Expression of MRP8 and MRP14 by macrophages is a marker for severe forms of glomerulonephritis

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Abstract: Expression of two S100 proteins, myeloid related protein (MRP)8 and MRP14, as well as their complex formation indicate proinflammatory properties of macrophages. We analyzed if the different forms of glomerulonephritis (GN) are associated with the appearance of certain phenotypes of infiltrating macrophages characterized by expression of MRP8 and MRP14 as well as their complex formation. Immunohistochemical analysis of 89 renal biopsies with different forms of nephritis revealed that expression and complex formation of MRP8 and MRP14 by infiltrating macrophages in the glomeruli correlated with the severity of the inflammatory process. As such, MRP8/MRP14-expressing monocytes prevailed in highly proliferating forms of GN, i.e., systemic lupus erythematosus GN and extracapillary GN. In contrast, a high percentage of macrophages in the renal interstitium expressed MRP8 and MRP14 without concomitant formation of their complex, and they indicated a chronic type of inflammatory reaction in GN. Immunosuppressive drugs had no direct effects on the expression of MRP8 and MRP14 in macrophages in vitro. The correlation of MRP8 and MRP14 expression with disease activity indicates that these calcium-binding proteins are of pathophysiological relevance in GN. In addition, our findings reflect differences in the inflammatory mechanisms underlying the various forms of GN, as they revealed that distinct macrophage subpopulations prevail in the different forms of GN. *J. Leukoc. Biol.* 75: 198–206; 2004.

Key Words: *systemic lupus erythematosus · interstitial nephritis · extracapillary GN · S100 protein · S100A8 · S100A9*

INTRODUCTION

Accumulation of macrophages in glomeruli and renal interstitium is a feature of all forms of human glomerulonephritis (GN) [1–6]. The intensity of this macrophage infiltrate correlates with histopathological damage and loss of renal function [1–5, 7]. Analysis of various animal models of GN has revealed that macrophages directly cause renal injury during these diseases [8–13]. Especially secretion of those cytokines for which macrophages are a major source appears to play an important role in the progression of GN [11, 14–17]. However, macrophages are not a homogeneous cell population, as they encompass distinct phenotypes that exhibit a wide range of inflammatory and anti-inflammatory activities [18, 19]. This heterogeneity is important for propagation or down-regulation of the initiated inflammatory process and the resulting glomerular injury [4, 18, 19]. Thus, analyzing different subtypes of monocytes and macrophages that represent distinct inflammatory effector functions in the infiltrate of GN will allow insight in the pathophysiology of these diseases and could provide good prognostic parameters for the course of renal disease [7]. In the present study, we therefore characterized the monocytic infiltrate of various GN with regard to the expression of two myeloic differentiation markers, myeloid related protein (MRP)8 and MRP14.

MRP8 and MRP14 are two calcium-binding proteins that belong to the S100 family [20, 21]. MRP8 and MRP14 represent the predominant calcium-binding capacity in early differentiation stages of monocytes and neutrophils [22], but both proteins cannot be detected in resting tissue macrophages or in lymphocytes [21, 23]. Noncovalently associated complexes of both molecules play a role in calcium-dependent modulation of cytoskeletal-membrane interactions [24–26]. Calcium-dependent translocation of MRP8 and MRP14 from the cytoplasm to the plasma membrane correlates with the inflammatory activation of these cells as shown by an elevated secretion of cytokines, e.g., tumor necrosis factor (TNF) or interleukin (IL)-1β, and activation of the respiratory burst [27, 28]. In various experimental models of inflammation, the expression of MRP8 and MRP14 by infiltrating cells showed a strong correlation with the inflammatory process [29–31]. Accordingly, MRP8 and MRP14 have been detected in monocytes and neutrophils in the infiltrate of various human diseases, e.g., chronic bronchitis, rheumatoid arthritis, Crohn's disease, or colitis ulcerosa [21, 32–35]. During renal allograft rejection, determination of a distinct expression pattern of these two calcium-binding proteins and their complex formation in monocytes in vivo allowed differentiation between acute and chronic courses of this disease [36]. Furthermore, in a prospective study, it has been shown that the intensity of the MRP8/MRP14-positive

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infiltrate is a prognostic factor for the course and outcome of acute renal allograft rejection [37]. No reliable marker has so far been identified to analyze inflammatory activity of the monocyte/macrophage lineage in GN. In the present study, we now demonstrate that expression of MRP8 and MRP14, concomitant with complex formation in infiltrating monocytes, is an indicator for a severe and acute inflammatory process in GN, whereas monocytic expression of these two proteins without concomitant complex formation rather indicates a chronic interstitial inflammation in GN.

MATERIALS AND METHODS

Patients

Eighty-nine patients with histologically proven nephritis were included in this study. Ten patients had minimal change disease (MCD), 13 patients had focal-segmental glomerulosclerosis (FSGS), 26 patients had systemic lupus erythematosus GN (SLE-GN), 13 patients had immunoglobulin-A GN (IgA-GN), six patients had extracapillary GN (ECGN) including four patients with Wegener's granulomatosis, 14 patients had idiopathic membrane-proliferative GN (MGN), and seven patients had interstitial nephritis (IN). The diagnosis was based on detailed clinical history, physical examination, laboratory tests, and pathological features, including conventional light and immunofluorescence microscopy. Disease activity of patients at the time of biopsy was documented by the following parameters: serum creatinine, blood pressure, and daily urinary protein excretion. In addition, we documented age, duration of disease at the time point of biopsy, duration of actual flares of disease (defined as an acute increase of blood pressure or elevation of serum creatinine of more than 25% within 4 weeks), and medical treatment.

Control tissue comprised five renal biopsies from the unaffected pole of kidneys removed for renal cell carcinoma from patients with no history of inflammatory disorders. The investigations were conducted in accordance with the guidelines proposed in the Declaration of Helsinki; informed consent was obtained.

Antibodies

Rabbit antisera against recombinant MRP8 (aMRP8) and MRP14 (aMRP14) were produced as described earlier [25]. Monospecificity of antibodies was analyzed by immunoreactivity against recombinant MRP8 and MRP14, Western blot analysis of lysates of monocytes and granulocytes, as well as by immunoreactivity against MRP8- and/or MRP14-transfected fibroblastic cell lines as described earlier [25]. In addition, the mouse monoclonal antibody (mAb) 27E10 was used, which detects exclusively the MRP8/MRP14 heterodimer but not single monomers [27]. Mouse mAb KP1 against the CD68 antigen, a 110-kDa transmembrane glycoprotein highly expressed by human monocytes and tissue macrophages, was used for single- and double-labeling experiments (Dako Diagnostika, Hamburg, Germany) [38, 39]. Peroxidase or alkaline phosphatase-conjugated second-stage antibodies were obtained from Dianova (Hamburg, Germany).

Cell culture

Human peripheral blood monocytes were isolated from buffy coats by Ficoll-Paque and Percoll (Pharmacia, Freiburg, Germany) density-gradient centrifugation. Purity of monocytes was >90%, as demonstrated by flow cytometry using mAb against CD14, CD15, and CD16 (Dianova). Monocytes were cultured for 1 day in Teflon bags using McCoy's 5A medium supplemented with 2 mM calcium chloride and 15% fetal calf serum as previously reported [25].

Immunoperoxidase technique

Cryostate sections of renal biopsies of GN obtained during diagnostic needle biopsies at the University of Heidelberg (Germany) were processed for immunoperoxidase staining as described previously. Briefly, acetone-fixed serial sections (5 μm) were placed in 0.1% hydrogen peroxide (v/v) and 0.12 M sodium azide (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) to destroy endogenous peroxidase activity. Nonspecific protein binding was blocked with 1% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) in PBS, and sections were subsequently incubated with 1 μg/ml antibodies against MRP8, MRP14, or the mAb 27E10, respectively, followed by peroxidase-conjugated second-stage antibodies (Dianova). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma Chemical Co.). Sections were counterstained with Mayer's haemalaun (Merck). For negative controls, isotype-matched antibodies of irrelevant specificity were used. For double-labeling experiments, tissue samples were incubated successively for 1 h with 1% BSA, 10% normal goat serum in PBS, aMRP14 rabbit antiserum, peroxidase-conjugated goat anti-rabbit IgG $F(ab')_2$, 1.5 mM 3-amino-9-ethylcarbazole (Sigma Chemical Co.), 0.02 mM H_2O_2 in acetate buffer (pH 5.2, 10 min) for color reaction, mAb KP1 against CD68, phosphatase-conjugated goat anti-mouse IgG $F(ab')_2$, 0.02% (w/v) naphtol AS-MX phosphate, 0.1% (w/v) levamisole, and 0.1% (w/v) fast blue RR salt (all Sigma Chemical Co.) in 0.1 M Tris buffer, pH 8.2, as substrate (30 min). Slides were not counterstained after double-labeling procedures. Two independent observers separately analyzed the expression patterns of leukocyte-specific molecules within glomeruli as well as in the interstitium. Glomerular expression of different leukocyte antigens is presented as positively stained cells per glomerulus. At least five cross-sections of glomeruli per biopsy were counted. For quantification of interstitial expression, at least 250 cells per biopsy were counted at the site of maximal inflammatory reaction. Under some conditions, MRP8 and MRP14 show a slightly diffuse staining pattern, which may be a result of extracellularily secreted proteins. Only cytoplasmatic stainings of MRP8 or MRP14 with a clear nuclear association were counted as positive cells. Two observers (M. Frosch and J. Roth) independently did all quantifications. The overall inter-observer variability was less than 10%.

Flow cytometry

For detection of surface expression of the MRP8/MRP14 heterodimer on monocytes, mAb 27E10 was used, which detects specifically the MRP8/MRP14 heterodimer [27]. Monocytes cultured for 1 day were treated with different anti-inflammatory drugs for 4 or 16 h as indicated. To analyze stimulatory as well as inhibitory effects on the surface expression of MRP8/MRP14, 0.1 mM arsenite was added to the medium in parallel sets of experiments during the last 2 h of the incubation period to up-regulate MRP8/MRP14 surface expression. After incubation, cells were harvested, washed, and resuspended in 20% normal goat serum for 30 min to block nonspecific binding. Primary antibodies (1 μg/ml) against the MRP8/MRP14 heterodimer (27E10) and against CD14 were allowed to react for 1 h at 4°C. After additional washing, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ fragments. Thereafter, propidium iodide (Sigma Chemical Co.) was added to permit determination of cell viability and exclusion of nonviable cells. Cell viability was found to be greater than 95% in all experiments presented. Expression of surface molecules on monocytes was analyzed using a FACScan equipped with Lysis-II software (Becton Dickinson, San Jose, CA). Purity of monocyte populations was controlled by high expression of CD14.

Northern blot analysis

Monocytes were treated with different immunosuppressive agents for 4 h as indicated. Total RNA of monocytes was prepared using a two-step method. Cells were lysed with sodium dodecyl sulfate in a citric acid-containing buffer. This procedure was succeeded by salt precipitation to remove contaminating DNA and protein and by a final alcohol precipitation of RNA [40]. Aliquots of the RNA (20 μg) were separated on a denaturing 1.5% agarose gel, blotted onto a nylon membrane, and probed with ^{32}P-labeled human MRP8 and MRP14 cDNA probes as described earlier [41]. Finally, the membranes were washed twice with 0.1% saline sodium citrate at 65°C and exposed to an X-ray film for 2 days. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control.

Determination of MRP8/MRP14 concentrations by sandwich enzyme-linked immunosorbent assay (ELISA)

Monocytes were incubated with different immunosuppressive drugs as indicated. Monocytes were harvested and lysed in PBS containing 1% Nonidet

P-40. Cellular debris was separated by centrifugation for 10 min at 10,000 rpm in an Eppendorf desk centrifuge, and a specific sandwich ELISA, as described earlier, determined concentrations of MRP8 and MRP14 in the fraction of soluble cellular proteins [42]. For calibration, different amounts (0.25–250 ng/ml) of the native complex of human MRP8 and MRP14 were solubilized in dilution buffer and applied to the system. MRP8 and MRP14 form noncovalently associated complexes, which are detected by our ELISA system [35]. We therefore calibrate our ELISA with the native MRP8/MRP14 complex and present our data as amount of MRP8/MRP14 complex per total cellular protein (μg/mg).

Statistical analysis

The U-test, according to Mann and Whitney (for values without normal distribution), was performed to determine significant differences in the number of MRP8- and MRP14-expressing cells in biopsies of GN. Values of $P > 0.05$ were considered not to be significant. Correlations of different disease parameters and MRP8/MRP14 expression are presented as Pearson's correlation coefficients (r).

RESULTS

Patient characteristics

Patients with GN showed marked proteinuria, which was most pronounced in MGN, MCD, and FSGS. Highest creatinine levels were found in patients presenting the histological picture of ECGN and in patients with IN, probably as a result of the long duration of disease at the time of biopsy. All other groups of GN showed only slightly elevated or normal serum levels of creatinine. **Table 1** summarizes the clinical and demographic data of our series. Differences of the mean age in the different GN groups are a result of the typical time of onset of some GN types, and a relatively high portion of juvenile patients were in MCD or FSGS and of young women, in SLE-GN.

Expression of MRP8 and MRP14 in cells infiltrating the glomerulus in different forms of GN

Expression of the calcium-binding proteins MRP8 and MRP14 was analyzed by immunohistochemical staining in 89 biopsies from the seven groups of histologically proven nephritis. Furthermore, serial sections were stained with the mAb 27E10, which detects exclusively an epitope formed by the complex of MRP8 and MRP14 without recognizing single monomers of MRP8 or MRP14 [27]. Data were related to the expression of a marker specific for human macrophages, i.e., CD68. The numbers of glomerular cells positive for MRP8 or MRP14, for the complex of MRP8/MRP14, or for CD68 are summarized in **Figure 1**. In general, forms of GN with high proliferative activity such as SLE-GN and ECGN showed a significantly higher number of MRP8- and MRP14-expressing cells ($P=0.05$) in the glomeruli than the less-aggressive form of GN, i.e., MCD. An intermediate number of cells expressing MRP8 and MRP14 were found in FSGS, IgA-GN, and MGN (**Figs.** 1 and **2**). The formation of the MRP8/MRP14 complex, as shown by 27E10 immunoreactivity, paralleled the expression of the single monomers. Consequently, there is a strong correlation of numbers of MRP8-, MRP14-, and 27E10-positive cells in the glomeruli from different forms of GN (r between 0.75 and 0.98). MRP8 and MRP14 expression could not be found in normal glomeruli, despite the presence of CD68-positive, resident macrophages (Fig. 1). Consequently, the increase of MRP8$^+$/MRP14$^+$ cells in GN was significantly higher in highly proliferative GN compared with the rise of CD68$^+$ cells (e.g., SLE: 38-fold increase for MRP8, 52-fold increase for MRP14, but only sevenfold increase for CD68 compared with controls). In FSGS and MGN, there were no marked increases of CD68$^+$ cells, whereas the percentage of MRP8$^+$ and MRP14$^+$ cells rose significantly ($P<0.05$). Thus, besides the infiltration of macrophages, the expression of MRP8 and MRP14 additionally reflects a qualitative change in the phenotype of renal macrophages during GN. It thus presents a significantly more sensitive parameter for inflammatory processes than merely the number of macrophages.

The question of whether the slightly stronger staining with aMRP14, when compared with aMRP8 and 27E10, would reflect a higher in vivo expression of this molecule or a stronger immunoreactivity of the aMRP14 antibodies cannot be answered by this phenotypical analysis. There are at least three different cellular phenotypes, i.e., MRP8/MRP14$^+$/CD68$^+$, MRP8/MRP14$^+$/CD68$^-$, MRP8/MRP14$^-$/CD68$^+$ in GN, as confirmed by double-labeling (Fig. 2). MRP8/MRP14$^+$/CD68$^+$ macrophages are the most abundant phenotype in those GN, presenting strong inflammatory reactions, e.g., SLE-GN or ECGN. Antibodies against CD14 showed similar staining patterns as those with CD68. There was no staining of lymphocytes by MRP8 or MRP14 or the other macrophage-specific antibodies.

TABLE 1. Clinical and Demographic Data of the Study Population

	MCD	FSGS	SLE-GN	IgA-GN	ECGN	MGN	IN
n	10	13	26	13	6	14	7
Male/female	4/6	7/6	4/22	6/7	5/1	11/3	2/5
Age (mean/years)	11.8	26.9	36.3	38.9	48.5	52.7	44.3
Disease duration (mean/years)	2.1	3.2	2.7	3.1	0.4	0.7	3.1
Serum creatinine (mean ± SD/mg/dl)	0.8 ± 0.1	1.6 ± 1.5	1.1 ± 0.6	1.5 ± 1.0	3.2 ± 1.6	1.1 ± 0.3	5.4 ± 2.9
Urinary protein (mean ± SD/mg/day)	2100 ± 990	6230 ± 5600	1880 ± 1400	2490 ± 1970	2500 ± 700	8990 ± 4580	1350 ± 560
Blood pressure: systolic/diastolic (mean/mmHg)	125/81	133/86	133/89	138/83	132/78	148/86	151/89

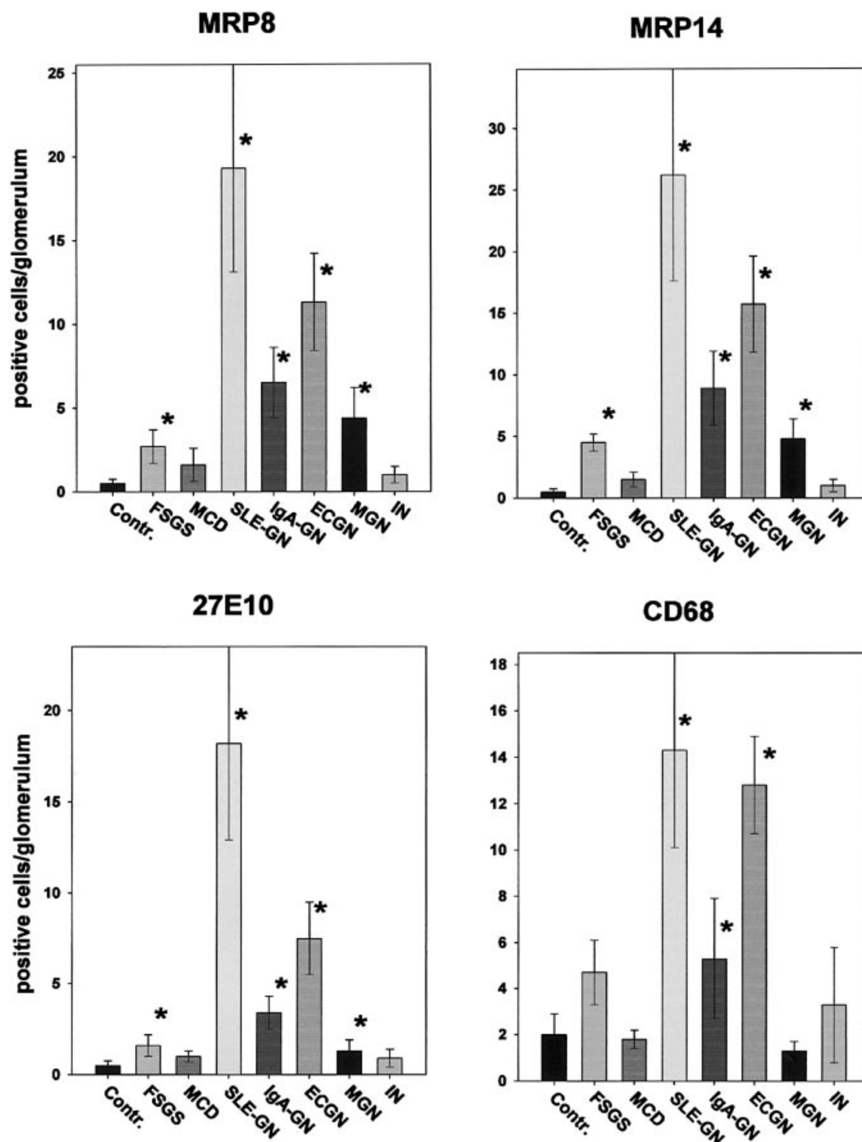

Fig. 1. Expression and complex formation of MRP8 and MRP14 in glomeruli of different GN. Serial sections of different GN were stained with antibodies specific for MRP8, MRP14, for the heterodimer MRP8/MRP14 (mAb 27E10), and for the macrophage-specific surface antigen CD68. Primary antibodies were detected by immunoperoxidase-conjugated second-stage antibodies. Data are presented as mean ± SEM of positively stained cells per glomerulus. *, Significant differences to controls ($P \leq 0.05$). Healthy controls, Contr.

Glomerular MRP8 and MRP14 expression and disease activity

In patients with SLE-GN, IgA-GN, or ECGN, there is a striking negative correlation between the number of MRP8- and MRP14-expressing cells in the glomeruli and the duration of acute flares after which the biopsy was obtained (r =−0.6 for SLE-GN, −0.7 for IgA-GN, and −0.7 for ECGN). Furthermore, there is a negative correlation between the number of MRP8- and MRP14-expressing cells and general duration of disease in IgA-GN (r=−0.8) and ECGN (r=−0.8). The observed relation of MRP8 and MRP14 expression with earlier stages of disease in these distinct subgroups of GN supports the assumption that a high abundance of MRP8 and MRP14 in the infiltrate is an indicator for an active type of inflammation in the glomerulus. This is in accordance with the finding that those GN that are known to present weaker inflammatory reactions in glomeruli, e.g., MCD, also show significantly lower numbers and lower percentages of MRP8- and MRP14-expressing cells. No correlation was observed between MRP8 or MRP14 expression in the glomeruli and long-term parameters of renal functions in the whole patient population, i.e., blood pressure, serum concentrations of creatinine, or daily urinary protein excretion.

MRP8 and MRP14 in infiltrating interstitial cells in different forms of GN

Immunohistochemical data of the interstitial infiltrate are presented as percentage of positively stained cells in the infiltrate. The expression pattern of MRP8 and MRP14 markedly differed between glomeruli and the interstitium (**Fig. 3**). IN, which by definition presented almost no inflammatory infiltrate in glomeruli and only a weak glomerular immunoreactivity against MRP8 and MRP14, showed dense infiltration and high percentage of positive cells for both proteins in the interstitium. In severe forms of GN, such as SLE-GN and ECGN, there was also a significantly raised expression of MRP8 and MRP14 in the interstitium, paralleling the findings in glomeruli, whereas MCD showed normal numbers of MRP8/MRP14-expressing monocytes in the interstitium. It is interesting that in IgA-GN and especially in SLE-GN, there was a lack of MRP8/MRP14 complex formation in infiltrating cells of the interstitium de-

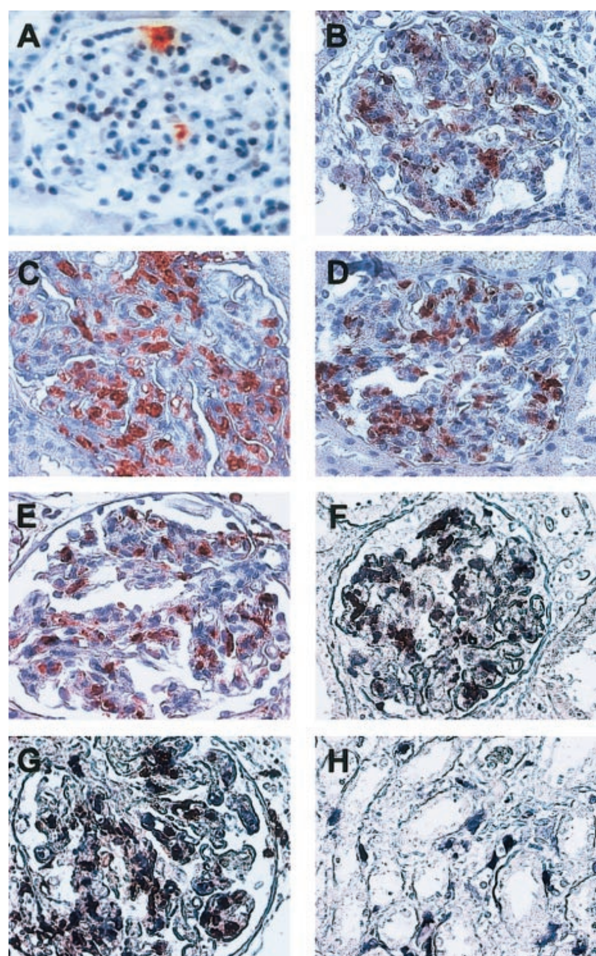

Fig. 2. Immunohistochemical stainings and double-labeling experiments. Serial sections of GN were stained with antibodies specific for MRP8, MRP14, the heterodimer MRP8/MRP14 (mAb 27E10), and CD68 (A–E). Primary antibodies were detected by immunoperoxidase-conjugated second-stage antibodies (red color), and slides were subsequently counterstained with hemalaun. Staining of MCD with mAb 27E10 (A) shows only a few MRP8/MRP8-expressing cells within the glomerulus. SLE-GN of World Health Organization class IV (B–H) shows a high abundance of MRP8 (B), MRP14 (C), and MRP8/MRP14 complex formation (D; mAb 27E10). CD68 shows an identical expression pattern in glomeruli of SLE-GN as MRP8 and MRP14 (E). Double-labeling experiments with aMRP14 (phosphatase staining, blue color) and aCD68 (peroxidase staining, red color) revealed that almost all $CD68^+$ cells within the glomeruli express MRP14 (dark brown color, F). Double-labeling with aMRP14 (phosphatase staining, blue color) and 27E10 (peroxidase staining, red color) showed an almost complete double-labeling of positive cells within the glomeruli of SLE (dark brown color, G), whereas infiltrating cells in the interstitium of the identical section does not form the 27E10 epitope despite staining for MRP14 (H, exclusively blue-color reaction). (F–H) Not counterstained after immunohistochemical double-labeling. Original bars represent 50 μM.

spite expression of the monomers and despite complex formation in the glomeruli (Fig. 2, G and H). Accordingly, r, between expression of single MRP8 and MRP14 monomers and complex formation, were relatively low in the interstitium of SLE-GN and IgA-GN (r between 0.38 and 0.68). IN also revealed lack of complex formation, i.e., lack of 27E10-positive cells, despite a high number of MRP8/MRP14-expressing cells in the interstitium. This suggests that the phenotype of $27E10^-$/$MRP8^+$/$MRP14^+$ cells reflects a more chronic, inflammatory reaction in the interstitium (r=0.40 for monomer expression and complex formation). This assumption is supported by the finding that the expression of MRP8 or MRP14 in the interstitium shows no relation to duration of acute flares in disease activity during GN. However, serum concentrations of creatinine as a parameter of chronic renal failure showed a significant correlation to expression of MRP8 and MRP14 in the interstitial infiltrate of all these forms of GN, which lacked complex formation (IgA-GN: r=0.91/0.86; FSGS: r=0.89/0.75; SLE-GN: r=0.57/0.60 for MRP8/MRP14, respectively). The parallel detection of MRP8, MRP14, and 27E10 reactivity in ECGN (r=0.93/0.97) probably points to a more acute type of inflammatory reaction in the interstitium in this special GN, but the number of cases examined is too small for a general statement.

Immunosuppressive therapeutics have no direct influence on MRP8 and MRP14 expression in monocytes

To exclude a direct influence of immunosuppressive agents on expression patterns, we investigated the effect of several drugs that are typically used for treatment of different forms of GN on the expression of MRP8 and MRP14 by monocytes in vitro. Peripheral blood monocytes cultured for 1 day were exposed to different immunosuppressive drugs for 4 h, and regulation of MRP8 and MRP14 expression was analyzed at mRNA and protein levels. Northern blots were hybridized with specific cDNA probes for MRP8 and MRP14. All bands were compared with the mRNA expression of GAPDH to exclude nonspecific toxic effects of the drugs. At protein levels, a specific sandwich ELISA determined concentrations of MRP8/MRP14 in monocytes, and data were related to the content of total cellular protein. We found that none of the immunosuppressants, dexamethasone (DEX; 10 nM), azathioprine (AZA; 1 μM), mycophenolic acid (MPH; 100 μM), and cyclosporine A (CyA; 10 μg/ml), causes significant changes in expression of MRP8 and MRP14 (**Fig. 4, A** and **B**).

Next, we excluded possible direct effects of different anti-inflammatory drugs on complex formation and surface expression of MRP8 and MRP14, which have been shown to form noncovalently associated complexes that are translocated to the plasma membrane in a calcium-dependent manner [25, 27, 28]. This process can also be induced by activation of mitogen-activated protein kinase p38 in monocytes via incubation with arsenite (own unpublished observation). We therefore incubated monocytes for 4 or 16 h with DEX (10 nM), AZA (1 μM), MPH (100 μM), and CyA (10 μg/ml). We observed no increase in baseline expression of MRP8/MRP14. In addition, none of the mentioned drugs showed an inhibitory effect on the arsenite-induced surface expression (0.1 mM arsenite for 2 h) of the MRP8/MRP14 heterodimer on monocytes (Fig. 4C).

Thus, differences of MRP8 and MRP14 expression as well as of MRP8/MRP14 complex formation observed in distinct GN are a result of the underlying inflammatory process and are not directly modified by immunosuppressive treatment.

DISCUSSION

Cells of the monocyte lineage play an important role in the inflammatory process of various GN. It has to be considered

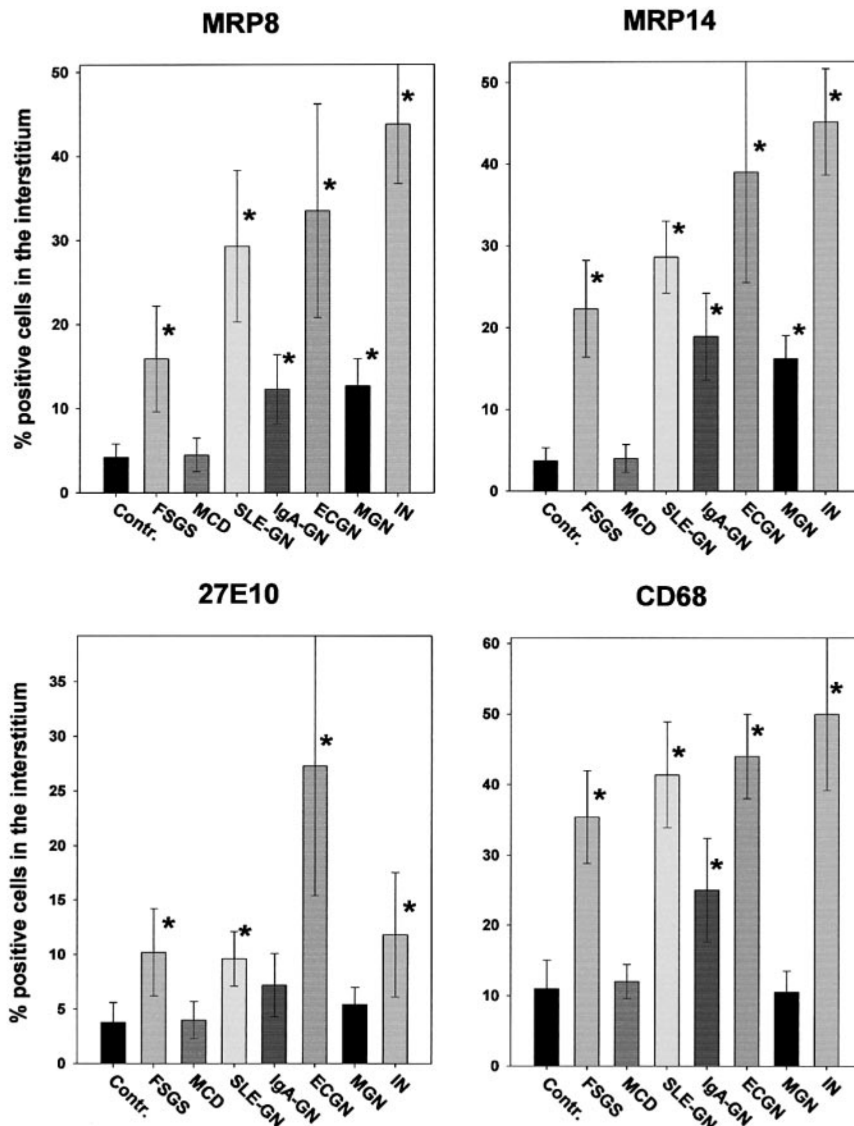

Fig. 3. Expression and complex formation of MRP8 and MRP14 in the interstitium of different GN. Serial sections of different GN were stained as described in Figure 1. Data are presented as percentage (%, mean±SEM) of positively stained cells in the interstitium. *, Significant differences to controls ($P \leq 0.05$).

that macrophages exhibit pro- as well as anti-inflammatory properties depending on their developmental stage and their level of activation [18, 19, 43]. Unfortunately, no reliable marker is currently available to characterize subpopulations of inflammatorily active macrophages or to monitor inflammatory activity of the monocyte/macrophage lineage in GN. However, in the present report, we demonstrate that expression of MRP8 and MRP14 and concomitant formation of the MRP8/MRP14 heterodimeric complex by infiltrating leukocytes within the glomerulus correlate with the activity of the inflammatory process. Accordingly, GN, with an aggressive type of inflammation such as SLE-GN and ECGN, exhibits the highest numbers of MRP8- and MRP14-expressing and complex-forming cells in the glomeruli. The finding that IN shows only limited expression in the glomeruli but a high abundance of both molecules in the interstitial infiltrate reflects the restriction of MRP8 and MRP14 expression to the site of active inflammation. As both proteins have been shown to be released at sites of inflammation [42, 44], determination of serum concentrations of MRP8 and MRP14 may be useful to monitor disease activity of GN.

Parallel expression of MRP8, MRP14, and CD68 represents a proinflammatory macrophage, which is found in highly active GN. The question of whether expression of MRP8 and MRP14 reflects infiltration of blood-derived monocytes or re-expression in resident tissue macrophages, as shown for peritoneal macrophages after lipopolysaccharide stimulation [45], cannot be answered completely by this phenotypical study. However, the high abundance of these molecules in SLE-GN and ECGN presents strong evidence for the recruitment of blood monocytes. The $MRP8^{-}/MRP14^{-}/CD68^{+}$ phenotype is compatible with a mature tissue macrophage, which is present in normal kidneys and in higher numbers in GN with less inflammatory activity such as MCD or FSGS. The phenotype of $MRP8^{+}/MRP14^{+}/CD68^{-}$ cells encompasses at least partially infiltrating granulocytes. The assumption that expression of MRP8 and MRP14 characterizes an active inflammatory macrophage phenotype is supported by the findings that MRP8 and MRP14 have been shown to be involved in calcium-dependent activation of macrophages and that translocation of these proteins to the plasma membrane correlates with release of inflammatory active substances such as TNF or IL-1β and with an increase in respiratory burst [27, 28]. Furthermore, in inflammatory bowel disease, it has been shown that MRP8- and MRP14-expressing cells are also the main sources for respiratory burst

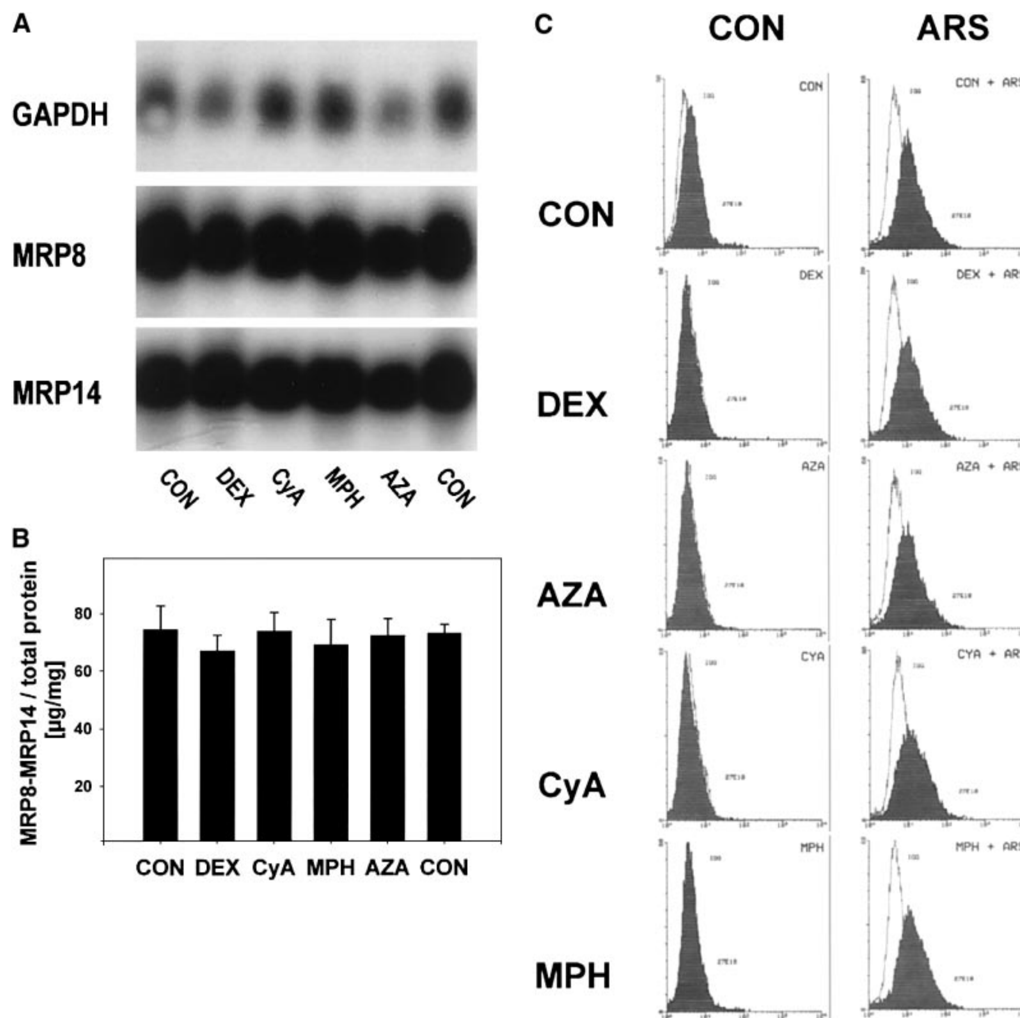

Fig. 4. Expression of MRP8 and MRP14 after treatment of monocytes with different anti-inflammatory drugs. Monocytes were incubated with different anti-inflammatory drugs for 4 h on culture day 1 (CON, medium as control). (A) Expression of MRP8 and MRP14 mRNA in monocytes was investigated by Northern blot analysis. Expression of GAPDH served as control for loading of RNA. (B) MRP8 and MRP14 expression at protein level was analyzed by a specific sandwich ELISA. Data are presented as content of MRP8/MRP14 complex/total cellular protein (µg/mg). (C) Monocytes cultured for 1 day were treated with different anti-inflammatory drugs for 4 h. Surface expression of the MRP8/MRP14 heterodimer on monocytes was detected by flow cytometry. Open graphs show the autofluorescence of cells treated with control IgG of nonrelevant specificity. Surface reactivity of mAb 27E10 is presented by the shaded histograms. None of the anti-inflammatory drugs increases basal expression of MRP8/MRP14 on nonstimulated monocytes (left column of histograms). Any anti-inflammatory agent investigated did not inhibit up-regulation of MRP8/MRP14 surface expression by treatment of monocytes with 0.1 mM arsenite for 2 h (histograms in the right column). Almost identical results were obtained after treatment of the monocytes for 16 h with the anti-inflammatory drugs mentioned above (data not shown).

and for IL-1β and TNF release in vivo [46]. Our in vitro data point against a direct influence of different anti-inflammatory drugs on de novo synthesis of MRP8 and MRP14 or on formation of their complexes. Thus, expression patterns reported here for different kinds of GN reflect differences in the underlying inflammatory processes rather than differences induced by anti-inflammatory drugs at a single-cell level.

Our findings are in accordance with the expression pattern of MRP8 and MRP14 in renal allograft rejection. During acute allograft rejection, the majority of infiltrating cells express MRP8 and MRP14, and both proteins have been shown to be a sensitive marker for the inflammatory activity of the rejection process [37]. In a previous paper, we have shown that absence of the MRP8/MRP14 complex formation, despite expression of both subunits in infiltrating macrophages, is in contrast to acute renal allograft rejections, characteristic for chronic rejections [36]. We now present evidence that there are similar differences in the complex pattern of MRP8 and MRP14 in distinct, inflammatory renal diseases. Macrophages in the interstitial infiltrate of IN show a phenotype identical to that found in chronic allograft rejections. It is interesting that this phenotype is also present in biopsies of patients with SLE-GN, IgA-GN, and FSGS, supporting the assumption that a chronic inflammatory process of the renal interstitium is an important pathogenetic factor in some forms of GN and that tubulointerstitial changes are major determinants in the progression of chronic renal damage [47, 48]. Thus, expression of MRP8 and MRP14 with simultaneous absence of their complex in the interstitial infiltrate of GN indicates an inflammatory pathomechanism distinct from the glomerular inflammation, which may also need specific anti-inflammatory therapies. Therefore, analysis of expression and complex formation of MRP8 and

MRP14 provide further information, in addition to the number of infiltrating macrophages, about the quality and activity of the distinct inflammatory processes involved in GN.

Besides this potential diagnostic tool, our data also point to novel aspects about the pathogenesis of GN. Secretion of MRP8 and MRP14 in vivo is induced by contact of monocytes with inflammatory, activated endothelium [42, 44]. A recent report demonstrated that extracellular MRP14 enhances CD11b/CD18 integrin-binding activity on phagocytes [49]. High expression of MRP8 and MRP14 during GN may thus point to a positive-feedback mechanism propagating the inflammatory reaction. In this model, contact of monocytes to activated endothelium induced secretion of MRP8 and MRP14, which leads to a higher affinity of integrin-adhesion molecules, thus inducing a switch from selectin-mediated rolling to a tight contact of leukocytes to the endothelium. Subsequently, MRP8/MRP14 mediates binding of leukocytes to specifically carboxylated glycans on endothelial cells and thus promotes leukocyte trafficking and extravasation [50]. Analyzing the molecular mechanisms of release and extracellular functions of MRP8 and MRP14 may thus offer molecular targets for novel, immunosuppressive strategies to modulate the important inflammatory response mechanisms of monocytes in GN.

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