The design of a liver-selective form of interleukin-10

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Chapter - 4

A study on the effects of IL-10 in anti-Thy 1-induced glomerulonephritis in rats

submitted

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Abstract

IL-10 is a well-tolerated anti-inflammatory cytokine and very effective in suppressing inflammatory processes in response to various stimuli. The rapid renal clearance of IL-10, however, prompted the question whether IL-10 might have a prolonged pharmacological effect. In the present study, we examined the effects of IL-10 after 24 h in a model of acute glomerulonephritis. One hour after the anti-Thy 1 antibody administration, a single iv dose of IL-10 was administered to rats. Normal rats, control nephritic rats, and nephritic rats treated with IL-10, were sacrificed 24 h after administration of the antibody. Samples of urine, blood and organs were subsequently collected. The effects of IL-10 were studied by quantification of various inflammatory parameters at the protein level after immunohistochemical stainings and at the mRNA level by a quantitative real-time PCR technique. Nitric oxide and protein content were determined in serum and in 24 h-excreted urine, respectively. The inflammatory parameters were reduced in the IL-10-treated group: the increment in glomerular CD14, ICAM-1 and MMP-13 staining induced by anti-Thy 1 injection was significantly attenuated by IL-10. In contrast, mRNA levels for CD14, IL-1β, TNF-α and MCP-1 were not different between IL-10-treated and control GN groups. In conclusion, a single iv dose of IL-10 suppresses the expression of several inflammatory parameters, 24 h after inducting of acute GN but at this time point mRNA levels of all parameters examined were not affected. Although its therapeutic efficacy needs further evaluation, anti-inflammatory effects of this short-lived cytokine can be found one day after its administration.

Introduction

Glomerulonephritis (GN) is the term applied to a group of diseases characterized by inflammatory changes in glomerular capillaries.[1,2] This disease is accompanied with signs and symptoms of an acute nephritis syndrom; particularly haematuria, proteinuria, and diminished renal function.[2] The causative agents in most forms of human GN are unknown yet. Several evidences indicate that an infectious challenge induces GN by triggering an autoimmune response that results in the formation of immune-complex deposits in glomeruli or elicits a cell-mediated immune response to antigens.[3-5] In response to this process, macrophages and neutrophils infiltrate into the glomeruli and play a pivotal role in mediating subsequent glomerular damage. Infiltrated macrophages and neutrophils in inflamed glomeruli are capable to produce a wide range of potentially cytotoxic products such as NO species, proinflammatory cytokines, chemokines, and proteolytic enzymes including matrix metalloproteinases (MMPs).[6-9]

Glomerulonephritis is the leading cause of end-stage renal failure.[1,2,12] Because the development of acute glomerulonephritis is initiated by intraglomerular macrophage and neutrophil infiltration, depletion or blockage of this process or blocking endothelial/leucocyte adhesion interactions potentially represent targets for therapy. Some current therapeutic agents for glomerulonephritis such as corticosteroids, cyclophosphamide or cyclosporine A however are not satisfactory because long-term exposure to these drugs induces significant side effects.[10-12] Therefore, a specific and well-tolerated intervention is urgently required to optimize the therapy for this disease.

IL-10 is a well tolerated cytokine that has a wide range of effects in controlling inflammatory responses.[13-15] It is very effective in suppressing macrophage functions and in inhibiting macrophage and neutrophil mediated injury in various experimental models of immune response including in the glomerulonephritis.[16,18-19] It has been reported that short-term multiple doses of IL-10 prevented glomerular injury through inhibition of proinflammatory cytokine production[16,19], mesangium cell proliferation[19], and macrophage recruitment.[16,18-19] In *in vitro* studies, IL-10 exhibited anti-inflammatory effects for several hours.[21-22] We previously observed that IL-10 inhibited LPS-stimulated

TNF-α release in a macrophage cell line in a dose-dependent manner. In addition, we showed that IL-10 was rapidly and predominantly distributed to the kidney after a single iv dose.[23] We also found that the IL-10 receptor is constitutively expressed in the glomeruli which could mediate the pharmacological actions of exogenous IL-10.[23] Taken together, we anticipated that IL-10 may have beneficial effects within the kidney. However, the short half-life in plasma of only 2 minutes elicited the question whether IL-10 might have effects 24 hour after its administration. Thus, the primary aim of this study is to determine effects of a single iv dose of IL-10 in the anti-Thy 1-induced glomerulonephritis model in rats.

Materials and Methods

Animals

Specific pathogen-free male Wistar rats (200 – 250 g), purchased from Harlan (outbred strain, Zeist, The Netherlands), were used in this study. The rats received a standard diet and were housed under standard laboratory conditions. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals and was performed according to strict governmental and international guidelines on animal experimentation.

Production, purification and affinity test of monoclonal anti-Thy 1 antibody

Production of anti-Thy 1 antibody was performed according to Bagchus WM, *et al.*[24] Briefly, monoclonal antibody (MAb) against Thy 1 (ER4) was produced *in vitro* by mouse hybridoma cells. IgG was collected from culture media and subsequently purified using HiTrap™ protein G column (Amersham Pharmacia Biotech AB, Upsala, Sweden). The specificity of this antibody was confirmed by immunohistochemical staining of mesangial cells in healthy rat kidneys.

Induction of glomerulonephritis in rats

To induce acute glomerulonephritis in rats, animals received a single iv dose of anti-Thy 1 IgG (5 mg/kg) via the penile vein, under anaesthesia with 40% O_2:60% N_2O combined with 0.5% isoflurane.

Experimental design

Six rats per group were used in this study. To examine the acute effects of IL-10, a single iv dose (8 μg/kg) of this cytokine (Peprotech, UK) with a specific activity of about 5 x 10^5 unit/mg, was administered to the rats one hour after anti-Thy 1 injection. Control rats received only vehicle (PBS). Subsequently, rats were housed individually in metabolic cages to collect the urine during the experiment. Twenty four hours after anti-Thy 1 administration, rats were sacrificed and samples of excreted urine, serum and kidneys were collected.

Immunohistochemical analysis

Since the monoclonal anti-Thy 1 antibody was detectable by a RAMPO/AEC staining in all glomeruli, 24 h after its administration, all primary antibodies used in this study for immunohistochemical analysis were polyclonal antibodies.

Detection of macrophage and neutrophil infiltrating the glomeruli by CD14 staining

Macrophage and neutrophil infiltration into glomeruli was determined by glomerular CD14 expression. CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein found on the surface of neutrophils and macrophages and can serve as a marker for these cell types.[25] The glomerular expression of CD14 was performed with a standard indirect immunostaining method, with amplification. Briefly, cryosections (5 μm) of kidney tissue were fixed in aceton. A goat polyclonal anti-CD14 (1:40, Santacruz Biotechnology, CA, USA) was applied as a primary antibody. A horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-goat (RAGPO 1:50, DAKO, Glostrup, Denmark) was used as a secondary antibody. To amplify the signal, a horseradish peroxidase (HRP)-conjugated goat polyclonal anti-rabbit (GARPO 1:50, DAKO) was subsequently added. The reaction was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma, St.Louis, USA). Semiquantitative evaluation of the staining for CD14 was performed by scoring of the average of the staining in 40 glomeruli per kidney section per rat using the following score: 0 (no staining within glomeruli), 1 (1 - 25%), 2 (26 – 50%) and 3 (> 50%) of total area/glomerulus.

Determination of glomerular ICAM-1 and MMP-13

The expression of glomerular ICAM-1 and MMP-13 was also determined on cryosections of kidney tissue with standard immunostaining methods with amplification, as described for the CD14 staining. Antibodies used to detect these proteins were respectively goat polyclonal anti-ICAM-1 (1:40, Santacruz Biotechnology, CA, USA) and anti-MMP-13 (1:40, Santacruz Biotechnology, CA, USA). Semiquantitative evaluation of the staining was performed in 40 glomeruli per kidney section per rat with the same score procedure as used for CD14.

Determination of nitric oxide (NO_x) in serum

Nitric oxide was determined in serum samples with a standard Griess reaction method.[26] The calculation of NO_x levels was corrected to endogenous nitrite in the serum which was determined in each sample in a parallel reaction in which the enzymatic nitrate reduction step was omitted.

Determination of urinary protein excretion

Total protein was determined from 24 h-excreted urine samples using a standard Bradford method according to manufacturer's instruction (Biorad kit protein assay, Biorad, Hercules, CA, USA).

Determination of the mRNA levels for TNF-α, IL-1β, MCP-1 and CD14

mRNA isolation from renal cortex

Total RNA was extracted from tissue samples of the renal cortex with a mini column system according to the manufacturer's instruction (RNeasy® mini kit, Qiagen Sciencer, Maryland USA). RNA concentrations in all samples were determined with NanoDrop® (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology,USA). Integrity of mRNA samples was checked by electrophoresis on a 2% agarose gel and the absence of DNA from the samples was verified by performing a PCR on the mRNA samples using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while omitting the reverse-transcriptase step. cDNA was prepared from 1 μg of total RNA in 25 μL with RT-PCR method according to the manufacturer's method (Promega Inc., USA).

Quantitative real-time RT-PCR (QRT-PCR) for TNF-α, IL-1β, MCP-1 and CD14 genes

Quantitative real-time RT-PCR was performed with a high-throughput real-time PCR system (ABI 7900HT sequence Detection System, Applied Biosystems, CA, USA).

The PCR mixture (20μL) contained 1.25 μL cDNA, primers (1 μM concentration of each primer, table I), and 2x SyberGreen master mix (Applied Biosystem). An initial denaturing step at 95°C for 10 minutes was followed by 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C (a measuring step) for 40 seconds. Each measurement was performed in three replicates. A single product was confirmed by checking the dissociation curve at the end of the PCR reaction. Data were analysed with the SDS software 2.1 (Applied Biosystems). The gene expressions were normalized to the signal of the house keeping gene β-actin.

Table I. Rat oligonucleotide primers used for the analysis of genes by quantitative real- time RT-PCR

Gene	Nucleotide sequences
IL-1β	Upstream: 5′-AGGCAGTGTCACTCATTGTG-3′
	Downstream: 5′-GGAGAGCTTTCAGCTCACAT-3′
TNF-α	Upstream: 5′-ATGTGGAACTGGCAGAGGAG-3′
	Downstream: 5′-GGCCATGGAACTGATGAGAG-3′
CD14	Upstream: 5′-CTTGTTGCTGTTGCCTTTGA-3′
	Downstream: 5′-CGTGTCCACACGCTTTAGAA-3′
MCP-1	Upstream: 5′-TTCCTTATTGGGGTCAGCAC-3′
	Downstream: 5′-TCCTCCACCACTATGCAGGT-3′
β-actin	Upstream: 5′-GGCATCCTGACCCTGAAGTA-3′
	Downstream: 5′-GGGGTGTTGAAGGTCTCAAA-3′
GAPDH	Upstream: 5′-CCATCACCATCTTCCAGGAG-3′
	Downstream: 5′-CCTGCTTCACCACCTTCTTG-3′

Statistical analysis

Data were presented as mean ± SD. All data were subjected to an unpaired, two-tailed distribution student t-test. Differences were considered significant at $p < 0.05$.

Results

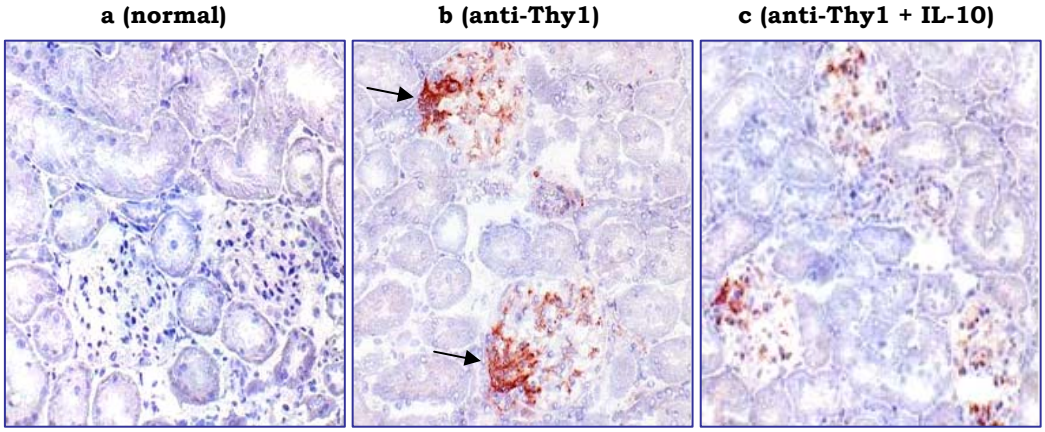
Effect of IL-10 treatment on the inflammatory responses in anti-Thy 1-induced GN

We examined the consequence of IL-10 treatment on inflammatory responses such as infiltration of inflammatory cells, expressions of adhesion molecules and matrix metalloproteinases, NO_x levels, genes expression of TNF-α, IL-1β, CD14 and MCP-1, and on glomerular integrity reflected by proteinuria.

Effect of IL-10 treatment on the intraglomerular infiltration of inflammatory cells

Detection of macrophage and neutrophil infiltration into glomeruli was performed by CD14 staining (fig.1). As shown in figure 1A, CD14 was not expressed by resident glomerular cells. Twenty four hours after anti-Thy 1 administration, this protein was markedly upregulated in the glomeruli. CD14 expression in inflamed glomeruli was distributed throughout the glomeruli and occasionally expressed in the vascular pole. Staining was also detected to a lesser extent in the interstitium of kidneys. The expression of anti-Thy 1-induced CD14 was clearly reduced in IL-10-treated nephritic group (fig.1.c). To quantify the effectivity of IL-10 on the reduction of this parameter, we used a score index as described in materials and methods section. According to this system, the effect of IL-10 on the glomerular CD14 expression, presented in figure 1B, revealed a reduction in score index from 2.5 ± 0.4 to 1.4 ± 0.2 ($p<0.05$).

A.



B.

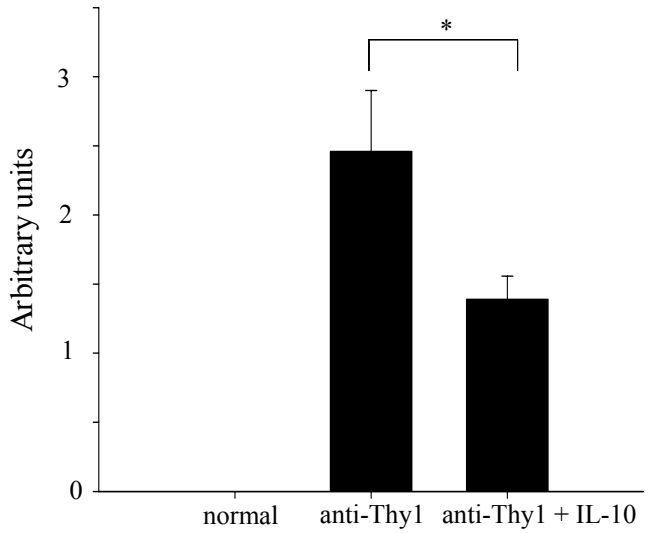
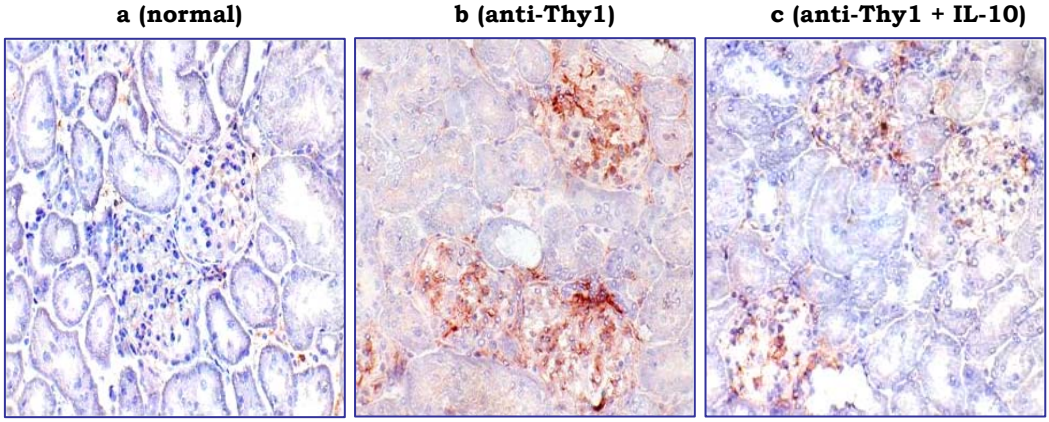


Fig.1.The effect of IL-10 on the intraglomerular macrophage and neutrophil recruitment. (**A**) Infiltrated macrophages and neutrophils were detected by CD14 staining. CD14 is not constitutively expressed in the glomeruli (a). This expression was markedly induced after anti-Thy 1 administration (b), in particular around the vascular pole (arrow). Treatment with IL-10 significantly reduced the staining (c). (**B**) Using a semiquantitative scoring system, 40 glomeruli per rat and 6 rats per group were analyzed according to the following score: 0 (no staining within glomeruli), 1 (1 - 25%), 2 (26 – 50%) and 3 (> 50%) of total area/glomerulus. Original magnification: 200x. *p < 0.05.

Effect of IL-10 on the protein expression of glomerular ICAM-1 and MMP-13

The immunostaining for glomerular ICAM-1 is shown in fig.2A. As can be seen, ICAM-1 was constitutively expressed in renal vascular endothelium and occasionally in the glomeruli at a very low level. A strong induction of the expression of glomerular, tubular and renal interstitial ICAM-1 was already detected 24 h after administration of anti-Thy 1 which is also reported by others.[27-29] The expression of ICAM-1 in particular in the mesangial area and in renal tubuli was clearly inhibited by IL-10 treatment. Semiquantitative evaluation of the effect of IL-10 on this expression, presented in figure 2B, revealed a reduction in score index from 1.9 ± 0.3 to 1.3 ± 0.2 ($p<0.05$).

A.



B.

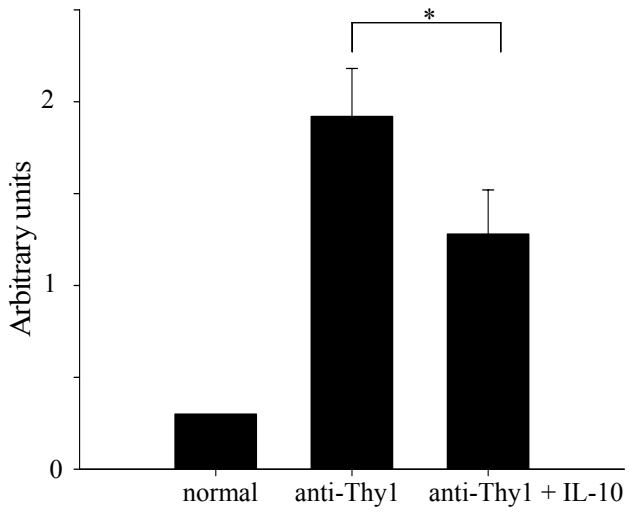
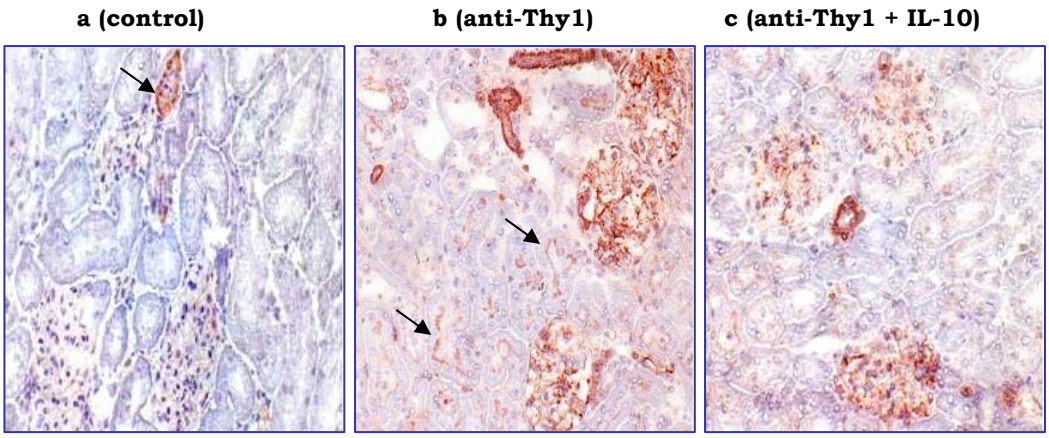


Fig.2. The effect of IL-10 on glomerular ICAM-1 staining. (**A**) Immunohistochemical staining of ICAM-1 in kidneys. ICAM-1 was expressed in the glomeruli at a very low level (a). This expression was markedly induced after anti-Thy 1 administration (b). Treatment with IL-10 significantly reduced the staining (c). (**B**) Using a semiquantitative scoring system, 40 glomeruli per rat and 6 rats per group were analyzed according to the following score: 0 (no staining within glomeruli), 1 (1 - 25%), 2 (26 – 50%) and 3 (> 50%) of total area/glomerulus. Original magnification: x200.
*$p < 0.05$.

A.



B.

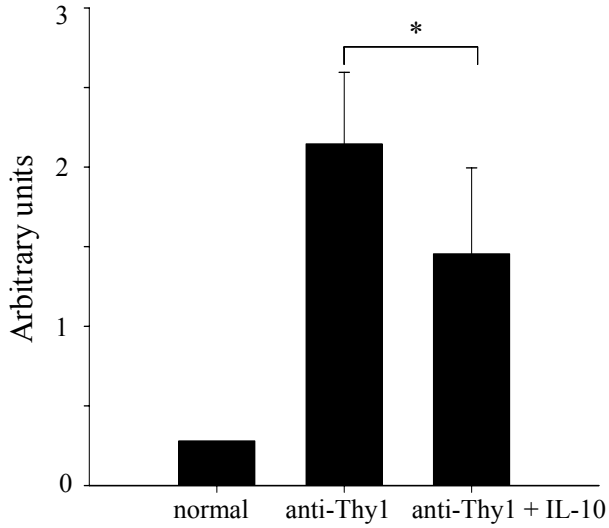


Fig.3. The effect of IL-10 on the glomerular MMP-13. (**A**) Immunohistochemical staining of MMP-13 in kidneys. MMP-13 was expressed in the glomeruli at a very low level (a) and strongly around renal blood vessels (arrow). This expression was markedly induced after anti-Thy 1 administration (b), and expressed in the vascular loops of glomeruli and in the tubuli (arrow). Treatment with IL-10 significantly reduced the staining (c). (**B**) Using a semiquantitative scoring system, 40 glomeruli per rat and 6 rats per group were analyzed according to the following score: 0 (no staining within glomeruli), 1 (1 - 25%), 2 (26 – 50%) and 3 (> 50%) of total area/glomerulus. The original magnification: x200. *p < 0.05.

In addition to the glomerular ICAM-1 staining, glomerular MMP-13 expression was determined. As shown in figure 3A, in normal rats, MMP-13 was highly expressed around renal blood vessels, and occasionally at a very low level in the glomeruli whereas the tubuli were negative. The glomerular and tubular

MMP-13 staining was however strongly enhanced 24 h after administration of anti-Thy 1 antibody. Expression of this protease was found around capillary loops of glomeruli, brush borders of distal and proximal tubuli and around renal blood vessels. Similar to the glomerular ICAM-1 staining, a reduced glomerular and tubular MMP-13 staining was seen in the IL-10-treated nephritic group. Semiquantitative evaluation of kidney sections revealed that IL-10 reduced the score index of this parameter from 2.1 ± 0.5 to 1.4 ± 0.5 ($p<0.05$, fig.3B).

Effect of IL-10 on serum levels of NO_x

NO_x is one of products of activated macrophages in response to various stimuli and is subsequently released into the circulation.

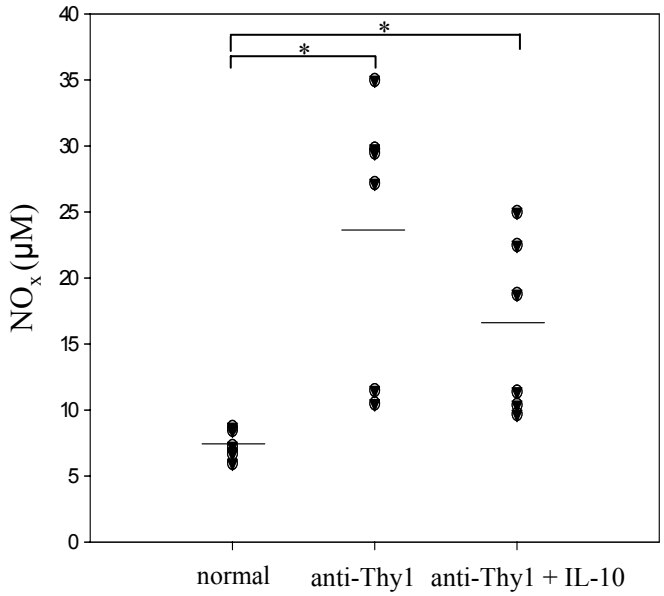


Fig.4. The serum NO_x levels after disease induction with anti-Thy 1 IgG. The serum NO_x levels significantly increased in nephritic rats as compared to normal rats. *p <0.05.

As shown in fig.4, the average serum NO_x level in normal rats was 7.3 ± 1.3 µM, whereas 24 h after anti-Thy 1 injection, serum NO_x levels rose to 23.9 ± 10.3 µM. In IL-10-treated nephritic rats, serum NO_x levels felt to 16.3 ± 6.7 µM. This difference was not significant compared to untreated nephritic rats.

The effect of IL-10 on the urinary protein excretion

Administration of anti-Thy 1 antibody to the rats resulted in a rapid onset of proteinuria within 24 h (113 ± 51.0 mg/24 h) compared to normal rats (21.0 ± 9.2 mg/24 h). Urinary protein excretion in IL-10-treated nephritic rats was 76.6 ± 59.4 (fig.5). This difference was not statistically significant as compared to untreated nephritic rats and also not significantly different from normal rats.

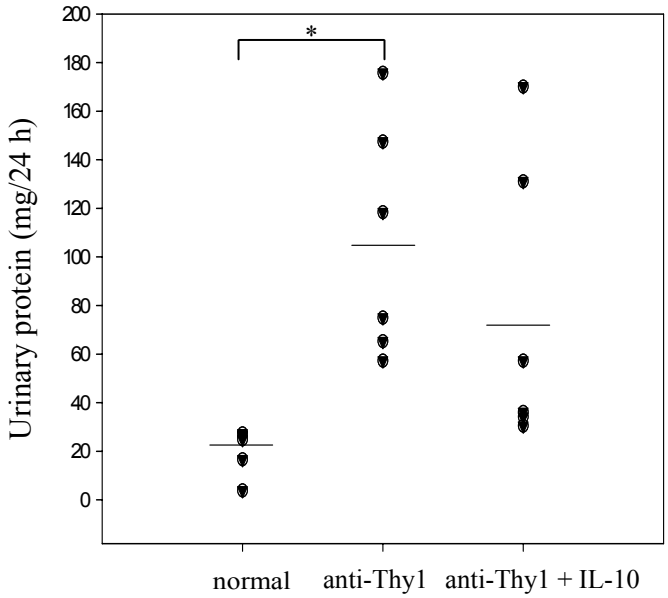


Fig.5. The urinary protein measurement, showing the development of proteinuria (mg/24 h) in anti-Thy 1 induced nephritic rats. *p <0.05.

Effect of IL-10 treatment on the genes expression of TNF-α, IL-1β, MCP-1, and CD14

We examined the mRNA levels for inflammatory parameters using quantitative real-time PCR techniques. mRNA levels for TNF-α and IL-1β were measured to assess proinflammatory activity, mRNA levels for CD14 were used as a marker for neutrophil and macrophage influx and MCP-1 mRNA levels were measured to assess chemotactic activity. β-actin expression was used as the house keeping gene. As shown in figure 6, all genes examined were significantly upregulated as compared to normal rats, in particular MCP-1 was substantially

increased. However, in IL-10-treated nephritic rats, expression of all these genes did not significantly change in comparison with the control nephritic group.

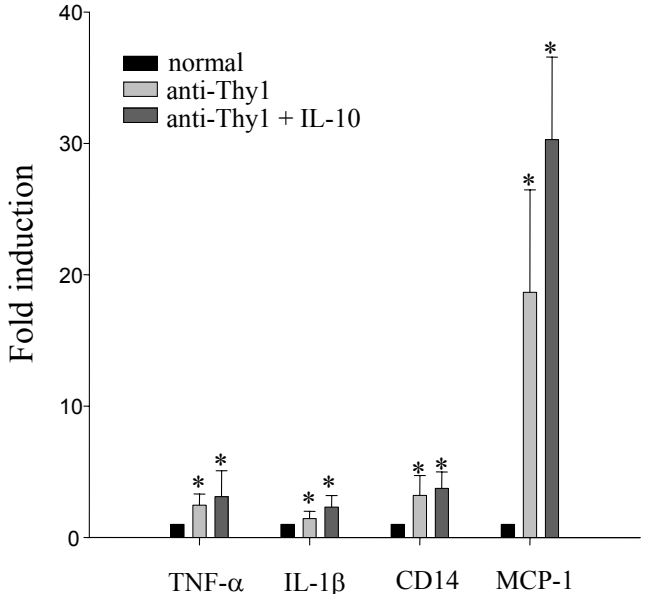


Fig.6. A quantitative real-time PCR of TNF-α, IL-1β, CD14, and MCP-1. A single shot injection of anti-Thy 1 IgG increased the mRNA levels for TNF-α, IL-1β, CD14, and markedly increased MCP-1 gene expression level. The expression of these genes was not different between control GN and IL-10-treated groups. *p <0.05 as compared to normal.

Discussion

IL-10 is an anti-inflammatory cytokine which suppresses the T helper 1 immune response and downregulates macrophage and monocyte activities. Because of its potent anti-inflammatory properties, IL-10 is now being tested in the therapy of inflammatory diseases like inflammatory bowel disease, rheumatoid arthritis and psoriasis.[30] In addition, several studies reported that IL-10 effectively prevented the development of glomerular damage both in acute and chronic nephritis models.[15,17-21] In a previous study, we found rapid accumulation of IL-10 in the kidney (30% of the injected dose within 10 min) and intraglomerular IL-10 receptor expression.[23] We therefore considered the possibility that IL-10 might be applicable for the treatment of in particular kidney diseases. However, the very short plasma half-life of approximately 2 minutes[23]

would not favour an IL-10-based therapy for chronic diseases. This rapid clearance of IL-10 from the plasma, prompted the question whether IL-10 might have a pharmacological effects after 24 h. For that reason we examined the effect of a single dose of IL-10 after 24 h in a model of acute GN.

Anti-Thy 1 nephritis is caused by the binding of Thy 1 IgG to the mesangial cell surface, which results in complement-dependent glomerular damage. Within hours, a destruction of mesangial cells occurs which is accompanied by acute inflammatory reactions.[3-7] As we presented in this study, mRNA levels for IL-1β, TNF-α, CD14 and MCP-1 in the kidney clearly increase in response to this anti-Thy 1 deposition. mRNA levels for IL-1β, TNF-α and CD14 in nephritic rats were moderately increased at 24 h, while mRNA level for MCP-1 was very strongly upregulated at this time point (fig.6). The increase of these proinflammatory factors was accompanied by a marked intraglomerular recruitment of macrophages and neutrophils, as reflected by the CD14 staining (fig.1). We stained CD14, one of macrophage and neutrophil markers, to identify the glomerular infiltration of these cells. Because, especially after 24 h of the disease induction, MAb anti-Thy 1 antibody was still clearly present, utilization of all monoclonal antibodies to identify the cells was excluded.

In this report, we demonstrate that a single iv injection of IL-10 significantly attenuated CD14 and ICAM-1 expression, macrophage and neutrophil recruitment, and MMP-13 expression. Proteinuria was reduced to a level intermediate between diseased and normal rats (not significantly changed compared to either group). So, the effect of IL-10 on proteinuria is yet inconclusive. In contrast to all this, we did not find any difference on the gene expression of CD14, IL-1β, TNF-α and MCP-1 between IL-10-treated and control GN groups. In particular for CD14, IL-10 attenuated this parameter at the protein levels but not at the mRNA level. We therefore conclude that the effect of a single injection of this short-lived cytokine is still notable after 24 h on many inflammatory parameters but at this time point the effect on the examined gene expression levels is not longer present.

In addition to the inhibition of glomerular ICAM-1 and CD14, a reduced glomerular MMP-13 expression was also noted. MMP-13 is one of matrix metalloproteinases (MMPs) with high collagenolytic activity.[31,32] The expression of this matrix degrading enzyme is associated with inflammatory activity and tissue

destruction. It was also found that MMP-2, MMP-3 and MMP-9 were induced in both patients with glomerulonephritis and in rat models of experimental glomerulonephritis, indicating a role for these enzymes in this disease. Accumulating evidence indicates that their expression is regulated by proinflammatory cytokines such as IL-1β, TNF-α, TGF-β and also proinflammatory mediators like nitric oxide.[33,35] Moreover, it has been reported that expression of the collagenase MMP-13, correlated with arthritis in patients and in experimental models.[36,37] To our knowledge, the induction of glomerular MMP-13 in rats with acute glomerulonephritis, reported here, is studied for the first time. MMP-13 is expressed around blood vessels in normal kidneys and in the glomeruli at very low level. Its high expression in anti-Thy 1 nephritic rats indicates a role in the pathogenesis of this disease. The mechanism underlying its upregulation in this model remains unclear, but previous studies showed that persistent accumulation of macrophages in the mesangium area resulted in glomerulosclerosis through expression and activation of MMPs[38], suggesting that infiltrated macrophages participate in this event. This is supported here by the glomerular CD14 expression.

In view of the effects of IL-10 on macrophage recruitment, we determined serum NO_x level as one of the products of activated macrophages. NO plays an important regulatory role in a variety of inflammatory conditions.[39] The generation of NO_x in serum was observed after anti-Thy 1 administration, reflecting the development of disease. However, administration of IL-10 did not result in a significant reduction of NO_x levels. Reduced macrophage recruitment by IL-10 seemed not to be associated with an effect of treatment on the NO_x levels.

In summary, this study demonstrates that despite its short plasma half-life of only 2 minutes, a single iv dose of IL-10 can suppress inflammatory processes during acute glomerulonephritis induced by anti-Thy 1 after 24 h. A possible anti-inflammatory mechanism underlying this acute effect of IL-10 may occur via inhibition of glomerular ICAM-1 expression, resulting in reduced macrophage recruitment reflected by reduced CD14 staining. The reduced MMP-13 after IL-10 treatment indicates that IL-10 may be beneficial to preserve glomerular integrity. This decrease of inflammatory parameters at the protein levels and the glomeruloprotective effect induced by IL-10 was however not associated with an effect of treatment on the mRNA levels for the parameters

examined. The effects of IL-10 demonstrated here call for further evaluation, but may indicate a role for this cytokine as a therapeutic compound during glomerulonephritis. In this framework, various dosage regiments should be investigated at different stages of the disease also using additional animal models. The anti-inflammatory effects of this short-lived cytokine found one day after its administration provide good perspectives for chronic applications.

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