of two extracellular Ig-like domains (206 aa) and carries one potential O-linked, and 6 potential N-linked glycosylation sites. The transmembrane part is 19 aa long and has a positive charged arginine which is necessary for association of CD89 with the FcR γ-chain. The FcR γ-chain is a homodimer-signaling unit. Like other Fc receptors lacking an intracellular signaling motif, signaling into the cell is initiated via this dimeric γ-chain with a size of 10 KD (57). Binding to CD89 leads to phosphorylation of the intracellular ITAM motifs on the γ-chains (58) activating the signaling pathways downstream into the cell.

CD89 is expressed on the surface of neutrophils (59,60), eosinophils (61,62) and cells of the monocyte/macrophage lineage (63). The predicted molecular weight of this receptor is 30 KD (54), however the molecular weight of CD89 on monocytes and neutrophils was found to be 55-75 KD and 70 – 100 KD for CD89 expressed on eosinophols.These results and further deglycosylation experiments (61,62,64) indicate that CD89 is highly glycosylated and that its glycosylation is dependent on the CD89 expressing cell.

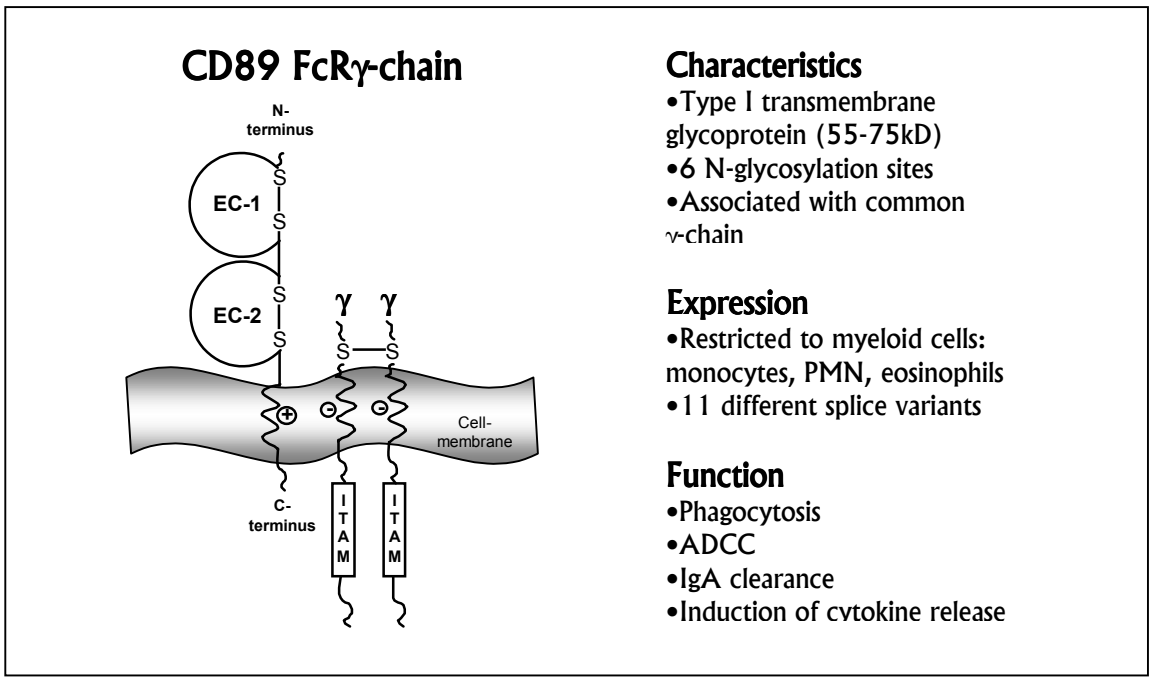


Figure 1: Structure and characteristics of the Fc-Receptor for IgA (FcαRI/CD89): The extracellular domain 1 and 2 (EC-1 and EC-2), as well as the associated pair of γ-chains with their signaling (ITAM) motifs are depicted. Furthermore the characteristics of CD89 the cellular distribution and its known functions are summarized.

The expression of CD89 on human renal MC is controversial. Expression of mRNA of the Fc receptor CD89 has been suggested on human MC (38,39). Activation of human MC with IL6 or TNFα led to increased binding of IgA accompanied by an increase of mRNA expression of CD89. However, no evidence was provided for the direct involvement in the binding of IgA to these cells via CD89 (39). Further mRNA expression of CD89 was demonstrated in whole human glomeruli in 40% of patients with IgAN. In healthy individuals and patients with mesangial proliferative glomerulonephritis distinct from IgAN no CD89-expression was found suggesting an absence of CD89 expression on human MC *in situ* (65,66). Even more CD89-surface expression was not detectable on human MC using known monoclonal antibodies against CD89. However binding of IgA let to activation of MC independent of CD89 (40).

CD89 has different functions dependent on the cell. In macrophages and neutrophils IgA mediated phagocytosis is evident as shown by several investigators (55,63,67-69), Furthermore release of cytokines initiated by crosslinking of CD89 with IgA or monoclonal antibodies has been shown. Release of TNFα, IL1α, IL-6 and some other cytokines from macrophages has been stated (70). All the cells mentioned above and expressing CD89 show antibody dependent cell-mediated cytotoxicity (ADCC) as another effector function of this receptor (71-75). CD89 dependent degranulation is reported for neutrophils and eosiniphils using aggregated IgA resulting in e.g. release of neurotoxins from eosinophils (55,76-80). The ligand for CD89, human IgA1, has been studied for its binding domain. It has been found that the Leu^{257}-Gly^{259} region in Cα2 and the Pro^{440}-Phe^{443} region in Cα3 is crucial for binding to CD89 (81,82).

VI.2 Asialoglycoprotein-receptor and mannose receptor

Other receptors, such as the asialoglycoprotein-receptor (ASGP-R) and mannose receptor could be involved in binding of IgA to MC. Since reliable poly- and monoclonal antibodies against the mannose receptor have been generated (83,84) and it has been suggested that activated mouse MC express this receptor (84), we investigated its expression on human MC. Neither on resting nor on activated human MC we detected protein expression of the mannose receptor using flowcytometry.

ASPG-R, a 44 KD single subunit receptor of the C-type lectin family, is known to be exclusively expressed on human and rat hepatocytes and involved in the clearance of galactose-terminal glycoproteins for example human IgA1 (85-87) which is eliminated via endocytosis (88). IgA1 has sialic acid residues in its O-linked oligosacharide side chains located in the prolanged hinge region of the immunoglobulin with which it can bind to the receptor. IgA2 on the other hand can not bind to this receptor (89-91). It is thought that clearence of IgA1 by ASGP-R is one of the main routes of clearance of this subclass of IgA.

Recently it was reported that ASGP-R is expressed on human and rat MC using RT-PCR (92). In this study binding and catabolism of IgA by human and rat MC was saturable and partly inhibitable by galactose but no other carbohydrates.

IV.3 Secretory component

Secretory component is expressed on the basolateral site of secretory epithelial cells and also known as poly-IgA receptor facing the blood compartment. This receptor specifically binds to J-chain associated dimeric/polymeric IgA and pentameric IgM. After binding of e.g. dimeric IgA it is transported through the cell to the apical site

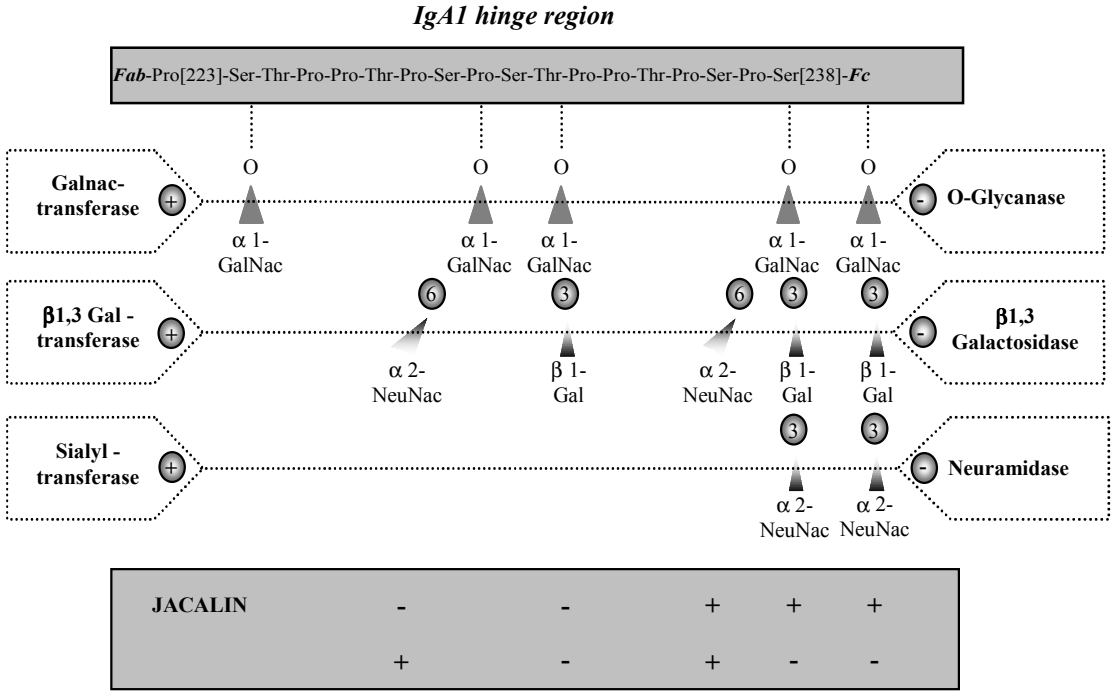
via endocytosis. There, after cleavage of the membrane-anchoring domain of the membrane bound secretory component sIgA is released into the glandular secretions (93,94). There it is responsible for neutralisation of microbes and toxins and prevents unwanted antigens to pass the mucosal barrier (95,96). Until now there is no evidence for expression of this receptor in the kidney.

V. IGA GLYCOSYLATION AND IGA-IMMUNECOMPLEX-FORMATION IN IGAN

Human IgA1 has an O-glycosylated hinge region unique to circulating immunoglobulins. There is raising evidence of changes of the glycosylation of the hinge region of IgA1 in IgAN. Observations of altered lectin binding to IgA1 in IgAN suggest that the O-glycan chains may be undergalactosylated (97-101). More precise structural investigations using fluorophore-assisted carbohydrate electrophoresis (FACE) to analyze IgA1 O-glycans in IgAN and controls demonstrates that in IgAN, the IgA1 O-glycan chains contain increased terminal GalNAc. This could provide evidence that the well-described alterations of lectin binding to IgA1 in IgAN are due to increased occurrence of ungalactosylated GalNAc units which may be due to an absolute increase in the number of O-glycans carried by IgA1, or to a reduced frequency of O-glycan galactosylation (102). So, the deficiency of galactose in the hinge region of the IgA1 molecule may result in the generation of antigenic determinants containing GalNAc residues that are recognized by naturally occurring IgG and IgA1 antibodies followed by the generation of immune complexes (97,98). The IgG antibodies detected in these immunecomplexes are mainly of the IgG2-subclass, however the mesangial IgG-deposits in human IgAN contain mainly IgG of the subclasses one and three. This failure of deposition of IgG2 in the renal mesangium is most likely dependent of the lack of human MC to express the Fc□RII. The relevance of these immunecomplexes is not clear because it has been shown that complement is activated in IgAN via the alternative pathway (103-105) Furthermore the formation of IgA1-IgGcomplexes may affect the plasma level of IgA1 by reducing the rate of its elimination and catabolic degradation by the liver (97,98). Another study found immunoglobulin A-fibronectin complexes to be elevated in patients with IgAN (106). In a recent study using two independent mouse models (gene knockout and antisense transgenic), both manifesting deficiency of an anti-inflammatory protein, uteroglobin (UG), were shown to develop almost all of the pathologic features of human IgA nephropathy. They demonstrate that fibronectin-uteroglobin heteromerization, reported to prevent abnormal glomerular deposition of fibronectin and collagen, also abrogates both the formation of IgA-fibronectin

complexes and their binding to glomerular cells. Moreover, UG prevents glomerular accumulation of exogenous IgA in UG-null mice (107).

Figure 2: Scheme of the IgA1 hinge region from proline [223] upto serine [238] (adapted from Emancipator 1999)



Furthermore it has been shown that the production of polymeric IgA is reduced in the mucosal immune system of patients with IgA-N. This effect could be due to a mucosal gamma delta T cell defect, and could explain the impaired mucosal IgA responses to immunisation, however polymer IgA1 production by the bone marrow is increased (108-110).

Therefore altered production rates of IgA and binding-characteristics of IgA/IgA-immunecomplexes to a suggested FcR for IgA on human MC in patients with IgAN could lead to an explanation on deposition of IgA(-immune)-complexes and inflammation in the renal mesangium. Furthermore, altered hinge region glycosylation may alter IgA1 structure, modifying interactions with matrix proteins, IgA receptors and complement, and therefore influence mesangial deposition and subsequent injury through mechanisms other than classical antigen-antibody reactions. Furthermore the clearance of IgA through the hepatic ASGP-receptor or Fc alpha receptors on circulating white cells may also be impaired (109).

VI. INFILTRATING CELLS DURING GLOMERULONEPHRITIS

VI.1 Neutrophils

There is accumulating evidence that neutrophils are involved in inflammatory injury in IgAN. Histologic examination revealed increased neutrophils and monocyte/macrophage infiltration in IgAN compared with other glomerulonephritides. Higher numbers of infiltrating neutrophils were associated with higher levels of serum levels of IL-8 and IL-8 autoantibodies and was correlated with more severe pathology (111). Furthermore it appeared that the increased renal infiltration of PMN which have a high potential for production of reactive oxygen species might induce the glomerular injuries in patients with IgA nephropathy (112,113). Others found a strong correlation between episodes of macroscopic hematuria and the glomerular influx of neutrophils and monocytes/macrophages. They conclude that, in the acute phase of mesangial IgA nephropathy, PMN and monocytes are present and presumably participate in glomerular injury (6,114).

Glomerular infiltration by neutrophils is also characteristic of acute experimental glomerulonephritis (115). Proteinuria resulting from neutrophil-mediated glomerular injury has been shown in nephrotoxic serum nephritis (116,117), crescentic glomerulonephritis (118) and after intra renal injection of phorbol myristate acetate (PMA) (119), or of cobra venom factor, which causes complement activation and subsequent PMN chemotaxis (120,121). In each of these described models, PMN depletion markedly diminished proteinuria and resulted in reduction or attenuation of renal disease (118).

VI.2 Monocytes/Macrophages

In idiopathic IgAN, infiltration of macrophages is rarely found or absent in the renal mesangium (115,122,123). However, in patients with IgAN with severe mesangial hypercellularity accompanied by glomerulosclerosis, numbers of infiltrating glomerular macrophages are increased (114,124).

Furthermore the involvement of monocytes and macrophages in various glomerulonephritides has been clearly established (125). In particular their involvement has been associated with proliferative forms of glomerulopathies (126,127). Experimental evidence has been provided by rodent models of glomerulonephritis (128-130). In contrast in some cases infiltrating monocytes may serve merely to remove immune complexes and do not contribute to the glomerular injury (131). Recent interest has focused on the role of macrophages in the pathogenesis of focal glomerulosclerosis (132,133). Both glomerular hypercellularity and expansion of the extracellular matrix are thought to be of

primary importance in the development of capillary obsolescence and glomerulosclerosis. In the remnant kidney model in the rat Van Goor and colleagues (125) showed that macrophages play a central role in the development of focal glomerulosclerosis. As described by Floege et al., the development of glomerulosclerosis in this model is preceded by mesangial proliferation and mesangial matrix expansion (134).

VII. SCOPE OF THIS THESIS

For a better understanding of the pathogenesis of human IgA-Nephropathy, we focussed on different questions in the course of this disease. We set up different experimental approaches to clarify the role of interleukin-6 during mesangial development and mesangial cell proliferative glomerulonephritis using an experimental rat anti-Thy-1 model (Chapter 2). During IgAN the role of infiltrating cells is not yet clear therefore we performed selective monocyte/neutrophil-depletion experiments to be able to point out the possible differential roles of infiltrating inflammatory cells during experimental mesangial cell proliferative glomerulonephritis (Chapter 3).

During IgA-N deposition of IgA and IgA-IC in the glomerulus takes place. To evaluate interactions of human IgA with its known Fc-receptor (CD89) which had been suggested to be expressed on human renal MC we developed novel specific reagents for further characterization of this receptor on mononucleated cells and found that the activated receptor CD89 is shedded from the cell surface. This process is FcR γ-chain dependent (Chapter 6). Furthermore we demonstrated that human mesangial cells fail to express CD89 in culture and in kidney sections(Chapter 4). After generating new monoclonal antibodies against IgA-binding proteins derived from neutrophils we observed also binding of this antibody to human MC. This and further experiments demonstrate the presence of an other molecule (s) able to bind IgA on the surface of human renal mesangial cells (Chapter 5).

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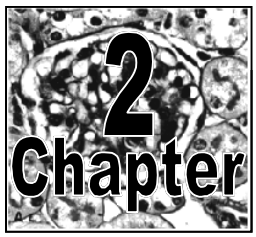

Role of interleukin-6 in mediating mesangial cell proliferation and matrix production in vivo[#]

Frank Eitner, Ralf Westerhuis, Michael Burg, Birgit Weinhold, Hermann-Josef Gröne, Tammo Ostendorf, Ulrich Rüther, Kapl-Martin Koch, Andrew J. Rees, and Jürgen Floege

Division of Nephrology, and Institute for Molecular Biology, Medizinische Hochschule, Hannover, and Department of Pathology, University of Marburg, Marburg, Germany; und Department of Medicine & Therapeutics, University of Aberdeen, Aberdeen, Scotland, United Kingdom

SUMMARY

Role of interleukin-6 in mediating mesangial cell proliferation and matrix production in vivo. Mesangial cell proliferation and matrix overproduction characterize many progressive glomerular diseases. Based on currently available data, the role of interleukin-6 (IL-6) in mediating mesangial cell proliferation and matrix production is controversial. The present study attempts to clarify this issue by showing that: (1) IL-6 knockout mice develop a normal glomerular architecture and in particular a normal mesangium. (2) Mesangioproliferative glomerulonephritis induced by Habu snake venom is equally severe in IL-6 knock out mice as in control mice. (3) A continuous seven-day intraperitoneal infusion of 50btg recombinant human IL-6 into rats with a prior minimal (subnephrito-genic) injury to mesangial cells does not induce glomerular cell activation, cell proliferation, matrix production, leukocyte influx, platelet influx or proteinuria. (4) A continuous seven-day IL-6 infusion into rats with mesangioproliferative nephritis (anti-Thy 1.1 nephritis) increases matrix protein transcription in the absence of detectable effects on matrix proteinaccumulation and otherwise has no effect on the natural course of the disease. We conclude from these findings that IL-6 is not an important mediator of mesangial cell proliferation and matrix overproduction in vivo, and that currently little rationale exists to advocate anti-IL-6 therapy in mesangioproliferative disease states.

[#] ***Kidney international 51: 69-78, 1997***

INTRODUCTION

Mesangial cell proliferation and mesangial matrix accumulation are key features of various human glomerular diseases, including IgA nephropathy, non-IgA mesangioproliferative glomerulonephritis, membranoproliferative glomerulo-nephritis, variants of idiopathic focal sclerosis, lupus nephritis, and possibly diabetic nephropathy (1,21). Mesangial cell proliferation and glomerular matrix accumulation may also contribute to the development of glomerulosclerosis independent of the underlying primary disease (3,4). Therefore, the study of factors that drive rnesangial cell proliferation and matrix production is important to understand the pathogenesis and to design new therapeutic approaches for mesangioproliferative glomerulonephritis and progressive glomerulosclerosis.

Interleukin-6 (IL-6) is produced in relatively large amounts by mesangial cells in response to a variety of stimuli, such as angiotensin II, lectins, matrix proteins, cytokines, and immunecomplexes (5-11). IL-6 has been reported to induce matrix protein transcription and autocrine growth in mesangial cells in vitro (12-14). In vivo, glomerular IL-6 overexpression was detected in human glomerulonephritis types characterized by mesangial hypercellularity such as IgA nephropathy and some types of lupusnephritis (15-19). Furthermore, IL-6 transgenic mice developed a mesangioproliferative glomerulonephritis (13) and the urinary excretion of IL-6 has been correlated with mesangial hypercellularity in patients with IgA nephropathy (13,20,21). It has therefore repeatedly been proposed that IL-6 is an important mediator of mesangial cell proliferation and matrix overproduction (2,8,16,22). This theory has been challenged by several observations. First, IL-6 transgenic mice develop a massive polyclonal B-cell activation, which in itself may be associated with mesangioproliferative disease (23). Second, the role of IL-6 in mediating mesangial cell proliferation in vitro is controversial, with follow-up studies showing either no effect (24,25) or even growth inhibition of mesangial cells treated with IL-6 (26). Third, increased urinary IL-6 excretion has been detected in a variety of renal abnormalities and several patients with mesangial hypercellularity failed to excrete detectable urinary IL-6 (21,27).

In the present study we have attempted to further define the role of IL-6 in the mediation of mesangial cell proliferation and matrix production in vivo. Four experimental approaches were chosen: (1) determine whether IL-6 knock out mice develop a normal mesangium; (2) investigate whether experimental mesangioproliferative glomerulonephritis can be generated in IL-6 knock-out mice in a similar manner as in control mice; (3) investigate the effects of an IL-6 infusion in rats with a prior minimal (subnephritogenic) injury to the rnesangial

cells; (4) investigate the effect of IL-6 infusion in rats with mesangioproliferative nephritis (anti-Thy 1.1 nephritis).

METHODS

Experimental design

All aninial experiments were approved by the local review boards.

Glomerular morphology in IL-6 knock-out mice

Kidneys were obtained from 6 homozygous IL-6 knock-out mice (28) (bred in the animal facilities of the Hannover Medical School, 3 males, 3 females, age 58 to 60 days), 2 heterozygous IL-6knock-out/wild-type mice (2 females, age 58 days) and 6 wild-type control mice (strain C57BL/6, obtained from the Zentralinstitut für Versuchstierkunde, Hannover Medical School; 4 males, 2 females, age 54 days). Prior to sacrifice, a 24-hour urine collection was performed and a serum sample was collected. The IL-6 genotype of each IL-6 knock-out mouse was verified by Southernblot analysis.

Mesangioproliferative glomerulonephritis in IL-6 knock-out mice

Mesangioproliferative glomerulonephritis was induced in 6 homozygous IL-6 knock-out mice (3 males, 3 females, age 90 days) and 6 wild-type control mice (3 males, 3 females, age 85days) by a single intravenous bolus injection of 4 mg/kg body wt Habu snake venom (Trimeresurus flavoviridis; Sigma, Deisenhofen, Germany). Following the injection, all mice were kept under an infrared light for the next three hours to reduce mortality. A 24-hour urine collection was performed from days 5 to 6 after disease induction. Mice were sacriticed at day 6 for the histological examination of the kidneys.

IL-6 infusion in rats following injection of a subnephritogenic anti-Thy 1.1 dose

Eleven male Wistar rats (Charles River) weighing about 180 g received an intravenous bolus injection of 0.2 mg/kg monoclonal anti-Thy 1.1 IgG, (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK). Microosmotic pumps (filling volume 200 μl, delivery rate 1 μl/hr; Alzet Corporation, Palo Alto,CA, USA) were then loaded with 50 μg recombinant human IL-6 (N = 5; kindly supplied by Dr. Amando Proudfood, Glaxo Institute for Molecular Biology, Geneva, Switzerland) or phosphate buffered saline (PBS; N = 6). The pumps were implanted into the peritoneal cavity at four hours after disease induction to avoid

any interference with glomerular anti-Thy 1.1 antibody binding, which is maximal at one hour after injection (29). One hundred microliters of citrate-anticoagulated plasma samples were collected at days 0, 2 and 7 after disease induction and 24 hour urine was collected from days 6 to day 7. Renal biopsies for histological examination of the kidneys were obtained at days 2, 4 and 7 (sacrifice) after disease induction. Prior to the day 2 renal biopsy, proliferating cells had been labeled with 5-bromo-2'-deoxyuridine (BrdU; Sigma) by an intraperitoneal injection of 100mg/kg body wt at 24, 32, and 40 hours after disease induction. Following the renal biopsies at day 7, glomeruli were isolated from the remaining pooled renal cortices of all rats per group by differential sieving and glomerular RNA was prepared.

IL-6 infusion in rats with anti- Thy 1. 1 mesangioproliferative glomerulonephtitis

Eleven male Wistar rats (Charles River) weighing about 180 g received an intravenous bolus injection of 1 mg/kg monoclonal anti-Thy 1.1 IgG, (clone OX-7). Fourty-eight hours later, that is, after the peak of mesangiolysis and at the start of the mesangioproliferative phase, a microosmotic pump (filling volume 200 μl, delivery rate 1 μl/hr) was implanted into the peritoneal cavity. Pumps were loaded with 50 μg recombinant human IL-6 (N = 5) or PBS (N = 6). One hundred microliter plasma samples were collected at days 2, 4 and 9 after disease induction and 24 hour urine was collected from days 8 to 9. Renal biopsies for histological evaluation were obtained at days 4, 6 and at sacrifice (day 9). Prior to the day 6 renal biopsy, proliferating cells had been labeled with BrdU by an intraperitoneal injection at 120, 128, and 136 hours after disease induction. At sacrifice (day 9) glomeruli were isolated by differential sieving from the pooled renal cortices of all rats per group and glomerular RNA was prepared.

Renal morphology

Tissue for light rnicroscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution (30) and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. In the PAS stained sections the total number of cells per glornerular cross section as well as the number of mitoses within the glomerular tuft (extrapolated to mitoses per 100 glomerular cross sections) was determined.

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by a direct or indirect immunoperoxidase technique as previously described (30). Primary antibodies included:

- 1A4, a murine monoclonal antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin (Dako, Glostrup,Denmark) (31).
- D33, a murine monoclonal IgG, antibody against human muscle desmin (Dako) (32).
- PC 10 (Oncogene Science Ine., Uniondale, NY, USA), a murine IgM monoclonal antibody against human PCNA, which is expressed by actively proliferating cells. We have previously shown, in angiotensin II infused rats, that cell proliferation as assessed by anti-PCNA antibody correlates with the cell proliferation as assessed by the conventional method of 'H-thymidineincorporation (33).
- BU-1, a murine monoclonal antibody against bromo-de-oxyuridine (34) containing nuclease in Tris buffered saline (Amersham, Braunschweig, Germany).
- ED1 (Serotec, Oxford, UK), a murine monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells (35).
- PL-1, a murine monoclonal antibody against rat platelets (36).
- affinity purified polyclonal goat anti-human/bovine type IV collagen (Southern Biotechnology Inc., Birmingham, AL, USA).
- an affinity purified IgG fraction of polyclonal rabbit anti-ratfibronectin (Southern Biotechnology).
- a polyclonal, biotinylated horse anti-mouse IgG antibody (Vector, Burlingame, CA, USA).

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit or goat IgG. Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

To obtain rnean nurnbers of proliferating cells or infiltrating leukocytes in glomeruli, more than 30 consecutive cross sections of glomeruli containing more than 20 discrete capillary segments were evaluated and mean values per kidney were calculated. To obtain total counts of proliferating cells or infiltrating leukocytes in the renal cortical tubulointerstitium over 40 grid fields (range 40 to 60), measuring 0.36 mm^2 each, were analyzed and, again, mean counts per kidney were obtained. For the evaluation of the immunoperoxidase stains for α-smooth muscle actin, desmin, type IV collagen, fibronectin and platelets, each glomerular area and tubulointerstitial grid field was graded semiquantitatively, and

the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of staining and dependend on the percentage of the glomerular tuft area or grid field showing positive staining: 0 = absent staining or less than 5% of the area stained; I = 5 to 25%; II = 25 to 50%; III = 50 to 75%; IV = >75%. We have recently described that this semiquantitative scoring system is not only highly reproducible among different observers, but that the data also are highly correlated with those obtained by computerized morphometry (37).

Staining for glomerular mouse IgG, that is, anti-Thy 1.1 antibody, was graded as 0 (negative), I (trace), II (moderate) or III (strong), and a mean glomerular score was calculated.

Electron microscopy

Renal tissue was cut into 1 mm slices and fixed for 24 hours in a 2.5% glutaraldehyde solution. Tissue was then embedded in araldite. Ultrathin sections were contrasted with lead citrate and viewed in a transmission electron microscope (Zeiss EM900).

Preparation of glomerular RNA and Northern analysis

Glomeruli were isolated by differential sieving (38). All glomerular isolates were checked microscopically and exhibited a purity of greater than 98%. Total RNA was extracted from glomeruli with guanidinium isothiocyanate and subsequent ultracentrifugation through caesium chloride using standard procedures (39,40). The RNA content of the samples obtained was determined by UV spectrophotometry at 260 and 280 nm. Samples with an OD260/280 nm ratio of < 1.8 were discarded. For Northern analysis the RNA was denatured and 3 or 10 μg/lane were electrophoresed through a denaturing l% agarose/formaldehyde gel (41). Integrity of the RNA was assessed by visualization of the 28S and 18S rRNA bands. Separated RNA was then transferred onto a nylon membrane (Hybond N; Amersham, Braunschweig, Germany) by capillary blotting and cross-linked using an UV-crosslinker (Stratagene, Heidelberg, Germany). Hybridization was performed using digoxigenin labeled riboprobes which were generated using a Digoxigenin RNA Labeling kit (Boehringer, Mannheim, Ger-many) and hybridized probe was detected using the Digoxigenin Nucleic Acid Detection kit (Boehringer) with minor modifications. Band intensities were scanned with a densitometer (Hero-lab, Wiesloch, Germany) and corrected for the relative intensities of the 28S rRNA signal as detected by hybridization with a 28S rRNA cDNA probe.

The fibronectin and α_1(IV) collagen probe were 420 bp and 296bp, respectively, cDNAs which were generated from total rat glomerular RNA by RT PCR. The sequence of the primers were:

5'-CGTGAATTCCAGGCACTGACTACAAGATC-3' (fibronectin sense);
5'-CGGTCACTCGAGCGATGACATAGATGGTGTAC-3' (fibronectin antisense);
5'-CGTGAATTCGTGCGGTTTGTGAAGCACCG-3' (α_1(IV) collagen sense); and
5'-AGCTCACTCGAGCTTCTTGAACATCTCGCTT-3' (α_1(IV) collagen antisense).

For in vitro transcription, the PCR products were cloned into bluescipt (Stratagene).
The 28S rRNA cDNA (a gift of L. Iruela-Arispe and U. Sage) (42) was labeled with digoxigenin by random priming using adigoxigenin random priming kit (Boehringer Mannheim) according to the manufacturer's instructions.

Monitoring of the IL-6 infusion

To examine whether the dosage regimen was appropriate, the infused human recombinant IL-6 and the biological effects were assessed by measuring plasma concentrations of α_2-macroglobulin (the major hepatic acute phase protein in rats) by immunoelectrophoresis as described (43). IL-6 concentrations were measured in the plasma samples using a commercially available kit (R&DSystems, Biermann, Bad Nauheim, Germany). Finally, all microosmotic pumps were explanted at the end of the infusion period (day 7) and cut open to ensure that all IL-6 had been delivered from the pumps.

Miscellaneous measurements

Urinary protein was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard. Creatinine and urea were measured in serum using an autoanalyzer (Beckman Instruments GmbH, München, Germany).

Statistical analysis

All values are expressed as mean $\pm$ sd. Statistical significance (defined as $P < 0.05$) was evaluated using the Mann-Whitney rank sum test. Correlations were assessed using linear regression analysis.

RESULTS

IL-6 knock-out mice develop a normal mesangium

As shown in Figure 1 A and B, IL-6 knock-out mice displayed a glomerular and mesangial architecture that did not differ from that of wild-type mice. Total glomerular nucleus counts in homozygous and heterozygous IL-6 knock-out mice were not different from those obtained in wild-type mice (Table 1).

Table 1. Glomerular characteristics of non-manipulated, 54- to 60-day-old homozygous or heterozygous IL-6 knock-out or wild-type mice

	IL-6 knock-out mice (homozygous) (N = 6)	IL-6 knock-out mice (heterozygous) (N = 2)	Wild-type mice (N = 6)
Number of nuclei per glomerular cross section	35 ± 13	40/33	36 ± 14
Glomerular α-smooth muscle actin staining score	0.20 ± 0.03	0.20/0.15	0.18 ± 0.04
Glomerular desmin staining score	1.02 ± 0.03	1.11/1.00	1.03 ± 0.04

Data are mean ± SD or individual values (heterozygous mice).

Similarly, glomerular expression of α-smooth rnuscle actin, a marker of activated mesangial cells (38), was equally absent in all three groups, while desmin, which is constitutively expressed by mesangial cells (38), was equally present in all three groups (Table 1). By electron microscopy glomeruli showed a regularly developed mesangium with a normal cell number. The glornerular basement membrane was typically structured and covered by podocytes with typical foot processes (Fig. 1 C, D). No significant proteinuria or hematuria was present in homozygous or heterozygous IL-6 knock-out mice, and serum urea and creatinine values were within the normal range in all mice (data not shown).

Mesangioproliferative glomerulonephritis similarly develops in IL-6 knock-out and wild-type mice

In pilot experiments 4 mg/kg Habu snake venom was identified as the maximally tolerated dose, since at 5 mg/kg of the venom a high mortality was observed. At the 4 mg/kg dose about 20% of the glomeruli exhibited hypercellular areas in both IL-6 knock-out and wild-type mice at day 6 after disease induction (Table 2).

In each group nodular mesangial lesions (Fig. 1 E, F) developed in 2 of 6 rats. The frequency of mesangial nodules did not differ significantly between IL-6 knock-out and wild-type mice (Table 2). Furthermore, scoring of glomerular markers of mesangial cell activation, namely the expression of desmin and the *de novo* expression of α-smooth muscle actin, did not reveal significant differences between the two groups (Tabie 2). Neither IL-6 knock-out nor wild-type mice developed signilicant proteinuria (Table 2).

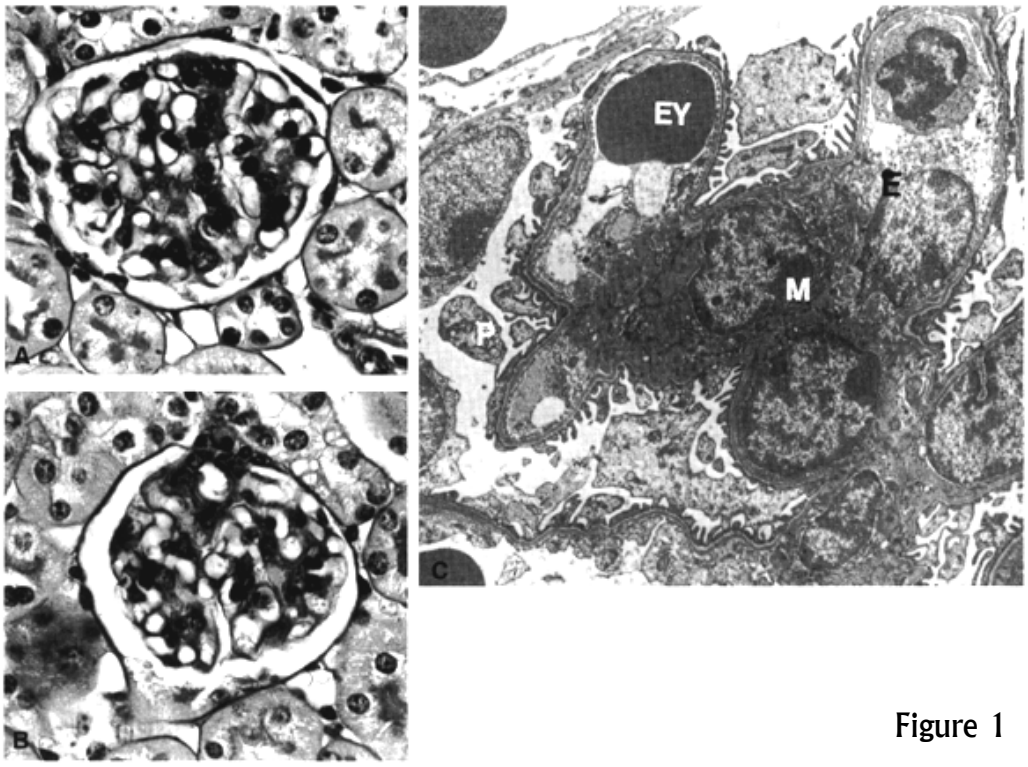

Figure 1

Fig. 1. Light microscopic appearance of a typical glomerulus of an IL-6 knock-out mouse (A) and a wild-type mouse (B). No abnormal morphological features are present (PAS stain; magnification x 1000). C and D show the electron microscopic appearance of glomerular segments of IL-6 knock-out mice. (C) Glomerular segment with regular mesangium, typical mesangial cells and no increase in mesangial matrix; the glomerular basement membrane and podocytes also exhibit a normal morphology. (D) Higher magnification of a mesangial cell with a typical nucleus and typical filaments at the plasmamembrane. Abbreviations are: M, mesangial cell; E, endothelium; EY, erythrocyte; P, podocyte. Light microscopic appearance of a glomerulus with a mesangial nodule in an IL-6 knock-out mouse (E) or wild-type mouse (F) at six days after the injection of Habu snake venom. Nodule formation appears similar in both mouse strains. (PAS stain; magnitication x 1000).

IL-6 infusion has no effects in rats with preceding minimal mesangial injury

In PBS infused rats the injection of a low dose of OX-7 anti-Thy1.1 antibody led to mild glomerular de novo α-smooth muscle actin expression, a minor increase in desmin expression, as well as a low grade increase in the number of glomerular mitotic figures, monocyte/macrophage influx and matrix protein accumulation, while no significant platelet influx was observed (Fig. 2).

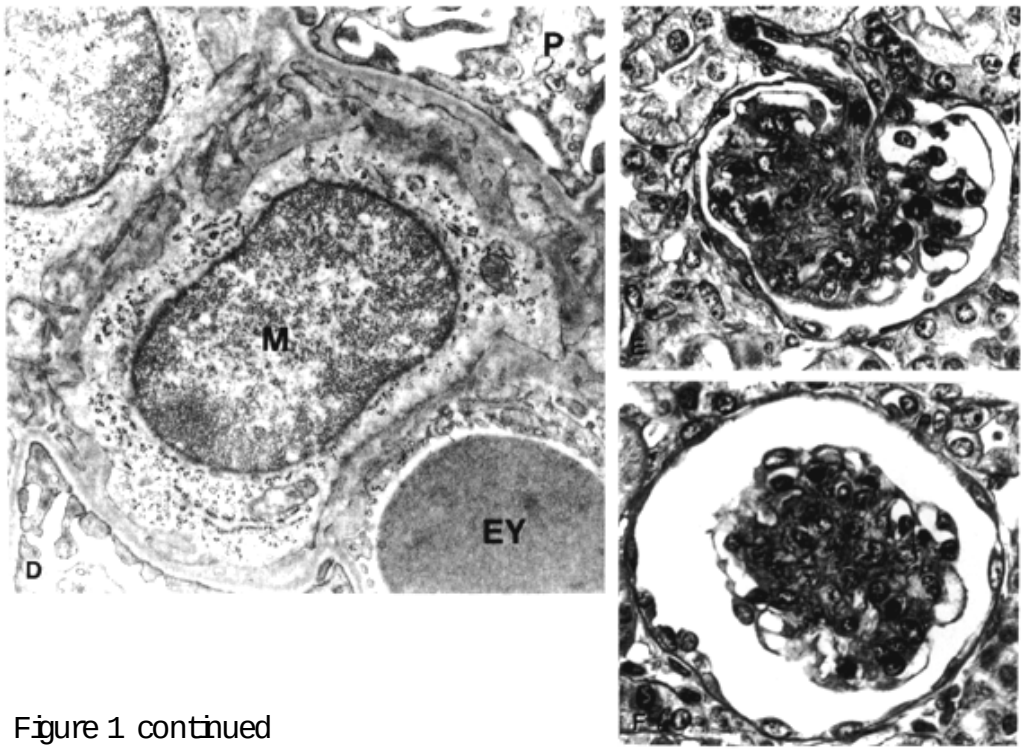

Figure 1 continued

Cell proliferation as assessed by counting of glomerular mitoses was correlated with the counts of PCNA-positive nuclei ($r = 0.75$, $P < 0.001$) as well as with counts of BrdU positive nuclei ($r = 0.56$, $P = 0.07$). None of the aforementioned parameters was significantly altered in IL-6 infused rats as compared to PBS infused rats (Fig.2). Northern analysis of glomerular RNA showed no detectable expression of fibronectin mRNA in both groups under our study conditions (data not shown), while α_1(IV) collagen mRNA was weakly expressed in both IL-6 and PBS infused rats (Fig. 3A). Densitometry and correction for the expression of 28S rRNA yielded an 1.4-fold (range 1.1- to 1.7-fold; N = 3) increase of α_1(IV) collagen mRNA in IL-6 infused rats as compared to PBS infused rats. In neither group did significant proteinuria or hematuria develop. Weight gain during the seven-day study period was similar (PBS infused rats, 5 ± 8 g; IL-6 infused rats, 7 ± 8 g). IL-6 infusion also had no apparent influence on the binding of anti-Thy 1.1

antibody in the mesangium since staining for murine IgG was not significantly different from that observed in PBS infused rats (Fig. 2).

Table 2. Characteristics of IL-6 knock-out or wild-type mice at 6 days after the induction of Habu snake venom mesangioproliferative glomerulonephritis

	IL-6 knock-out mice ($N = 6$)	Wild-type mice ($N = 6$)	P
Hypercellular glomeruli	20.5 ± 13.7%	22.2 ± 21.0%	NS
Glomeruli with mesangial nodule formation[a]	0;0;0;0;10;13%	0;0;0;0;2;45%	NS
Glomerular α-smooth muscle actin staining score	0.33 ± 0.07	0.63 ± 0.77	NS
Glomerular desmin staining score	1.44 ± 0.27	1.65 ± 0.70	NS
Proteinuria *mg/24 h*	3.9 ± 1.9	3.1 ± 1.8	NS

NS is not significantly different.
[a] Individual frequencies per animal are shown

No tubulointerstitial damage occurred in PBS and IL-6 infused rats as judged by PAS-staining of the sections and by the absence of interstitial α-smooth muscle actin and desmin expression, normal PCNA counts, monocyte/macrophage counts and normal staining for type IV collagen and fibronectin (data not shown).

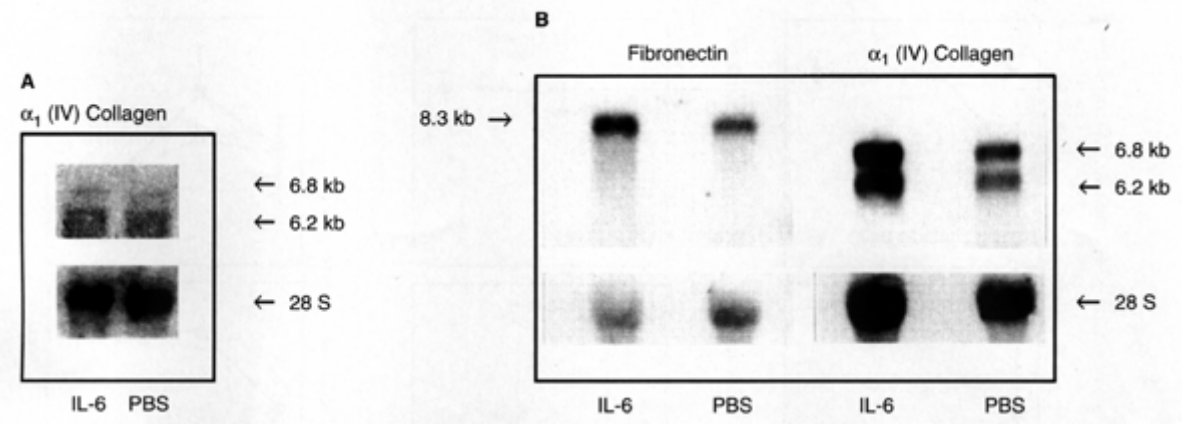

Fig. 2. Glomerular changes in rats with subnephritogenc mesangial injury (Methods) infused from days 0 to 7 with either PBS (; N = 6) or 50 μg recombinant human IL-6 (●; N = 5). Values are means ± SD.

Table 3 shows that the infused IL-6 was biologically active, since all day 2 of the infusion period plasma concentrations of the acute phase protein α2-macroglobulin were significantly elevated over those observed in PBS infused rats. Comparable

levels of plasma α2-macroglobulin have been measured previously after a bolus injection of 4 μg IL-6 or 0.25 μg lipopolysaccharide into normal rats (43). Similar to findings in nephrotoxic nephritis (43), the induction of immune-mediated glomerular injury also induced to some degree the acute phase response, since α2-macroglobulin concentrations increased with time in the plasma of PBS infused rats (Table 3).

Table 3. Plasma concentrations of α_2-macroglobulin and IL-6 in IL-6 or PBS infused rats with either subnephritogenic mesangial injury or anti-Thy 1.1 mesangioproliferative glomerulonephritis

	Plasma α_2-macroglobulin concentration *μg/ml*		Plasma IL-6 concentration *pg/ml*	
	IL-6 infused	PBS infused	IL-6 infused	PBS infused
Subnephritogenic anti-Thy 1.1 dose; *day after disease induction*				
day 0	135 ± 33	119 ± 32	0 ± 0	0 ± 0
day 2	5123 ± 1137	1374 ± 699[a]	52 ± 21	0 ± 0[a]
day 7	1678 ± 474	1142 ± 368	5 ± 2	0 ± 0[a]
Nephritogenic anti-Thy 1.1 dose; *day after disease induction*				
day 2	550 ± 158	852 ± 357	0 ± 0	0 ± 0
day 4	3634 ± 1431	1573 ± 407[a]	42 ± 15	3 ± 3[a]
day 9	3744 ± 1835	2636 ± 526	12 ± 10	2 ± 5

[a] $P < 0.05$ versus PBS infused rats

Measurement of the IL-6 plasma concentrations obtained during the infusion period confirmed that high circulating levels were achieved at day 2, and that they dropped rapidly after the end of the infusion, that is, at day 7 (Table 3).

IL-6 infusion induces matrix protein transcription but has no other effects in rats with mesangioproliferative glomerulonephtitis.

In PBS infused rats the injection of the regular dose of OX-7 anti-Thy 1.1 antibody led to marked glomerular de novo α-smooth muscle actin expression, a marked increase in desmin expression, as well as increased cell proliferation, monocyte/macrophage influx, platelet influx and matrix protein accumulation (fig.

4) similar to previously described findings (30,38,44). Again, none of the aforementioned parameters was significantly altered in IL-6 infused rats as compared to PBS infused rats (Fig. 4).

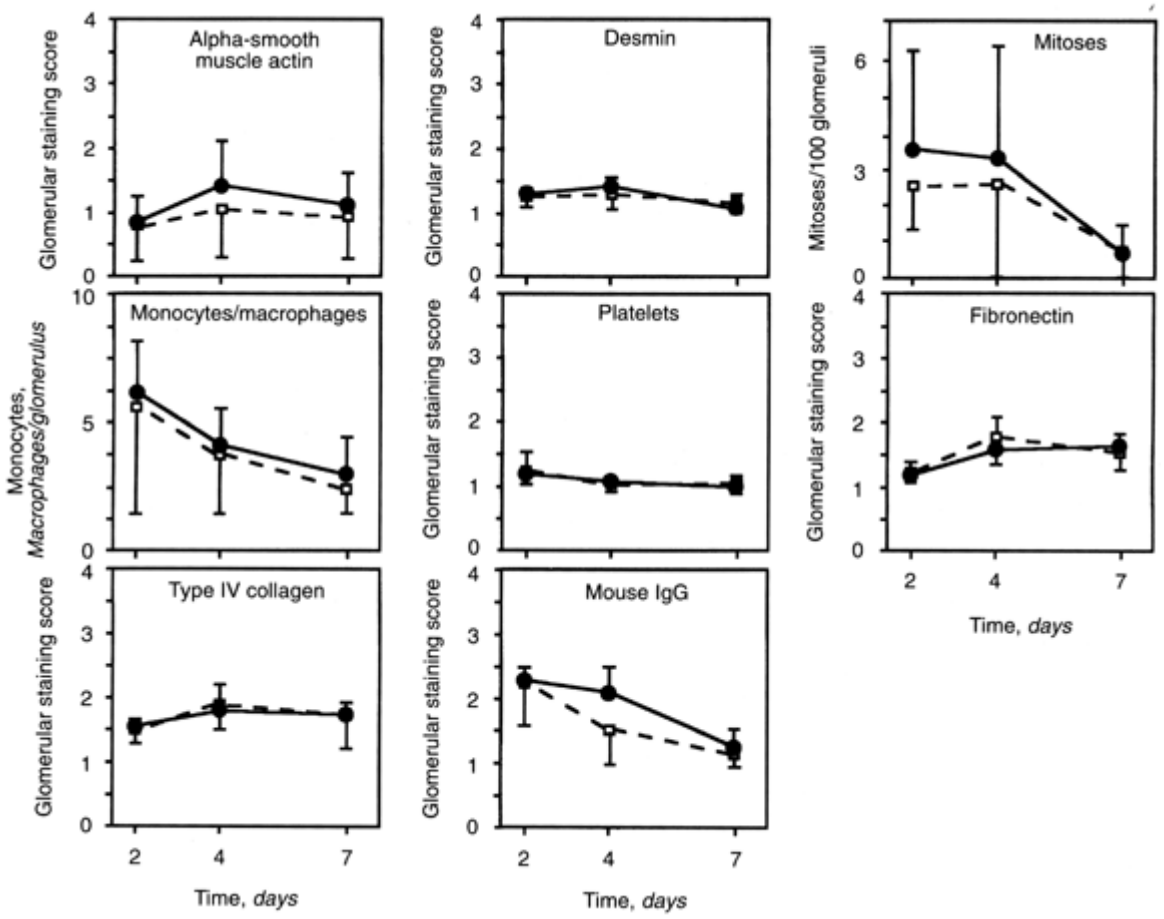

Fig. 3. (A) Demonstration of α_1(IV) collagen mRNA in total glomerular RNA isolated from rats with subnephritogenic mesangial injury and infused from days 0 to 7 with either PBS or 50 μg recombinant human IL-6. (B) Demonstration of fibronectin and α_1(IV) collagen mRNA in total glomerular RNA isolated from rats with mesangioproliferative anti-Thy 1.1 nephritis infused from days 2 to 9 after disease induction with either PBS or 50 μg recombinant human IL-6.

Cell proliferation was also not signifcantly different between IL-6 and PBS infused rats when assessed by PCNA staining or BrdU incorporation (data not shown). Northern analysis of glomerular RNA for the expression of fibronectin or α_1(IV) collagen mRNA showed an upregulation of both RNA species in the IL-6 infused rats as compared to PBS infused rats (Fig. 3B). Densitometry and correction for the expression of 28S rRNA yielded 6.0-fold (range 2.2- to 11.2-fold; N = 3) increases of fibronectin mRNA in IL-6 infused rats as compared to PBS infused rats, and 2.0-fold (range 1.8- to 2.1-fold; N = 3) increases of α_1(IV) collagen mRNA. Proteinuria (54.2 ± 34.4 mg/24 hr) but no hematuria was present at day

8 in PBS infused rats and was not significantly different from that observed in IL-6 infused rats (64.8 ± 26.9 mg/24 hr). Weight gain during the seven-day study period was not significantly different (PBS infused rats, 5 ± 33 g; IL-6 infused rats, 27±8 g). IL-6 infusion also had no apparent influence on the binding or clearance of anti-Thy 1.1 antibody in the mesangium since staining for murine IgG was not significantly different from that observed in PBS infused rats (Fig. 4).

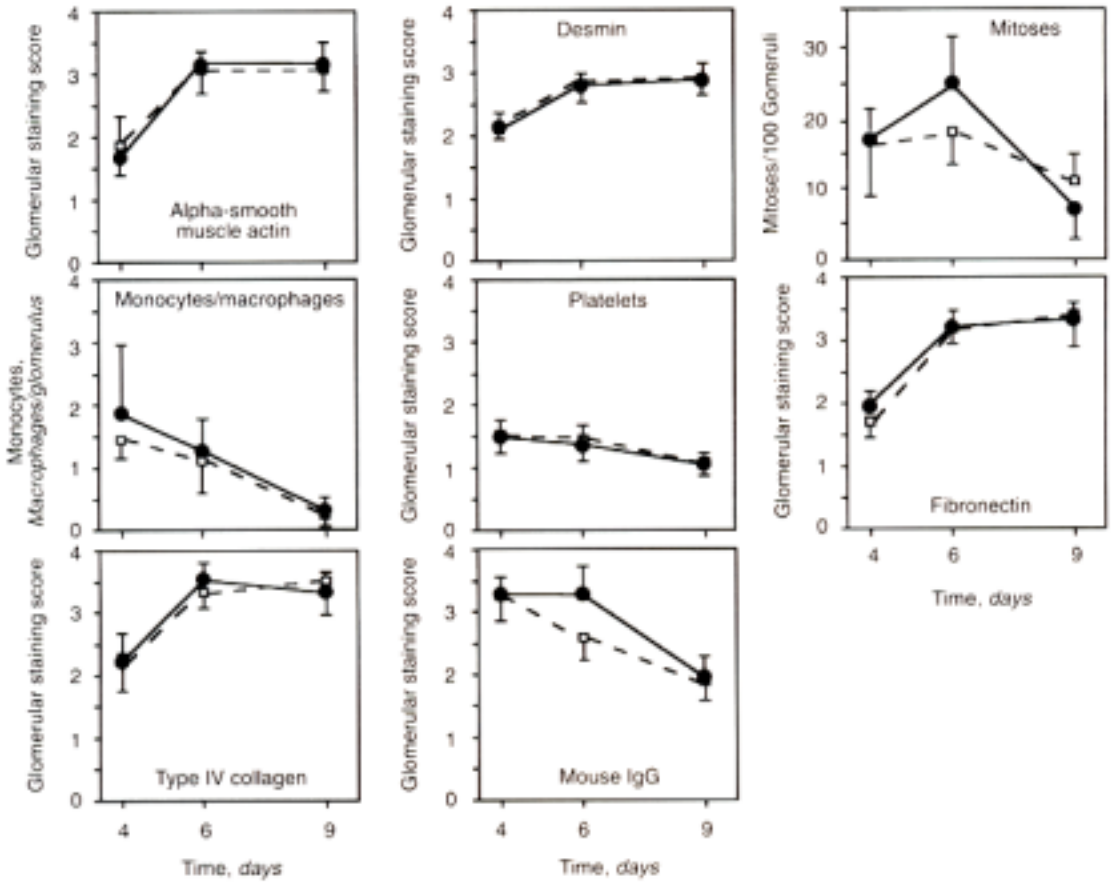

Fig. 4. Glomerular changes in rats with mesangioproliferative anti-Thy 1. 1 nephritis infused from days 2 to 9 after disease induction with either PBS (; N = 6) or 50 μg recombinant human IL-6 (●; N = 5). Values are means ± SD.

No tubulointerstitial damage occurred in PBS and IL-6 infused nephritic rats as judged by PAS-staining of the sections and by the absence of interstitial α-smooth muscle actin and desmin expression, normal PCNA counts, monocyte/macrophage counts and normal staining for type IV collagen and fibronectin (data not shown).

As in the experiments with subnephritogenic anti-Thy 1.1 antibody doses, Table 3 again confirms that the infused IL-6 was biologically active in the

nephritogenic rats and that high circulating levels of IL-6 were achieved during the infusion period.

DISCUSSION

Glomemlar morphology in IL-6 knock-out mice

To evaluate the potential role of IL-6 in mediating mesangial cell proliferation and matrix production, we first examined the glomerular morphology of IL-6 knock-out mice. The rationale for this approach is provided by observations that ontogenetic events are frequently recapitulated during glomerular disease (45). Therefore, mesangial maldevelopment in IL-6 knock-out mice would point to an ontogenetically important role of IL-6 and would imply that IL-6 may play a similarly important role in glomerulonephritis. However, we could not detect any significant difference in the mesangial morphology of IL-6 knockout mice by light and electron microscopy when compared to wild-type mice. Furthermore, the immunostaining pattern in these mice for two cytoskeletal proteins was normal, namely desmin, whose expression is restricted to normal mesangial cells, and α-smooth muscle actin, which is only expressed by activated mesangial cells in vivo (38). It could be argued that these findings do not exclude an important role of IL-6 in mesangial ontogenesis since other growth factors might have compensated for the lack of IL-6. However, it is noteable that in the case of another well established mesangial cell mitogen, platelet-derived growth factor (PDGF), both PDGF B-chain knock-out mice as well as PDGF ß-receptor knock-out mice completely failed to develop a mesangium (46,47).

Mesangioproliferative glomemlonephritis in IL-6 knock-out mice

To further define the role of IL-6 in regulating mesangial cell behavior in vivo, we adapted the rat model of Habu snake venom-induced mesangioproliferative nephritis to mice. Similar to findings in rats (48), Habu snake venom induced a focal glomerulonephritis in wild-type mice, with less than one third of the glomeruli involved. Again, genetic IL-6 deficiency had no effect on the manifestation of the Habu nephritis, suggesting that IL-6 is not of central importance in mediating mesangial cell proliferation and matrix accumulation in this murine model of mesangioproliferative glomerulonephritis.

IL-6 infusion in rats following injection of a subnephritogenic anti-Thy 1.1 dose

In normal rats, toxicity studies performed by others using very high IL-6 doses (daily subcutaneous injections of up to 500 μg/kg for 30 days) have failed to induce any renal abnormalities in rats or mice (49,50). However, failure to detect a cytokine effect in normal animals does not exclude a potential role in disease. Hence, we have described that other mesangial cell growth factors, including basic fibroblast growth factor (bFGF) and PDGF also exhibited no or little proliferative activity in rat glomeruli when infused into normal rats (51-53). However, following minor (subnephritogenic) mesangial injury, similar to that induced in the present study, the mesangial cells became susceptible to the mitogenic action of bFGF and PDGF in vivo and marked proliferation could be observed (51,52). This "priming"of the mesangial cells by subnephritogenic injury likely involved modulation of receptor expression and/or altered post-receptor responses (52,54).

The above observations led us to test the effects of IL-6 in rats with prior minimal mesangial injury rather than in normal rats. As opposed to bFGF and PDGF, under these circumstances IL-6 had no detectable effect on glornerular cell activation (as assessed by the expression of α-smooth muscle actin and desmin (38), cell proliferation or matrix accumulation. This was not due to biological inactivity of the infused IL-6 since it led to a considerable induction of the acute phase protein α_2-macroglobulin, similar to that observed in systemic inflammation induced by endotoxin injection (43). However, we can not exclude that higher, that is, pharmacological, doses of IL-6 might have yielded different biological responses under our experimental conditions.

IL-6 infusion in rats with anti-Thy 1.1 mesangioproliferative glomerulonephritis

In a fourth experimental approach, we tested whether or not the biological activity of IL-6 may depend on a synergism or interactions with other cytokines, such as IL-1, PDGF, bFGF or TGF-ß, all of which are overexpressed in glomeruli of rats with anti-Thy 1.1 nephritis and/or have been invoked in its pathogenesis (51,55-57). Again, IL-6 had no significant effect on a large variety of damage parameters in rats with a fully established mesangioproliferative glomerulonephritis. The only detectable effect of the IL-6 infusion, apart from augmenting the acute phase protein synthesis, was an upregulation of glomerular matrix protein mRNA levels. These data corroborate previous *in vitro* findings in which IL-6 stimulation of

mesangial cells also led to increased matrix protein transcription (14). The lack of a detectable parallel increase of matrix protein deposition in our study may be due to either one of three possibilities: (a) relative insensitivity of our semiquantitative immunostaining scores to small increases in matrix protein deposition, (b) a posttranscriptional block in the matrix protein synthesis or effects of IL-6 on one of the various steps of matrix protein assembly, or (c) a concomitant increase in proteolytic activity. Independent of this latter issue, our data do not support a major role of IL-6 in mediating glomerular extracellular matrix accumulation *in vivo*. Similarly, in a murine model of mesangioproliferative IgA nephropathy, IL-6 administration had no adverse effects on the overall glomerular morphology or on the short-term course of the disease (58). However, in this later study a roughly 35-fold lower total amount of IL-6 was administered (about 7.5 μg/kg body wt/5days vs. 250 μg/kg/7 days in the present study). When given at the same dose as in the present study, IL-6 reduced proteinuria and macrophage activation in rats with nephrotoxic nephritis (59). Part of this action may have resided in an IL-6 induced up-regulation of renal IL-1 type II receptor (59), which acts as a functional antagonist for the action of IL-1. In contrast, in murine models of lupus IL-6 has been identified as an important mediator of the nephritis (50,60). However, additional experiments demonstrated that this effect was largely mediated via IL-6 actions on the immune system rather than direct renal actions of the cytokine (50,60).

In conclusion, using four different experimental approaches, we have failed to detect any evidence for a role of IL-6 in mediating mesangial cell activation or growth in vivo. Despite an induction of glomerular matrix protein transcription by IL-6 under special circumstances, we also failed to establish IL-6 as an important mediator of matrix protein accumulation. While each of the four different approaches individually can not provide definitive evidence against a major role of IL-6 in modulating mesangial cell behavior *in vivo*, the consistency of our findings in four very diverse experimental situations strongly argues against an important role of IL-6 in the pathogenesis of mesangioproliferative diseases. This conclusion receives further support from recent observations in a murine model of IgA nephropathy (58). Taken together, these experimental data at present do not support that neutralization or antagonism of IL-6 should be a therapeutical goal in mesangioproliferative glomerulonephritis.

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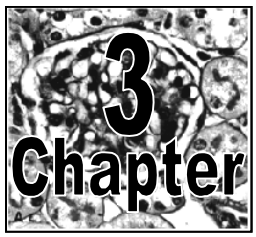

Distinctive Roles of Neutrophils and Monocytes in Anti-Thy-1 Nephritis[#]

Ralf Westerhuis,* Suzanne C. van Straaten, † Mieneke G. A. van Dixhoorn,* Nico van Rooijen, ‡ Nicole A. M. Verhagen,* Christine D. Dijkstra, ‡ Emile de Heer, † and Mohamed R. Daha*

From the Departments of Nephrology * and Pathology †, Leiden, University Medical Center, University of Leiden, Leiden; and, Department of Cell Biology and Immunology ‡, Medical Faculty, Free University, Amsterdam, The Netherlands

SUMMARY

Anti-Thy-1.1 glomerulonephritis as an experimental model for mesangial proliferative glomerulonephritis was induced in Wistar rats by a single injection of monoclonal IgG2a–anti-Thy-1.1 antibody (ER4G). This transient model is complement-mediated and leads to mesangial-cell (MC) lysis followed by MC proliferation, glomerular microaneurysm formation, glomerular influx of polymorphonuclear leukocytes (PMNs) and macrophages, proteinuria, and hematuria. In this study we investigated the distinctive roles of infiltrating PMNs or monocytes/macrophages by treating rats with an antibody against rat integrin CD11b/CD18 (ED7) or by depletion of monocytes with multilamellar clodronate liposomes, respectively. ED7 administration resulted in reduction of the influx of PMNs in glomeruli during the first 6 days after induction of Thy-1.1 nephritis, whereas treatment with an isotype-matched irrelevant antibody (PEN9) or with phosphate-buffered saline had no effect on macrophage influx. Increased glomerular C3 and C6 deposition on days 1 and 3 was seen in the ED7-treated rats but not seen in the control groups. In addition, the ED7-treated group showed an increased number of aneurysmatic glomeruli and more severe hematuria. Monocyte/macrophage depletion led to a significant reduction of mesangial matrix expansion, although mesangial proliferation, proteinuria, and hematuria remained unaltered. These results, together with the known effects of PMN-derived enzymes on C3 cleavage, suggest that a reduction in the influx of PMNs results in sparing of C3 and consequently of more complement activation in the glomerulus with increased complement-mediated damage. Our data indicate that infiltrating PMNs and monocytes/macrophages play distinctive roles during inflammation in this model of MC glomerulonephritis.

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INTRODUCTION

The contribution of polymorphonuclear leukocytes (PMNs) and macrophages in glomerular injury has been well established. The influx of neutrophils during anti-thymus nephritis is well documented (1), but its role in the pathogenesis is largely unexplored(2). Injection of antibodies against Thy-1.1, a transmembrane glycoprotein on mesan-gial cells (3,4), results in complement-dependent mesangial-cell lysis (1,5,6), apoptosis (7), and subsequent mesangial proliferation (8) and extracellular-matrix expansion(9). Previous studies have shown that mesangial-cell injury and the sub-sequent proliferative response depend on complement activation and that the response can be suppressed by decomplementation with cobra venom factor (2,10). Similar results were demonstrated by Bagchus et al (11) who found no glomerular lesions after the injection of a non-complement-fixing monoclonal anti-Thy-1 antibody. During the development of mesangioproliferative glomerulonephritis, the glomeruli are infiltrated by neutrophils (1,2,12) and monocytes (13) Glomerular infiltration by neutrophils is characteristic of acute experimental glomerulonephritis (14,15) Proteinuria resulting from neutrophil-mediated glomerular injury has been shown in nephrotoxic serum nephritis (9,16) and cres-centic glomerulonephritis (17) and after intrarenal injection of phorbol myristate acetate (PMA),(18) or of cobra venom factor, which causes complement activation and subsequent PMN chemotaxis (12,19) In each of these described models, PMN depletion markedly diminished proteinuria and resulted in reduction or attenuation of renal disease (17) The β 2 -integrin CD11b/CD18 has been shown to be involved in the influx of PMNs and monocytes/macro-phages in different reperfusion models in rabbit (20) and experimental allergic encephalomyelitis (EAE) and acute colitis in rats (21,22). In the latter models, a mouse monoclonal antibody (mAb) against CD11b (ED7) was able to reduce the influx of inflammatory cells (predominantly PMNs) into the inflamed tissues and also the severity of the induced disease. In EAE, no effect of administration of anti-CD11b antibodies ED7 or ED8 was observed on the cellular infiltration (predominantly monocytes), but clinical severity was significantly reduced in the anti CD11b-treated EAE rats (21). The involvement of monocytes and macrophages in various glomerulonephritides has been clearly established (23). In particular their involvement has been associated with proliferative forms of glomerulopathies (24,25). Ex-perimental evidence has been provided by rodent models of glomerulonephritis (26–28) However, in some cases infiltrating monocytes may serve merely to remove immune complexes and not to contribute to the glomerular injury (29) Recent interest has focused on the role of macrophages in the pathogenesis of focal glomerulo-sclerosis (30,31) Both

glomerular hypercellularity and expansion of the extracellular matrix are thought to be of primary importance in the development of capillary obsolescence and glomerulosclerosis. In the remnant kidney model in the rat, Van Goor and colleagues (23) showed that macrophages play a central role in the development of focal glomerulosclerosis. As described by Floege et al, the development of glomerulosclerosis in this model is preceded by mesangial proliferation and mesangial matrix expansion (32) In this study we investigated the role of glomerular monocytes and PMNs in the development of mesangial injury and subsequent glomerular hypercellularity and mesangial matrix expansion. The PMN infiltration into the glomeruli during Thy-1 nephritis was affected by pretreatment with monoclonal antibody ED7. Monocyte depletion was performed using a macrophage suicide technique, by injecting liposomes, in which clodronate was encapsulated (33) This study shows that each of these inflammatory cells plays a distinctive role in the pathogenesis of mesangioproliferative glomerulonephritis.

MATERIALS AND METHODS

Animals

Female inbred Wistar rats (160–185 g) were obtained from the animal facilities of the Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. The rats were housed in accredited animal facilities and fed pelleted food, and they had access to water *ad libitum.* Experiments were performed in accord with Dutch legislation for the care and use of laboratory animals.

Antibodies

mAb against Thy-1.1 (mouse IgG2a/k against rat CD90) was derived from hybridoma ER4G as previously described (11). It was purified from ascitic fluid by affinity purification on protein A-Sepharose 4B (Pharmacia, Upp-sala, Sweden). Rabbit anti-mouse IgG was obtained from Jansen (Beerse, Belgium), and goat anti-rat fibrinogen-fluorescein isothiocyanate (FITC) was from Nordic (Tilburg, The Netherlands). The ED1 antibody is a murine monoclonal IgG1 to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (34). PC10 (Dako, Glostrupp, Denmark) is a murine immunoglobulin M (IgM) mAb against proliferating cell nuclear antigen (PCNA), which is expressed by actively proliferating mesangial cells (35) Mouse mAb directed against C6 was kindly provided by Dr. W. G. Couser (Division of Nephrology, University of Washington, Medical Center, Seattle, WA). FITC-conjugated rabbit anti-rat C3 antibodies were generated in our own laboratory. Rat mAb anti-mouse IgG1-horseradish

peroxidase (HRP) was from Sanbio (Uden, The Netherlands). The accumulation of platelets was detected by mAb PI-1 (33) The glomerular influx of PMNs was assessed with FITC-labeled mAb W3/13. After the original description of its tissue distribution (36) and purification (37), the antigen recognized by mAb W3/13 has been identified as leukosialin (38), which is present on T cells, neutrophils, and brain from the rat. After molecular cloning of leukosialin, it has been designated as CD43. The expression and function of CD43 on neutrophil granulocytes have been described in several studies (39,40). It is well established that, during the course of anti-Thy-1 nephritis, an influx of neutrophils and monocytes/macrophages takes place (1). However, there has been no T cell infiltration, during this type of experimental mesangial cell-proliferative glomerulonephritis, that has been detectable with mAb R73 against the T cell receptor, although CD43-positive PMNs were observed with mAb W3/13. Therefore, our study and several earlier ones chose mAb W3/13 for immunohistochemical detection of PMNs in kidney sections of rats (1). ED7 is an mAb against cell adhesion molecule CD11b/CD18 and was prepared as described earlier (41). PEN9 is specific for penicillin and was used as an isotype control for ED7. PEN9 has been characterized (13)

Preparation of Liposomes

Multilamellar liposomes were prepared as described earlier (33). In brief, 86 mg phosphatidylcholine (a gift from Lipoid KG, Ludwigshafen, Germany) and 8 mg cholesterol, molar ratio 6:1, were dissolved in 20 ml methanol/ chloroform (1:1) in a round-bottomed flask. The thin film that formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed in 10 ml phosphate-buffered saline (PBS; 10 mmol/L, pH 7.4), containing either 2.5 g dichloromethylene diphosphonate (Cl2 MDP; a gift from Boehringer Mannheim GmbH, Mann-heim, Germany) or PBS, by gentle rotation for 10 min. Free Cl2 MDP was removed by rinsing the liposomes with PBS and centrifuging them for 30 min at 100,000 3 *g* at 16°C. The liposomes were then resuspended in 4 ml of PBS; 2 ml were intravenously injected per rat. Controls received 2 ml of PBS-encapsulated liposomes. The efficacy of depletion was evaluated by immunohistology with ED1 on kidney biopsies taken after 24 hours. Pilot experiments had shown that administration of higher concentrations of Cl2 MDP-liposomes led to a profound complement depletion, resulting in a decrease of proteinuria development and subsequent reduction of anti-Thy-1–induced pathology (data not shown). Therefore a lipo-some dose was chosen that did not affect the CH50 levels in the blood.

Induction of Anti-Thy-1 Glomerulonephritis

Mesangial proliferative glomerulonephritis was induced by injection of mAb against Thy1.1 (ER4G) at a dose of 1mg/kg intravenously in the rat-tail vein. The animals were housed in metabolic cages, and 24-hour urine protein excretion was measured daily, using the biuret standard method. Urine containing 0.1% merthiolate was used.

Hematuria was assessed with dipsticks (Hema-Combist-icks; Bayer Diagnostics, Mijdrecht, The Netherlands), which have been shown to be as sensitive as direct microscopic enumeration of erythrocytes in full urine. Moreover, the used dipstick can be examined to distinguish between small differences in the amounts of erythrocyturia. Because the values are comparable with those obtained by a colorimetric method and a direct counting of erythrocytes, we used the values obtained from dipsticks (1).

Experimental Design

For ED7-treatment, 21 young female Wistar rats were divided into three groups of 7 rats each. To determine the role of PMNs during anti-Thy-1.1 nephritis, one group of rats was injected, 2 hours before and 3 days after injection of ER4G, with 0.5 mg of mAb ED7. As controls, we used two groups of seven rats each, injected with PBS alone or with an isotype-matched irrelevant antibody, PEN9 (0.5 mg dissolved in equal amounts of PBS as for ED7). All antibodies were administered by tail vein injection. From all rats, renal biopsies were taken 1, 3, 6, and 10 days after injection of ER4G. Urine samples (24 hours) were collected before the experiment and on days 1, 2, 4, 6, 8, and 10.

For macrophage depletion studies, 54 female Wistar rats were used. One group of rats (n = 24) was injected with 1 ml/100 g of body weight liposome-encapsulated Cl2 MDP at day 21 and with 1 mg/kg mAb ER4G at day 0. Kidney biopsies (n = 8 at each time point) were per-formed at 1, 4, and 24 hours after ER4G injection. At days 2, 6, and 14, eight animals were sacrificed. Corresponding groups of rats served as controls; six animals received liposome-encapsulated Cl2 MDP followed by saline injection; in nine animals, only ER4G was injected at day 0; eight animals received liposome-encapsulated PBS followed by ER4G on day 0; four animals received liposome-encapsulated PBS followed by saline injection; six animals received saline only. Blood samples were collected from the tail vein before the injection of Cl2 MDP and immediately before the administration of ER4G to evaluate CH50 levels. All intravenous injections, blood samples, and biopsies were performed under ether anesthesia. At sacrifice, the

kidneys were perfused *in situ* with PBS. Cortical tissue was processed for light and immunofluorescence micros-copy.
All experiments were carried out in two distinct sets. One complete set of experiments is presented in this study.

Histological Examination

Kidney tissue was obtained for light and immunofluorescence microscopy. For light microscopy, tissues were fixed in methacarn solution, dehydrated in graded ethanols, and embedded in paraffin. Sections (4- mm each) were stained with periodic acid/Schiff (PAS) reagent. The degrees of glomerular mesangiolysis (glomerular aneurysms) 6 and glomerular extracellular matrix expansion were graded semiquantitatively in 25 representative glomerular cross sections per rat, as described previously by Floege et al (42). Mean values per biopsy were calculated for the number of proliferating ($PCNA^+$) cells and monocytes/macrophages per glomerular cross section. An indirect immunoperoxidase method was used for the identification of monocytes with mAb ED1 and for PCNA with PC10. Specific antibody binding was revealed by the reaction with hydrogen peroxide and diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Normal rat spleen served for ED1 as positive control. Immunofluorescence microscopy was performed on tissue samples snap-frozen in CO2–ice-cooled isopentane and stored at 270°C. Cryostat sections of 3 mm each were obtained and, after air-drying, fixed in acetone for 10 minutes at room temperature. The slides were washed twice in PBS and examined for the presence of rat C3 by a directly FITC-conjugated antibody. For the detection of PMNs and C6, tissue sections were preincubated with PBS containing a 1:300 dilution of a 30% H2 O2 solution (Merck, Darmstadt, Germany), to block endogenous peroxidases. Thereafter the slides were washed and incubated with digoxigenin (DIG)-conjugated mouse monoclonal anti-rat C6 or the DIG-conjugated mAb W3/13 (1) in 0.5% Boehringer blocking reagent (TNB) after incubation with HRP-conjugated Sheep F(ab') anti-DIG fragments. The slides were subsequently incubated with tyramide-FITC (NEN-Dupont Research Products, Boston, MA) for 30 minutes at room temperature (1). Mean values per biopsy were calculated for the number of PMNs per glomerular cross-section. Glomerular C3, C6, fibronectin, and IgG2a depositions were scored by a semiquantitative method as previously described (42), with scores ranging from 0 to 4 (0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75%, 4 > 75% of the glomerular cross-section). The slides were coded, and 20 glomerular cross-sections per rat were scored independently for each parameter by two experienced microscopists.

Photographs were taken on Kodak TX-400 film on a Leitz microscope equipped with a 4-mm BG 38 1 5-mm BG 12 filter for FITC.

Statistical Analysis

Mean values (6SD) for each parameter were calculated and compared in an independent Student's t-test. A *P* value smaller than 0.05 was considered significant.

RESULTS

Anti-CD11b Treatment

Glomerular binding of mouse mAb ER4G showed a mesangial pattern with no differences between the ED7- treated and control groups. The antibody was detectable in the rat glomerulus until day 3. The influx of PMNs into the glomerular cross-section as assessed by detection with W3/13 was maximal on day 1. The PBS- and the PEN9-treated groups revealed 2.0 $\pm$ 0.2 and 2.1 $\pm$ 0.2 PMNs per glomerular cross-section, respectively. The number of PMNs detected in the ED7-treated group was significantly reduced to 1.6 $\pm$ 0.1 PMNs per glomerular cross-section *(P* $<$ 0.05). A comparable reduction of PMNs was measured on days 3 and 6 of the experiment with highly significant values (Figures 1 and 2). Equal results of glomerular PMN counts were obtained by using PAS-stained sections.

The influx of monocytes/macrophages into the glomerular area did not reveal any differences between the various groups. In all rats, we observed a peak influx of this cell type at day 1, followed by a subsequent decline to baseline levels at day 10 (data not shown).

The glomerular deposition of C3 and C6 was scored semiquantitatively after indirect immunohistochemical staining of frozen kidney sections of each rat. Significantly higher scores for C3 and C6 deposition were found on day 1 for the rats treated with ED7 as compared with the PBS- and the PEN9-injected rats. C6 deposition was seen only in rats treated with ED7 on day 3. At later time points, no C6 deposition was detected in any of the experimental animals. Glomerular C3 deposition was highest on day 1 in the ED7-treated group and significantly increased as compared with the controls. On day 6, only ED7-treated rats exhibited some C3 deposition (Figures 1 and 2).

When the biopsies of the rats were analyzed by light microscopy, we observed a significantly higher maximum of glomerular microaneurysms (37.1 $\pm$ 16%) on day 3 for the rats receiving ED7. The glomerular lesions of the control

groups were approximately 50% less compared with ED7-treated animals (Figures 2 and 3). The extracellular matrix/fibronectin score was not significantly different between the ED7-treated and the control groups. Also, the number of proliferating glomerular cells was not changed after reduction of glomerular influx of PMNs.

Figure 1

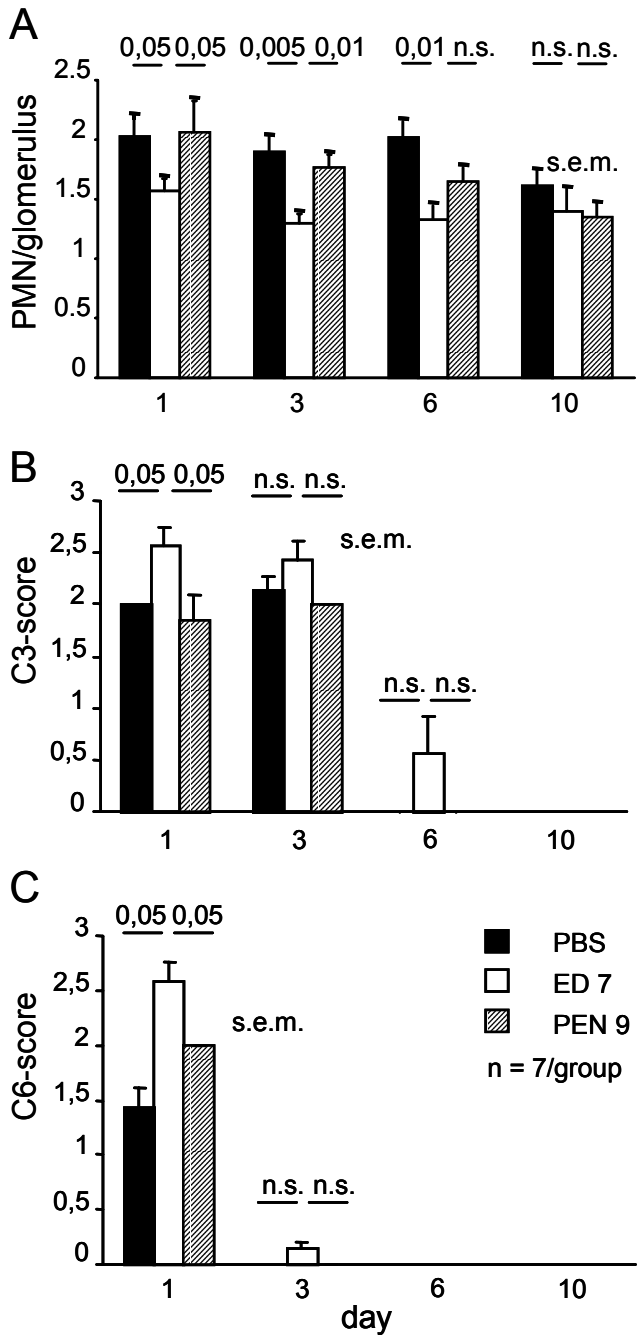


Figure 1. A: *PMN influx in ED7-treated* ***(white bars)****, PEN9-treated* ***(pat-terned bars)****, and PBS-treated* ***(black bars)*** *rats. Immunohistochemical staining for intraglomerular PMNs is shown as mean 6 SEM number of W3/13-positive cells/glomerular cross section per group. ED7 pretreatment led to significant reduction of PMN influx during anti-Thy-1.1 nephritis until day 6. At day 10, no significant difference was detected.* ***B:*** *Immunohisto-chemical staining for glomerular C3 deposition after induction of anti-Thy-1.1 nephritis, using a semiquantitative scoring system (shown as mean 6 SEM). Higher scores for C3 were observed on days 1 and 3 for the ED7-pretreated group as compared with the control groups. Only the ED7-pretreated rats showed residual C3 deposition on day 6.* ***C:*** *Immunohistochemical detection for glomerular C6 deposition was found to be higher on day 1 in the ED7 group (shown as mean 6 SEM). On day 3 only little C6 deposition was still found in this group, whereas the control groups did not show positivity for C6.*

Hematuria and proteinuria

Hematuria from day 4 and later in this experiment showed, for the ED7-treated rats, higher values as compared with the control groups. Because of the high standard deviations in all groups, this increase was significant only on days 2, 8, and 10 (Figure 3). For proteinuria we did not find significant differences between the groups (data not shown).

Monocyte and Macrophage Depletion Studies

Rats treated with clodronate liposomes exhibited a nearly complete reduction of glomerular monocyte infiltration. Although control rats showed about 4.2 ± 1.8 cells per glomerulus, the monocyte-depleted rats had 0.2 ± 0.25 ED1^{+}1 cells *(P* < 0.001; Figure 4). A reduction of 15 ± 23% of circulating monocytes was found. It is interesting that no additional effect of ED7 was seen on the clodronate-mediated reduction in circulating monocytes.

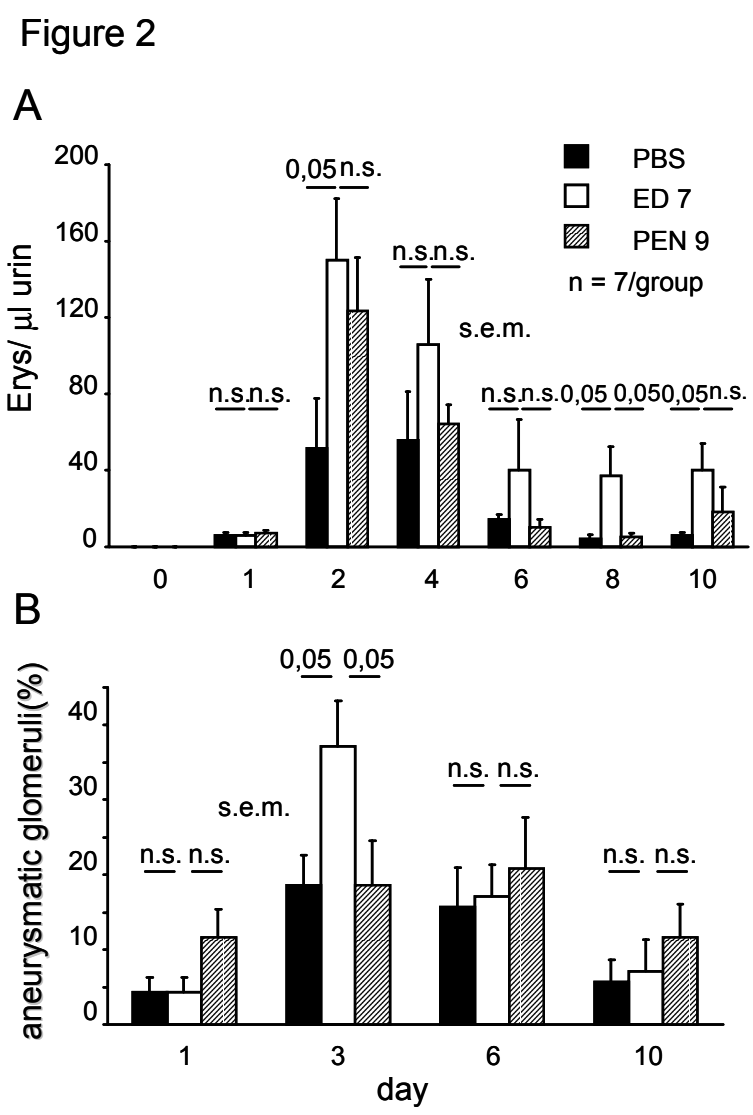
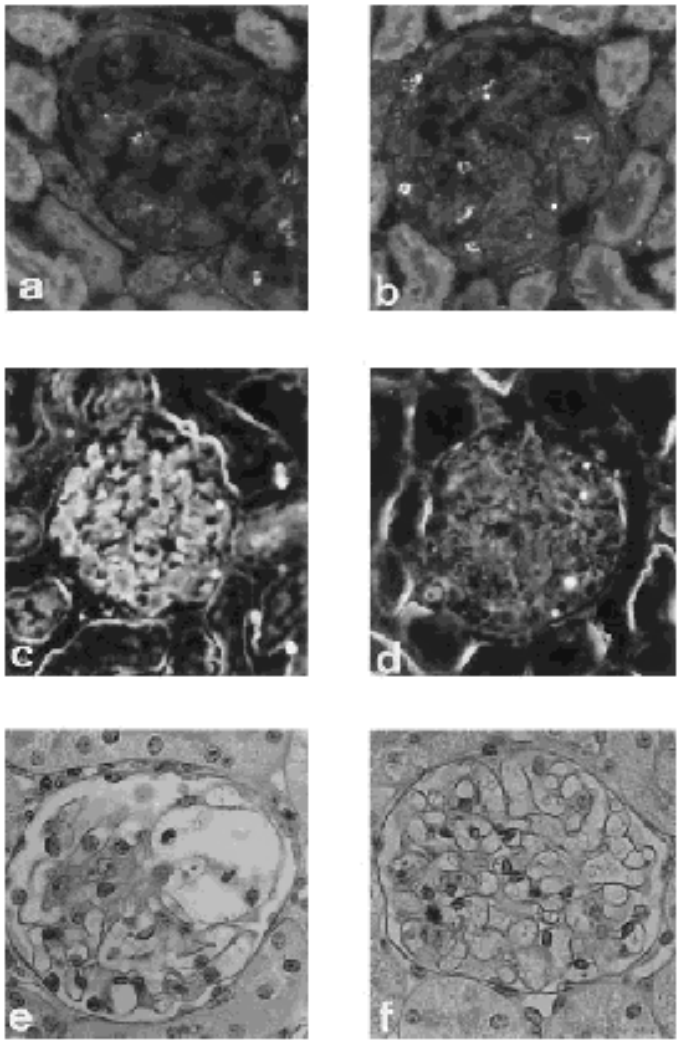


Figure 2. A: Course of hematuria during the experiment detected by dip stick. At all time points, hematuria of the ED7-treated group was highest compared with the control groups. Because of the high standard deviations, only the differences on days 2, 8, and 10 were significant. The data show mean values per group 6 SEM. **B:** Detection of glomerular microaneurysms by scoring PAS-stained kidney sections (mean values per group 6 SEM). The ED7-pretreated group developed a signifycantly higher percentage of glomeruli with microaneurysms on day 3 after induction of nephritis. The aneurysms detected on the other days of the experiment were not significantly different.

The influx of PMNs into the glomeruli was not affected by the monocyte/macrophage depletion. Glomerular deposition of C3 and C6 was not changed at all by macrophage depletion. All groups showed the expected degree of complement deposition as described for the control groups in the ED-7 experiment.

Figure 3. Immunohistochemical staining for PMN influx in ED7- and PEN9-/PBS-treated rats was performed using mAb W3/13. **A:** ED7 treatment led to significant reduction of PMN influx during anti-Thy-1.1 nephritis. A representative glomerulus obtained on day 1 is shown. **B:** The control groups showed significantly higher values of W3/13-positive cells. **C** and **D:** Immunohistochemical staining for glomerular C3 deposition on day 1 after induction of anti-Thy-1.1 nephritis are shown. **D:** Rats treated with control antibody PEN9 or PBS showed less deposition of C3 as compared with rats treated with ED7 **(**C). **E** and **F:** Glomerular microaneurysms were scored in PAS-stained kidney sections. The ED7-treated group of rats developed significantly more glomerular microaneurysms **(**E) on day 3 as compared with rats treated with PBS or PEN9 **(**F).



In the PAS-staining, we observed no differences for glomerular micro-aneurysms /mesangiolysis. However, a significant difference in extracellular matrix scores was found between the two groups (Figure 4). The glomerular deposition of fibronectin corresponded to the difference in mesangial extracellular matrix expansion (data not shown).

We did not observe significant differences in proliferating ($PCNA^+$) mesangial cells between the different groups of rats (data not shown).

Proteinuria and Hematuria

In monocyte-depleted Wistar rats, proteinuria and hematuria were comparable with that in the control rats (data not shown).

DISCUSSION

In this study we demonstrate that treatment of rats with ED-7, a mouse anti-CD11b mAb, selectively reduces glomerular PMN influx during the course of anti-Thy-1.1 ne-phritis. PMN reduction was associated with enhanced hematuria, a higher percentage of glomeruli with micro-aneurysms, enhanced glomerular deposition of C3 and C6, and no significant change in proteinuria, suggesting that microaneurysm formation is an important factor in determining the degree of hematuria.

Figure 4

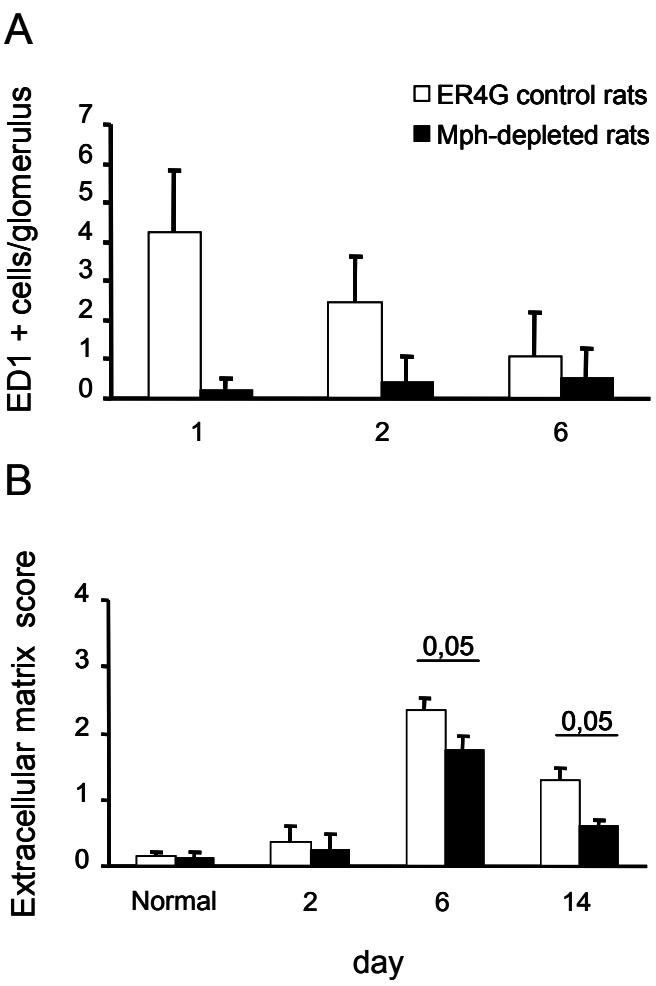


Figure 4. A: Macrophage influx in macrophage-depleted **(black bars)** and control **(white bars)** rats. Immunohistochemical staining for intraglomerular macrophages is shown as mean number of ED1-positive cells per glomerulus per group 6 SEM. Macrophage depletion led to an almost complete reduction of infiltrating macrophages on days 1 and 2 after induction of anti-Thy-1.1 nephritis. At day 6, no significant difference was detected. **B:** Extracellular matrix expansion was scored using PAS staining of rat kidneys. During anti-Thy-1.1 nephritis, glomerular extracellular matrix expansion (shown as mean ± SEM) was significantly reduced at days 6 and 14 after depletion of macrophages as compared with rats not depleted **(white bars)**.

It is known that PMNs contribute to tissue injury during ischemic reperfusion of the pulmonary, coronary, cerebral, and splanchnic circulation. Injection of antibodies against CD18 confers protection against ischemic-reperfusion injury of these organs in many experimental models (43). Previously it was shown that ED7 is able to reduce the influx of neutrophils during experimental acute colitis (22) in rat. Also the damage in the colon was found to be much less. In a peritoneal recruitment assay, ED7 treatment reduced the influx of myelomonocytic cells (21). Also, in a lung

reperfusion model, an anti-CD18 antibody prevented vascular injury associated with a reduction of myeloperoxidase in the examined tissues (20,44).

No effect of ED7 treatment on monocyte influx during anti-Thy-1.1 nephritis was observed. The reason for the different behaviors of the two cell types in this model is unknown. A possible explanation could be the major pathogenetic role of complement in this model, in which several chemoattractants are released during the initial phase of injury, which may contribute to the attraction of monocytes.

Earlier experiments assessed the influence of mAb against CD18, CD11a, CD11b, very late antigen-4 (VLA-4), intercellular adhesion molecule-1 (ICAM-1), and E-selectin on glomerular neutrophil accumulation and proteinuria in Long-Evans rats with nephrotoxic-serum nephritis (45). This model is characterized by up-regulation of vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and E-selectin expression, rapid neutrophil infiltration into the glomerulus, and proteinuria. Treatment of animals with antibodies against CD18, CD11b, and ICAM-1 caused 63%, 46%, and 54% reduction, respectively, in proteinuria and 79%, 66%, and 54% reduction, respectively, in glomerular neutrophil counts, suggesting an important role for these adhesion molecules in this model of experimental nephritis (45). Moreover, these data indicate that neutrophils play a major pathogenetic role in experimental nephrotoxic nephritis, specifically in glomerular permselectivity, whereas other factors, for example, complement activation, seem to have a secondary role. These findings are further supported by another study investigating nephrotoxic-serum nephritis (46).

Our own results for the reduction of glomerular PMN counts are in agreement with the above mentioned studies. On the other hand, we did not find a reduction of hematuria or proteinuria in this complement-mediated experimental glomerulonephritis, suggesting that neutrophils do not significantly contribute to loss of permselectivity in this model. This is in agreement with earlier studies. (2,6,10).

In contrast, PMNs seem to contribute to the repair process during anti-Thy-1.1 nephritis. Maybe proteolytic enzymes released by these cells have a complement-clearing function, and consequently a reduction in PMN influx may be responsible for the observed pronounced and sustained C3 and C6 deposition in rats treated with ED7, compared with the controls. In the literature a complement-cleaving function of proteases released from PMNs has been described. It was found that proteolytic enzymes derived from PMNs in pleural empyema or from isolated PMNs can inactivate C3 bound to Sepharose (47). Furthermore, cleavage of C3b and C3bi bound to human erythrocytes was shown to depend on purified

leukocyte enzymes or crude extracts from human PMNs (48,49), indicating a complement-clearing function of PMN-related enzymes. Because complement is the major pathogenic factor leading to MC lysis in this experimental model, this could be an explanation, on one hand, for the increased damage and hematuria observed in this study. On the other hand, it was found (50) that incubation of highly purified human C1 inhibitor with equally pure human leucocyte proteinase 3 resulted in a dose- and time-dependent inactivation of C1 inhibitor hemolytic activity, which may lead to an increase of activated complement factors.

The major difference in the above mentioned nephrotoxic nephritis is that, during Thy-1 nephritis, neutrophils and complement together play a role, whereas, in nephrotoxic nephritis, neutrophils seem to be the main factor affecting the permselectivity of the damaged rat kidneys. Neutrophils in nephrotoxic nephritis seem to contribute to mesangial injury, whereas neutrophils during Thy-1 nephritis do not seem to play a major role.

Concerning the role of macrophages, we found that selective depletion of macrophages led to a reduced glomerular matrix expansion. In contrast to ED7 treatment, glomerular complement deposition, microaneurysm formation, and hematuria remained unaffected.

These data are in agreement with recently published investigations (51). It was found that, after induction of anti-Thy- 1.1 nephritis, influx of macrophages is strain dependent. Lewis rats showed high amounts of infiltrating macrophages into the rat glomerulus, whereas F344 rats showed no enhancement of these infiltrating cells. Moreover, after transplantation of F344 kidneys in Lewis rats and vice versa, there was influx of macrophages only in kidneys transplanted to Lewis rats and not in kidneys transplanted to F344 rats. In F344 rats, the influx of macrophages was low, and the extracellular matrix expansion was marginal. In contrast, high amounts of infiltrating macrophages led to a highly significant increase of extracellular matrix in Lewis rats (51). The mechanisms leading to these differences are still unclear; all grafts showed an equal induction of monocyte chemotactic protein-1 in both rat strains. These data indicate a predominant factor located outside the kidney that leads to mononuclear infiltrates in this experimental disease. The potential mechanisms by which macrophages mediate glomerular-cell proliferation and the development of mesangial matrix expansion could involve release of cytokines by the macrophage itself or could involve stimulation by the macrophage of other cell types to release cytokines within the glomerulus (52,53). During tissue repair processes, macrophages can release TGF-β1 (54), a growth factor that has clearly been shown to be involved in mesangial matrix expansion (54–56). A second macrophage-derived product with a possible high impact is nitric oxide, which is

abundantly secreted by infiltrating monocytes in this model (57). Monocytes have been shown to induce increased transcription of TGF-β and fibronectin (58).

In coculture studies, Mosquera demonstrated that monocyte-derived culture supernatants could induce mesangial cells to synthesize fibronectin in vitro (59). As shown in Figure 3, the mesangial expansion in monocyte-depleted rats was not completely inhibited. This may be because 1) especially major histocompatibility complex-II neg resident macrophages are less sensitive to liposome-mediated elimination (E De Heer, V Cattell, unpublished results), and 2) cytokines from other sources, for instance platelet-derived growth factor, may be able to induce mesangial matrix expansion, albeit less effectively (52).

In conclusion our study presents evidence that monocytes are specifically involved in the expansion of the mesangial extracellular matrix, whereas other immunopathological processes in the mesangium (complement activation, platelet aggregation, and mesangial proliferation) remain unaffected. These findings indicate that the mesangial alterations occur through distinct signaling pathways.

Taken together we hypothesize that a reduced influx of PMNs and a subsequent reduction in generation of C3-cleaving enzymes from PMNs may lead to a relative sparing of activated C3, which, on its own, results in more injury (glomerular aneurysms) and up-regulation of hematuria via a complement-dependent mechanism. Furthermore, we found monocytes being selectively involved in mesangial extracellular matrix expansion, whereas the induction of mesangial proliferation is induced through a separate pathway.

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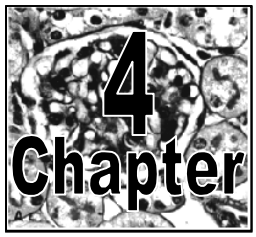


Human mesangial cells in culture and in kidney sections fail to express Fc alpha receptor (CD89) [#]

Ralf Westerhuis, Ger van Zandbergen, Nicole A.M. Verhagen, Ngaisah Klar-Mohamad, Mohamed R. Daha and Cees van Kooten *Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands*

SUMMARY

The mechanism of deposition of IgA in the renal mesangium in primary IgA-nephropathy is poorly understood. It has been suggested that membrane receptors for IgA on mesangial cells (MC) of the kidney may be involved. To obtain more insight in the occurrence of the myeloid receptor for IgA (CD89) on MC, both *in situ* and in culture, rabbit- and goat polyclonal antibodies and mouse monoclonal antibody against recombinant CD89 were raised. Kidney sections from five control subjects and five patients with primary IgA-nephropathy failed to be positive for CD89 in the mesangium, using our polyclonal and monoclonal antibodies. Also, five primary human MC cultures assessed for CD89 expression showed no protein expression of CD89. Furthermore reverse transcription-PCR failed to detect mRNA expression of CD89 in the cultured MC. It was demonstrated that all five human primary MC bound human IgA in a dose-dependent manner, which was not inhibitable by blocking monoclonal anti-CD89 antibody (My43). In contrast, binding of IgA to U937 cells was blocked efficiently by My43. Finally, incubation of human MC with either human or rat IgA, led to increased interleukin-6 production, whereas only human IgA, but not rat IgA, was able to bind to human CD89. Therefore, it is concluded that human MC do not express CD89 (to a significant extent). These results strongly suggest that binding of IgA to human MC occurs via an IgA receptor distinct from CD89.

[#] ***Drs Westerhuis and van Zandbergen contributed equally to this study. J Am Soc Nephrol 10:770, 1999***

INTRODUCTION

Primary IgA-nephropathy (IgAN) is the most common form of glomerulonephritis in humans, leading to progressive renal failure in nearly half of the patients. IgAN is characterized by deposition of mainly IgA1 in the mesangial area often associated with higher serum IgA1 levels. IgA deposition in the mesangium is thought to play a crucial role in the inflammatory process in this disease (1,2) but the mechanism responsible for IgA deposition remains unknown.

Different investigators have showed that IgA binds to rat mesangial cells (MC) (3,4). It was also shown that binding of IgA to MC leads to uptake and degradation of IgA and that IgA induces release of interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) (3). Previous results have shown that IgA binds to human MC (5-7), however, no specific receptor could be identified.

The DNA sequence of a receptor for the Fc tail of IgA expressed on myeloid cells was published earlier (8). Further investigations found expression of this IgA receptor, designated Fcα receptor or CD89, on the surface of neutrophils (9,10), eosinophils (11) and cells of the monocyte/macrophage lineage (12).

Very recently, mRNA expression of the Fc receptor CD89 has been suggested on human MC (13). Activation of human MC with IL-6 or TNFα led to increased binding of IgA accompanied by an increase of mRNA expression of CD89. However, no evidence was provided for the direct involvement in the binding of IgA to these cells via CD89 (13). Two additional studies showed an increase of mRNA expression of monocyte chemoattractant protein-1, IL-8, nuclear factor-κB, and also an increase of intracellular calcium release following interaction of MC with aggregated IgA (14,15). Further mRNA expression of CD89 was demonstrated in whole human glomeruli in 40% of patients with IgAN. In healthy individuals and patients with mesangial proliferative glomerulonephritis distinct from IgAN, no CD89 expression was found, suggesting an absence of CD89 expression on human MC in situ (16).

Because of these contradictory results, the aim of the present study was to determine whether CD89 is expressed on human MC *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cell culture

The CD89-expressing human monocytic cell line U937 was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Breda, The Netherlands).

The murine B-cell line IIA1.6 (17) was cultured in RPMI 1640 supplemented with 10 % FCS. IIA1.6 was transfected with CD89 cDNA, and stable cell surface expression was maintained by cotransfection of human γ-chain cDNA, as described previously (17). The CD89 transfectants were grown in the same medium supplemented with geneticin (G418, 0.8 mg/ml; Life Technologies) and methotrexate (MTX, 10 mmol/L; Pharmachemie, Haarlem, The Netherlands).

In the present study, primary human MC were obtained from five different normal donor kidneys. MC were cultured and characterized as reported in detail elsewhere (18,19). After outgrowth of the MC, the hillocks formed 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, 100 U/L penicillin and 100 μg/ml streptomycin (Life Technologies). Primary MC-cultures were used between subculture 3 and 10.

Monoclonal anti CD89 antibodies

The generation and production of recombinant soluble CD89 (sCD89) by transfected Chinese Hamster Ovary (CHO) cells using the pEE14 expression system will be described in detail elsewhere *(G. v. Zandbergen, Manuscript in preparation)*. Soluble recombinant CD89 was isolated from the culture supernatant by affinity chromatography, using Sepharose-bound human IgA.

To generate new monoclonal antibodies, female BALB/C mice were immunized with purified sCD89 (8). Splenocytes isolated from immunized mice were fused with myeloma cells (SP20), using 50% polyethylene glycol. The cell suspension was diluted in RPMI 1640 supplemented with 10% FCS, hypoxantine (100 μmol), aminopterin (0.4 μM), thymidine (16 μM), 100 U/L penicillin and 100 μg/ml streptomycin. Cells producing anti-CD89 antibodies were subcloned by limiting dilution. Five clones producing anti-CD89 antibodies were expanded and the specificity determined by Western blotting. For this purpose, recombinant sCD89 was electrophoresed on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under reducing conditions and blotted onto nitrocellulose (20) and subsequently interacted with culture supernatant from A77, a well defined mouse monoclonal antibody (mAb) anti-CD89 (21), and the five new clones (7D7, 7G4, 2D11, 2E6, 2H8, 1/100). Finally, bound antibodies were detected using goat anti-mouse IgG-horseradish peroxidase, and detected by chemiluminescence.

Polyclonal antibodies

A rabbit and a goat were immunized with 100 μg of purified sCD89 at 4 weekly intervals. After 3 months, serum was tested for anti-CD89 reactivity by enzyme-linked immunosorbent assay (ELISA). The IgG fractions of the polyclonal antibodies were isolated by $(NH_4)SO_4$ precipitation followed by diethylaminoethyl Sephacel (Pharmacia, Uppsala, Sweden) anion exchange chromatography and tested on Western blots. For control purposes, rabbit- and goat IgG were also isolated from normal sera.

Immunohistochemistry

Pretransplant kidney tissue and biopsies from patients with proven IgAN (Department of Nephrology, University Hospital Leiden, The Netherlands) were snap-frozen in liquid nitrogen. Tissue specimens were processed for immunofluorescence according to standard procedures. As a control for tissue with CD89 antigen, normal donor spleen was used.

For analysis of CD89 on MC, primary MC were grown on glass coverslips for 24 h, washed in phosphate-buffered saline (PBS), and air-dried. As a positive control, U937 cells cultured on glass coverslips were used. Cryostat sections and coverslips were fixed in acetone for 10 min at room temperature.

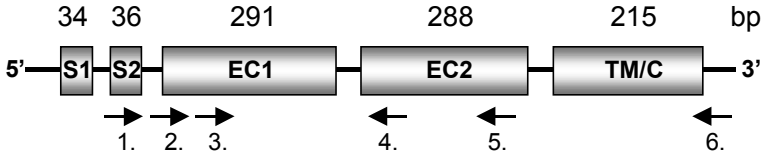
The tissue sections and the glass coverslips were washed 3 times for 5 min with PBS and incubated with either polyclonal or monoclonal anti-CD89 antibodies for 1 h. As a positive control, we used W6-32, a mouse mAb anti-MHC-class I. After washing the tissue sections and coverslips the preparations were incubated with goat anti-mouse-FITC, rabbit anti-goat-FITC, and with goat anti-rabbit-FITC, respectively (Dako, Glostrup, Denmark).

RNA Isolation and Reverse Transcription-PCR

Total RNA from U937, IIA1.6 CD89-transfected cells, and human MC in the same passage as used for fluorescence-activated cell sorter (FACS) analysis. Also MC cultured in 20% FCS or stimulated with phorbol 12-myristate 13-acetate (10ng/ml)/Ionomycin (1 μg/ml) for 24 h were used. Total cellular RNA was isolated from 1 x 10^6 cells, using RNAzol B (Cinna/Biotecx, Houston, TX), according to manufacturer's instructions (22). Fixed amounts of total cellular RNA (1 μg) were reverse-transcribed into cDNA by oligo-(dT) priming, using Moloney murine leukemia virus reverse transcriptase (Life Technologies).

The amplification of cDNA by PCR was performed using the primers as shown in Figure 1. We tested seven different combinations of the above-described forward and reverse primers as shown. Furthermore we performed a nested PCR in which

we first used the forward primer (bp 90 to 109) combined with the reverse primer (bp 614 to 633). The product of this PCR was checked on agarose gel and then 1, 0.1, and 0.01 μg of the PCR product were again amplified by PCR using the forward primer (bp 119 to 138) and the reverse primer (bp 441 to 460). PCR amplification was performed under standard conditions (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 20 mmol/L $MgCl_2$, 0.06 mg/ml bovine serum albumin, 0.25 mM dNTP, 25 pmol of each primer, and 1 U of *Taq* polymerase: Perkin Elmer, Norwalk, CT) by 35 cycles of the following scheme: 1.5 min at 95°C, 2.5 min at 60 °C, 1.5 min 72°C, followed by 10 min of primer extension at 72 °C. $MgCl_2$ concentrations of 20 mmol/L was found to be superior compared with 15 or 25 mmol/L for the amplification of CD89. PCR products were analyzed on a 1% agarose gel containing ethidium bromide. Results were registered using a digital camera (Eagle eye: Stratagene, San Diego, CA), and for reason of clarity, the images were black/white inverted.



Primers No.	Primer sequence 5'- 3'	Primer position	forward/ reverse
GAPDH	ccAcccATggcAAATTccATg	185 - 205	forward
GAPDH	cTAgAcggcAggTcAggTcc	762 - 781	reverse
for CD89:			
1.	ccTccTgTgTcTTgTgcTcT	60 - 79	forward
2.	gAgGATTcAggcAcAggAAg	90 - 109	forward
3.	ccATgccTTTcATATcTgcc	118 - 138	forward
4.	TcTcTccTggcATcAAcAcc	460 - 441	reverse
5.	gTTgTAccAAccgTAgcAcc	633 - 614	reverse
6.	cTTgcAgAcAcTTggTgTTc	900 - 881	reverse

Figure 1. Gene of CD89 and the used primers. *At the top, the gene of CD89 is shown containing five exons: S1, S2, extra-cellular-1 (EC1), EC2, and TM/C (transmembrane/ cytoplasmic). The six different primers shown at the bottom are indicated by an arrow below the CD89 gene to illustrate their location. Three forward and three reverse primers were used in different combinations. Primers for GAPDH were used as positive control.*

Isolation of human and rat IgA

Human IgA1 was isolated from serum of an IgA1 myelomatosis patient as described previously (23). Rat hybridoma anti-dinitrophenol (DNP) IgA was isolated as described (24). Briefly, IgA containing ascites was precipitated using $(NH4)_2SO_4$, resuspended, and dialyzed against PBS-2mM ethylenediaminetetra-acetic acid (EDTA). Specific IgA-anti-DNP was obtained by immunoabsorption using a DNP-lysine-coupled Sepharose affinity column. After washing, anti-DNP-specific IgA was eluted from the column with 0.1 M DNP. After removal of free DNP by chromatography on Dowex (1x2 to 400), the IgA-containing fractions

were pooled, concentrated, and subjected to gel filtration chromatography on a Sephacryl S-300 column to yield monomeric, dimeric and polymeric IgA. The purified IgA preparations were dialyzed against PBS and were shown to be devoid of detectable IgG and IgM by sandwich ELISA.

FACS analysis

To evaluate a possible trypsin sensitivity of CD89, U937 cells were treated with 0,05% trypsin/0,02% EDTA (all from Sigma) for 3 min at room temperature and assessed for CD89 expression by FACS (see below). No differences between trypsinized and nontrypsinized U937 cells were observed. Human MC were tested for binding of IgA after using the different detaching procedures described above and again no differences were detected. This suggests that the IgA binding molecules present at the surface of MC are not affected by the different detaching protocols. Therefore, 0.05% trypsin/0.02% EDTA was used for the detachment of MC.

FACS analyses for CD89 expression on U937 and MC were performed as follows: MC were detached with trypsin/EDTA, and portions of 0.25 x 10^6 cells per sample were washed twice in FACS buffer (PBS/1% bovine serum albumin/ 0.02% NaN_3) and incubated 1 h with the five different mouse mAb anti-CD89 (culture supernatant diluted 1:10 in FACS buffer) and the rabbit and goat polyclonal anti-CD89 IgG (both 5 μg ml). As a positive control, W6-32 was used (culture supernatant 1:10, IgG2a mouse mAb). After incubation, cells were washed twice with FACS buffer and incubated for 1 hour with goat anti-mouse IgG1-phycoerythrin (PE) polyclonal antiserum, goat anti-rabbit IgG-PE polyclonal antiserum, rabbit anti-goat–FITC and goat anti-mouse IgG-PE polyclonal antiserum, respectively (all from Dako). All staining procedures were performed at 4°C.

The binding of human IgA1 to U937 and MC was analyzed as follows: cells were washed twice with FACS buffer and incubated for 1 h with 400, 200 and 100 μg/ml purified IgA1. After incubation, the cells were washed and bound IgA was detected by incubation with mouse monoclonal antihuman IgA antibody (4E8) and subsequently with PE-labeled goat anti-mouse IgG1 polyclonal antiserum.

To demonstrate the specificity of binding of IgA to cellular CD89, studies were performed in which U937 and MC were incubated with IgA in the presence and absence of the CD89 blocking mouse mAb My43 (25). An isotype-matched irrelevant mouse mAb served as a control for My43.

To determine the specificity of rat and human IgA for human CD89, IIA1.6 CD89- transfected cells were tested for binding of human and rat IgA. A total of 100 μg/ml human IgA1 or rat dimeric IgA was added to the cells for 1 h. Then

cells were washed and bound IgA was detected by incubation with mouse monoclonal antihuman IgA antibody (4E8)-biotin or rabbit polyclonal anti-rat IgA-biotin, respectively. After washing, cells were incubated for 1 h with streptavidin PE (Dako). The percentage positive cells were used in all FACS-experiments as a measure for CD89 expression and IgA binding.

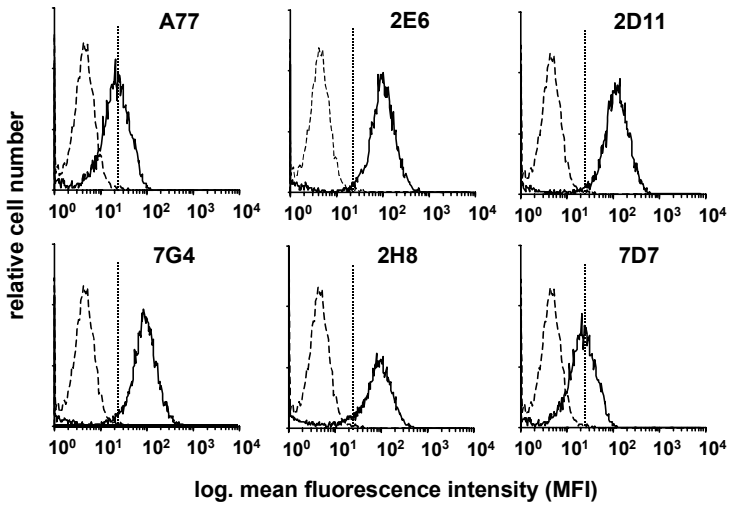


Figure 2. Fluorescence-activated cell sorter (FACS) analyses of CD89 expression on U937 cells by six mouse monoclonal antibodies (mAb). *A77, a defined Ms mAb anti-CD89, and five new mouse mAb anti-CD89 (2E6, 2D11, 7G4, 2H8, and 7D7) were tested on FACS using CD89-expressing U937 cells. Broken lines show the staining of the cells by an isotype-matched control antibody: the solid lines show the staining with the anti-CD89 mAb. The dotted lines in all panels indicate the mean staining obtained with A77.*

IL-6 production by human MC in vitro

Subconfluent 48-well plates with human MC were washed 3 times with PBS and cultured for another 48 h in Dulbecco's modified Eagle's medium/0.5% FCS to bring the cells to a quiescent state. After washing the cells three times with medium, 100 μg of dimeric human and rat IgA was added to triplicate wells. After 72 h of incubation, supernatants were harvested and assessed for IL-6 production, using the IL-6 dependent murine hybridoma cell line B9 (26,27). Serial dilutions of human recombinant IL-6 were used as a standard.

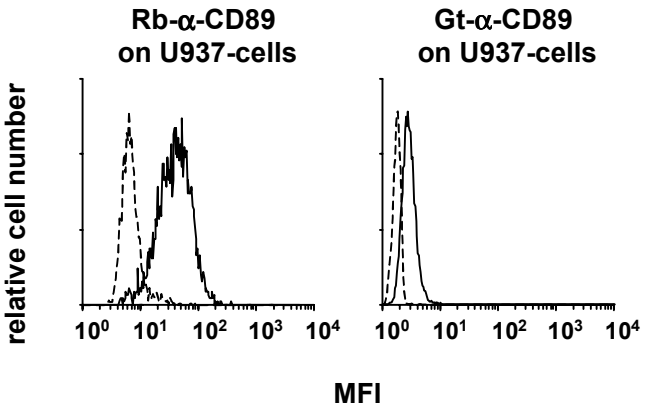


Figure 3. FACS analysis of CD89 expression on U937 cells. *Two polyclonal anti-CD89 antibodies (rabbit and goat) were tested on U937 cells. The broken lines show the staining of the cells using a normal control antibody, the solid lines show the staining with the polyclonal antibodies.*

Statistical analyses

All values are expressed as mean ± SD unless stated otherwise. Statistical analysis was performed using an unpared *t* test. A *P* value <0.05 was considered significant.

RESULTS

Generation of anti-CD89 antibodies

Five mouse hybridoma cell lines producing anti-CD89 antibodies designated 7D7, 2D11, 2E6, 7G4, and 2H8 were selected for the present studies. In addition, two polyclonal Ab (goat and rabbit) were raised. The whole set of antibodies was tested for CD89 specificity by FACS, using CD89 expressing U937 (Figures 2 and 3) and IIA1.6 CD89-transfected cells. Besides the well-known mAb anti-CD89 A77, all of the new monoclonals were able to recognize CD89 to the same extent or better.

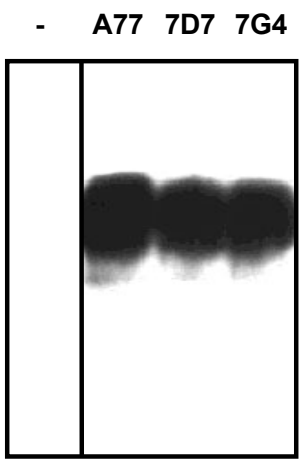


Figure 4. Western blot analysis for the characterization of the monoclonal and polyclonal anti-CD89 antibodies. *Soluble recombinant CD89 was blotted and stained using the five new mouse mAb anti-CD89, A77, and two polyclonal anti-CD89 antibodies. As an example, results using A77, 7D7 and 7G4 are shown. As the negative control, we used an isotype-matched irrelevant mouse mAb. The other monoclonal and polyclonal antibodies were also positive using this method.*

Furthermore, the new antibodies were tested by Western blot. All of the monoclonal and polyclonal antibodies showed a strong reaction with soluble CD89. The results for two of the monoclonal antibodies and A77 are shown in Figure 4.

Immunofluorescence of kidney and spleen cryostat sections

Kidney sections of control pretransplant biopsies and those of patients with IgAN failed to show any CD89-expressing cells in the mesangium: however, a strong reaction was observed in normal spleen cryostat sections using the above- described set of anti-CD89 antibodies (Figure 5). The positive cells morphologically resembled macrophages and monocytes. Staining of the control antigen MHC class I was positive for nearly all cells in glomeruli of controls, IgAN patients, and the spleen sections (Figure 5).

Immunofluorescence of cells grown on glass coverslips

MC from five of five primary MC cultures grown for 24 h on glass coverslips revealed no positive staining for CD89, using all our antibodies and A77 (Figure 5). On the other hand, CD89 expression on the monocytic cell line U937 was clearly present. As expected, expression of MHC class I was positive for both U937cells and MC (Figure 6).

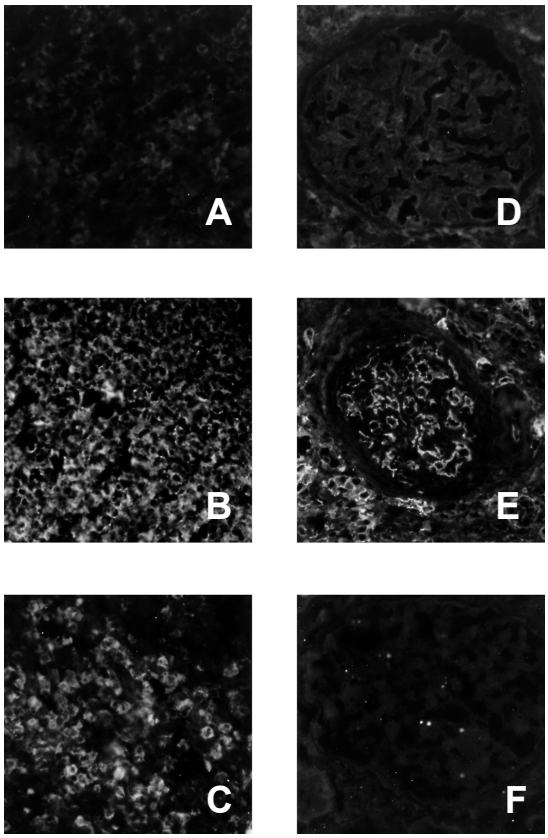


Figure 5. Tissue staining of human kidney and spleen cryostat sections. *Cryostat sections of human kidney from patients with IgAN* ***(D through F)*** *and normal spleen* ***(A through C)*** *were stained for MHC-class I* ***(B and E)****, CD89* ***(C and F)****, and control mAb* ***(A and D)****. For MHC class I staining, W6-32 was used and for CD89 the whole set of our monoclonal and polyclonal antibodies was used. As an example, stainings with one mouse mAb anti-CD89 (7G4) is shown* ***(C and F)****.*

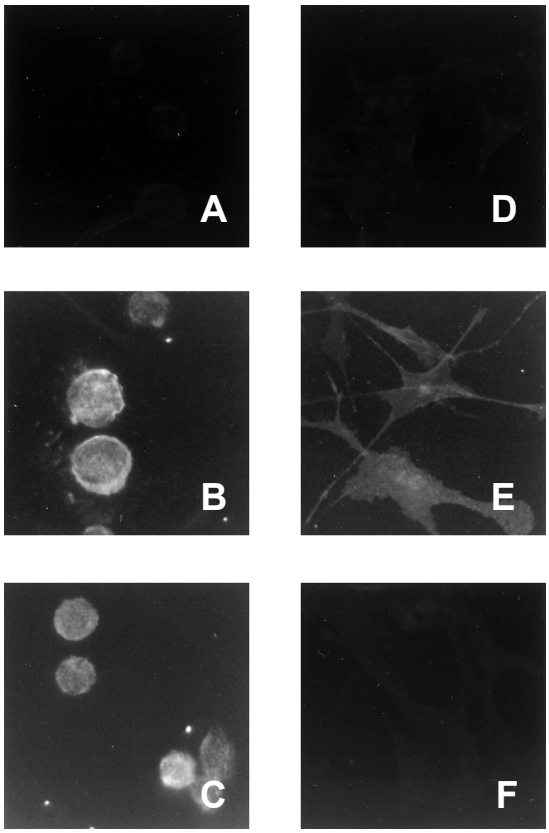


Figure 6. CD89 stainings for mesangial cells (MC) and U937 cells grown on glass coverslips. *Human MC* ***(D through F)*** *derived from five different individuals and U937 cells* ***(A through C)*** *were stained for MHC class I* ***(B and E)*** *and CD89* ***(C and F)****. For MHC class I staining, W6-32 was used and for CD89 the whole set of our new monoclonal and polyclonal antibodies was used. As an example, a staining with mouse mAb 7G4 is shown* ***(C and F)****.* ***A*** *and* ***D*** *show the negative control stained with an isotype-matched antibody.*

FACS analysis for CD89 expression

U937 and MC were also analyzed for CD89 protein expression by FACS analysis. Although U937 cells were strongly positive for CD89, all of the five primary MC were completely negative with all monoclonal and polyclonal antibodies (Figure 7). In contrast, MHC class I expression was clearly positive for both U937 and MC.

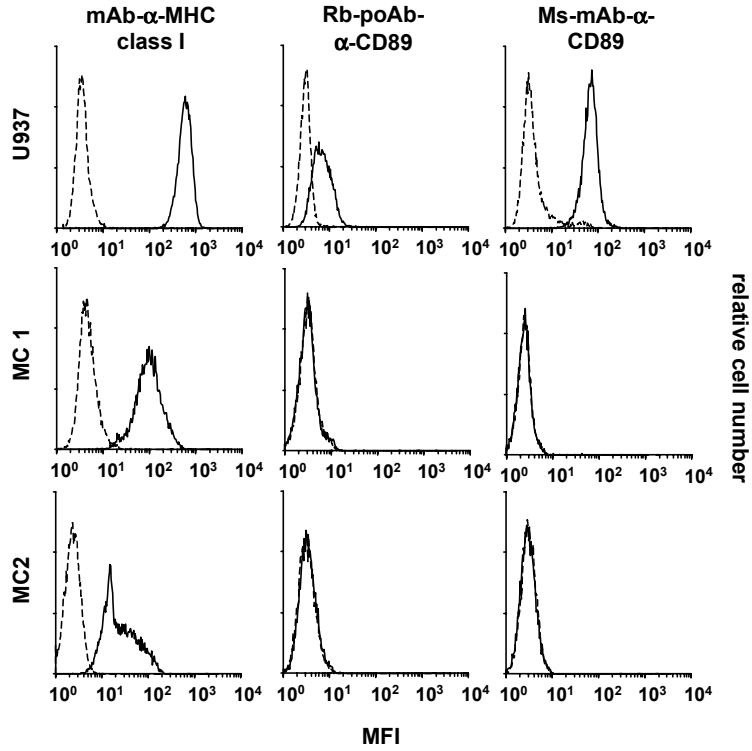


Figure 7. FACS analysis of CD89 expression on U937 and MC. *CD89 expression was analyzed with five mouse mAb anti-CD89 and two polyclonal antibodies directed against CD89. Histograms of one monoclonal and one polyclonal antibody are shown as representative examples for two of the five examined primary MC cultures. As a positive control MHC class 1 detection by W6-32 is shown.*

Reverse Transcription PCR for CD89

The results above indicate the absence of protein expression of CD89 on MC. Therefore, we analyzed U937, IIA1.6 CD89-transfected cells, and MC for mRNA expression of CD89, using reverse transcription (RT)-PCR. Reverse transcriptase and PCR of the mRNA showed for U937 cells and IIA1.6 CD89- transfected cells the expected positive band in each primer combination except primer combination 3+6 (Figure 8). In contrast, CD89-PCR analysis of cDNA from five primary MC cultures revealed no PCR product with the expected amplicon sizes of all primer combinations. Expression of GAPDH was detectable in all cases (Figure 8). Also, when cDNA was derived from MC cultured in 20% FCS or stimulated with phorbol 12-myristate 13-acetate (10ng/ml)/Ionomycin (1 μg/ml), no mRNA from CD89 was found. Finally, we performed a nested PCR using three different concentrations (1, 0.1, and 0.01 μl) of the product of a first RT-PCR in a second PCR with internal primers. The CD89-expressing control cells were positive in all cases, whereas mRNA isolated from human MC failed to be positive for CD89, even after this second round of amplification (Figure 8).

Binding of human IgA to MC

Incubation of MC with IgA and subsequent analysis by FACS revealed that all five MC bound human IgA1 with a mean of 19.9 ± 3.2% positive cells (ranging from 17.4 to 25.1%). To determine whether the binding of IgA to the MC was mediated by CD89, MC were exposed to IgA in the presence and absence of the mAb anti-CD89 My43 and subsequently assessed for binding of IgA. My43 did not reduce the binding of IgA1 to human MC (18.1 ± 4.4%), but it inhibited the binding of IgA to U937 cells by 94.8 % (Figure 9). The U937 cells without preincubation with IgA showed a basal MFI of 3.05. Cells incubated with IgA increased their mean fluorescence intensity (MFI) to 28.2 and those cells preincubated with the blocking anti-CD89 antibody (My43) showed minimal binding of IgA indicated by an MFI of 5.81.

(A)

Primer set	1+4	1+5	2+4	2+5	3+4	3+5	3+6	2+5/ 3+4
U937	+	+	+	+	+	+	-	+
IIA1.6 CD89	+	+	+	+	+	+	-	+
5 hu MC	-	-	-	-	-	-	-	-

(B)

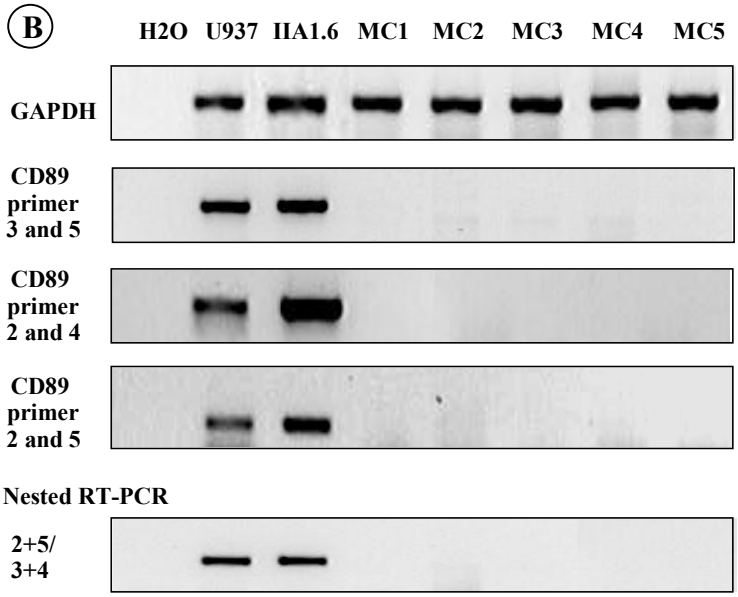


Figure 8. Reverse Transcription (RT)-PCR analysis to detect CD89 mRNA expression. (A) *Results of RT-PCR obtained with eight different primer sets. None of the primer combinations for CD89 was able to detect mRNA of this receptor in five of five primary MC cultures, whereas only primer combination 3+6 was negative for U937- and IIA1.6-CD89-transfected cells. All other combinations showed positive bands. Control primers detecting mRNA expression of GAPDH was positive for all the examined cell lines. As a negative control, water was used.* ***(B)*** *Three examples of a primer combination as well as the result of the nested RT-PCR for CD89. GAPDH control is shown at the top.*

Production of IL-6 after incubation of MC rat and human IgA

To determine the effect of dimeric rat and human IgA, MC were incubated with 100 μg of rat or human IgA and assessed for IL-6 production. The basal production was 1280 ± 400 U IL-6 per 10^5 cells. Incubation of MC with 100 μg of

human IgA resulted in enhancement IL-6 production to 3170 ± 1400 U IL-6 per 10^5 cells ($P<0.05$). Interestingly, rat IgA also induced an enhancement of IL-6, and resulted in production of 3586 ± 1471 U IL-6 per 10^5 cells ($P<0.01$). Both results are significantly higher compared with MC cultured in medium alone (Figure 10).

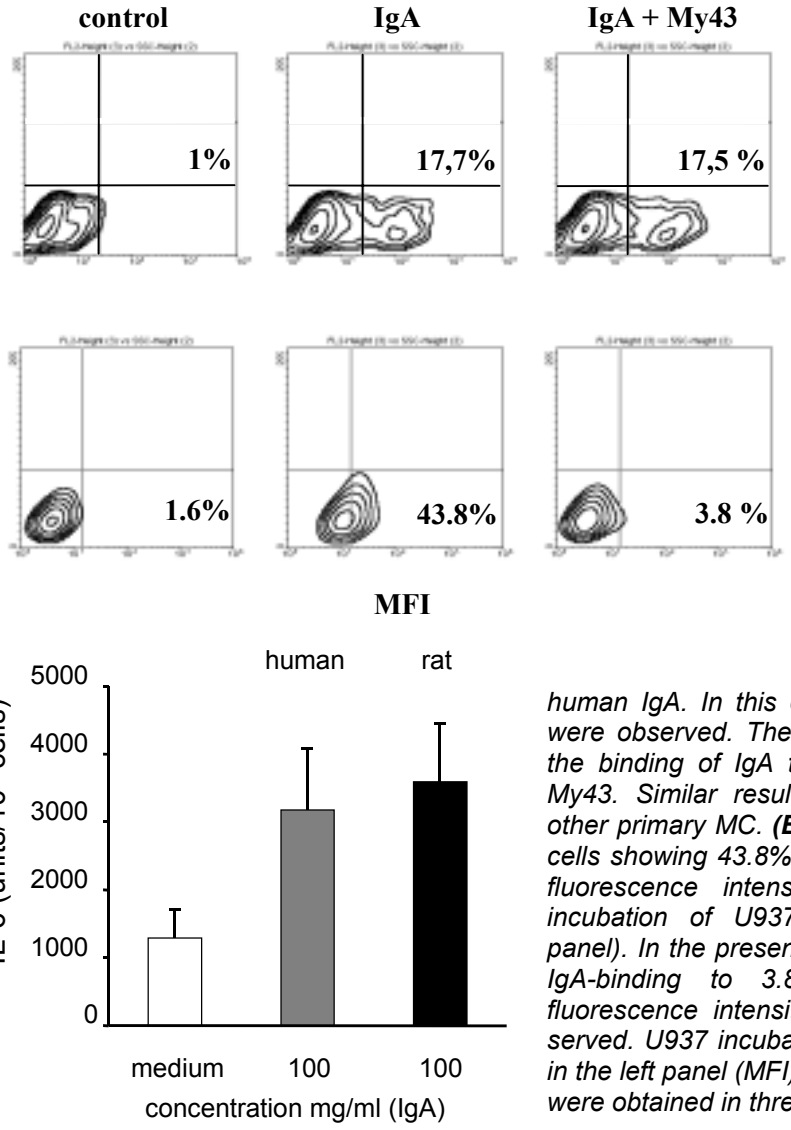


Figure 9. Effect of My43 on the binding of IgA1 to U937, and MC. (A) *Contour profiles of MC incubated without (left panel) and with (middle panel) human IgA. In this case, 17.7% positive cells were observed. The panel on the right shows the binding of IgA to MC in the presence of My43. Similar results were found using four other primary MC.* ***(B)*** *Contour profiles of U937 cells showing 43.8% positive cells with a mean fluorescence intensity (MFI) of 28.2 after incubation of U937 cells with IgA (middle panel). In the presence of My43, a reduction of IgA-binding to 3.8% respective a mean fluorescence intensity (MFI) of 5.81 was observed. U937 incubated without IgA are shown in the left panel (MFI) 3.05). Comparable results were obtained in three different experiments.*

Figure 10 IgA-induced enhancement of interleukin-6 (IL-6) production by human MC. *The effect of human (100 μg/ml) and rat IgA (100 μg/ml) on production of IL-6 by human MC compared to MC incubated in medium alone is shown. The results are means ± SD of three experiments.*

FACS analysis of binding of human IgA1 and rat dimeric IgA to CD89-transfected cells

To test the specificity of human IgA for CD89, FACS-analysis using IIA1.6 CD89-transfected cells expressing recombinant human CD89 on their surface was performed. After incubation IIA1.6 CD89-transfected cells with human and rat IgA, a difference in binding was found.

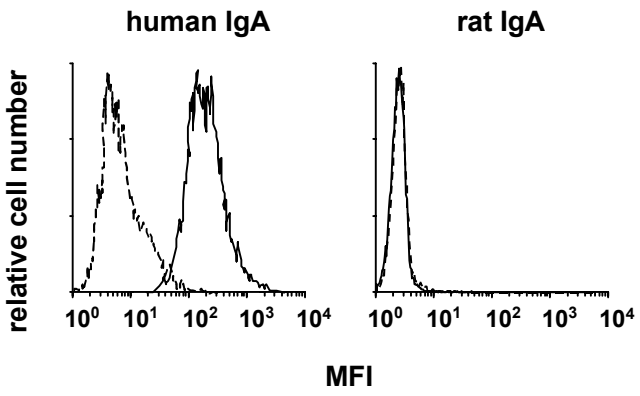


Figure 11. FACS analysis of the binding of human and rat IgA to IIA1.6-CD89-transfected cells. *IIA-1.6 CD89-transfected cells were incubated with 100 μg of human or rat IgA, respectively. Almost 100 % of the CD89-transfected cells bound human IgA (left histogram) in contrast to the cells incubated with rat IgA (right histogram). Control stainings without IgA are depicted as dotted lines. On the other hand, rat IgA was able to bind to human MC (data not shown).*

Human IgA1 bound to approximately 100% of the transfected cells, whereas dimeric rat IgA did not show any detectable binding (Figure 11). No binding to nontransfected cells was found (data not shown).

DISCUSSION

Although deposition of IgA in the mesangium of IgAN patients is well documented, the involvement of the Fc receptor for IgA (CD89) on MC in the pathogenesis of the disease is controversial. In this study, we investigated whether CD89 is expressed on the surface of human MC *in vivo* and *in vitro*. Therefore, we developed five mouse mAb and two polyclonal antibodies directed against CD89. These antibodies were shown to be specific for CD89 and able to stain CD89 on cells in cryostat sections of spleen. However, we did not find CD89 reactivity in kidney sections of control subjects and patients with IgAN by using our new set of monoclonal and polyclonal antibodies completed with a well-known mouse mAb anti-CD89 A77. MC grown on glass coverslips were also negative for CD89. Furthermore, we performed FACS analysis and again CD89 protein expression was not detectable on human MC. However, various tissue and cell controls were positive for CD89.

To exclude a possible processing defect of CD89 by MC, we determined mRNA expression of CD89. Seven combinations of three forward and three reverse primers (Figure 7A) were used recognizing different parts of the extracellular domain 1 and extracellular domain 2 of CD89. This should also allow the detection of possible expression of splice variants of CD89 in human MC. All of these primer sets failed to reveal expression of CD89 in human MC, while with primer combination 1-6 U937 and IIA1.6 CD89-transfected cells were positive. Also, the very sensitive nested RT-PCR failed to detect CD89 mRNA expression in five of five human MC, whereas the control cells were clearly positive. Only primer

combination 7, which is identical to the primers used in an earlier publication (13), did not show an amplification band either in the controls or the MC. Taken together, these data strongly indicate that CD89 is neither expressed as a protein on the surface nor as mRNA in human MC. To exclude that MC derived from patients with IgAN are in contrast to "normal MC" able to express CD89, we stained five kidney sections from five different IgAN patients with our set of two polyclonal and five monoclonal anti-CD89 antibodies. They also did not show any positivity for CD89; furthermore, the "normal MC" showed binding of IgA independent of CD89.

These results are in contrast with earlier findings demonstrating CD89 mRNA expression in human and rat MC by Northern blot and RT-PCR analysis, respectively (6,13). Also, experiments performed with the primers used in the above mentioned studies (13), failed to detect CD89 mRNA in either quiescent or activated MC, or in our CD89-expressing controls (U937 and IIA1.6 CD89-transfected cells) as stated above. Based on the finding that 40% of patients with IgAN have CD89 mRNA expression in whole glomeruli, it has been suggested that MC in IgAN might express CD89 mRNA (16). However, it is known that influx of monocytes may occur in IgAN (28-30), which may explain these positive results. Furthermore, no CD89 expression was found in normal glomeruli and those isolated from patients with non-IgA mesangial proliferative glomerulonephritis, supporting our findings of the absence of CD89 mRNA in isolated human MC. However, in the present study, we could not find any CD89-positive monocyte in the mesangial area of patients with IgAN. We suggest that the sensitivity of the immunohistochemical staining is too low to detect CD89 expression on this infiltrating cell.

Recently, it was shown that stimulation of human MC with IL-6, TNFα, and interferon-γ enhances binding of IgA in vitro to MC (13). The investigators interpreted the enhanced binding of IgA and a simultaneous upregulation of mRNA of CD89 as evidence for enhanced CD89 protein expression. However, no direct evidence of CD89 receptor involvement in the binding of IgA was presented, because they did not perform blocking experiments with anti-CD89 antibodies.

A number of earlier investigations showed binding of IgA to human and rat MC (4-6,13,31); however, the direct involvement of CD89 on MC in the binding of IgA to the cells has not been addressed until now. Therefore, we examined the binding of IgA to human MC in the presence and absence of a blocking monoclonal antibody (My43) against CD89. My43 anti-CD89 inhibited the binding of IgA to U937, whereas no reduction of binding of IgA to MC was observed, indicating that the binding of IgA to MC is CD89-independent.

To confirm specificity of CD89 for human IgA, it was shown that only human but not rat IgA reacts with CD89-transfected IIA1.6 cells. Similar results were obtained using an ELISA-system, using recombinant soluble CD89 (data not shown). On the other hand, it was found that not only human but also rat IgA is able to enhance IL-6 production by human mesangial cells *in vitro*, again indicating the presence of another IgA binding moiety on the surface of human MC.

A number of possible receptors, such as the asialoglycoprotein receptor (ASGP-R) and mannose receptor, could be involved. Since reliable poly- and monoclonal antibodies against the mannose receptor have been generated (32,33), and it has been suggested that activated mouse MC express this receptor (34), we investigated its expression on human MC. However, we did not find mannose receptor expression using FACS analysis either on resting or on activated human MC (data not shown).

ASPG-R is known to be expressed on human and rat hepatocytes and is involved in the clearance of galactose-terminal glycoproteins for example human IgA1 (35-38). Recently, it was reported that ASGP-R is expressed on human and rat MC using RT-PCR (31). In the above-mentioned study (31) binding and catabolism of IgA by human and rat MC was saturable and partly inhibitable by galactose but not by other carbohydrates.

Taken together, we have shown for the first time that CD89 is not expressed on human MC either *in situ* or *in vitro*. Furthermore, we found that binding of IgA to MC is not mediated via CD89 and that activation of MC after binding of IgA is independent of CD89. Therefore, we postulate that another receptor, different from CD89, is responsible for binding of IgA to MC.

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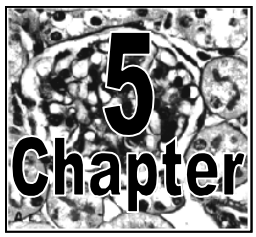

A novel receptor for IgA expressed on human renal mesangial cells[#]

R. Westerhuis, G. van Zandbergen, A.M. Woltmann, B.E. van Zandbergen, N.A.M. Verhagen, N. Klar-Mohamad, M.R. Daha and C. van Kooten.
Dept. of Nephrology, Leiden University Medical Center, The Netherlands

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.

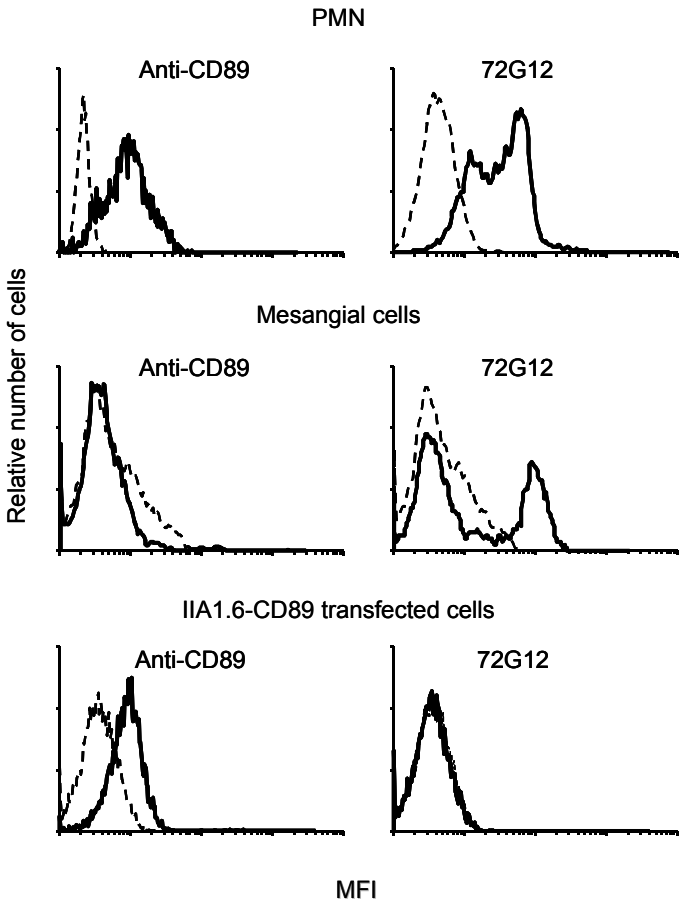

Figure 1: FACS analysis of MCs and IIA1.6 CD89 transfected cells. Top: Human PMN showed positivity after staining with the anti-CD-89-mAb My43 and the new developed mAb 72G12. Middle: Human MC were stained for CD89 (My43, left) and with 72G12 for expression of a new IgA-binding protein (right). Bottom: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

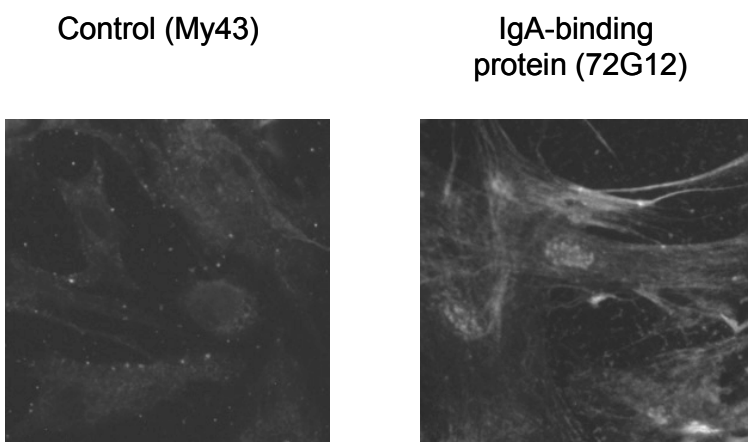

Figure 2: Stainings of MC grown on glass cover slips. Human MC derived from 4 different individuals were stained with 72G12. As an example a staining of one MC is shown. The negative control staining was performed using My43; an isotype matched anti-CD89 mAb

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.

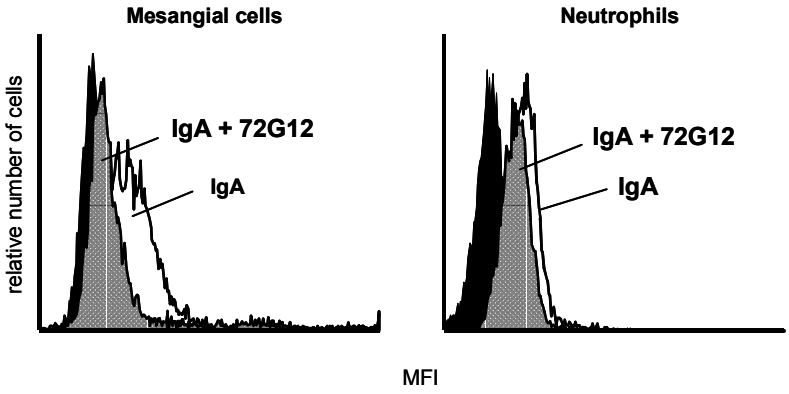

Figure 3: IgA-blocking effect of 72G12 and My43 to human MCs and PMN's. Histograms for binding of IgA to MC (left) and PMN (right) are shown. The empty histogram showed binding of IgA to these cell types. After preincubation of MC with 72G12 binding of IgA was almost completely reduced (gray patterned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

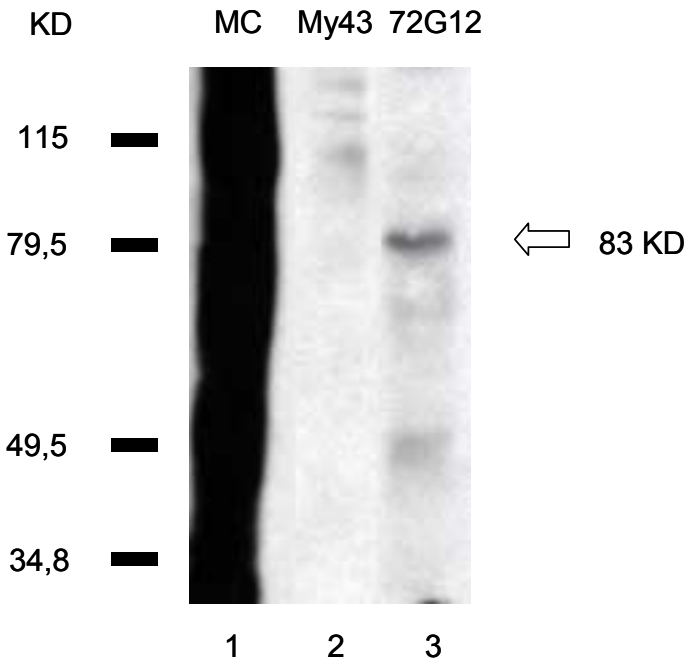

Figure 4: Immunoprecipitation of protein isolated from human MC using 72G12. Precipitation experiments of MC-protein using 72G12 revealed an enriched band at the size of 83 Kd using the chemiluminescence method (lane 3) whereas no comparable enriched protein was found using My43 (lane 2). Starting material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

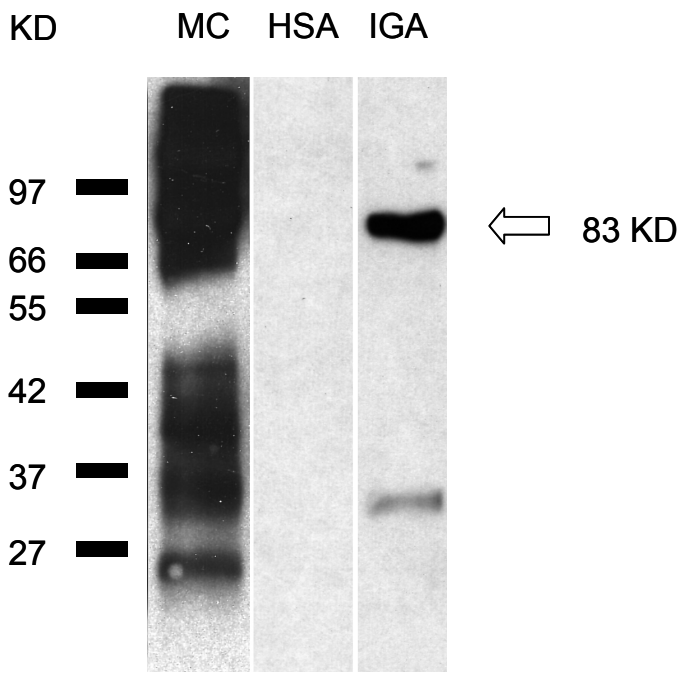

Figure 5: Immunoprecipitation of protein isolated from human MC with IgA Precipitation experiments of MC-protein using HSA revealed no enrichment of a specific protein (HSA-lane). In contrast precipitations using human IgA revealed a strong enhancement of an 83 kD protein (IgA-lane) using the chemiluninescence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

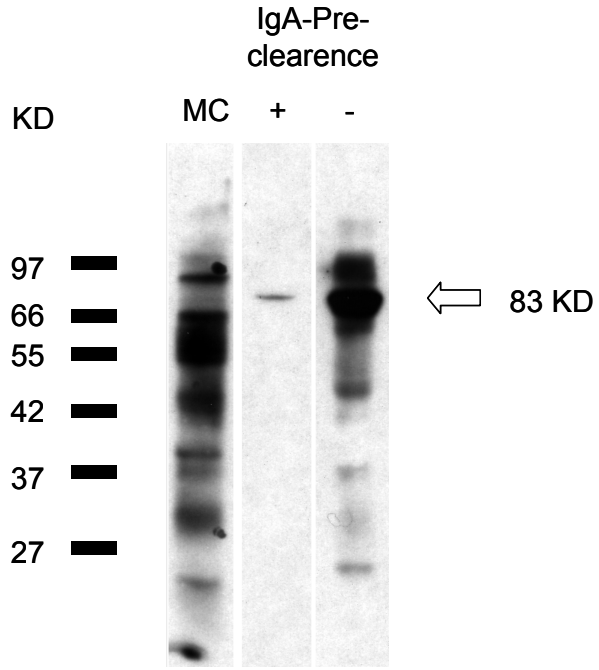

Figure 6: Effect of preclearence with human IgA on immunoprecipitations by 72G12 of proteins isolated from human MC After preclearence using human IgA BiogelA5 of proteins derived from human MC 72G12 precipitated much less protein with the expected size of 83 Kd (lane +). The result of a precipitation 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.

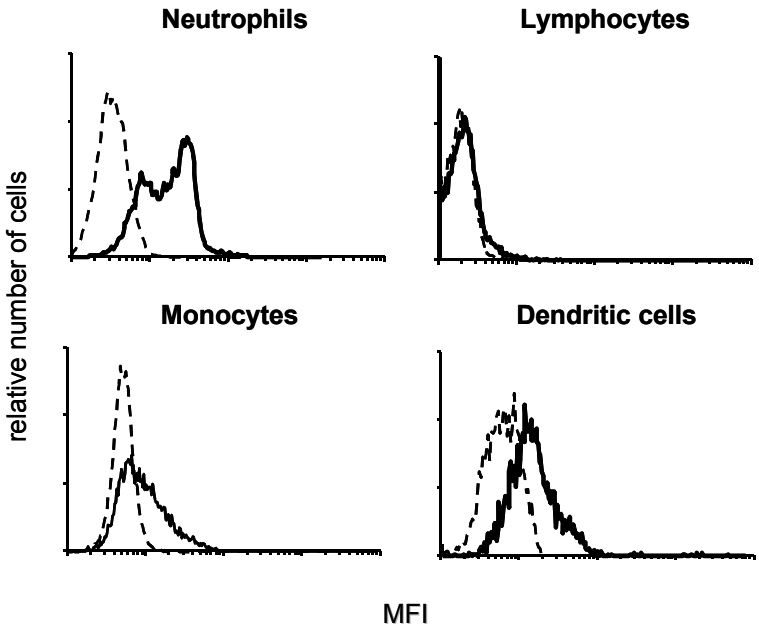

Figure 7: FACS analysis of expression of the new receptor for IgA on white blood cells. At the top a strong reactivity of freshly isolated PMN with 72G12 is shown. Monocytes revealed only a slight expression of the investigated protein whereas dendritic cells showed an increasing expression of the new 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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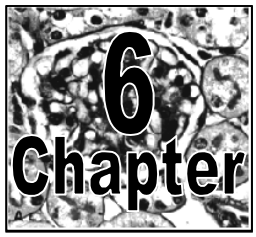

Crosslinking of the human Fc receptor for IgA (CD89) triggers FcR gamma-chain dependent shedding of soluble CD89 [#]

Ger van Zandbergen[‡], Ralf Westerhuis[‡], Ngaisah Klar-Mohamad[‡], Jan G.J. van de Winkel[|], Mohamed R. Daha[‡] and Cees van Kooten[‡]

[‡]Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands, [|]Department of Immunology and Medarex Europe, University Hospital Utrecht, Utrecht, The Netherlands

SUMMARY

CD89/FcαRI is a 55-75 kD type I receptor glycoprotein, expressed on myeloid cells, with important immune effector functions. At present, no information is available on the existence of soluble forms of this receptor. We developed an ELISA for the detection of soluble CD89 forms (sCD89) and investigated the regulation of sCD89 production. PMA/ionomycin stimulation of monocytic cell lines (U937, THP-1 and MM6), but not of neutrophils, resulted in release of soluble CD89. Crosslinking of CD89, either via its ligand IgA or with anti-CD89 mAb's similarly resulted in sCD89 release. Using CD89 transfected cells, we showed ligand-induced shedding to be dependent on co-expression of the FcR γ-chain subunit. Shedding of sCD89 was dependent on signaling via the γ-chain and prevented by addition of inhibitors of protein kinase C (staurosporine) or protein tyrosine kinases (genistein). Western blotting revealed sCD89 to have an apparent molecular mass of 30 kD, and to bind IgA in a dose-dependent fashion. In conclusion, the present data document a ligand-binding soluble form of CD89 that is released upon activation of CD89-expressing cells. Shedding of CD89 may play a role in fine-tuning CD89 immune effector functions.

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INTRODUCTION

Immunoglobulin A (IgA) plays a critical role in protecting the host against environmental pathogens and antigens encountered at mucosal surfaces. In humans IgA is the predominant isotype produced (±66mg/kg/day), with 80 % of all B cells committed to IgA production (1). Receptors for the Fc portion of IgA (FcαR) have been identified on a variety of cell types within the immune system and provide a crucial link between the humoral and cellular branches of the immune system *(2)*. Compared to the Fc receptors for IgE (FcεRI and FcεRII) and IgG (FcγRI, FcγRII and FcγRIII), relatively little is known about the nature and function of Fcα receptors. The best characterized human FcαR described until now, FcαRI/CD89, is a type I transmembrane glycoprotein that binds both IgA1 and IgA2 subclasses with similar affinity ($K_a \sim 10^6$ M^{-1})(3). Molecular cloning demonstrated CD89 to be a member of the Ig superfamily (4). The site of interaction between IgA and CD89 was identified on the junction of Cα2 and Cα3 of the IgA molecule (5), and in the membrane distal EC-1 domain of CD89 as shown by both mutagenesis (3) and domain swapping (6). Comparison of the primary amino acid sequence showed CD89 to be more closely related to killer cell inhibitory receptors (KIR) than to human FcγR (7).

CD89 is constitutively expressed as a 50-70 kD protein on neutrophils and monocytes/macrophages, or as a 70-100 kD glycoprotein on eosinophils due to increased glycosylation (2,8). The CD89 molecule is associated through a charge-based mechanism with the common FcR γ-chain, which connects CD89 to intracellular signaling pathways via ITAM signal motifs located within the cytoplasmic tail of the FcR γ-chain (9,10). Crosslinking of CD89 on myeloid cells can trigger diverse processes including phagocytosis, superoxide generation, ADCC and release of inflammatory mediators (2).

Several signals have been shown to modulate surface expression of CD89. Cytokines (TNF-α, GM-CSF, IL-1β, IL-8), LPS, PMA and aggregated IgA can induce increased CD89 expression on cells (8,11,12). In contrast, TGFβ (13) and suramin (14) were shown to downregulate its expression. Altered CD89 expression may directly affect the effector function of CD89-expressing cells. Soluble forms have been identified for various Fc receptors for IgG (FcγRII/CD32, and FcγRIII/CD16) and IgE (FcεRII/CD23) (15-17). Furthermore, it has been proposed that these soluble Fc receptors have a pathophysiological role in several diseases (18-20). No information is available on the existence of soluble forms of FcαR. In the present study we demonstrate the existence of a soluble CD89 protein (sCD89), a 30 kD glycosylated protein with retained ability to bind human IgA.

MATERIALS AND METHODS

Production of recombinant CD89 protein and anti-CD89 reagents

A recombinant soluble form of CD89 was produced by expressing the cDNA encoding the extracellular part of CD89 (21) in CHO-K1 cells using the pEE14 expression system (Celltech, Slough, UK). The stable CHO-K1 transfectant produced approximately 15 μg/ml of recombinant soluble CD89. Using columns of Immobilized IgA, more than 99% of the recombinant soluble protein was recovered from culture supernatants (22). The purity of the preparations was checked by SDS-page and a single band by Coomassie-brilliant-blue-staining was detected. The recombinant sCD89 protein was used to immunize mice, a rabbit and a goat. CD89-reactive rabbit (Rb) and goat (Gt) antisera were raised and used as purified IgG fractions. Using standard hybridoma technology we raised novel mouse mAb's specific for CD89. The specificity of these reagents was confirmed by FACS analysis on CD89-transfected cells and by immunoprecipitation and Western blotting (6,23).

ELISA for sCD89

Rabbit anti-CD89 IgG (2 μg/ml) was coated to ELISA plates (NUNC Maxisorb, Life Technologies, Gaithersburg, MD) by overnight incubation at room temperature in coating buffer (0.1M $NaHCO_3/Na_2CO_3$, pH9.6). The wells were washed 3 times using washing buffer (PBS, 0.02% Tween20) and subsequently varying concentrations of the recombinant sCD89 protein or BSA (as a control) were added. All samples were diluted in ELISA buffer (PBS, 0.02% Tween20, 1% FCS) and incubated for 1 hour at 37 °C. Following incubation, wells were washed as above and incubated first with digoxigenin (DIG)-conjugated rabbit $F(ab`)_2$ anti-CD89 (1$\mu$g/ml), followed by Horseradish peroxidase (HRP)-conjugated $F(ab`)_2$ anti-DIG (1/5000, Boehringer Mannheim; both for 1 hour at 37 °C and washed in between as above). OD415 was measured after addition of $ABTS/H_2O_2$ as substrate. The optical density at 415 nm was assessed using a Microplate Biokinetics Reader EL 312e (Bio-tek).

We also used a sandwich ELISA of monoclonal and polyclonal antibodies for the quantification of CD89 in supernatants of PMA/ionomycin activated cells and found similar values as measured in an ELISA with polyclonal antibody coating. However, since 4 out of 5 mAb's recognize the IgA binding site on CD89 (6), this hampers the study of IgA- or anti-CD89 induced shedding. Therefore, for consistency in our work, we have chosen to present all data from one type of ELISA were we used the polyclonal antibody as a coating.

Soluble CD16 was measured by ELISA (24).

Cell culture and activation

PMN's and monocytes were isolated from whole blood of healthy donors by Ficoll density centrifugation. The following CD89-expressing cell lines were used: U937 (ATCC nr CRL-1593.2)(25), THP-1 (ATCC nr TIB-202)(26) and MonoMac-6 (kindly provided by Dr. H.W.L. Ziegler-Heitbrock, Institut fur Immunologie, Universitat Munchen, Germany)(27). All cells were cultured at 37 °C with 5% CO2 in a humidified atmosphere in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies). IIA1.6 cells were grown in the same medium supplemented with geneticin (G418, 0.8 mg/ml; Life Technologies) for CD89-transfected cells, or geneticin and methotrexate (MTX, 10 mM; Pharmachemie, Haarlem , The Netherlands) for cells co-transfected with CD89 and FcR γ-chain (10). Cell viability was greater than 95% for all cell preparations used.

For activation PMN's were cultured at a concentration of $1.0x10^7$ cells/ml (24). Monocytes and myeloid cell lines were activated at a concentration of $2.0x10^6$ cells/ml. All activation experiments were performed in triplicate. After the indicated times, cells were harvested and tested by FACS analysis or supernatants were harvested and tested by ELISA. The following stimuli were used: PMA (10 ng/ml), Ionomycin (1 μg/ml) and LPS (*Salmonella thyphosa*, 100 ng/ml) (all from Sigma, St Louis, MO). In addition various purified IgA preparations isolated from normal human serum, and sera from myeloma patients and anti-CD89 monoclonal antibodies and goat anti-mouse-Ig antibodies were used (all prepared in our laboratory) (6,22,23,28).

For inhibition of γ-chain-induced signal transduction, we have used inhibitors of protein kinase C (staurosporine; 50 ng/ml) or protein tyrosine kinases (genistein; 100μM)(both from Sigma). These concentrations were non-toxic for the cells as determined by trypan-blue exclusion.

FACS analysis

For FACS analysis, cells ($5x10^5$) were incubated with the CD89 mAb 2D11 (IgG1) or an isotype matched control, diluted in FACS buffer (PBS/0.5% BSA/0.02% Azide). Following incubation for 1 hour at 4 °C, cells were washed with FACS buffer and incubated for 1 hour with PE-conjugated goat anti-mouse IgG1 polyclonal IgG (Southern Biotechnology, Birmingham, AL). After washing, cells were fixed with 1% paraformaldehyde in PBS and analyzed on a FACScan

(Becton Dickinson, San Jose, CA). Data acquisition and analysis were conducted with Lysis II (Becton Dickinson).

Immuno-precipitation and Western blotting

As a positive control for intact CD89, U937 cells ($1x10^8$) were lysed, using PBS/ 1% NP40. Cell lysates and culture supernatants were separated on 10% SDS polyacrylamide gels under reducing conditions, and blotted onto PVDF membrane (Millipore, Bedford, MA). Using standard Western blotting protocols, different forms of CD89 were detected with a mixture of Rb- and Gt IgG anti-CD89 (both 10 μg/ml). After incubation and washing, followed by subsequent incubation with HRP-conjugated Swine anti-Rabbit-IgG (1/50,000; Dako, Denmark) and HRP-conjugated Rabbit anti-Goat-IgG (1/50,000; Dako). Signals were visualized using Super Signal Chemiluminescence substrate, according to manufacturers' instructions (PIERCE, Rockford, IL).

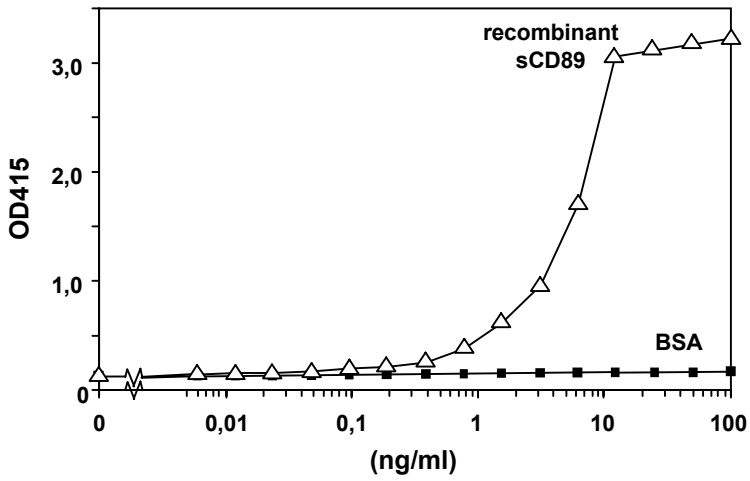

Figure 1. Detection of soluble CD89 protein by ELISA. Rabbit anti-CD89 IgG (2μg/ml) was coated to ELISA plates and samples, containing different concentrations of either recombinant soluble CD89 (open triangles) or BSA (closed squares), were added and after incubation wells were washed and bound CD89 detected with digoxigenin (DIG)-conjugated rabbit F(ab`)$_2$ anti-CD89 (1$\mu$g/ml), followed by Horseradish peroxidase (HRP)-conjugated F(ab`)$_2$ anti-DIG. OD415 was measured after addition of ABTS/H_2O_2 as substrate. The detection limit of soluble CD89 ELISA was reproducibly found to be 50 pg/ml (extinction background +2x standard deviation in 6 independent experiments).

Isolation of sCD89 and IgA binding ELISA

The sCD89 protein was isolated from culture supernatant of PMA/ionomycin stimulated U937 cells using an affinity column of human IgA isolated from normal serum (22). Preparations of purified IgA from normal human serum (2μg/ml) were coated to an ELISA plate and binding of sCD89 was detected using DIG-conjugated Rb F(ab`)$_2$ anti-CD89, similar to the CD89 ELISA described above.

RESULTS

Establishment of an ELISA for sCD89

To study the presence of a soluble form of CD89, we developed a CD89-specific ELISA, using rabbit polyclonal antibodies. As a positive control a recombinant sCD89 protein produced in CHO cells was employed.

Increasing concentrations of recombinant sCD89 resulted in a dose-dependent signal in this ELISA. The detection limit of this ELISA was reproducibly found to be ~ 50 pg/ml (Figure 1).

Monocytic cells release sCD89 upon activation

We investigated the release of soluble CD89 in supernatants of activated human cells. First, the release of CD89 was studied in the pro-monocytic cell line U937, a cell type expressing high levels of CD89. Stimulation of U937 with PMA and ionomycin consistently induced release of a soluble form of CD89 (Figure 2A).

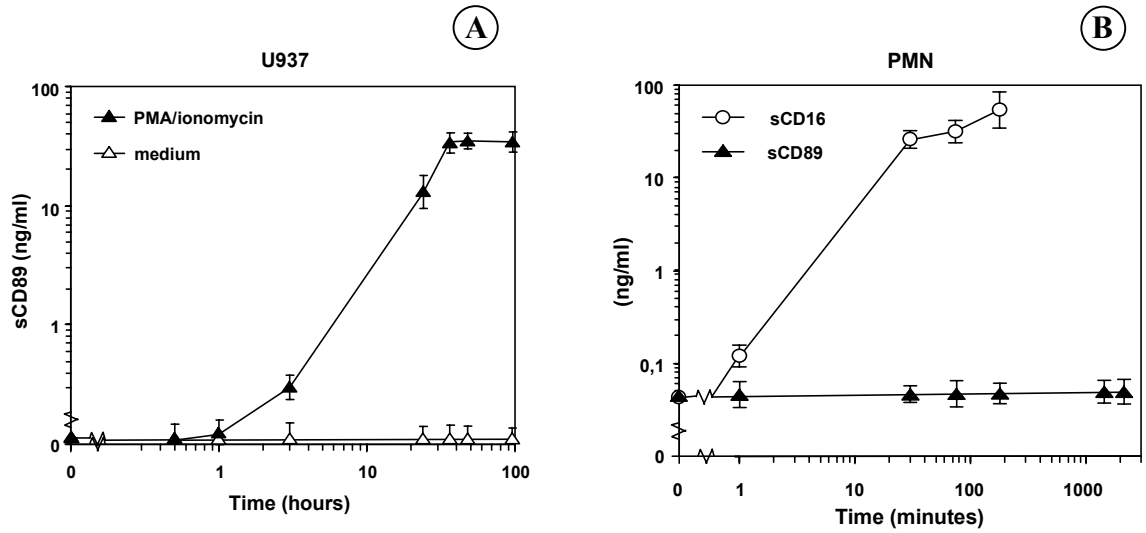

Figure 2. Detection of sCD89 in supernatants of PMA/ionomycin-stimulated cells. A. U937 cells were cultured in medium alone (open triangles) or medium supplemented with PMA/ionomycin (closed triangles). At indicated time points supernatants were tested for sCD89. Results are expressed as the mean ± SEM of 3 independent experiments. **B.** Freshly isolated PMN were cultured in medium with PMA/ionomycin. At the indicated time points supernatants were harvested and tested for sCD89 (closed triangles) and sCD16 (open circles). Results are expressed as a mean ± SEM of 3 independent experiments, using cells from 3 different donors

Soluble CD89 was first detectable after 3 hours, and reached a maximum at 36 hours. In 5 independent experiments the amount of sCD89 detected ranged from 25-43 ng/ml. When the same number of U937 cells were cultured in the absence of PMA/ionomycin, no sCD89 could be detected (Figure 2A). Next PMN, a cell that expresses similar amounts of CD89 to U937, were stimulated with PMA and ionomycin. Irrespective of the time points tested, varying from 1 minute to 36 hours, soluble CD89 could not be detected in the supernatants (Figure 2B). As a positive control for the activation conditions, the same supernatants were tested for the release of CD16. In accordance with the literature (16,24), after 1 minute soluble CD16 was detectable in supernatants of activated PMN, and this production further increased in time (Figure 2B).

Regulation of CD89 surface expression

Since modulation of surface expression might contribute to the release of CD89, we investigated the effect of PMA/ionomycin on CD89 membrane expression as detected with monoclonal antibodies. FACS analysis showed that PMA/ionomycin induced a 3-10 fold (range of 4 independent experiments) increase in CD89 surface expression on U937 cells compared with cells cultured in medium alone (Figure 3).

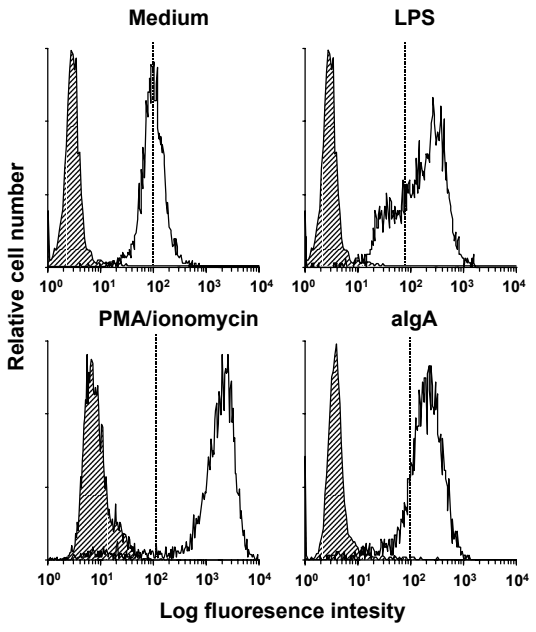

Figure 3. FACS analysis of CD89 surface expression. U937 cells were cultured in medium alone or in medium supplemented with PMA/ionomycin, LPS or heat aggregated IgA. After a 36-hour culture, CD89 surface expression was assessed with CD89 mAb 2D11 (1/1,000) followed by PE-conjugated goat anti-mouse-IgG1. Hatched histograms represent conjugate controls; open histograms represent CD89 expression. Out of 3 independent experiments one histogram is shown per condition. The dotted line represents the median fluorescence for CD89 expression of cells cultured in medium alone.

This upregulation was not unique for PMA/ionomycin and could also be observed with other stimuli, which have previously been found to affect CD89 expression (12). Both LPS and heat-aggregated human IgA (aIgA) enhanced CD89 surface expression on U937 cells.

Crosslinking of CD89 induces release of a soluble form

To verify whether the release of sCD89 is correlated with upregulated surface expression, supernatants of stimulated U937 were tested. Next to PMA/ionomycin, also high molecular weight forms of both IgA1 and IgA2 (polymeric or heat aggregated) consistently induced release of sCD89 from U937 cells (Figure 4A). In contrast, little activation was observed with preparations containing monomeric IgA. No sCD89 was detected after LPS stimulation of U937 (Figure 4A). We then tested FcαRI-crosslinking using different concentrations of the anti-CD89 mAb 2D11 and found a dose-dependent induction of sCD89 release (Figure 4B). An isotype matched control antibody did not induce the release of CD89, whereas crosslinking 2D11 using goat anti-mouse IgG antibodies increased CD89 shedding.

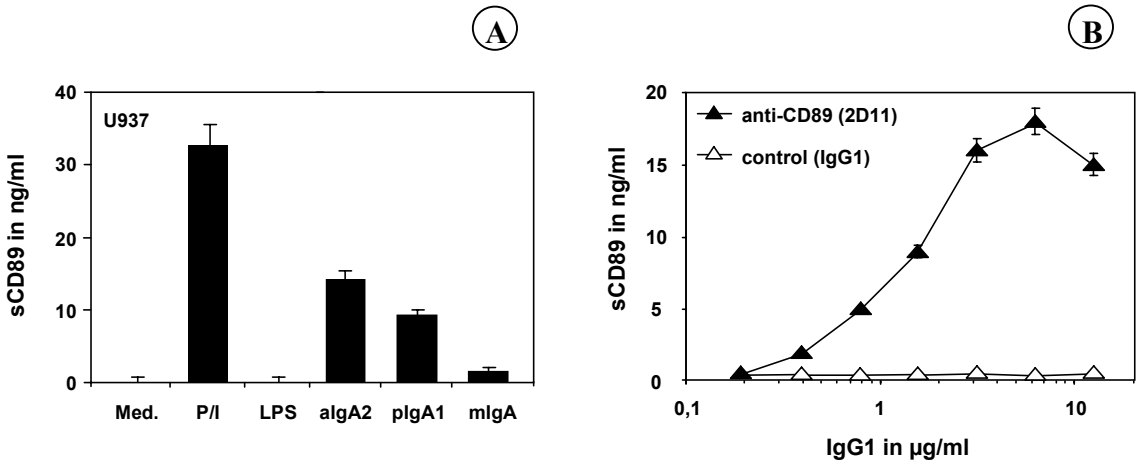

Figure 4. CD89-specific crosslinking triggers sCD89 release from U937. A. U937 cells were cultured in medium alone or in medium supplemented with PMA/ionomycin, LPS or IgA preparations consisting of heat aggregated IgA2 (aIgA2) from a patient with an IgA2 myeloma, polymeric IgA1 (pIgA1) from a patient with an IgA1 myeloma or monomeric serum IgA (mIgA) as indicated. After 36 hours of culture, supernatants were collected and tested for sCD89. Results are expressed as the mean ± SEM of 3 independent experiments. **B.** U937 cells were cultured in medium supplemented with different concentrations of the anti-CD89 mAb 2D11 (closed triangles) or an isotype matched control (open triangles). After 36 hours supernatants were collected and tested for sCD89. Results are expressed as the mean ± SEM of 3 independent experiments.

Similar activation conditions were applied to freshly isolated peripheral blood monocytes and two other myeloid cell lines, MonoMac-6 and THP-1. Activation with PMA/ionomycin or IgA stimulated an increased surface expression on all three cell types (data not shown). In addition, both PMA/ionomycin and aIgA, as well as anti-CD89 antibodies, induced the release of soluble CD89 (Figure 5).

The FcR γ-chain is essential for CD89-triggered release of sCD89

To study the mechanism of CD89-triggered release of sCD89 in more detail, we used murine IIA1.6 cells transfected with human CD89 alone, or with human CD89 in combination with the FcR γ-chain subunit. Both transfectants were previously shown to have a comparable CD89 expression and both cell lines displayed a similar IgA binding (29). Activation of CD89/γ-chain transfected cells with increasing amounts of heat aggregated human IgA (aIgA) triggered a dose-dependent release of sCD89. No sCD89 could be detected in supernatants of aIgA-stimulated cells transfected with CD89 alone (Figure 6A).

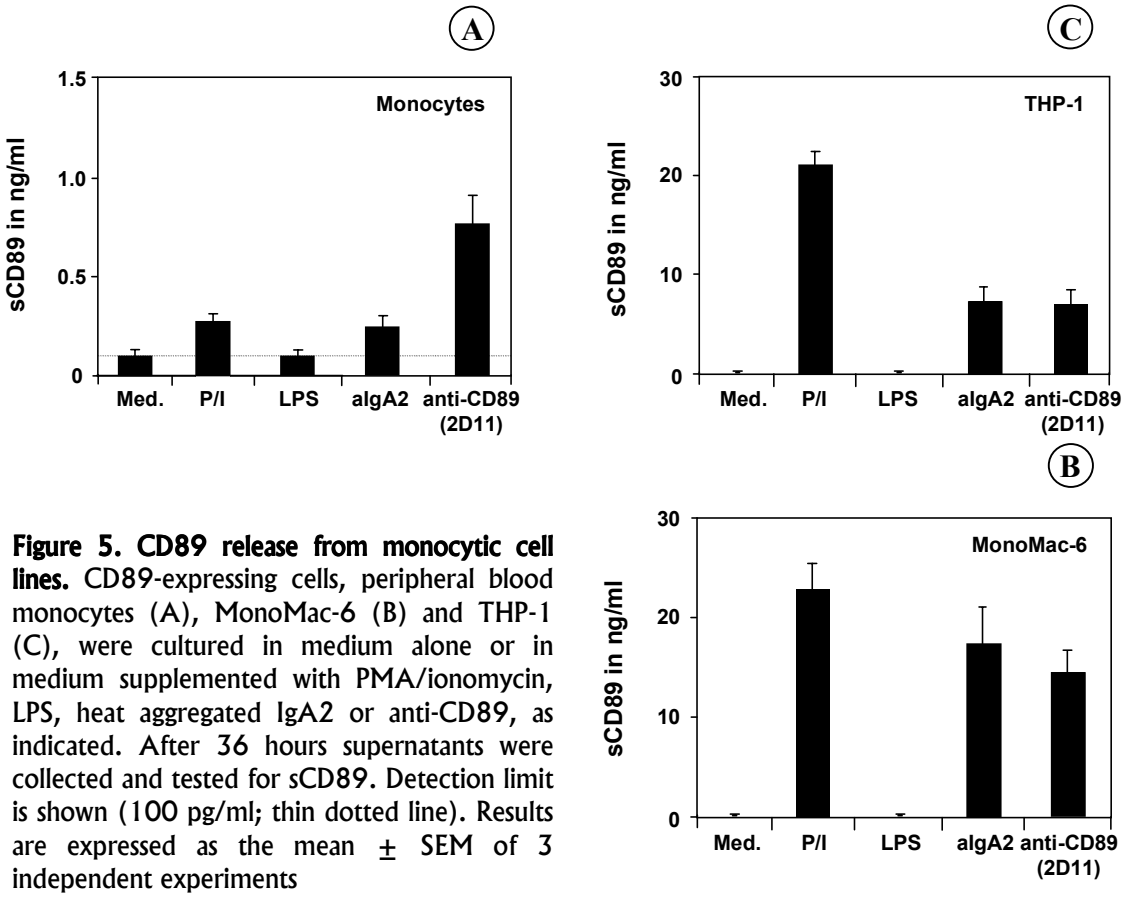

Figure 5. CD89 release from monocytic cell lines. CD89-expressing cells, peripheral blood monocytes (A), MonoMac-6 (B) and THP-1 (C), were cultured in medium alone or in medium supplemented with PMA/ionomycin, LPS, heat aggregated IgA2 or anti-CD89, as indicated. After 36 hours supernatants were collected and tested for sCD89. Detection limit is shown (100 pg/ml; thin dotted line). Results are expressed as the mean ± SEM of 3 independent experiments

Similarly, anti-CD89 antibodies induced release of sCD89 only in cells co-expressing the γ-chain (Figure 6B). PMA/ionomycin stimulation led to release of sCD89 in both cell types (Figure 6B).

To investigate whether signaling via the γ-chain is important for the release of sCD89, two specific inhibitors were used. Addition of either an inhibitor of protein kinase C (staurosporine) or an inhibitor of protein tyrosine kinases (genistein) prevented the shedding of sCD89 from the surface of aIgA-stimulated IIA1.6 CD89/γ chain transfectants or U937 cells (Figure 7).

Biochemical characterization of soluble CD89

To further analyze the nature of soluble CD89, Western blotting was performed on cell lysates and supernatants of myeloid cells. Utilizing rabbit and goat polyclonal anti-CD89 antibodies, a broad band ranging from 55-75 kD was detected on U937 cell lysates (Figure 8A, lane 2).

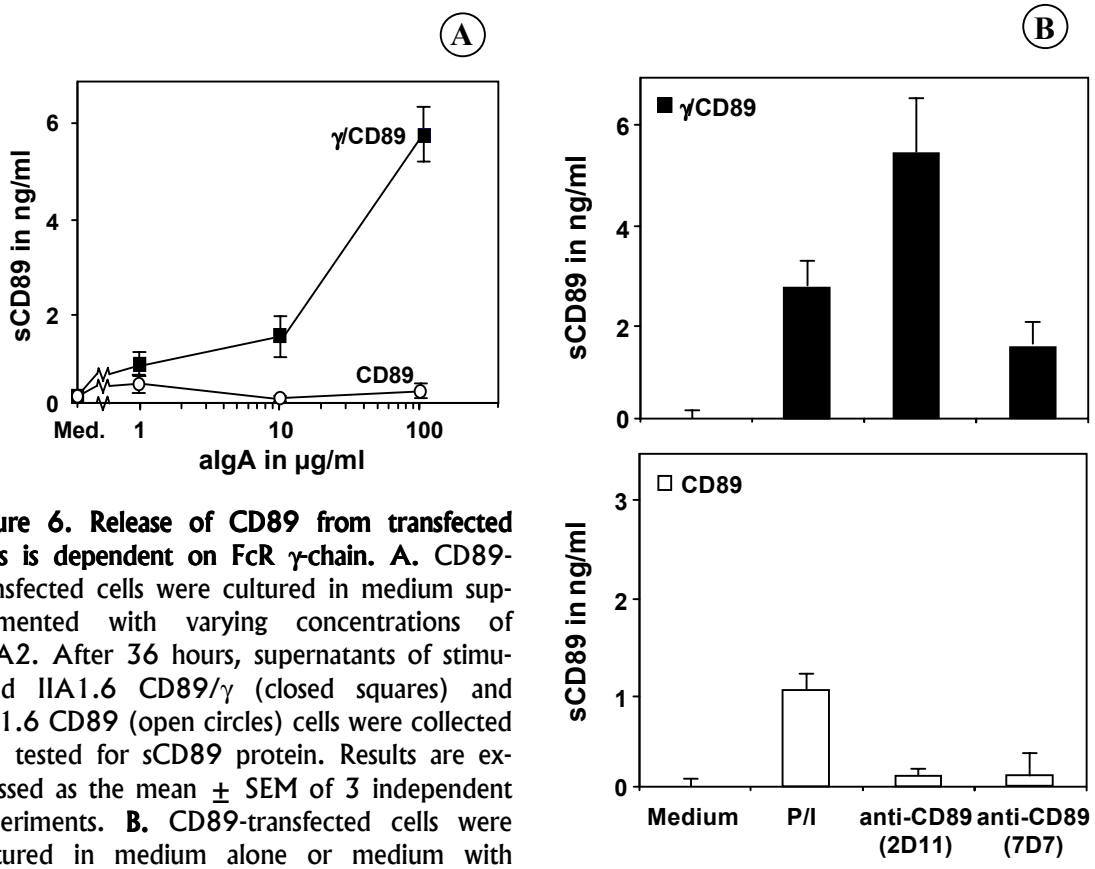

Figure 6. Release of CD89 from transfected cells is dependent on FcR γ-chain. A. CD89-transfected cells were cultured in medium supplemented with varying concentrations of algA2. After 36 hours, supernatants of stimulated IIA1.6 CD89/γ (closed squares) and IIA1.6 CD89 (open circles) cells were collected and tested for sCD89 protein. Results are expressed as the mean ± SEM of 3 independent experiments. **B.** CD89-transfected cells were cultured in medium alone or medium with PMA/ionomycin, or anti-CD89 mAb's 2D11 and 7D7. After 36 hours supernatants of the stimulated IIA1.6 CD89/γ (closed bars) and IIA1.6 CD89 (open bars) cells were collected and tested for sCD89 protein. Results are expressed as a mean ± SEM of 3 independent experiments.

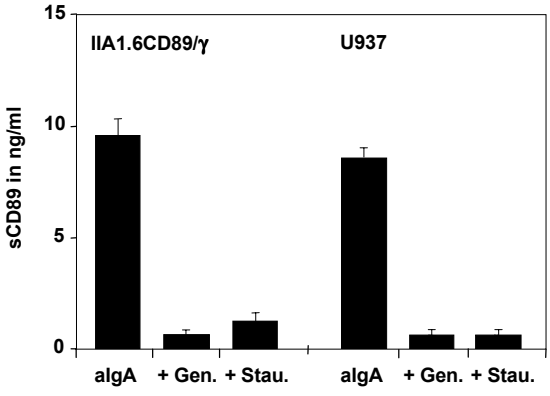

Figure 7. Shedding of sCD89 is prevented by genistein and staurosporine. Both the CD89/γ transfectants (left) and U937 cells (right) were cultured stimulated with algA2 in the absence or presence of an inhibitor of protein tyrosine kinases (gen. = genistein; 100µM) or an inhibitor of protein kinase C (stau. = staurosporine; 50 ng/ml). After 36 hours supernatants were collected and tested for sCD89 protein. Of three independent experiments, one representative example is shown.

When analyzing the supernatants of U937 cells, a specific product of approximately 30 kD was found after PMA/ionomycin stimulation, which was not observed under non-stimulated conditions (lanes 3,4). Reactivity against the 30 kD protein was completely blocked by preincubating the antisera with recombinant sCD89 (lane 5). A similar 30 kD protein was found in the supernatant of PMA/ionomycin stimulated THP-1 cells (lanes 6,7) and in supernatant of aIgA stimulated CD89/γ transfected IIA1.6 cells (lanes 8,9).

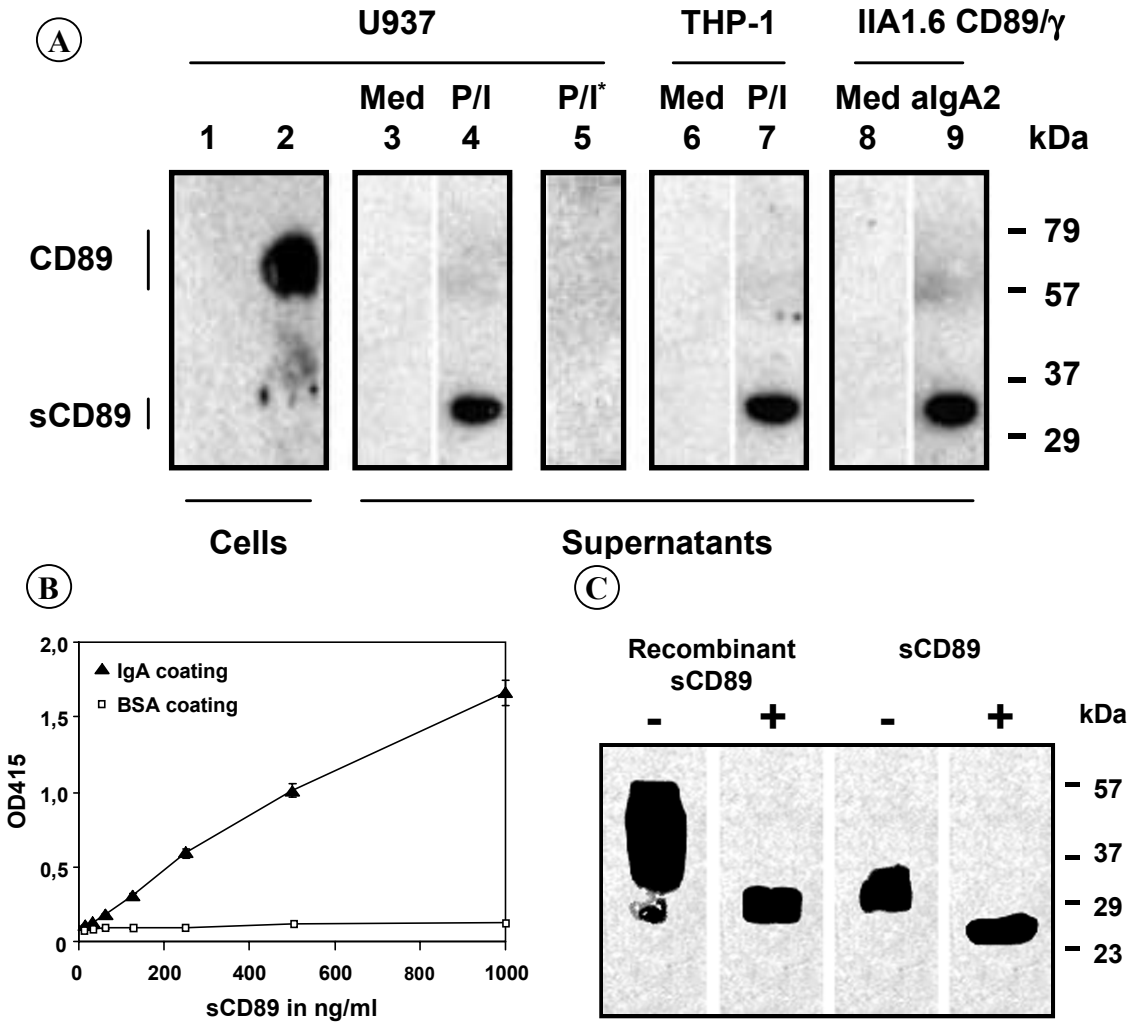

Figure 8. Immuno-chemical analysis of sCD89 protein. A. Cell lysates from U937 cells were blotted with normal rabbit and goat sera as a control (lane 1), or CD89 specific antisera (lane 2). Supernatants of U937 and THP-1 cells cultured in medium alone or stimulated for 36 hours with PMA/ionomycin (as indicated) were blotted with CD89 specific sera (resp. lane 3, 4 and 6, 7). As a specificity control (lane 5), rabbit and goat polyclonal anti-CD89 antibodies, were preincubated with 25 μg recombinant soluble CD89 and used for blotting of supernatant from PMA/ionomycin stimulated U937 (P/I*). Similar as for U937 and THP-1 supernatants of IIA1.6 CD89/γ cultured in medium alone or stimulated with aIgA2 were blotted (resp. lane 8, 9). **B.** Purified sCD89 was tested at the indicated concentrations for binding to IgA (coated 2 μg/ml, closed triangles) or BSA (coated 2 μg/ml, open squares)(as a specificity control). Results are expressed as the mean ± SEM of 3 independent experiments. **C.** Recombinant soluble CD89 and purified sCD89 were treated with N-Glycosidase F for removal of N-linked sugars. Deglycosylated or untreated samples, as indicated, were separated on 10 % SDS-PAGE and blotted specific for CD89.

In order to obtain more detailed information on the structure of sCD89, the protein was purified from supernatant of PMA/ionomycin activated U937 cells. Soluble CD89 could be purified using an IgA-affinity absorbent, which showed the natural form of sCD89 to be capable of binding IgA. This was confirmed in an ELISA system, with IgA coated to ELISA wells, where a dose-dependent binding of sCD89 to IgA was observed (Figure 8B). When the purified sCD89 was analyzed by Western blotting, we observed a 30 kD molecule. Treatment with N-glycanase reduced the MW to 25 kD. For comparison, the recombinant soluble form (37-55 kD) was treated in the same way, and predictably showed a 27 kD backbone after removal of the N-linked glycans (Figure 8C) (21).

DISCUSSION

In the present study we demonstrate that upon activation, myeloid cells can release a soluble form of the FcαRI/CD89. Biochemical analysis showed this soluble receptor to represent a 30 kD glycosylated protein, which is capable of binding IgA. Both aIgA and anti-CD89 antibodies induced the release of sCD89, which suggests that IgA antibodies produced during a mucosal immune response might have a regulatory effect on the CD89 effector functions.

In our experiments we concentrated on the myeloid cell line U937, although similar effects were found with other monocytic cell lines (THP-1 and MonoMac-6). Activation of PMN with PMA/ionomycin results in a strong and fast release of sCD16, but does not result in release of sCD89, suggesting that regulation of receptor shedding is different between CD16/FcγRIII and CD89/FcαRI. Comparing peripheral blood monocytes and myeloid cell lines, we found that the regulation of sCD89 shedding was qualitatively similar. However in peripheral blood monocytes, we detected lower amounts of sCD89 in our ELISA. This might be partially explained by a low-level expression of CD89 on monocytes, or by differences in regulation between these myeloid cell lines and monocytes. We showed that the release of sCD89 is dependent on an active signaling event, which might be quantitatively different in cell lines. It was demonstrated that release of CD89 after CD89 crosslinking is dependent on the presence of the common γ-chain, which is also associated with FcεRI, FcγRIIIa/CD16 and FcγRIIa/CD32 (2) (Figure 6A). Signaling via this subunit, induces PKC activation (9), as well as tyrosine phosphorylation of the γ-chain by members of the Src family (*Lyn*, *Syk*), PI-3 kinase activation and Bruton tyrosine kinase (Btk) activation (30,31). In accordance, we were able to block the release of sCD89 by inhibition of protein kinase C or protein tyrosine kinases.

In contrast to sCD16 shedding (16), release of sCD89 was rather slow, suggesting the involvement of secondary processes. Induction of sCD89 release was accompanied by upregulation of surface expression, although increased surface expression not always resulted in sCD89 shedding. Previous experiments have shown that the IIA1.6 transfectants we have used in our experiments have a comparable CD89 expression and display a similar IgA binding (32). Therefore, the presence of the γ-chain seems to have no effect on the affinity for IgA. These findings are different from data published for FcγR (33) and require further investigation.

The molecular weight of 30 kD of CD89 rules out the possibility that the products measured in ELISA are released membrane vesicles containing full length CD89. Recently, at least 11 different splice variants of CD89 have been identified (34-38). It seems unlikely that they are responsible for the sCD89 molecule, as most of them showed partial or complete deletions of EC1 or EC2, while still containing the predicted transmembrane region. Finally, we found that IIA1.6 cells transfected with full length CD89 cDNA, excluding alternative splicing, also release a similar 30 kD molecule upon activation (Figure 7A, lane 8). These data suggest a role for proteolytic cleavage, as demonstrated for various molecules, including cytokines (TNF-α), cytokine receptors, adhesion molecules and Fc receptors (18,24,39).

The cleavage site of CD16/FcγRIII was identified between Val196 and Ser197 after C-terminal sequencing large amounts of sCD16 purified from human serum (40). In preliminary experiments we found that both EDTA and 1,10 phenantriolin, which are inhibitors of metalloproteinases, prevented the release of sCD89 (data not shown), suggesting the involvement of metalloproteinases in cleavage of CD89. The difference in core size between recombinant sCD89 and sCD89 cleaved from U937 shows that the cleavage site is N-terminal from Tyr^{207}, the C-terminal amino acid of the recombinant product.

An important question concerns the (patho-) physiological role of soluble CD89. Release of soluble receptors has been suggested to represent a universal mechanism of receptor regulation, which might be dysregulated in various human diseases (18). Shedding of CD89 will uncouple the receptor from its signaling transduction pathways and therefore represents a means of effector function downregulation. Quantification of CD89 in cell lysates of U937 as compared to their supernatants suggested that upto 5% of the receptor might appear in soluble form after stimulation with PMA/ionomycin (data not shown). It is likely that soluble CD89 might immediately interact with circulating IgA and influences the function of IgA. We have obtained preliminary evidence that sCD89 is present in

the circulation. It is possible that IgA-CD89 complexes might have "nephritogenic" activities as has been suggested recently (41).

Levels of sCD16 (FcγRIII) have been proposed to be a measure for the number of neutrophils (16). Our *in vitro* data suggest that PMN do not release CD89 and that monocytes might be the most important source of soluble CD89. Therefore sCD89 levels might represent a measure for monocyte numbers and/or activation. Recently, monocytes (but not neutrophils) of patients with primary IgA nephropathy, were found to display a marked reduction of surface CD89 expression that correlated with the increased levels of serum IgA (42). At present it is unclear whether the negative regulation of monocytic CD89 expression might be associated with an increased release of soluble CD89.

In conclusion, in the present study we have shown that the myeloid FcαRI/CD89 can be released as a 30 kD soluble molecule. The release of sCD89, which can bind IgA, is induced upon activation of myeloid cells. This may provide a mode of "fine-tuning" effector functions of CD89 expressing cells. In recent years the CD89 molecule has evolved as a candidate target for bispecific antibody therapy (43,44). It will be important to unravel the mechanisms of CD89 shedding, not only to potentially improve the efficacy of therapy, but also to monitor immune activation.

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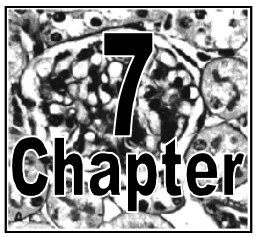

GENERAL DISCUSSION AND CONCLUSIONS

I. INTRODUCTION

A multitude of pathogenetic mechanisms has been suggested to be relevant and may be crucial during the course of IgA nephropathy (IgAN). Defective B cell activation after mucosal infection leads to an aberrant immunoglobulin production especially for the IgA-isotype and may result in increased levels of IgA in the circulation of patients with IgAN. Accordingly clinical relapses of IgAN, indicated by (macro-) hematuria, are associated with mucosal infections (1-3). On the other hand in the last years interest has focussed on abnormal IgA1 glycosylation of the hinge region of this immunoglobulin subclass. Structural investigations to analyze IgA1 O-glycans in IgA nephropathy and controls demonstrate that in IgAN, the IgA1 O-glycan chains are truncated, with increased terminal GalNAc (4). Other investigators found that the deficiency of galactose in the hinge region of the IgA1 molecule results in the generation of antigenic determinants containing GalNAc residues that are recognized by naturally occurring IgG and IgA1 antibodies. Nevertheless the relevance of immunecomplexes is not clear (5,6). Elevated plasma IgA1 in patients with IgAN could also be related with its different glycosylation of the hinge region. This is suggested to be important for interaction of IgA1 with receptors such as the asialoglycoproteinreceptor (7). Reduced binding of IgA1 to the asialoglycoproteinreceptor expressed on hepatocytes could be responsible for a slower clearance of IgA from the circulation and could result in higher serum IgA-levels (8). The other main clearance pathway is via Fc receptors for IgA expressed on circulating myeloid cells, which are downregulated in IgAN (9). This could lead to deposition of IgA in the renal mesangium. Furthermore it has been speculated that deficient glycosylation could lead to a higher capacity of IgA1 to aggregate to macromolecular forms of IgA1. Also its capacity to interact with extracellular matrix proteins as well as its capability in activating complement could be impaired and could therefore be an explanation of chronic inflammatory processes during IgAN (10,11).

The deposition of IgA in the glomerulus considered, as the major characteristic of IgA-nephropathy is still not explained (12,13). Deposition of IgA leads to complement activation via the alternative pathway followed by influx of inflammatory cells increased cytokine release by resident and infiltrating cells. This inflammatory response is followed by upregulation of mesangial matrix-production and mesangial cell proliferation (10,14,15).

II. RECEPTOR-MEDIATED BINDING OF IGA TO MESANGIAL CELLS

Increasing evidence on the existence of a mesangial receptor for IgA is supported by the observation that IgA is able to bind to rat and human mesangial cells and that bound IgA can be internalized and degraded by these cells (16,17,17,18,18,19,20). Binding of 125I-IgA to quiescent human MC showed 2.55 x 10^5 sites/cell with an affinity (Ka) of 3.2 x 10^7 M^{-1}. Addition of selected recombinant cytokines had no significant influence on Ka, but increased the number of sites/cell relative to unstimulated cells (24). MC incubation with aggregated IgA (aIgA) elicited a dose-dependent increase in cytosolic Ca^{2+}. This effect was dependent on the Fc region of IgA, because Fc, but neither Fab fragment nor carbohydrates, inhibited the Ca^{2+} rise. The initial induction of Ca^{2+} rise was due to Ca^{2+} mobilization from inositol trisphosphate (IP3)-sensitive intracellular stores, while sustained levels were maintained through extracellular Ca^{2+} influx. Protein tyrosine kinase inhibitors abolished Ca^{2+} rise, indicating that tyrosine phosphorylation of some substrates is required for Ca^{2+} mobilization (21). Furthermore binding of IgA to MC leads to activation of MC and subsequently to increased expression of the transcriptional factor NF-$_{kappa}$B (22), c-jun (23), interleukin-6 (IL-6) (16-20,20), IL-8 (22), MCP-1 (22) and Tumor Necroses Factor-α (TNF-α) (16).

Also most of these effects of IgA seems to be related to the Fc part of IgA: Investigations of Duque et al. (22) shows that aggregated IgA induces activation of the transcriptional factor NF-κB and increases the expression of monocyte chemoattractant-protein 1, interleukin-8 and gamma-interferon induced protein 10. This effect was absent after preincubation of mesangial cells with Fc-fragments of IgA indicating that binding of IgA to mesangial cells is mediated via the Fc portion of this immunoglobulin. These findings strongly indicate the existence of a Fc-receptor for IgA on human MC.

The involvement of the known myeloid Fc receptor for IgA (CD89) on mesangial cells in the pathogenesis of IgA-nephropathy has been controversial (22-25). In chapter 4 a study is presented, investigating the expression of CD89 on human mesangial cells *in vivo* and *in vitro*. IgA blocking experiments demonstrated that IgA do not bind to this cell type via CD89. Even more immunohistochemical and flowcytometric experiments revealed no protein expression of CD89 neither on mesangial cells in vitro nor in human kidney section in vivo using known antibodies for CD89 as well as new generated monoclonal and polyclonal antibodies against this receptor. Furthermore also mRNA expression was not

detectable using RT-and nested RT-PCR. In summary the know Fc-receptor for IgA as well as other potentially IgA-binding proteins as stated in the introduction are not expressed on human mesangial cells.

Recently, a British group showed expression of mRNA transcripts with partial identity to CD89 on human MC in vitro using RT-PCR(26). The detected transcripts were located in the area of the extracellular domain 2 of CD89 which makes it unlikely that binding of IgA is mediated via theses transcripts because it has been shown that binding of IgA to CD89 is mediated via the extracellular domain 1(27). Even more there was no protein expression detectable on the surface of mesangial cells as well as in culture medium using Western blot. However, the transcript fragments they identified may encode a new protein with partial homology to CD89. This hypothesis originates from the recent discovery of genes mapping to chromosome 19Q13.4 and encoding a family of type I transmembrane proteins with significant homology: CD89, paired Ig-like receptors (PIRs), killer inhibitor receptors (KIRs), leukocyte Ig-like receptor (LIRs), and Ig-like transcripts (ILTs) (28).

To identify the putative IgA-Fc receptor we generated an antibody directed against an IgA-binding protein expressed on human neutrophils as well as on human mesangial cells (Chapter 5). This monoclonal mouse antibody designated 72G12 is able to block binding of IgA to human mesangial cells. The recognized antigen is furthermore expressed on monocytes and even more on monocyte derived dendritic cells. Precipitation studies of membrane surface proteins from human mesangial cells demonstrated an 83 KD protein recognized by 72G12, which is also precipitated by human IgA. Further characterization of this potential IgA-receptor is needed to clarify its potential role during IgAN. Most important questions are possible difference in expression of this receptor in the glomerulus of patients with IgA-nephropathy and controls. It is of great interest to investigate its ability in binding of IgA, especially those with defective glycosylation of the hinge region and IgA-immuncomplexes as described in IgAN (5,6).

III. SHEDDING OF CD89/FCαRI

Soluble forms have been identified for various Fc receptors for IgG (Fc gRII/CD32 and FcgRIII/CD16) and IgE (Fc eRII/CD23) (29-32). Furthermore, it has been proposed that these soluble Fc receptors have a pathophysiological role in several diseases (33-35).

We investigated mechanisms involved in release of soluble CD89 from the surface of monocytic cell lines (Chapter 6). It was found that upon activation, myeloid cells could release a soluble form of FcαRI/CD89. Western blot analysis

showed this soluble receptor to represent a 30-kD glycosylated protein that is capable of binding IgA. Both IgA and anti-CD89 antibodies induced the release of sCD89, which suggests that IgA antibodies produced during a mucosal immune response might have a regulatory effect on the CD89 effector functions. The 30 kD soluble CD89 molecule is released in a FcRγ-chain dependent manner from monocytic cell lines as indicated by experiments done with the CD89 transfected mouse cell line IIA1.6 which was alternatively cotransfected with the FcRγ-chain. Shedding of sCD89 was dependent on signaling via the γ-chain and prevented by addition of inhibitors of protein kinase C (staurosporine) or protein tyrosine kinases. Next to ligand-induced release of CD89 also pharmacologic activation by ionomycin and phorbol-myristate acetate as well as activation of the cell by tumor necrosis factor α leads to shedding of this receptor.

Recently Monteiro and coworkers reported about the presence of circulating soluble CD89-IgA complexes in patients with IgAN. Soluble CD89 was identified as a glycoprotein with a 24-kD backbone that corresponds to the expected size of CD89 extracellular domains. To demonstrate their pathogenic role, transgenic (Tg) mice expressing human CD89 on macrophage/monocytes were generated. These mice spontaneously developed massive mesangial IgA deposition, glomerular and interstitial macrophage infiltration, mesangial matrix expansion, hematuria, and mild proteinuria. The molecular mechanism was shown to involve soluble CD89 released after interaction with IgA. In contrast to our own results release of CD89 was independent of CD89 association with the FcRγchain in this mouse model. The disease was induced in recombination activating gene mice by injection of serum from CD89-Tg mice, and in severe combined immunodeficiency -Tg mice by injection of IgA from patients. Depletion of soluble CD89 from serum abolished this effect. These results could reveal an important pathophysiologic aspect of soluble CD89 in the pathogenesis of IgAN (36).

IV. ROLE OF INTERLEUKIN-6 IN MEDIATING GLOMERULAR DAMAGE

Mesangial cell proliferation and mesangial matrix accumulation are key features of various human glomerular diseases, including IgA nephropathy (37,38). Interleukin-6 (IL-6) is produced in relatively large amounts by mesangial cells in response to a variety of stimuli, such as angiotensin II, lectins, matrix proteins, cytokines, as well as IgA-immuncomplexes and immune complexes of other isotypes of antibodies (16,19,39-44). It was described that IL-6 contributes to induction of matrix protein transcription and autocrine growth in mesangial cells in vitro (45,46). In vivo, glomerular IL-6 overexpression was detected in human

glomerulonephritis types characterized by mesangial hypercellularity such as IgA nephropathy and some types of lupus nephritis (47-51). Furthermore, IL-6 transgenic mice developed a mesangioproliferative glomerulonephritis (46) and the urinary excretion of IL-6 has been correlated with mesangial hypercellularity in patients with IgA nephropathy (46,52,53). It has therefore repeatedly been proposed that IL-6 is an important mediator of mesangial cell proliferation and matrix overproduction (38,41,54). The results described in chapter 2 analyze the role of interleukin 6 in mediating mesangial cell proliferation and matrix production in vivo. We demonstrated that IL-6 knock out mice develop a normal glomerular architecture and in particular a normal mesangium. Mesangioproliferative glomerulonephritis induced by Habu snake venon is equally severe in IL-6 knock out mice as in control mice. A continuos seven-day intraperitoneal infusion of 50 μg recombinant IL-6 into rats with a prior minimal (subnephritogenic) injury to mesangial cells does not induce glomerular cell activation, cell proliferation, matrix production, leukocyte influx, platelet influx or proteinuria. A continuos seven-day IL-6 infusion into rats with mesangioproliferative nephritis increases matrix protein transcription in the absence of detectable effects on matrix protein accumulation and otherwise has no effect on the natural course of the disease. Therefore we conclude that IL-6 is not a predominant mediator of mesangial cell proliferation and matrix overexpression in vivo.

Lots of other factors have been investigated in their opportunity to affect secretory/synthetic activity of mesangial cells. Change of their phenotype results in the acquisition of a proinflammatory and profribrotic phenotype of this cell. In vitro and animal data suggest that platelet-derived growth factor (PDGF) B-chain has a particular important role in this respect (55). MC produces PDGF and PDGF B-chain and its receptor are overexpressed in glomerular diseases. Infusion of PDGF-BB or glomerular fransfection with PDGF B-chain cDNA induce mesangial proliferative changes in vivo. PDGF B-chain or β-receptor knock-out mice fail to develop a mesangium. Furthermore, antagonisation of PDGF B-chain reduce mesangial proliferation during experimental mesangial proliferative glomerulonephritis (56). Another factor playing a well-established role in experimental glomerulonephritis is transforming growth factor-β (TGF-β). Similar to PDGF, its release from the MC is induced by other growth factors, in particular angiotensin-II. TGF-β I is overexpressed in IgAN and transgenic overexpression of TGF-β in the kidney results in progressive fibrosis (57).

V. INFILAMMATORY CELLS DURING MESANGIOPROLIFERATIVE GLOMERULONEPHRITIS

The involvement of monocytes and macrophages in various glomerulonephritides has been clearly established (58). In particular their involvement has been associated with proliferative forms of glomerulopathies (59,60). Rodent models of glomerulonephritis (61-63) have provided experimental evidence. However, in some cases infiltrating monocytes may serve merely to remove immune complexes and do not contribute to the glomerular injury (64). Recent interest has focused on the role of macrophages in the pathogenesis of focal glomerulosclerosis (65,66). Both glomerular hypercellularity and expansion of the extracellular matrix are thought to be of primary importance in the development of capillary obsolescence and glomerulosclerosis. In the remnant kidney model in the rat Van Goor and colleagues (58) showed that macrophages play a central role in the development of focal glomerulosclerosis. As described by Floege et al., the development of glomerulosclerosis in this model is preceded by mesangial proliferation and mesangial matrix expansion (67).

To clarify the distinctive roles of inflammatory cells during mesangial cell proliferative glomerulonephritis we depleted selectively monocytes and neutrophils in a rat anti-Thy-1.1 model described in chapter 3. In this model PMN reduction was associated with enhanced hematuria, a higher percentage of glomeruli with micro-aneurysms, enhanced glomerular deposition of C3 and C6, and no significant change in proteinuria, suggesting that microaneurysm formation is an important factor in determining the degree of hematuria. So PMNs seem to contribute to the repair process during anti-Thy-1.1 nephritis. Maybe proteolytic enzymes released by these cells have a complement-clearing function, and consequently a reduction in PMN influx may be responsible for the observed pronounced and sustained C3 and C6 deposition in rats treated with ED7. In the literature a complement-cleaving function of proteases released from PMNs has been described (68-70). Because complement is the major pathogenic factor leading to MC lysis during anti-Thy-1.1 nephritis, this could be an explanation for the increased damage and hematuria observed in this study.

In contrast to our results obtained in a complement mediated anti-Thy-1.1 model earlier experiments investigated the influence of mAb against various adhesion molecules on glomerular neutrophil accumulation and proteinuria in rats with nephrotoxic serumnephritis (71). This model is characterized by upregulation of different adhesion molecules, rapid neutrophil infiltration into the glomerulus, and proteinuria. Treatment of animals with antibodies against CD18, CD11b, and ICAM-1 caused 63%, 46%, and 54% reduction, respectively, in proteinuria and

79%, 66%, and 54% reduction, respectively, in glomerular neutrophil counts, suggesting an important role for these adhesion molecules in this model of experimental nephritis (71). Moreover, these data indicate that neutrophils play a major pathogenetic role in experimental nephrotoxic nephritis, specifically in glomerular permselectivity. These findings are further supported by another study investigating nephrotoxic-serum nephritis (72). Recently Waddington and coworkers described a significant glomerular iNOS induction and high output nitric oxide production in the acute phase of neutrophile-dependent acute immunecomplex nephritis. Selective iNOS inhibition in vitro inhibited nephritic glomerular and neutrophil NO2 synthesis (73).

Concerning the role of macrophages, we found that selective depletion of macrophages lead to a reduced glomerular matrix expansion. In contrast to ED7 treatment, glomerular complement deposition, microaneurysm formation, and hematuria remained unaffected.

These data are in agreement with recently published investigations (51). It was found that, after induction of anti-Thy-1.1 nephritis, influx of macrophages is strain dependent. High amounts of infiltrating macrophages led to a highly significant increase of extracellular matrix in Lewis rats (74). The potential mechanisms by which macrophages mediate glomerular-cell proliferation and the development of mesangial matrix expansion could involve release of cytokines by the macrophage itself or could involve stimulation by the macrophage of other cell types to release cytokines, e.g. transforming growth factor β within the glomerulus (75-77). For transforming growth factor β involvement in mesangial matrix expansion has clearly been shown (77-79). Another macrophage-derived product with a possible high impact is nitric oxide, which is abundantly secreted by infiltrating monocytes in this model (64). Monocytes have been shown to induce increased transcription of TGF-β and fibronectin (80).

In coculture studies, Mosquera demonstrated that monocyte-derived culture supernatants could induce mesangial cells to synthesize fibronectin in vitro (81). As shown in Figure 3, the mesangial expansion in monocyte-depleted rats was not completely inhibited. This may be because 1) especially major histocompatibility complex-II negative resident macrophages are less sensitive to liposome-mediated elimination (E De Heer, V Cattell, unpublished results), and 2) cytokines from other sources, for instance platelet-derived growth factor, may be able to induce mesangial matrix expansion, albeit less effectively (57).

Taken together monocytes are specifically involved in the expansion of the mesangial extracellular matrix, whereas other immunopathological processes in the mesangium (complement activation, platelet aggregation, and mesangial

proliferation) remain unaffected. These findings indicate that the mesangial alterations occur through distinct signaling pathways.

VI. CONCLUDING REMARKS

The work described in this thesis has contributed to a better understanding of the differential role of infiltrating inflammatory cells as well as the relevance of interleukin-6 during the course of experimental mesangial cell proliferative glomerulonephritis. It was found that interleukin-6 does not play a major role in the pathogenesis of the mentioned form of gomerulonephritis, furthermore interleukin-6 is not crucial for the development of a normal mesangium. In contrast to other examinations in other forms of experimental nephritis neutrophils seems to have a complement clearing function during anti-Thy-1.1 nephritis. This function is contributed to worsening of this form of complement mediated glomerulonephritis after depletion of neutrophils. Infiltrating macrophages on the other hand are associated with an increase of mesangial matrix accumulation.

Concerning the role of the suggested IgA-receptor expressed on human mesangial cells CD89/FcαRI we failed to show its expression neither at the protein nor at its mRNA-level in vitro and in vivo. However, recent observation described in this thesis demonstrates the existence of a novel IgA-binding moiety expressed on the surface of human renal mesangial cells. This finding could be an important connecting link between IgA-deposition in the renal mesangium and the defective glycosylation or/and the existence of IgA/IgG immuncomplexes in IgA-nephropathy.

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SAMENVATTING EN CONCLUSIES

Primaire Immuunglobuline A (IgA) nefropathie (IgAN) wordt veroorzaakt door depositie van IgA vanuit de circulatie in het mesangium van de nier. Waarom en hoe IgA neerslaat in de nier is nog steeds niet bekend. Een opmerkelijke bevinding is dat het IgA in de nier uitsluitend bestaat uit één subklasse, n.l. IgA1. Depositie van IgA in de nier leidt tot een ontsteking en gaat vaak gepaard met (macro) hematurie. Eerdere studies hebben aangetoond dat het neergeslagen IgA voor ten minste een deel bestaat uit dimeer- en polymeer IgA. Recente studies suggereren dat patiënten met IgAN, IgA produceren met een defect in O-glycosylering. Dit IgA zou in de eerste plaats slechter geklaard worden uit de circulatie en ook beter met mesangium cellen reageren. Het is al enige tijd bekend dat patiënten met IgAN gemiddeld een hogere concentratie van circulerend IgA hebben. De hypothese is dat abnormale glycosylering van het IgA en de relatief hogere concentraties van dimeer- en polymeer IgA bijdragen tot een verhoogde kans van depositie van IgA in het mesangium. Er is verder voldoende bewijs dat interactie van IgA met mesangiumcellen leidt tot binding van IgA aan deze cellen en activatie van de cellen met als gevolg productie van verschillende cytokinen en chemokines. Tevens kan door activatie van complement de ontsteking ter plaatse versterkt worden. De verhoogde mate van activatie van mesangium cellen leidt tenslotte tot verhoogde productie en neerslag van matrix eiwitten die weer de filtreerfunctie van de glomerulus verstoren. Op termijn leidt dit tot gradueel verlies van nierfunctie en uiteindelijk tot nierfalen.

In hoofdstuk 2 werden verschillende aspecten van activatie van mesangium cellen bestudeerd. Aangetoond werd dat interleukine (IL-6) geen cruciale rol speelt bij de proliferatie van mesangium cellen in de muis en de rat, in tegenstelling tot eerdere publicaties dat het instaat is om mesangium cellen te zetten tot verhoogde matrix productie. Omdat mesangium cellen na stimulatie zelf IL-6 produceren bestond de gedachte dat IL-6 en autocrien effect zou kunnen hebben in de nier. Gebaseerd op onze huidige vondsten dient een dergelijke conclusie echter afgezwakt te worden.
Interessant was onze bevinding dat blokkering van granulocyten (hoofdstsuk 3) in een model van membranoproliferatieve glomerulonefritis n.l. in het anti-Thy.1 model, leidt tot verergering van proteïnurie en schade in de glomerulus. Het bleek dat ratten met anti Thy.1 nefritis die anti-integrine antilichamen kregen toegediend, meer depositie van de complement componenten C3 en C6 vertoonden en verhoogde proteinurie. Wij veronderstellen dat onder condities van infiltratie en activatie van granulocyten, de mate van depositie en/of activatie van complement beïnvloedt wordt door proteolytische enzymen van de geactiveerde granulocyten.

Wij denken dat deze proteolytische enzymen geactiveerd C3 afbreken waardoor versterking van de amplificatie route van complement wordt onderbroken. Verder onderzoek is nodig om deze hypothese nader te onderbouwen.

Het tweede gedeelte van dit proefschrift richt zich meer op de directe effecten van IgA op het mesangium.
Eerdere bevindingen van ons laboratorium hebben laten zien dat IgA kan binden aan mesangium cellen en deze cellen aanzet tot verhoogde productie van groeifactoren. Deze studies zijn ook door anderen bevestigd. De wijze waarop IgA bindt aan mesangium cellen is nog steeds een open vraag. Er zijn 3 membraan moleculen bekend waarmee IgA kan reageren n.l.: de pIgA receptor of secretoir component (SC). Dit molecuul komt voor op epitheel cellen waar het functioneert als een transporteur van dimeem IgA van het weefsel compartiment naar bijvoorbeeld het lumen van de darm. Tot nu toe zijn er geen bevindingen die het voorkomen van SC op mesangium cellen ondersteunen. Het tweede molecuul waar IgA aan kan binden is de zgn. asialoglycoproteïne receptor dat voorkomt op o.a. lever epitheel. Ook voor dit molecuul zijn er bij de mens geen sterke aanwijzingen op mesangium cellen van de mens. Tenslotte is er een derde receptor voor IgA beschreven, n.l. CD89. Deze receptor komt voor op monocyt/macrofragen, granulocyten en eosinofielen. Eerdere studies hebben gesuggereerd dat mesangium cellen van de mens een hoge mate van mRNA expressie voor CD 89 hebben. In hoofdstuk 4 hebben wij uitgebreide studies beschreven waarin gebruik is gemaakt van specifieke polyclonale- en monoclonale anti-CD89 antistoffen voor het aantonen van CD89 in vitro op gekweekte mesangium cellen, en in coupes van nieren en milt van patiënten met IgAN of controles.
Terwijl de binding van IgA aan mesangium cellen duidelijk optrad, vonden wij geen enkele aanwijzing, noch in vitro noch in weefsel biopten, voor het voorkomen van CD89 op nier mesangium cellen. Ook met zeer gevoelige PCR-methoden konden wij geen mRNA voor CD89 in mesangium cellen bevestigen. Onze studies worden gesteund door tenminste 3 andere onderzoeksgroepen en aangenomen mag worden dat de eerder gepubliceerde gegevens van de groep van Egido op een technische fout moet berusten.
Zoals eerder vermeld treedt ondanks de afwezigheid van CD89 en de andere receptoren voor IgA toch binding van IgA op aan mesangium cellen. Verder onderzoek beschreven in hoofdstuk 5 van het proefschrift levert bewijzen dat IgA bindt aan een mesangium celmembraan eiwit van ongeveer 83 Kd. Wij zijn er nog niet in geslaagd om de identiteit van dit molecuul te bepalen. In het hoofdstuk 6 wordt beschreven dat oplosbaar CD89 (S CD89) kan vrijkomen van cellen die

CD89 tot expressie brengen, wanneer deze cellen worden geactiveerd. Wij vonden dat SCD89 alléén vrijkomt van cellen als het CD89 in de membraan gassocieerd is met een andere peptide keter, nl de J. Chain die ook gebruikt wordt door andere membraanmoleculen zoals FC-gammareceptoren. Het SCD89 is net als CD89 in de membraan in staat om IgA te binden. Wij hypothetetiseren dat SCD89 een regulerende rol speelt op de klaring en functie van IgA en daardoor mogelijk een rol zou kunnen spelen bij IgAN. Verdere studies op dit onderdeel zijn van essentieel belang voor het nader definiëren van de depositie van IgA in het mesangium. Indien dit lukt kan een deel van de puzzel worden opgelost, van hoe en waarom IgA neerslaat in het mesangium van de nier bij patiënten met IgAN.

CURRICULUM VITAE

De schrijver van dit proefschrift, Ralf Westerhuis, werd geboren op 26 mei 1966 te Hildesheim. In 1986 werd het Atheneum diploma behaald aan het Scharnhorst Gymnasium te Hildesheim. In het volgende jaar begon hij aan zijn medische studie aan de Medizinische Hochschule Hannover, Duitsland. Naar het behaalen van het einddiploma tot arts gegon hij 1994 met zijn interne opleiding in de afdeling nierziekten, Medizinische Hochschule Hannover (hoofd Prof. Dr. K.M. Koch). 1997 tot 1999 werkte hij als onderzoeker bij de afdeling Nierziekten van het Leids Universitair medisch Centrum (hoofd Prof. Dr. L.E. van Es, sinds 1 juli 1998 Prof. Dr. L.C. Paul). Het onderzoek werd gedaan onder leiding van Prof. Dr. M.R. Daha en Dr. C. van Kooten. Van maart to juni 1999 vervolgde hij zijn opleiding tot internist in Hannover en daarna sinds juni 1999 aan de universiteitskliniek Aken, afdeling Nierziekten (hoofd Prof. Dr. J. Floege).

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