Single Application of Low-Dose Mycophenolate Mofetil-OX7-Immunoliposomes Ameliorates Experimental Mesangial Proliferative Glomerulonephritis


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ABSTRACT
IgA nephropathy, one of the most frequent forms of glomerulonephritis, characterized by mesangial hypercellularity and glomerular extracellular matrix (ECM) expansion, often leads to end-stage renal disease over a prolonged period. We investigated whether antiproliferative treatment in a single low dose specifically targeted to the glomerular mesangium by immunoliposomes (ILs) results in an amelioration of mesangial proliferative glomerulonephritis in rats (anti-Thy1.1 nephritis). Mycophenolate mofetil (MMF) containing ILs was generated that targets the Thy1.1 antigen (OX-7) in rat mesangial cells. Treatment benefit of a single intravenous dose of these ILs given 2 days after disease induction was investigated by stereology, immunohistochemistry, and functional analyses (creatinine, albuminuria) until day +9 and was compared among untreated and free MMF-treated rats using six male Wistar rats per group. MMF-loaded OX7-IL prevented creatinine increase and albuminuria. Stereological analyses of MMF OX7-IL-treated animals yielded 30% reduction of mesangial cells on day +9 and a 40% reduction of glomerular ECM volume on day +5, compared with all of the other nephritic animals. Furthermore, at days +5 and +9 we observed decreased ECM content and decreased glomerular volume (day +5) in the MMF-OX7-IL-treated group compared with the nephritic group treated with free MMF. In conclusion, MMF-OX7-IL-based directed drug delivery represents a novel approach for treating mesangial cell-mediated forms of glomerulonephritis.

Introduction
IgA nephropathy (IgAN), also known as Berger’s disease, is the most frequent form of primary glomerular disease in the Western world and significantly contributes to end-stage renal disease (Dillon, 2001). Up to now, no consistently effective treatment for IgAN has existed. Kidneys with IgAN are characterized by accumulation and expansion of extracellular matrix (ECM) and by mesangial hypercellularity. Typically, diffuse deposits of mainly IgA, but also to a lesser extent IgG and C3, are found in the glomerular mesangium. Current treatments include glucocorticosteroids, cyclophosphamide, azathioprine, ω-3 fatty acids, anticoagulants, and antithrombotic agents that are often accompanied by severe toxicity (Pozzi et al., 2004). Systemic application of mycophenolate mofetil (MMF) failed to show consistent benefits with respect to amelioration of kidney function and reduction of proteinuria (Maes et al., 2004). However, an-
other investigation showed a marked long-term effect of MMF with respect to the preservation of kidney function in Asian patients (Tang et al., 2010).

Therefore, novel therapeutic approaches with more efficacy and potentially fewer side effects are mandatory. In this respect MMF still remains a candidate drug because of its potent antiproliferative effect on mesangial cells (MCs), which has been demonstrated in experimental mesangial proliferative glomerulonephritis (Ziswiler et al., 1998) in vivo and in human MC cultures in vitro (Dubus et al., 2002). Because a therapeutic effect in humans may well depend on a sufficiently high intracellular concentration of MMF in MCs a targeted drug delivery system might be attempted. A drug delivery system, such as immunoliposomes (ILs), may represent such an approach, because ILs with covalently linked antibody (fragments) bind to specific antigens on designated cells and allow the delivery of drugs to these targeted cells.

In the past, IL technology has been preferentially applied in antitumor therapy (Goren et al., 2000); however, it was also used to target nonmalignant tissues, such as brain (Huwiler et al., 1996), lung (Maruyama et al., 1999), or cells of the immune system (Dufresne et al., 1999).

Because mesangial hypercellularity is an important feature of IgAN, MCs represent promising targets for specific directed drug delivery via IL (Tuffin et al., 2005). In this respect, the Thy1.1 antigen, a cell surface glycoprotein belonging to the Ig-like supergene family, is highly specific for rat glomerular MC (Paul et al., 1984). Using the presence of the Thy1.1 antigen, we targeted rat MCs using OX7-IL-containing doxorubicin. A visible pharmacological effect in the mesangium without noticeable toxicity in other organs was observed (Tuffin et al., 2005).

The principal aim of our study was to treat experimental mesangial proliferative glomerulonephritis in a more specific and effective manner by the application of MMF delivered in OX7-ILs and compare these data with results obtained from conventional systemic administration of free MMF.

First, the time course of pathological alterations in anti-Thy1.1 nephritis was characterized. Then the therapeutic potential of MMF-containing OX7-ILs was assessed to ameliorate anti-Thy1.1 nephritis-associated pathology. To perform statistical analysis of between-group differences, structural alterations were quantified by design-based stereology (Nyenggaard, 1999).

Materials and Methods

Experimental Animals

Male Wistar rats (150–250 g body weight) were purchased from Charles River (Sulzberg, Germany) and housed in individual metabolic cages according to institutional guidelines. On a daily basis, behavior, body weight, eating attitude, mobility, and appearance were registered for each animal using a score sheet. Approval for animal studies was obtained from the local commissions for animal studies.

Reagents

Anti-Thy1.1 IgG (OX7)-synthesizing hybridoma cells (European Collection of Animal Cell Cultures, Porton Down, Wiltshire, UK; cell line ECACC 84112008) were a gift from J. Floege (Medizinische Hochschule, Hannover, Germany). Antibodies were isolated and purified according to standard procedures (Lindmark et al., 1983; Falkenberg et al., 1993). MMF was kindly provided by Roche Pharma (Reinach, Switzerland). For liposome production, hydrogenated soybean phosphatidylcholine (HSPC; Northern Lipids, Burnaby, Canada), cholesterol, streptavidin, and N-hydroxysuccinimidy-biotin were purchased from Sigma-Aldrich (Buchs, Switzerland), and di-
tearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG) and biotin-PEG-DSPE MW 2000 were purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of Conventional Liposomes

HSPC and cholesterol (90:10 M ratio) were dissolved in chloroform (50 mg/ml HSPC and 10 mg/ml cholesterol) using a conic-bottom glass flask containing 100 glass balls (n = 0.03 mg each). Chloroform was evaporated under rotation in a water bath at 70°C using a nitrogen stream.

MMF (10 mg) was dissolved in 1 ml of 0.01 M HCl and PBS (1:1), and 600 μl of this solution were added to the conical flask containing dissolved lipids. Using a rotavapor in a water bath at 70°C, a multilayer vesicular suspension of MMF-loaded conventional liposomes was formed and subsequently extruded through a membrane of 100-nm pore size using an extrusion system (Avanti Polar Lipids). Thereafter the resulting liposome suspension was immediately cooled down on ice.

Preparation of OX7-Immunoliposomes

Preparation of Pegylated and Biotinylated Liposomes. For the preparation of ILs, conventional liposomes were pegylated and biotinylated using lipoplexes. Pegylated (DSPE-PEG) and biotinylated (DSPE-biotin) lipids were dissolved in chloroform (2:1). Chloroform was evaporated as described above, resulting in the formation of a homogenous lipid film at the surface of the flask. Thereafter, 1 ml of water was added to the lipid film and vigorously shaken until all lipids were resuspended in the aqueous phase, forming a suspension of pegylated and biotinylated lipids (lipoplex), which was concentrated using a Speed-Vac (Vaudaux-Eppendorf AG, Schönenbuch/Basel, Switzerland) device to a final concentration of 50 mg/ml.

Pegylipoxes (6%) were added to the liposome suspension (empty or MMF-loaded) under gentle vortex mixing, incubated in a 65°C water bath for 30 min, and cooled down on ice. The resulting biotinylated and pegylated liposomes were purified by an Akta Purifier system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) using a G50 Sephadex column (GE Healthcare) and PBS and concentrated to 1 mM.

Preparation of Biotinylated OX7 F(ab′2) Fragments. F(ab′2)2 fragments were produced as described previously (Waelti et al., 2002). In brief, a OX7 F(ab′2)2 fragment solution (3 mg/ml in Tris buffer, pH 7.6) was incubated with cystein (30 mM) for 20 min at 37°C and purified by size exclusion chromatography (Sephadex G50M, GE Healthcare; 0.1 M Tris, pH 7.4). The purified OX7 F(ab′)2 fragment solution (0.5 mg/ml) was mixed with a N-hydroxysuccin- imidy-l-biotin solution (Sigma-Aldrich; 5 mg/ml in dimethylsulfoxide) at a molar ratio of 1:50 for 3 h at room temperature and overnight at 4°C. The resulting biotinylated OX7 F(ab′)2 fragments were purified with an Akta Purifier system and concentrated to 110 μg/ml.

Preparation of Empty and MMF-Loaded Pegylated OX7 Immunoliposomes. OX7 F(ab′)2 fragments were conjugated to empty and MMF-loaded pegylated liposomes using the binding of the biotinylated OX7 F(ab′)2 and the biotinylated liposomes to the same streptavidin molecule. A total of 3.6 ml of a 100 μg/ml solution of biotinylated OX7 F(ab′)2 (molecular mass 55 kDa) solution was mixed under gentle vortexing, with 3.6 ml of a 110 μg/ml solution of streptavidin (60 kDa) in water (molar ratio 1:1) in a 50-ml Falcon tube (BD Biosciences, San Jose, CA) at room temperature for 30 min. Then, 18 ml of a 1 mM total lipid liposome suspension was added to the OX7 F(ab′)2-streptavidin complex, incubated at room temperature for 30 min, and concentrated, resulting in a IL suspension containing 2 mM total lipids.
Characterization of Empty and MMF-Loaded OX7 Pegylated Immunoliposomes. MMF and empty loaded IL were completely dried and solubilized in methanol (Fig. 1, A and B). MMF concentration was calculated after measurement of the optical density at 305 nm with the help of a standard curve (Fig. 1C). Size and polydispersity index (PDI) of the empty IL and the MMF-loaded IL were determined using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). Empty IL and MMF-loaded IL had a size of 138 and 203 nm and a PDI of 0.066 and 0.241, respectively (Fig. 1, D and E).

Experimental Design

Sixty male Wistar rats (approximately 200 g body weight at day 2) were divided into five experimental and control groups: group A (n = 6), healthy rats; group B (n = 18), nephritic rats; group C (n = 12), nephritic rats treated with empty OX7-IL (1.8 μmol lipids/ml with 2.5 μmol/kg body weight); group D (n = 12), nephritic rats treated with OX7-IL containing MMF (MMF 21.67 mg/kg body weight); and group E (n = 12), nephritic rats treated with free MMF (MMF 21.67 mg/kg body weight).

To determine the area under the curve (AUC) of the albumin/creatinine ratio, each animal was kept separately in a metabolic cage throughout the study with free access to food and water after an initial period of 2 days for cage adjustment. Anti-Thy1.1 nephritis was induced at day 0 by injection of anti-Thy1.1 IgG (1 mg/kg body weight in PBS) into the tail vein of groups B to E as described previously (Ziswiler et al., 1998). Healthy rats (group A) received only PBS. Groups C to E were treated with a single intravenous injection of 180 μl of either OX-IL (groups C and D) or free MMF (group E) at day +2.

Nephrectomy was performed in anaesthetized rats (pentobarbitone, 50 mg/kg body weight intraperitoneally; Abbott Laboratories, Abbott Park, IL) at day +2 (group B, n = 6), day +5 (groups B–E, n = 6 per group), and day +9 (groups A–E, n = 6 per group). Blood samples at the time of sacrifice were obtained by cardiac puncture. Animals were first perfused with Ringers’ solution containing liquemin (Braun, Crissier, Switzerland) for 5 min, followed by 4% paraformaldehyde/0.5% glutaraldehyde in 0.2 mM HEPES for 20 min with a flow rate of 20 ml/min.

The experiment was performed once for each group (n = 6). In the cases of empty OX7-IL and MMF OX7-IL (groups C and D), ILs from the same batch were used.

Processing of Renal Tissues

Paraffin Embedding. After at least 24-h fixation at 4°C kidney slices were processed for paraffin embedding with dehydration starting with 50% ethanol (4 h), followed by 70% ethanol (2 h), 80% ethanol (2 h), 95% ethanol (1 h), and 95% ethanol (2 h). Before embedding, slices were incubated three times in isopropanol (2 h) and two times in xylene and paraffin (1 and 2 h). Subsequently, 1.5-μm sections were stained with periodic acid-Schiff reagent.

Methacrylate Embedding and Toluidine Blue Staining. Embedding was performed according to the manufacturer’s guidelines...
lated by N 100% ethanol three times for 10 min, in acetone two times for 10 min, at room temperature. Subsequently, samples were immersed in four times for 5 min and subsequently incubated with 70% acetone for 2 h. Thereafter, slices were washed with 0.15 M HEPES two times for 5 min, postfixed with 1% OsO4 in 0.1 M Na-cacodylate for 2 h. Subsequently, slices were washed again with 0.1 M Na-cacodylate four times for 15 min and with bidistilled water two times for 5 min before being incubated overnight with 0.5% uranyl acetate in distilled water. Thereafter slices were washed with bidistilled water two times for 5 min and incubated with 70% acetone (2 h), 90% acetone (2 h), and 100% acetone (2 h). The acetone fixation solution was changed every 30 min. After 48 h, samples were ready to be examined; 1.5-μm sections were stained with toluidine blue.

Epon Embedding. After systematic uniform random sampling as described in Kidney Volume Determination, Sectioning, and Sampling, slices were further subsampled in 1-mm³ cubes out of the kidney cortex and placed into a buffer of 1.5% paraformaldehyde/1.5% glutaraldehyde in 0.15 M HEPES for 24 h at 4°C. Then slices were washed with 0.15 M HEPES two times for 5 min, postfixed with 1% OsO4 in 0.1 M Na-cacodylate at 4°C for 2 h, washed with 0.05 M maleate-NaOH buffer three times for 5 min, and then stained in 0.5% uranyl acetate in 0.05 M malat-NaOH buffer at 4°C for 2 h. Thereafter tissue slices were washed in 0.05 M malat-NaOH buffer three times for 5 min and dehydrated in 70, 80, and 96% ethanol for 15 min at room temperature. Subsequently, samples were immersed in 100% ethanol three times for 10 min, in acetone two times for 10 min, and finally in acetone-epoxy (1:1) overnight at room temperature. The next day, slices were embedded in epon and left to harden at 60°C.

Stereological Analyses

Kidney Volume Determination, Sectioning, and Sampling. Kidney volume was determined by the water displacement method (Scherle, 1970). The kidneys embedded in 3% agar were cut into equally thick slices, beginning at a random start point. Thereafter, the slices were assigned by systematic uniform random sampling (Gundersen and Jensen, 1987) to paraffin, methacrylate, or epon embedding. On average, three slices for each embedding technique were obtained. This procedure ensured that each part of the kidney had approximately the same probability to be evaluated.

Light Microscopy Stereology: Physical Disector. For microscopic stereological evaluations, an Olympus (Tokyo, Japan) light microscope equipped with a digital camera and Cast stereology software (Visiopharm, Horsholm, Denmark) were used. To determine the number of MCs per kidney, a physical disector was exploited (Sterio, 1984). It consisted of a pair of thin physical sections separated by a known distance and an unbiased two-dimensional counting frame (Gundersen, 1978).

The first and the fourth sections of a consecutive row of 1.5-μm methacrylate sections were mounted on a glass slide, thus providing a disector height of 4.5 μm. Fields of view were sampled by systematic uniform random sampling. An unbiased counting frame (1728.2 μm²) was superimposed on the field of view. MCs lying within the counting frame were counted if their nucleus was present in the first counting events (see above) and (vis) is the volume of the physical disector. The latter is calculated by: (dis) = half the length of a single line segment. The equation for the arithmetic mean of the BUB was calculated by: (BUB) = (P) × V/glomerulus)/2L, where L is the total length of the line segment hitting glomerular basement membrane. This formula can be facilitated as: (BUB) = (P) × V/glomerulus)/2L, where (l(p)) is half the length of a single line segment. The equation was derived from a morphometric study on the thickness of the pulmonary air-blood barrier (Weibel and Knight, 1964).

Immunohistochemistry

Immunostaining and Quantification of α-Smooth Muscle Actin ED-1 and PCNA. Kidney sections were stained with mouse monoclonal antibodies against α-smooth muscle actin (α-SMA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ED-1 (CD68 antibody; Millipore Bioscience Research Reagents, Temecula, CA), and proliferating cell nuclear antigen (PCNA; PC-10, mouse monoclonal; Abcam Inc., Cambridge, MA). In brief, 1.5-μm sections were deparaffinized and rehydrated with a gradient of ethanol concentrations. Antigen retrieval was performed by heating the samples in a water bath of 90°C in 10 mM citrate buffer, pH 6.0. Samples were washed with 0.1% Tween-PBS, pH 7.3; this buffer was used for all subsequent washes. Thereafter, sections were blocked for endogenous peroxidase activity with 3% H2O2 in PBS 15 min at room temperature in the dark. PAP pens were used to identify the boarder of the microscope slides to minimize drainage of reagents and edge effects on staining. Next, sections were blocked with 1% BSA in PBS for 1 h at room temperature. Incubation with primary antibody diluted in PBS supplemented with 1% BSA was carried out for 60 min at room temperature. In two negative control experiments, the primary antibody was replaced by buffer or normal serum (same species as primary antibody). Afterward, sections were incubated in a 1:200 dilution (same buffer as primary antibody) of biotinylated rabbit anti-mouse Ig and thereafter with avidin-biotin-complex/alkaline phosphatase (Dako Denmark A/S, Glostrup, Denmark) diluted 1:200 in PBS. Finally, sections were developed using 3,3′-diaminobenzidine for 5 min and counterstained with Mayer’s hematoxylin (Dako Denmark A/S).

Glomerular monocye/macrophage influx and proliferating cells were determined by counting ED-1-positive cells (Ziswiler et al., 1998) and PCNA-positive cells (Floege et al., 1992) in 120 cross-sections per animal group (A-E) at days +5 and +9.

Staining for α-SMA was evaluated in 120 glomerular cross-sections per animal group (A-E) at days +5 and +9, according to a semiquantitative grading system (0–4), as described previously (Floege et al., 1991). The mean scores were determined for the different experimental groups.
Immunostaining and Quantification of Collagen IV and Fibronectin. Kidney sections (1.5 μm) were deparaffinized and rehydrated with a gradient of ethanol concentrations. Antigen retrieval was performed by heating the samples in a water bath of 90°C in 10 mM citrate buffer, pH 6.0. Samples were washed three times with PBS containing 0.3% Triton X-100 and incubated with a permeabilization buffer (0.1% saponin and 0.009% sodium azide) at room temperature for 1 h and with a blocking buffer (3% BSA, 0.3% Triton X-100, in PBS) containing 0.1% saponin at room temperature for 1 h. Thereafter sections were incubated with either collagen IV (Abcam Inc.) or fibronectin (Abcam Inc.) in blocking buffer at 4°C overnight, washed, and further incubated with a fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody at room temperature for 1 h. Negative controls included the replacement of the primary antibody by buffer or normal serum (same species as primary antibody). Washed sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Immunofluorescent staining for fibronectin and collagen IV was analyzed by adapting a previously published method (Vetterlein et al., 2006). In brief, the integrated density of three randomly chosen images of glomeruli per animal in each group was measured. The mean integrated density per glomerulus was determined for the different experimental groups. For image analysis, ImageJ software 1.44 (National Institutes of Health, Bethesda, MD) was applied.

Histocytochemical Study to Determine Apoptosis. Cytotoxicity, reflected by apoptosis, of MMF OX7-IL was assessed by in situ end-labeling of DNA fragments (TUNEL, Roche Applied Science, Indianapolis, IN). Paraffin-embedded kidneys were cut into 1.5-μm thick sections, mounted on slides, and rehydrated before TUNEL staining. For negative controls, label solution without terminal deoxynucleotidyl transferase was used instead of the TUNEL reaction mix. Positive control slides were incubated for 10 min at room temperature with DNase I recombinant (3000 U/ml–3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) to induce DNA strand breaks, before labeling procedures. Samples were processed with Vectashield and directly analyzed under a fluorescence microscope [Nikon (Tokyo, Japan) Eclipse E600; excitation wavelength of 488 nm and detection in the range of 515–565 nm].

Determination of Plasma Creatinine. Blood samples were taken at time point of nephrectomy on days +5 and +9 for the analysis of plasma creatinine in groups A through E. All analyses were performed in the laboratories of the Clinical Chemistry Department of the University Hospital of Bern.

Quantification of Albuminuria. Total urinary intact albumin from days −2 to +9 was measured by high-pressure liquid chromatography (HPLC) in aliquots of urine that had been stored at −80°C using a BioSuite 250, UHR size exclusion chromatography column (4.6 × 3000 mm, 4 μm; Waters, Milford, MA) and a Waters 2695 HPLC system equipped with a UV-visible detector at 220 nm. Urinary specimens were centrifuged and diluted before 20 μl were injected onto the HPLC system. The mobile phase consisted of PBS (9 g/l NaCl, 0.775 g/l Na2HPO4, and 0.165 g/l KH2PO4, pH 7.4). Albumin excretion in 24-h urine specimens was expressed as albumin-to-creatinine ratios (μg/mM). The mean baseline values of albuminuria from days −2 to 0 were subtracted from all subsequent measurements. The AUC of the albumin-to-creatinine ratio was calculated from days +1 to +9 for each rat separately. A mean AUC(1–9), for each animal group was obtained.

Statistical Analysis

Results are expressed as mean ± S.E.M. Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA). For statistical analysis of the natural disease progression (see Fig. 5), Student’s unpaired t test was applied. The Student’s unpaired t test was applied. For the investigations of the treatment effects with respect to albuminuria (see Fig. 4) and histology (see Figs. 6 and 7), statistical analyses were performed using ANOVA with Newman-Keuls multiple comparison test. For all experiments, p values < 0.05 were considered significant. The total number of animals studied was 60, with a minimum of 12 animals per treatment group.

With respect to renal function, the 4 × 2 ANOVA design with a cell size of six would not have missed a deterioration of renal function (main effect) of more than 20% with a power of 83% or more than 25% with a power of 96%.

Results

Histopathology

After injection of OX7-Ab, natural progression of anti-Thy1.1 proliferative mesangial glomerulonephritis with increasing mesangial hypercellularity and ECM accumulation was observed from days +2 to +5 and +9. In this study, we investigated the therapeutic effect of MMF-loaded OX7-IL on days +5 and +9 and compared the results with those obtained after application of conventional free MMF.

The MMF-OX7-IL-treated group (D) showed a milder form of nephritis with less MC proliferation and mesangial matrix accumulation in comparison with all other nephritic animals of groups B (untreated nephritic rats), C (nephritic rats treated with empty OX7-IL), and E (nephritic rats treated with free MMF).

Fig. 2 depicts representative examples of glomerular histology obtained from animals of group A (healthy controls), group B (untreated nephritic rats), group D (nephritic rats treated with MMF OX7-IL), and group E (nephritic rats treated with free MMF) at day +9, demonstrating a visible attenuation of nephritis by single application of MMP-loaded OX7-IL at day +2 after induction of disease.

Therapeutic Effects of MMF-OX7-IL

To analyze the therapeutic effects of MMF-OX7-IL, we looked at the influx of monocytes/macrophages. In the pres-
ent study we confirmed the presence of monocytes/macrophages in nephritic animals by ED-1 staining (Fig. 3B). The treatment of nephritic rats with MMF OX7-IL (group D) resulted in an obvious amelioration of the glomerular influx of monocytes/macrophages at day +9, as shown in Fig. 3C.

To quantify the effect of MMF OX7-IL (group D) on the invasion of monocytes/macrophages, we counted the ED-1-positive cells in glomerular cross-sections (Table 1). The healthy animals of group A demonstrated only 0.1 ± 0 ED-1-positive cells per glomerular cross-section. In comparison with group A, all nephritic animals of groups B, C, and E showed increased detection of ED-1-positive cells per glomerular cross-section. It is noteworthy that we were able to show that MMF OX7-IL treatment (group D) significantly attenuated the glomerular influx of monocytes and macrophages, as reflected by the significant reduction of ED-1-positive cells per glomerular cross-section (3.7 ± 0.8 and 2.4 ± 0.6 on days +5 and +9, respectively) in comparison with all other nephritic animals (group B, 9.6 ± 0.7 and 7.2 ± 1.1; group C, 9.1 ± 1.8 and 6.4 ± 1.9; group E, 8.9 ± 0.9 and 6.8 ± 0.7 on days +5 and +9, respectively).

Furthermore, we demonstrated the antifibrotic effect of MMF OX7-IL (group D) with immunohistochemical staining for α-SMA (Fig. 3I) and immunofluorescence analyses of collagen IV and fibronectin (Fig. 4, C and F) at day +9. Nephritic rats treated with MMF OX7-IL (group D) showed far less staining for α-SMA (Fig. 3I), collagen IV, and fibronectin (Fig. 4, C and F) in comparison with untreated nephritic rats (group B).

In addition, because in rat MCs the level of expression of α-SMA may be associated with cell proliferation and represents a marker of cell activation (Johnson et al., 1991), the expression of α-SMA was quantified at days +5 and +9. A semiquantitative scoring system (0–4) was used for these analyses, as depicted in Table 1. Healthy rats (group A) displayed a mean α-SMA score of only 0.1 ± 0. Predictably, the α-SMA score in glomerular cross-sections of nephritic rats (groups B, C, and E) significantly increased to 3.6 ± 0.3 and 3.9 ± 0.2, 3.5 ± 0.4 and 3.8 ± 0.3, and 3.6 ± 0.5 and 3.9 ± 0.1 on days +5 and +9, respectively.

In contrast, MMF OX7-IL treatment of nephritic animals resulted in a decreased expression of α-SMA, reflected by significantly lower scores of 2.5 ± 0.3 on day +9 in group D (p < 0.001 versus nephritic rats of groups B, C, and E). On day +5 only a slightly lower α-SMA score (2.6 ± 0.2) was observed by MMF-OX7-IL-treated nephritic animals (group D) in comparison with the nephritic animals of groups B, C, and E (3.6 ± 0.3, 3.5 ± 0.4, and 3.6 ± 0.5) on day +5.

No staining was observed when the specific primary antibody was replaced with PBS or normal serum. Immunofluorescent staining for fibronectin and collagen IV was also quantified (Table 1). Animals treated with MMF OX7-IL (group D) showed significantly less staining intensity for fibronectin and collagen IV in comparison with all other nephritic animals (groups B, C, and E) on days +5 and +9. It is noteworthy that staining intensities of fibronectin and collagen IV in the MMF OX7-IL-treated group (group D) did not differ significantly from healthy control animals.

Kidney Function

Plasma creatinine was measured in all animals at sacrifice (Table 2). Healthy animals (group A) had average creatinine levels of 17 ± 1 μM. In comparison, nephritic animals of groups B, C, and E displayed elevated creatinine levels (22–26 μM; group A versus group C and E of day +9, p < 0.05; group A versus group B, p < 0.01). No difference was observed within these groups between days +5 and +9 after injection. MMF-loaded OX7-IL prevented an increase in plasma creatinine concentration. Thus, animals of group D (nephritic rats treated with MMF OX7-IL) had almost equal mean values of 19 μM similar to control animals at day +9 (group D versus group A, p > 0.05).

Albuminuria

Albuminuria expressed as albumin-to-creatinine ratio obtained from 24-h urine specimens was analyzed from all animals in each group from days −2 to +9 (Fig. 5). Before induction of nephritis on day 0, baseline albuminuria from days −2 to 0 was minimal. These baseline values were subtracted individually from all subsequent measurements. In the nephritic groups B, C, and E, the disease progression of Thy1.1 nephritis as indicated by proteinuria was obvious. Compared with control animals (group A), albuminuria increased in these three nephritic groups on day +1, peaked around day +5, and then slowly declined (groups B, C, and E versus group A, p < 0.05).

In contrast, the animals treated with MMF OX7-IL (group D) showed an almost complete eradication of albuminuria. Their albumin levels did not differ from the healthy control animals of group A (p > 0.05) and were significantly lower than the levels in nephritic animals (groups B, C, and E, p < 0.05 for all three comparisons).

![Fig. 3. Immunohistostaining for ED-1, PCNA, and α-SMA in anti-Thy1.1 nephritis at day +9. Therapeutic effects of MMF-OX7-IL were assessed by immunohistology, A, D, and G, healthy controls stained for ED-1, PCNA, and α-SMA are shown. B, E, and H, untreated nephritic animals show positive staining for ED-1, PCNA, and α-SMA. C, F, and I, amelioration of these features in nephritic animals treated with MMF-loaded OX7-IL is shown. Formalin-fixed, paraffin-embedded tissue specimens were used. Magnification, 60×.](image-url)
fibronectin (F). Formalin-fixed paraffin-embedded tissue specimens were assessed by immunofluorescence staining. A and D, healthy control (group A), the mean glomerular volume of a kidney was 64.92 m\(^3\), 64.92 \(\pm\) 6.4 m\(^3\), and 81.47 \(\pm\) 2.4 m\(^3\) in comparison with all other nephritic animals of groups B, C, and E days +5 and +9, respectively. On day +9, the volume of the glomeruli increased to 56.86 \(\pm\) 7.2 m\(^3\), 64.92 \(\pm\) 6.4 m\(^3\), and 81.47 \(\pm\) 2.4 m\(^3\) after disease +9, as expected, nephritic animals treated with MMF OX7-IL showed a significant reduction of PCNA-positive cells (7.6 \(\pm\) 0.3*, **p = 0.05 vs. control, **p = 0.05 vs. control (group A), **p = 0.05), as shown in Fig. 6A. Increased detection of PCNA staining (Fig. 3F) reflected MC proliferation in nephritic animals. In contrast, PCNA staining in MMF OX7-IL-treated animals (group D) was minimal (Fig. 3F).

In addition, we quantified the PCNA staining by counting PCNA-positive cells in glomerular cross-sections (Table 1). As expected, nephritic animals treated with MMF OX7-IL showed a significant reduction of PCNA-positive cells (7.6 \(\pm\) 0.3) in comparison with all other nephritic animals of groups B, C, and E (11 \(\pm\) 0.8, 10.9 \(\pm\) 0.6, and 11 \(\pm\) 0.9, p < 0.001 versus nephritic rats of groups B, C, and E, respectively) on day +9. On day +5, only a slightly lower number of PCNA-positive cells was observed in MMF-OX7-IL-treated nephritic animals (group D) in comparison with the nephritic animals of groups B, C, and E (14.1 \(\pm\) 0.1, 14.3 \(\pm\) 1.7, and 13.6 \(\pm\) 1.2, respectively).

**Glomerular Volume per Kidney.** In healthy animals (group A), the mean glomerular volume of a kidney was 45.51 \(\pm\) 2.12 \(\times\) 10\(^4\) \(\mu\)m\(^3\). During anti-Thy1.1 nephritis (group B), the volume of the glomeruli increased to 56.86 \(\pm\) 0.38 \(\times\) 10\(^4\) \(\mu\)m\(^3\), 64.92 \(\pm\) 0.78 \(\times\) 10\(^4\) \(\mu\)m\(^3\), and 81.47 \(\pm\) 4.64 \(\times\) 10\(^4\) \(\mu\)m\(^3\) respectively.

### TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Monocyte/Macrophages (ED-1) per Glomerular Cross-Section (\times 10^6)</th>
<th>Proliferating (PCNA+) Cells per Glomerular Cross-Section (\times 10^6)</th>
<th>α-SMA Expression (Score)</th>
<th>Fibronectin (Integrated Density/Glomerulus) (\times 10^4)</th>
<th>Collagen IV (Integrated Density/Glomerulus) (\times 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>9</td>
<td>0.1 (\pm) 0.**</td>
<td>0.4 (\pm) 0.1**</td>
<td>0.1 (\pm) 0.**</td>
<td>13.1 (\pm) 5.4**</td>
<td>4.7 (\pm) 0.5**</td>
</tr>
<tr>
<td>B Nephritic</td>
<td>5</td>
<td>9.6 (\pm) 0.7*</td>
<td>14.1 (\pm) 1.4*</td>
<td>3.6 (\pm) 0.3*</td>
<td>31.1 (\pm) 2.6*</td>
<td>16.0 (\pm) 0.8*</td>
</tr>
<tr>
<td>C Empty OX7-IL</td>
<td>5</td>
<td>7.2 (\pm) 1.1*</td>
<td>11 (\pm) 0.8*</td>
<td>3.9 (\pm) 0.2*</td>
<td>49.5 (\pm) 3.9*</td>
<td>18.2 (\pm) 0.4*</td>
</tr>
<tr>
<td>D MMF OX7-IL</td>
<td>5</td>
<td>3.7 (\pm) 0.8***</td>
<td>12.5 (\pm) 0.6*</td>
<td>2.6 (\pm) 0.2*</td>
<td>15.5 (\pm) 1.1**</td>
<td>4.7 (\pm) 0.1**</td>
</tr>
<tr>
<td>E Free MMF</td>
<td>9</td>
<td>8.9 (\pm) 0.9*</td>
<td>13.6 (\pm) 1.2*</td>
<td>3.6 (\pm) 0.5*</td>
<td>31.7 (\pm) 1.9*</td>
<td>15.4 (\pm) 0.9*</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control, **p < 0.05 vs. nephritic on days +5 and +9, respectively.

### Stereological Analyses of Anti-Thy1.1 Nephritis

**Mesangial Cells.** MC number per kidney was determined by applying the physical disector (Sterio, 1984) on sections from methacrylate-embedded samples (Bertram et al., 1992; Bertram, 2001). We evaluated the natural progression of anti-Thy1.1 nephritis on days +2, +5, and +9, after disease induction and compared the results with healthy animals.

Healthy Wistar rats (group A) had an average of 6 \(\times\) 10\(^6\) MCs per kidney (5,953,618 \(\pm\) 591,052). On the second day after anti-Thy1.1 nephritis induction, we observed a slight augmentation of MC number of more than 7 \(\times\) 10\(^6\) MC per kidney (7,186,525 \(\pm\) 597,620). MC per kidney further increased to 12 \(\times\) 10\(^6\) (11,130,810 \(\pm\) 971,162) and 14 \(\times\) 10\(^6\) (13,792,021 \(\pm\) 11,391,88) on days +5 and +9, respectively (control versus nephritic days +5 and +9: p < 0.05), as shown in Fig. 6A. Increased detection of PCNA staining (Fig. 3E) reflected MC proliferation in nephritic animals. In contrast, PCNA staining in MMF OX7-IL-treated animals (group D) was minimal (Fig. 3F).

### TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Creatinine ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>9</td>
<td>3.3 (\pm) 0.2*</td>
</tr>
<tr>
<td>B Nephritic</td>
<td>9</td>
<td>3.3 (\pm) 0.2*</td>
</tr>
<tr>
<td>C Empty OX7-IL</td>
<td>9</td>
<td>3.3 (\pm) 0.2*</td>
</tr>
<tr>
<td>D MMF OX7-IL</td>
<td>9</td>
<td>3.3 (\pm) 0.2*</td>
</tr>
<tr>
<td>E Free MMF</td>
<td>9</td>
<td>3.3 (\pm) 0.2*</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control, **p < 0.05 vs. nephritic on days +5 and +9, respectively.
μm³ on days +2, +5, and +9, respectively (group A versus group B; \( p < 0.05 \), days +5 and +9), as depicted in Fig. 6B. As expected, these results indicate an increase of glomerular size and volume during disease progression in comparison with healthy animals.

**Extracellular Matrix Volume per Kidney and Blood-Urine Barrier.** Healthy animals (group A) had an ECM mean volume per kidney of 2.17 ± 0.07 × 10⁴ μm³. We monitored an increase of ECM throughout the disease progression with nearly double amounts of ECM observed at day +2 (3.90 ± 0.07 × 10⁴ μm³; control versus nephritic day +2, \( p < 0.05 \)), thereafter 5.15 ± 0.24 × 10⁴ μm³ at day 5 (control versus nephritic day +5; \( p < 0.05 \)) and reaching up to 7.55 ± 0.26 × 10⁴ on day +9 (control versus nephritic day +9; \( p < 0.05 \)), as shown in Fig. 6C. Healthy animals exhibited a mean BUB of 0.45 ± 0.02 μm (Fig. 6D), which was not altered during the untreated progression of anti-Thy1.1 nephritis.

**Effect of MMF-Loaded OX7-IL on Mesangial Cell Proliferation.** At day +5, only a slightly lower MC content was observed for MMF OX7-IL-treated animals (group D) in comparison with the other nephritic animals (groups B, C, and E) (Fig. 7A).

Animals treated with MMF OX7-IL (group D) showed a 30% MC reduction on day +9, compared with the other nephritic animals (group D versus groups A or C, \( p < 0.05 \); group D versus group E, \( p < 0.05 \)). Groups treated with empty OX7-IL (group C) or free MMF (group E) did not differ from untreated nephritic animals (group B) at day +9 (Fig. 8A).

**Effect of MMF-Loaded OX7-IL on Glomerular Volume.** MMF OX7-IL-treated animals (group D) showed a significant difference in glomerular volume compared with nephritic animals (group B) and nephritic animals treated with empty OX7-IL (group C) on day +5 (MMF OX7-IL versus nephritic and empty OX7-IL, \( p < 0.05 \)) as depicted in Fig. 7B. No significant glomerular volume reduction by MMF OX7-IL (group D) in comparison with nephritic animals treated with either free MMF (group E) or empty OX7-IL (group C) was observed on day +9 (Fig. 8B).

**Effect of MMF-Loaded OX7-IL on ECM Volume.** Approximately 40% reduction of glomerular ECM volume was observed in MMF OX7-IL-treated animals (group D) on day +5 compared with nephritic animals treated with empty OX7-IL (group C) and free MMF (group E) (group D versus groups C and E, \( p < 0.05 \)) (Fig. 7C). On day +9, a reduction of ECM content by the MMF-OX7-IL-treated group compared with nephritic group E (\( p < 0.05 \)) (Fig. 8C) was determined.

**Effect of MMF-Loaded OX7-IL on Blood-Urine Barrier.** In all nephritic groups (B–E), the thickness of the BUB remained unchanged at days +5 and +9 (Fig. 7D).

**Lack of Side Effects after OX7-IL Treatments.** All OX7-IL formulations and the free form of MMF were well tolerated according to the information recorded on score sheets (see Materials and Methods). No clinically relevant side effects such as decreased water or nutrition intake or weight loss were registered. Furthermore, in all nephritic groups (B–E), essentially no apoptotic cells were observed on days +5 and +9, which argues strongly against a non-specific cytotoxic effect of free MMF and MMF OX7-IL formulations (Fig. 9B). There was only occasionally faint
positive staining in small parts of glomeruli on day +2 (data not shown).

Discussion

Design-based stereology provides an unbiased and robust method to quantitatively analyze the natural course of anti-Thy1.1 nephritis as well as treatment efficacy. Our data demonstrate that MMF-loaded OX7-IL treatment results in amelioration of glomerular histology by reduction of MC proliferation, glomerular volume, and ECM accumulation without affecting the thickness of the BUB. Moreover, MMF-loaded OX7-IL prevented the occurrence of albuminuria in nephritic rats and preserved their kidney function. Thus, MMF-loaded OX7-IL might be considered as a promising approach for treating mesangial cell-mediated forms of glomerulonephritis, such as IgAN.

Because anti-Thy1.1 nephritis in rats is not a consequence of deposition of abnormal IgA containing deposits in the glomeruli the results cannot be directly extrapolated to human IgAN. Nevertheless, anti-Thy1.1 nephritis still represents a relevant model for studying interventions directed to MC proliferation and mesangial matrix deposition (Jefferson and Johnson, 1999).

So far, only semiquantitative scoring systems have been used for the characterization of Thy1.1 nephritis, such as the investigation by Floege et al. (1991) assessing the ECM accumulation by semiquantitative scoring (0–4) of glomerular cross-sections.

Similar scoring systems have been used by our group to quantify therapeutic effects of a synthetic matrix metalloproteinase inhibitor and MMF (Steinmann-Niggl et al., 1998; Ziswiler et al., 1998; Daniel et al., 2001). In this respect, design-based stereology provides unbiased data and therefore greatly facilitates the quantitative evaluation of the therapeutic effects with the aim to reducing MC proliferation and ECM deposition (Nyengaard, 1999).

Fig. 7. Stereological analysis of effect of OX7-IL at day +5: mesangial cells per kidney (A), glomerular volume per kidney (B), ECM content per kidney (C), and thickness of blood-urine barrier (D). ANOVA with Newman-Keuls multiple comparison test was used. *, p < 0.05; **, p < 0.01. Mean values ± S.E.M., groups A–E, n = 6, one experiment.

For more than a decade, MMF has proven to be a potent immunosuppressive drug for the treatment and prophylaxis of renal allograft rejection (Villarreal et al., 2009). In general, this drug has a favorable effect profile because of its efficacy and relatively benign adverse effects, and it has presented evidence of benefits to some individuals refractory to conventional therapies of various glomerulopathies (Sahin et al., 2007).

MMF is hydrolyzed by esterases to form the active moiety mycophenolic acid. Mycophenolic acid inhibits the enzyme inosine monophosphate dehydrogenase, which is responsible for the de novo synthesis of guanosine and is essential in the proliferation pathway of B and T lymphocytes (Allison and
Eugui, 1996). In separate in vitro experiments, we were able to demonstrate that MMF inhibits in a dose-dependent fashion the proliferation of cultured rat MC (data not shown). This finding is compatible with an intracellular activation of MMF in these cells.

Furthermore, Ziswiler et al. (1998) showed a significant amelioration of glomerular histology, assessed by glomerular cellularity, synthesis of α-SMA, ECM deposition, and glomerular hypertrophy by the application of oral MMF. Based on our hypothesis that the application of MMF in IL is more efficient than in its conventional free form we aimed therefore to test the efficacy of MMF-loaded OX7-IL in acute anti-Thy1.1 nephritis. We have chosen, as primary therapeutic endpoints, MC number, ECM accumulation, glomerular volume, plasma creatinine levels, and albuminuria. In addition, we have also analyzed, as secondary endpoints, monocyte/macrophage influx, MC proliferation and apoptosis, and deposition of certain ECM proteins.

In the present study, we used only a single application of low-dose MMF in OX7-IL (22 mg/kg body weight) in contrast to the full therapeutic MMF dose (40 mg/kg body weight), which was given daily for up to 11 days in our previous investigation (Ziswiler et al., 1998). Treatment was applied after induction of the disease at the time of already overt glomerular damage reflected by mesangiolysis. However, during the period of mesangiolysis a sufficient number of intact MCs remained to proliferate and thereafter become susceptible for targeted interventions.

Furthermore, two other research groups have reported a beneficial effect of MMF in anti-Thy1.1 nephritis and chronic-progressive glomerulosclerosis. Chiara et al. (2005) used the same dose of MMF as in our previous study (40 mg/kg body weight p.o.) given from days 3 to 7. They observed a decrease
in MC proliferation and matrix expansion. It is noteworthy that Krämer et al. (2005) showed in a chronic Thy1.1-induced glomerulosclerosis model that the same low dose of MMF (20 mg/kg body weight) used in the present study was very efficient. However, in their study MMF was given with food in a daily dose of 20 mg/kg body weight compared with the single dose in our study.

Because MMF interferes with the synthesis of membrane glycoproteins (Allison and Eugui, 1996), MMF may decrease tissue invasion of leukocytes. Previous investigations demonstrated that MMF did not attenuate the glomerular influx of monocytes/macrophages (Ziswiler et al., 1998). However, it has also been shown previously that MMF inhibits infiltration of lymphocytes and monocyte/macrophages into renal transplants, in a rat model of chronic kidney allograft rejection (Azuma et al., 1995). In the present study the therapeutic and antifibrotic effect of MMF-OX7-IL was confirmed by a decrease in the deposition of α-SMA, fibronectin, and collagen IV and the decrease of ED-1-positive cells.

In our present study it became evident that MMF OX7-IL attenuated functional and histological inflammatory features. The most significant finding was the nearly complete abolition of albuminuria assessed by size-exclusion HPLC (Comper et al., 2004). Furthermore, MC proliferation was inhibited reflected by the reduction of MC number per kidney and decreased detection of PCNA-positive cells per glomerular cross-section. It is noteworthy that the decrease of MCs was not caused by an increased rate of apoptosis.

To support the validity of our approach, the numbers of MCs per kidney measured in our healthy control animals were well in accordance with the work by Bertram et al. (1992), who showed a similar value of a mean of approximately 7.8 × 10^6 MC per kidney in 215-g Sprague-Dawley rats that was obtained by an optical disector analyzing electron microscopic images.

In addition to MC hyperplasia, ECM accumulation remains one of the main features in experimental mesangial proliferative glomerulonephritis. Previously, we analyzed ECM deposition in the mesangium by the semiquantitative grading method published by Floege et al. (1991) and observed a decreased mesangial matrix score by systemic application of MMF (Ziswiler et al., 1998). However, these results are related to limited areas of tissue and provide no information on the score in the whole kidney, therefore possibly leading to biased data. In addition, processing kidney tissue for paraffin embedding may lead to a range of dimensional changes. In the present study, we show unbiased stereological assessment of ECM volume per kidney at the electron microscopic level, revealing significant differences of ECM accumulation during natural anti-Thy1.1 nephritis progression. Significantly, the application of MMF-loaded OX7-IL also reduced ECM accumulation in nephritic rats.

The glomerular volume showed a significant reduction after the MMF-OX7-IL therapy on day +5 and only a slight reduction after the MMF-OX7-IL therapy on day +9; the latter result might be caused by the rather small number of animals investigated. In accordance with the present study, we described previously a significant reduction of glomerular hypertrophy after systemic application of MMF, by analyzing maximal diameters of glomerular cross-sections. Other nephropathies, such as a diabetic nephropathy, present glomerular hypertrophy and enlargement as main characteristic features. Guo et al. (2005) performed a stereological study of the renal glomerular vasculature in the db/db mouse model of diabetic nephropathy.

We analyzed possible changes in the thickness of the blood-urine barrier, which, however, remained essentially unaffected.

Several liposomal drugs have already been approved for clinical applications but, despite the encouraging results obtained to use liposomes as vehicles in numerous disease models, the application of liposomes and IL has been more or less restricted to tumor and cancer therapy rather than to inflammatory disease models such as anti-Thy1.1 nephritis (Torchilin, 2005).

The limitation of the present study is, despite the encouraging results, that the complete therapeutic potential of MMF OX7-IL in experimental mesangial proliferative glomerulonephritis has not been fully explored. In this respect, additional studies using multiple dose regimens may help to maximize the therapeutic value of MMF OX7-IL. Furthermore, it would be attractive to use a multitherapy approach with coinjection of OX7-IL loaded with different drugs or with OX7-IL loaded with multiple drugs. With the focus on future human studies, transcriptome analyses and related techniques may be helpful to identify accessible antigens preferentially expressed on the surface of human MCs (Sengøege et al., 2002). The identification of suitable antigens might provide the potential to target MC successfully and improve the destiny of patients suffering from MC-mediated types of glomerulonephritis.

In summary, this is, to the best of our knowledge, the first stereological study demonstrating therapeutic effects of low-dose MMF encapsulated in OX7-IL in experimental mesangial proliferative glomerulonephritis. MMF OX7-IL caused an almost complete abolition of albuminuria, a reduced ECM accumulation at day +5, and a successfully reduced MC proliferation on day +9. Because a therapeutic effect was achieved with only one dose of OX7-IL at 2 days after induction of nephritis, we conclude that directed one-dose delivery of low-dose MMF to MC represents a novel efficient and promising approach for the treatment of experimental mesangial proliferative glomerulonephritis. These results open up a new perspective in view of a therapeutic intervention of glomerular inflammatory disease in humans.

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Authorship Contributions

Participated in research design: Suana, Tuffin, Frey, Knudsen, Mühlfeld, and Marti.

Conducted experiments: Suana, Tuffin, and Frey.

Contributed new reagents or analytic tools: Tuffin and Knudsen.

Performed data analysis: Suana, Tuffin, and Marti.

Wrote or contributed to the writing of the manuscript: Suana, Tuffin, Frey, Mühlfeld, Rödder, and Marti.

Other: Marti acquired funding for the research and is the principal investigator.

References


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