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# Not the ED1$^+$ but the ED3$^+$ Macrophage Participates in the Pathogenesis of Irreversible Glomerular Changes

**Yumi ITO[1,2], Yoshio MORIOKA[1], Hiroko KOIKE[1], Yohei IKEZUMI[1], Hideo KOBAYASHI[3], Fumitake GEJYO[2], Fujio SHIMIZU[1] and Hiroshi KAWACHI[1]**

[1]Department of Cell Biology, Institute of Nephrology, Faculty of Medicine, Niigata University, [2]Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, [3]Kidney Center, Department of Medicine, Tokyo Women's Medical University, Tokyo, Japan

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***Summary.*** **We compared the acute phase responses of mesangial cells and inflammatory cells in reversible and irreversible mesangial alterations. We used a reversible model that induced by a single injection of anti-Thy 1.1 monoclonal antibody (mAb) 1-22-3, and an irreversible model of glomerulonephritis induced by two consecutive injections of mAb 1-22-3 with an interval of 2 weeks. Similar mesangiolysis occurred in both models. However, pathological phenotypic changes in the mesangial cell began more rapidly and markedly after the second wave of mesangiolysis. Glomerular mRNA expression of platelet-derived growth factor (PDGF) was higher in the irreversible model than in the reversible model, whereas mRNA expression of monocyte chemoattractant protein-1 (MCP-1) was lower in the irreversible model. The numbers of polymorphonuclear cell (PMN) and ED1$^+$ monocytes/macrophages were lower in the irreversible model than in the reversible model. By contrast, the recruitment of ED3$^+$ cells into the glomeruli was dominant in the irreversible model. A dual labeling study showed that major parts of ED3$^+$ cells in the glomeruli of the irreversible model were ED1 negative. ED3$^+$ ED1$^-$ cells were not detected at any time in the reversible model. The ED3$^+$ cell should be regarded as an important candidate for acting on the mesangial cell to lead to irreversible mesangial alterations.**

**Key words**—Thy 1.1. nephritis, mAb 1-22-3, ED3, MCP-1.

## INTRODUCTION

The mesangial alteration induced by anti-thymocyte serum (ATS) has been widely investigated as a model of mesangial proliferative glomerulonephritis. Binding of the anti-Thy 1.1 antibody to mesangial cells causes mesangiolysis, inflammatory cell infiltration, and mesangial cell proliferation, followed by mesangial matrix expansion[1,2,3]. Many investigators have reported that the inflammatory cells, such as monocytes and polymorphonuclear cells (PMNs), and the cytokines produced by these cells are essential for the development of Thy 1.1 nephritis[4,5]. However, as mesangial cell proliferation and mesangial matrix expansion are temporary in this model and normalize within 2 months, it is still unclear whether these cells and cytokines are important for the progression of glomerular changes or for recovery of the disease. We have already reported that the anti-Thy 1.1 monoclonal antibody (mAb) 1-22-3 was capable of inducing severe proteinuria as well as morphological change[6] and also reported that two consecutive mAb 1-22-3 injections were capable of inducing irreversible sclerotic change accompanied with persistent proteinuria[7]. From our preliminary observations, we hypothesized that the inflammatory cells and the intrinsic glomerular cells responded alternatively after the second immune injury and that these alternative responses initiated the irreversible sclerotic changes. In this study, we compared the kinetics of mesangial alterations and the inflammatory parameters after the second injection with those after the first injection. We report here that the ED 3$^+$ macrophage is an important candidate for acting on mesangial cell to lead to irreversible mesangial alterations.

Correspondence: Hiroshi Kawachi, MD, Ph. D, Department of Cell Biology, Institute of Nephrology, Faculty of Medicine, Niigata University 1-757 Asahimachi-dori, Niigata 951-8510, Japan.

## MATERIALS AND METHODS

### Induction of animal model

All experiments were performed using female Wistar rats weighing 150-200 g, purchased from Charles River Japan (Atsugi, Japan). mAb 1-22-3 was prepared as described previously[6]. The experimental model of irreversible mesangial proliferative glomerulonephritis was induced in Wistar rats by two consecutive injections of 1.0 ml of saline containing 500 $\mu$g of mAb 1-22-3 with an interval of 2 weeks. Reversible mesangial proliferative glomerulonephritis was induced by a single injection of mAb 1-22-3. All rats were sacrificed under ether anesthesia, and kidney specimens were removed. Glomeruli were isolated by the sieving method[8].

### Experimental protocol

*Experiment 1*

To compare the long-term kinetics of the reversible and the irreversible models, five rats each were treated with a single injection of mAb 1-22-3 or with two consecutive injections of mAb 1-22-3. All rats were sacrificed 2 months after the last mAb 1-22-3 injection. Urinary protein was measured by the Biuret method using bovine serum albumin as a standard[9]. The kidneys were perfused from the heart with ice-cold, sterilized phosphate-buffered saline (PBS). To quantify the mesangial matrix and the extension of IF staining, 30 full-sized glomeruli (80-100 mm in diameter) per rat were examined by an observer who was unaware of the experimental protocol. The degree of glomerular matrix expansion was expressed as 0 to 4 according to the percentage of each glomerulus occupied by the mesangial matrix detected with periodic acid-Schiff (PAS) staining basically according to the method described by Raij et al.[10]. The degree of the anti-type I collagen Ab (Chemicon International Inc., Temecula, CA, USA) staining and anti-$\alpha$-smooth muscle actin ($\alpha$SMA) Ab (Sigma Bioscience St. Louis, MO, USA) staining was evaluated basically according to the method described by Floege et al.[11].

*Experiment 2*

To compare the parameters in the acute phases of the reversible model with a single injection and the irreversible model with two consecutive injections, five rats each of the reversible and the irreversible models were sacrificed at 30 min, 2, 24 h, 3, 7, 10 and 14 d after the last injection. The kidneys were perfused from the heart with ice-cold, sterilized PBS, and half of the left kidney was used for histological studies. The remaining one and a half kidneys were used for preparation of the glomerular total RNA of individual rats. To compare the binding of injected mAb 1-22-3 and the complement fixation in glomeruli after the first and the second mAb 1-22-3 injection, the fluorescence intensity of mouse IgG 3, rat C 3, and C 9 were analyzed. The sections were stained with FITC-conjugated anti-rat C 3 (Cappel, West Chester, Pa., USA) or anti-rat C 9 (kindly donated by Dr. Matsuo, Nagoya University, Japan)[12] and FITC-conjugated anti-rabbit IgG (Dakopatts, Glastrup, Denmark). To compare the glomerular cell damage, the degree of mesangiolysis, and the immunofluorescence intensities of anti-$\alpha$SMA were scored. In each kidney sample, more than 30 full-sized glomerular cross sections were examined. Mesangiolysis was semi-quantitatively graded from 0 to +4 according to the method by Johnson et al.[13]. To compare the numbers of recruited PMN and monocyte/macrophages in glomeruli, the kidney sections were incubated with Rp-1 (kindly donated by Dr. Sendo, Yamagata University, Japan)[14], anti-pan monocyte ED 1 (Chemikon International Inc.) or anti-activated macrophage ED 3 (Chemikon International Inc.), and then respectively stained with FITC-conjugated anti-mouse IgM (Southern Biotechnology Associates, Birmingham, USA), FITC-conjugated anti-mouse IgG 1 (Southern Biotechnology Associates), and FITC-conjugated anti-mouse IgG 2 a (Southern Biotechnology Associates). The numbers of positive cells per full-sized glomerulus were counted in 30 glomeruli/rat. For dual labeling study of ED 1 and ED 3, TRITC-conjugated anti-mouse IgG 1 (Southern Biotechnology Associates) and FITC-conjugated anti-mouse IgG 2 a were used. To compare mRNA expressions of glomeruli for MCP-1 and platelet-derived growth factor (PDGF), a semi-quantitative reverse transcriptinal-polymerase chain reaction (RT-PCR) method was performed.

### Morphological and immunohistological studies

Light microscopy (LM) and immunofluorescence (IF) were performed as described previously[6,15,16].

### Semi-quantitative RT-PCR

RT-PCR was performed basically according to the method described previously[17]. Total RNA was extracted from isolated glomeruli with TRIZOL (Gibco BRL Life Technologies Inc., Gaithersburg, MD, U.S.A) as standard protocol. The final product

was air dried, dissolved in DEPC-treated water, and stored at −80℃. Sample RNA levels were quantitated by reading the absorbance at 260 nm. First strand complementary DNA (cDNA) was synthesized using the SuperScript Preamplification System (Gibco BRL Life Technologies Inc., Gaithersburg, MD, U.S. A). 5 μg RNA were reverse transcribed at 42℃ for 50 min. in 20 μl buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μg oligo (dT) and 50 U SuperScript II. Reactions were stopped by heat inactivation at 90℃ for 5 min, and chilled on ice. Subsequently, 3 μl of cDNA mixture were amplified by PCR in 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM each of 5' and 3' primers and 2.5 U AmpliTaq Gold.

The following rat sequences were used as primers:
(1)monocyte chemoattractant protein-1 (MCP-1): 5' primer: CTCTTCCTCCACCACTATGC,
3' primer: CTCTGTCATACTGGTCACTTC.
(2)BB-isomer of PDGF: 5' primer: GAAGCCAGTCTTCAAGAAGGCCAC, 3, primer: AACGGTCACCCGAGTTTGAGGTGT,
(3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'primer: CTCTACCCACGGCAAGTTCAA, 3'primer: GGATGACCTTGCCCACAGC.

After the initial denaturing step (95℃ 9 min.), the mixture was subjected to a round of denaturation (95℃ for 30 sac), annealing (60℃ (MCP-1, GAPDH), 57℃ (PDGF) for 30 sec), and extension (72℃ for 1 min.). The samples were subjected to the following cycle numbers: 25 (MCP-1, PDGF and GAPDH). The products of PCR were separated by electrophoresis on a 1.0% agarose gel with ethidium bromide staining. The band intensities were determined by image analysis using a Macintosh computer and the software of DENSITOMETRY (ATTO, Tokyo, Japan).

### Statistical analysis

All values are expressed as means±SD. Statistical significance (defined as $p<0.05$) was evaluated using the unpaired t test or Mann Whitney U test.

## RESULTS

### Experiment 1

The glomerular findings at 2 months after a single injection of mAb 1-22-3 showed near normalization. By contrast, glomerular alterations were still evident at 2 months after two consecutive injections of mAb.

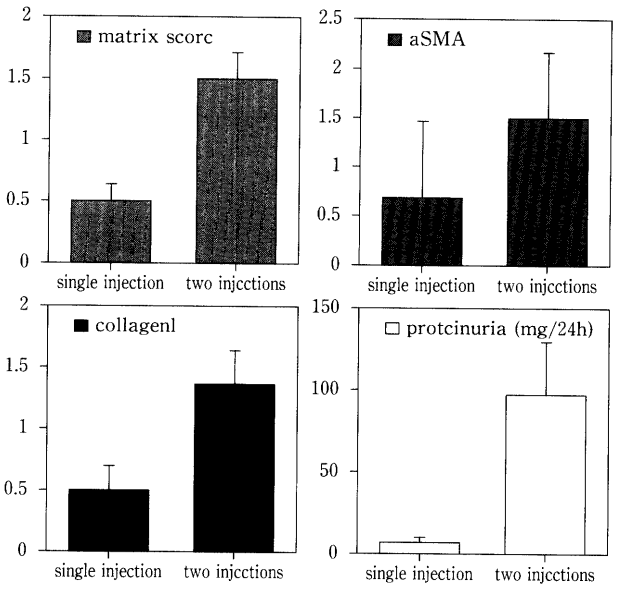


**Fig. 1.** Matrix score, IF staining scores of α-SMA, and collagen type I, and the amount of proteinuria at 2 months after disease induction. The mesangial alteration and proteinuria were normalized by 2 months in the reversible model caused by a single injection of mAb 1-22-3, whereas severe mesangial alterations and abnormal proteinuria were still detected in the irreversible model caused by two consecutive injection of mAb. (matrix score: $p<0.005$, collagen type I: $p<0.05$, proteinuria: $p<0.005$)

The matrix score and IF staining score of collagen type I at 2 months after two consecutive injections of mAb 1-22-3 were much higher than those after a single injection of mAb 1-22-3 (matrix score: 1.50±0.23 vs. 0.57±0.12, $p<0.005$, collagen type I score: 1.35±0.35 vs. 0.472±0.2, $p<0.05$). The matrix score and the IF score of collagen type I and αSMA were shown in Fig. 1. Abnormal proteinuria was still detected at 2 months after two consecutive injections of mAb (98.0±3.2 mg/24 h) (Fig. 1). Representative LM findings and IF finding of αSMA are shown in Fig. 2.

### Experiment 2

No differences in immunofluorescence intensity of bound mAb 1-22-3, rat C 3 or C 9 deposition were detected between the reversible and the irreversible models. Similar mesangiolysis occurred in both the reversible and irreversible models. Mesangiolysis scores on day 1 after the first and the second injection of mAb 1-22-3 were 1.5±0.71 and 1.1±0.55, respectively. However, pathological phenotypic

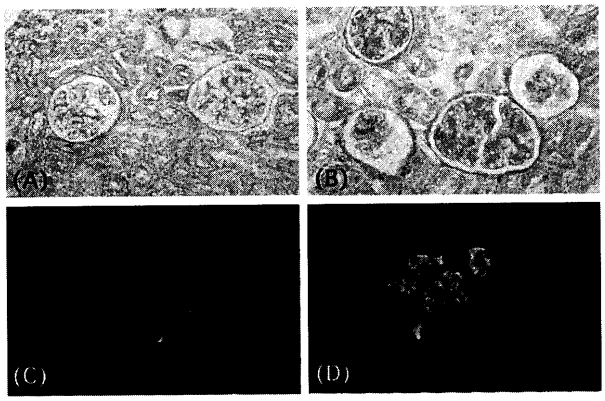


**Fig. 2.** Representative LM finding and the IF finding of $\alpha$-SMA at 2 months. PAS staining (**A**) and $\alpha$SMA IF staining (**C**) at 2 months after a single injection of mAb 1-22-3 showed that glomerular findings were almost normal. By contrast, glomerular alterations were still evident at 2 months after two consecutive injections of mAb (PAS staining (**B**) and $\alpha$SMA IF staining (**D**)). ×200)

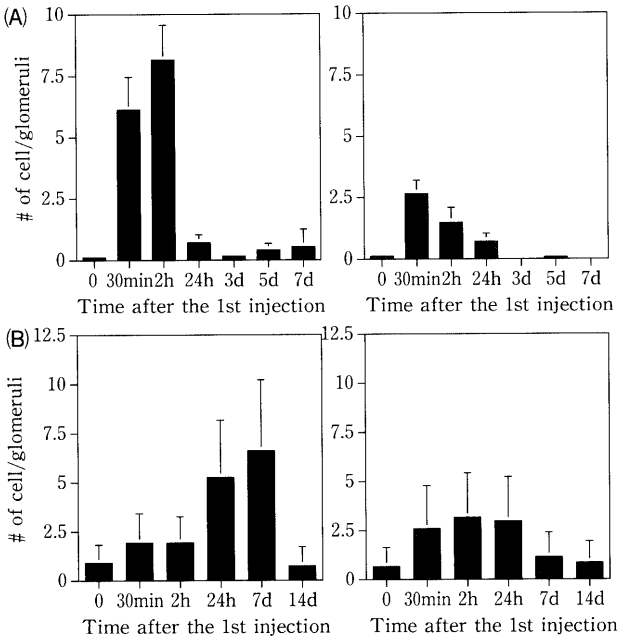


**Fig. 4.** Time course of PMN (**A**) and ED $1^+$ monocyte (**B**) infiltration into a glomerulus after the first and the second injection of mAb 1-22-3. The numbers of PMN and ED $1^+$ cell infiltrated in glomeruli were smaller after the second injection of mAb 1-22-3 than after the first injection. (*, $P<0.05$ versus the corresponding time after the first injection)

changes of mesangial cells began more rapidly and markedly after the second wave of mesangiolysis than after the first mesangiolysis. $\alpha$SMA staining

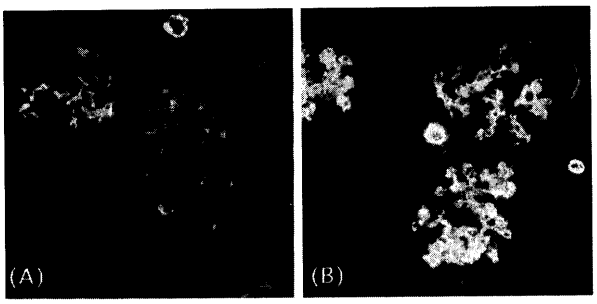


**Fig. 3.** Representative IF finding of $\alpha$-SMA at 10 days after the first (**A**) and the second (**B**) injection of mAb 1-22-3. Mesangial cell proliferation began more rapidly and markedly after the second injection of mAb 1-22-3. × 200)

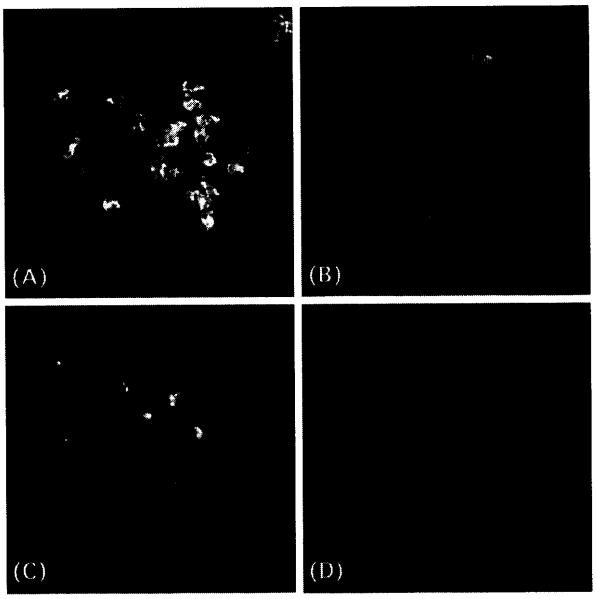


**Fig. 5.** IF findings of Rp 1 (**A, B**) at 2 h and ED 1 (**C, D**) at 7 days after the first and the second injection of mAb 1-22-3. The infiltration of PMN and ED $1^+$ monocytes/macrophages was greater in the reversible model caused by a single injection of mAb 1-22-3 (**A, C**). Smaller numbers of PMN and ED $1^+$ cells had infiltrated after the second mAb injection (**B, D**). ×200)

score on day 3 and day 10 after the first mAb 1-22-3 injection was $0.6\pm0.55$, and $2.1\pm0.45$, respectively. By contrast, $\alpha$SMA staining score on day 3 and day 10 after the second mAb 1-22-3 injection was $2.8\pm0.45$, and $4.0\pm0.0$. $\alpha$SMA findings on day 10 after the first (A) and the second (B) mAb 1-22-3 injection are shown in Fig. 3. The numbers of PMN and ED $1^+$ cell were smaller in the irreversible model than in the reversible model (PMN, 2 h after the first

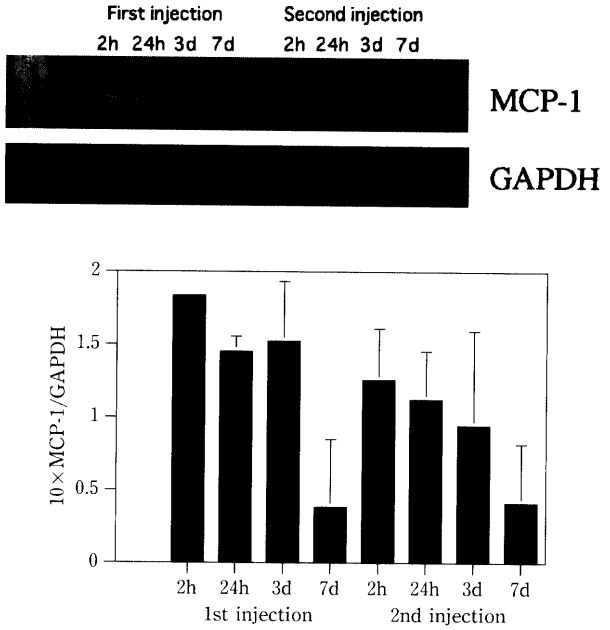


**Fig. 6.** Time course of MCP-1 mRNA expression in glomeruli after the first and the second injection of mAb 1-22-3. The data shown are representative semi-quantitative measurements made by RT-PCR. The characteristic agarose gel electrophoretic pattern from one of three independent experiments is shown (*top*). Ratios of the densitometric signal of MCP-1 to the internal control (GAPDH) are shown at the bottom.

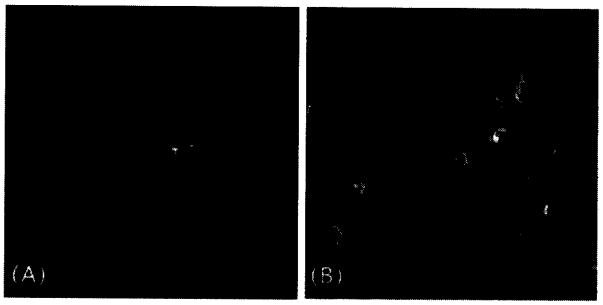


**Fig. 8.** IF findings of ED 3 at day 7 after the first (**A**) and the second (**B**) injection of mAb 1-22-3. The infiltration of ED $3^+$ cells was greater in the irreversible model caused by two consecutive injections of mAb 1-22-3. ×400)

injection vs. the second injection: 7.98±1.44 vs. 1.45±0.54, $p<0.005$, ED $1^+$ cells, day 7 after the first injection vs. the second injection: 6.48±3.05 vs. 1.18±1.65, $p<0.05$). The kinetics of the numbers of Rp $1^+$ PMN and ED $1^+$ monocyte/macrophages in glomeruli of the reversible and the irreversible models are summarized in Fig. 4. Representative IF

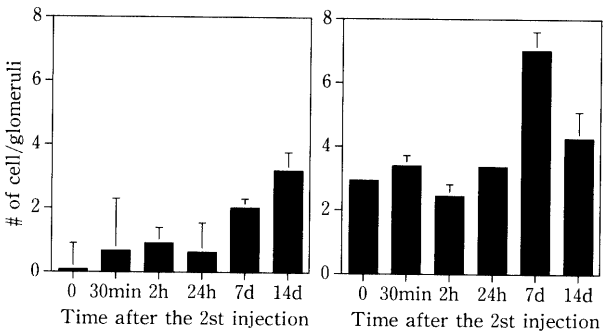


**Fig. 7.** Time course of ED $3^+$ cell infiltration into a glomerulus after the first and the second injection of mAb 1-22-3. The numbers of ED $3^+$ cells infiltrating into glomeruli were larger after the second injection of mAb 1-22-3 than after the first injection. (#, $P<0.005$ versus the corresponding time after the first injection)

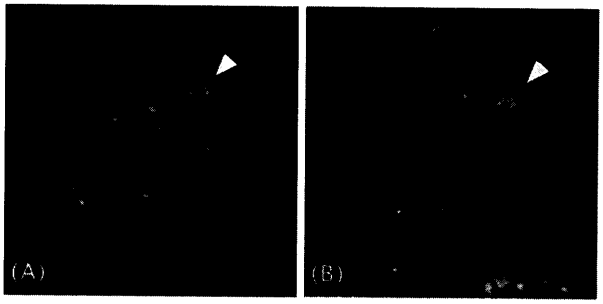


**Fig. 9.** Dual labeling IF findings with ED 3 (**A**) and ED 1 (**B**) in glomeruli 7 days after the second injection of mAb 1-22-3. The major portions of ED $3^+$ cells in glomeruli of the irreversible model were ED 1 negative, although some ED $3^+$ cells were ED 1 positive (*arrowheads*). ×400)

findings of Rp 1 and ED 1 in glomeruli of the reversible and the irreversible models are shown in Fig. 5. No MCP-1 mRNA expression was detected in normal rat glomeruli by RT-PCR analysis even in 35 PCR cycles. The semi-quantitative analysis of mRNA expression in pathologic glomeruli was performed in 25 PCR cycles. The optimal cycle number (25 cycles for MCP-1 and PDGF) was determined in a preliminary trial to be in the linear phase of amplification. MCP-1 mRNA expression increased just after the injection and had already decreased at day 7 after the first injection, when the number of ED $1^+$ cells peaked. MCP-1 mRNA expressions after the second injection were lower than those after the first injection. The representative findings of RT-PCR of MCP-1 are shown in Fig. 6 (Top). The mean values of the ratios to GAPDH of the 3 experiments are

shown in Fig. 6 (Bottom). By contrast, PDGF mRNA expression after the second injection was higher than that after the first injection. mRNA expression of PDGF on day 7 after the first mAb injection was 5.0±1.4 (10×PDGF expression/GAPDH expression), whereas that after the second mAb injection was 9.03±1.1. (PDGF mRNA expression of normal rat glomeruli was 4.02±0.32). The number of ED 3$^+$ cell in glomeruli was greater in the irreversible model than in the reversible model (day 7: 6.86±0.91 vs. 2.01±0.27, $p<0.005$). The kinetics of the number of ED 3$^+$ cells in glomeruli is shown in Fig. 7. Representative IF findings of ED 3 are shown in Fig. 8. A dual labeling study showed that major parts of the ED 3$^+$ cells in glomeruli of the irreversible model were ED 1$^-$ (Fig 9). By contrast, no ED 3$^+$ED 1$^-$ cells were detected in the reversible model at any time examined.

## DISCUSSION

We succeeded in developing the irreversible model by two consecutive injections of mAb 1-22-3 with an interval of 2 weeks. In this study, we confirmed that the severe mesangial alteration with abnormal proteinuria was still present at 2 months after inducing the disease (Figs 1 and 2). The treatment for induction of this irreversible model is simple, if compared with other methods that induce the irreversible alterations using OX 7 or polyclonal anti-thymocyte serum[18]. The reason why the irreversible alterations could be induced by only two injections of mAb 1-22-3 might be explained by its epitope specificity. mAb 1-22-3 has a reactivity to the limited site of the mesangial cell which faces the endothelial cell. mAb 1-22-3 is capable of inducing more marked mesangiolysis and mesangial cell proliferation by a single injection than other reported anti-Thy 1 antibodies[6,19,20]. Thus, the epitope recognized by mAb 1-22-3 is considered to be the critical site of the Thy 1.1 molecule, which regulates the mesangial cell function.

Morita et al. tried to determine the threshold for bringing about irreversible mesangial alterations, based on the hypothesis that repeated injuries cause cumulative damage which are beyond the threshold of the self limited alterations[18]. On the other hand, we hypothesized that the response of mesangial cell after the second injury is different from that seen after the first injury, and that the response after the second injury may sometimes be destructive and lead to irreversible changes.

In this study, we observed that pathological phenotypic changes of mesangial cells began more rapidly and markedly after the second wave of mesangiolysis than after the first mesangiolysis. We also observed that PDGF mRNA expression was higher after the second mAb injection than after the first mAb injection. These differences in the acute-phase responses are not considered to be derived from the difference in the initiation events, because mesangiolysis occurred similarly after the first and the second mAb 1-22-3 injection. These differences are considered to arise from different responses of mesangial cells and/or inflammatory cells. In this study, we then compared the kinetics of the recruitment of PMN and the subpopulations of macrophages. Several markers were reported to identify the subpopulation of macrophages such as ED 1, ED 2, ED 3 and Mar 3[21,22]. Neither ED 2$^+$ cells nor Mar 3$^+$ cells were detected in glomeruli at any time after the single injection or two consecutive injections of 1-22-3 (data not shown). Interestingly, the numbers of both Rp 1$^+$ PMN and ED 1$^+$ macrophages infiltrating into glomeruli after the second mAb 1-22-3 injection were smaller than those seen after the first injection. Some reports have shown that the macrophage plays an important role in the development of glomerulosclerosis in several kinds of experimental models[23,24]. On the other hand, it has been pointed out that ED 1$^+$ cells have a role in the rearrangement of capillaries because they are scavengers[25,26,27]. Some reports showed that apoptotic cells were detected in glomeruli in Thy 1.1 nephritis[28,29]. It is thought that macrophages play a role in phagocytosis of the apoptotic leukocyes that infiltrate into glomeruli just after 1-22-3 injection. Fadoc et al. showed with an in vitro study that macrophages that had ingested apoptotic neutrophils expressed an anti inflammatory phenotype[30]. Futamura et al. reported that ED 1$^+$ cells inhibit mesangial proliferation[31]. The findings in this study suggest that PMN and ED 1$^+$ cells are not essential for the induction of the irreversible glomerular alterations. We also detected here that MCP-1 mRNA expression was much lower 2 h after the second mAb injection than after the first mAb injection. Stahl et al. reported that the mesangial cell produced MCP-1 in Thy 1.1 nephritis[32]. Our findings also suggest that glomerular MCP-1 was produced by mesangial cells and not by monocytes in anti-Thy 1.1 nephritis, because glomerular MCP-1 mRNA expression was detected 2 h after the first mAb injection, when ED 1$^+$ cells were not yet present in the glomeruli. The lower MCP-1 expression after the second mAb injection might result in fewer ED 1$^+$ cells infiltrating the glomeruli. These observations suggest that mesangial cells

produced PDGF or some cytokines to promote mesangial proliferation instead of producing the MCP-1 or some chemotactic factors to attract PMN or ED $1^+$ macrophages in the second wave of mesangiolysis.

In contrast with ED $1^+$ cells, the ED $3^+$ cells infiltration into glomeruli was more marked after the second mAb 1-22-3 injection than after the first injection. This finding might suggest that the ED $3^+$ cell was recruited by a chemokine other than MCP-1. ED 3 was originally reported to be confined to lymphoid organs[21]. ED 3 is reported to bind to the sialoadhesion antigen on macrophages[33]. It is understood that the ED $3^+$ cell is an activated macrophage that actively produces some cytokines, whereas the ED $1^+$ cell is thought to be a pan-monocytes/macrophage[21]. However, it should be noted that the number of the ED $3^+$ cells was larger than that of ED $1^+$ cells in the acute phase after the second 1-22-3 injection. This finding means that ED $3^+$ ED $1^-$ cells were recruited into the glomeruli. A dual labeling IF study clearly showed that ED $3^+$ ED $1^-$ cells were recruited in glomeruli in the acute phase after the second 1-22-3 injection (Fig. 8), whereas ED $3^+$ ED$^-$ cells were not detected at any time in the reversible model induced by a single injection of 1-22-3. Although the characteristics of this subpopulation of macrophages have not been completely understood, it is suggested that ED $3^+$ cells participate in the pathogenesis of inflammatory disease[34,35,36]. The mechanism of the recruitment of ED $3^+$ cells also remains unclear. The mesangial cells might produce some chemotactic factors to attract ED $3^+$ into the glomeruli. On the other hand, the ED $1^+$ ED $3^-$ cell might change its phenotype to ED $1^-$ ED $3^+$ cells in the glomeruli. Another possibility is that ED $3^+$ cells proliferate in the glomeruli. Whatever the mechanism of the recruitment of ED $3^+$ cells into glomeruli might be, ED $3^+$ cells represent an important candidate for acting on the mesangial cell to lead to irreversible mesangial alterations.

In conclusion, the numbers of PMN and ED $1^+$ macrophage were smaller in the irreversible model than in the reversible model, whereas the number of ED $3^+$ cells recruited into the glomeruli was larger in the irreversible model. ED $3^+$ ED $1^-$ macrophages may participate in the pathogenesis of irreversible mesangial alterations.

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