Clinical and Experimental Rheumatology 2005; 23: 239-242.

Monocyte chemoattractant protein-1 activates a regional Th1 immunoresponse in nephritis of MRL/lpr mice

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Received on September 10, 2004; accepted in revised form on January 4, 2005.

Key words: Monocyte chemoattractant protein-1(MCP-1), MRL/lpr mice, Th1/Th2 balance, SLE.

ABSTRACT

Objective. *Monocyte chemoattractant protein-1 (MCP-1) is upregulated and recruits and activates inflammatory cells in nephritis of MRL lpr mice. It has been shown that anti-MCP-1 gene therapy is specifically effective in nephritis, while it was apparent that an imbalance towards Th1 predominance accelerates nephritis in MRL/lpr mice. The aim of this study was to clarify whether blockade of the MCP-1 signal by anti-MCP-1 gene therapy influences the Th1/Th2 balance in MRL/lpr mice.*

Method. *An NH2-terminal deletion mutant of the MCP-1 gene (7ND) was injected into the skeletal muscles of MRL/lpr mice with advanced stage nephritis to suppress MCP-1 and its receptor (CCR2) signaling pathway. We evaluated the local tissue production of cytokines in splenocytes and microdissected infiltrating cells within the glomeruli or interstitium.*

Result. *Although the production of cytokines in splenocytes was not influenced by anti-MCP-1 gene therapy, kidney glomeruli IL-12 mRNA production and interstitium-infiltrating cell production of IL-12 and IFN-γ mRNA were significantly reduced.*

Conclusion. *The blockade of MCP-1 gene therapy does not influence helper T cell polarization, but acts directly on the regional Th1 immunoreaction in MRL/lpr mice.*

Introduction

MRL lpr mice are a particularly valuable model of human systemic lupus erythematosus (SLE). Nephritis in MRL lpr mice consists of glomerular, interstitial, and vascular components mediated by infiltrating macrophages and T cells, and shows similar findings to human diffuse proliferative lupus nephritis (DPLN). An imbalance towards Th1 predominance accelerated nephritis in MRL lpr mice, and the presence of Th1 cytokines, such as IFN-γ and IL-12, was linked to the severity of glomerulonephritis (1). IFN-γ gene deficiency (2), IFN-γ R gene deficiency (3) and treatment with a cDNA encoding IFN-γ R/Fc to block the IFN-γ signal (4) ameliorated glomerulonephritis in MRL lpr mice, and apparently reduced the production of IgG2a which is an Ig subclass associated with the Th1 phenotype. Conversely, Th2 cytokine IL-10-deficient MRL lpr mice developed severe lupus with an earlier appearance of skin lesions, increased lymphadenopathy, and more severe glomerulonephritis, and showed higher mortality than their IL-10-intact littermate controls (5). However, Th2 cytokine IL-4-deficient MRL lpr mice showed reduced lymphadenopathy and glomerulonephritis, as did IFN-γ deficient MRL lpr mice. These mice produced significantly less IgG1 and IgE serum immunoglobulins, but maintained comparable levels of IgG2a, IgG2b and autoantibodies in comparison with controls (6). Although these conflicting findings may have resulted from differences in the experimental approaches, it is suggested that Th1 and Th2 responses play prominent roles in the pathogenesis of lupus-associated tissue injury.

In the chemokine family, the monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant for monocytes, T cells, and natural killer cells (7-9). Tissue expression of MCP-1 in MRL lpr mice has been reported to be upregulated (10, 11), and MCP-1 may also be responsible for inflammation in nephritis (12). In addition, the fact that MCP-1 deficiency resulted in the improvement of glomerulonephritis in MRL lpr mice (13) and that treatment with a MCP-1 antagonist results in reduction of arthritis in MRL lpr mice, suggests that MCP-1 may be responsible for inflammation in tissues during the progress of an autoimmune disease (14). It remains, however, controversial whether a blockade of the MCP-1/CCR-2 signaling pathway influences Th cell polarization.

We have shown that the application of anti-MCP-1 gene therapy by a mutant gene (7ND) is effective in treating nephritis in MRL/lpr. Although RT-PCR examination and immunohistologic staining of kidney tissues showed that 7ND transfection did not influence intrinsic MCP-1 transcription or production, histological findings of the kidneys in treated mice which received more than four injections of 7ND showed protection against renal injury. The

Table I. Sequences and amplicon sizes of primers and probes that were used for real-time quantitative PCR.

Products	Oligonucleotide name	Amplicon size	Sequences
IFN-γ	Forward		5'-CTCTTTCCATTTTTGCATCAAGTTC-3'
	Probe	135bp	5'-CTCCGCCCACTCATCTTCGGTGG-3'
	Reverse		5'-CCCATCTTTAACCGATCTAGAGTCA-3'
IL-12	Forward		5'-AGACCCTGCCCATTGAACTG-3'
	Probe	81bp	5'-TCAGGGACATCATCAAACCAGACCCG-3'
	Reverse		5'-CTGACCTCCACCTGTGAGTTCTT-3'
IL-18	Forward		5'-GGCTGCCATGTCAGAAGACTCT-3'
	Probe	135bp	5'-CCTGGAATCAGACAACTTTGGCCGACT-3'
	Reverse		5'-TTCCGTATTACTGCGGTTGTACA-3'
IL-4	Forward		5'-TCTCATGGAGCTGCAGAGACTCT-3'
	Probe	184bp	5'-TCGATAAGCTGCACCATGAATGAGTCCA-3'
	Reverse		5'-TCCAGGAAGTCTTTCAGTGATGTG-3'
IL-10	Forward		5'-AGAGAAGCATGGCCCAGAAAT-3'
	Probe	197bp	5'-CGCTGTCATCGATTTCTCCCCTGTGA-3'
	Reverse		5'-TGGCCTTGTAGACACCTTGGT-3'
GAPDH	Forward		5'-GCAGTGGCAAAGTGGAGATTG-3'
	Probe	95bp	5'-CCATCAACGACCCCTTCATTGACCTC-3'
	Reverse		5'-ATTTGCCGTGAGTGGAGTCAT-3'

numbers of macrophages and T cells in the interstitium and the number of macrophages in the glomeruli were significantly reduced in 7ND-treated MRL-lpr mice. (15).

In this study we analyzed the influence of blockade of the MCP-1/CCR2 signaling pathway by anti-MCP-1 gene therapy on the Th1 immunoresponse in MRL/lpr mice.

Materials and methods

Mice and gene transfer

In a procedure described in a previous study, human 7ND cDNA, which codes mutant human MCP-1 lacking NH_2-terminal amino acids 2-8, was inserted in the pcDNA3 expression vector plasmid and amplified. Treatments were initiated in 16-week-old MRL/lpr mice whose kidneys showed an advanced stage of glomerulonephritis. Under anesthesia by intra-peritoneal injection of pentobarbital, one group (7ND-treated mice) was injected with the 7ND gene [50μg in 50μl phosphate-buffered saline (PBS)], and another group (empty plasmid-treated mice) was injected with empty plasmid (50μg in 50μl PBS) in the femoral muscle using a 27-gauge needle. The control group mice (PBS-treated mice) were injected with 50μl PBS. Immediately after the injections into the femoral muscle, *in vivo* electroporation was performed using an electric pulse generator CUY201 (Nepagene Co. Ltd, Chiba, Japan). The three groups received injections at biweekly intervals until they reached 28 weeks of age.

Laser capture microdissection (LCM) and RNA isolation

Glomeruli and infiltrating cells in the interstitium were microdissected from consecutive tissue sections as described (16) using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Total RNA was extracted

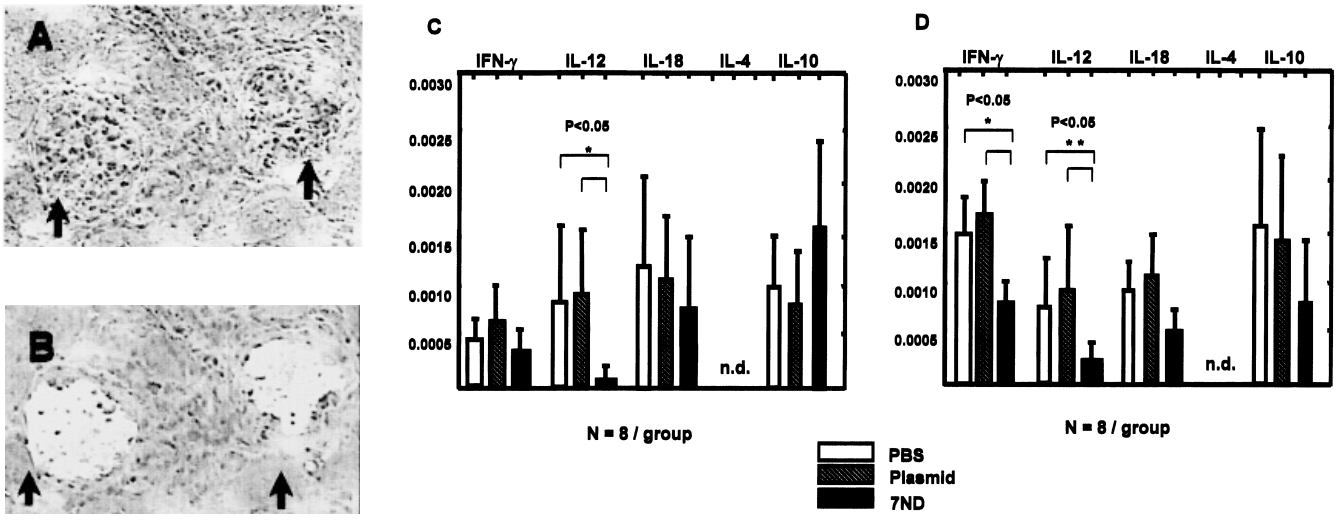

Fig. 1. Laser capture microdissection (LCM) of glomeruli in kidney sections. Tissue sections of the same specimen are shown before (**A**) and after (**B**) LCM. **Arrows** indicate glomeruli. (**C**) Quantities of cytokine mRNA expressions in glomeruli and (**D**) interstitium of kidney sections from PBS-treated, empty plasmid-treated and 7ND-treated MRL lpr mice at 2 months after the initial treatment. Template mRNAs were prepared from 100 microdissected glomeruli. The values are the mRNA product ratio against GAPDH mRNA. (**C**) The relative IL-12 mRNA expressions in the glomeruli from PBS-treated, empty plasmid-treated and 7ND-treated mice were 0.0010 ± 0.00070, 0.0011 ± 0.00051 and 0.00032 ± 0.00046, respectively. There was a significant difference among the three groups ($P < 0.05$). (**D**) Relative IL-12 and IFN-γ mRNA expression in the interstitium from PBS-treated, empty plasmid-treated and 7ND-treated mice were: IL-12: 0.00080 ± 0.00050, 0.00092 ± 0.00055 and 0.00023 ± 0.00021, and IFN-γ: 0.00150 ± 0.00044, 0.00175 ± 0.00039 and 0.00084 ± 0.00043, respectively. There were significant differences among the three groups ($P < 0.05$).

from the captured cells using a Picopure RNA Isolation Kit (Arcturus Engineering, Mountain View, CA).

Real-time quantitative PCR and Taqman primers and probes

We assessed the transcription levels of IFN-γ, IL-12, IL-18, IL-4, and IL-10 relative to GAPDH in the kidney and spleen tissues. Reverse transcription reactions and TaqMan-PCRs were performed according to the manufacturer's instructions (Applied Biosystems Japan Ltd., Tokyo, Japan). Sequence-specific amplification was detected with an increased fluorescent signal of 5-[(N-(3'-diphenylphosphinyl-4'-methoxycarbonyl) phenylcarbonyl) aminoacetamido] fluorescein (FAM) during the amplification cycles using an ABI prism 7700 sequence detection system (PerkinElmer Japan Co. Ltd., Yokohama, Japan). Oligonucleotide primers and probes were designed using the Primer Express program and synthesized (Applied Biosystems Japan Ltd., Tokyo, Japan). These sequences (5' to 3') are listed in Table I.

Statistical analysis

Data are expressed as the mean ± SE. Statistical differences were compared by Mann-Whitney U-tests. A level of $P < 0.05$ was considered statistically significant.

Results

7ND treatment had little influence on systemic inflammatory cytokine production. However, kidney glomeruli IL-12 mRNA production and interstitium-infiltrating cell production of IL-12 and IFN-γ mRNA were significantly reduced by 7ND injections. Comparative measurements of mRNA expression for Th1/Th2-associated cytokines (IFN-γ, IL-12, IL-18, IL-4, IL-10) in splenocytes from the three groups of mice revealed that there were no significant differences in IFN-γ, IL-18, or IL-10 transcription (data not shown). Nor could we detect IL-4 or IL-12 mRNA.

To investigate whether 7ND treatment had an effect on local tissue production of cytokines, we used laser capture microdissection to study images of infiltrating cells in the glomeruli (Fig. 1 A, B) and interstitium, and then isolated their mRNA. In the glomeruli, IL-12 transcripts were greatly reduced in the 7ND-treated mice, although no significant difference in IFN-γ mRNA was observed (Fig. 1C). However, the signal for the production of cells infiltrating the interstitium revealed reduced message levels for both IFN-γ and IL-12 (Fig. 1D). Three groups of mice showed comparable levels of IL-18 and IL-10 expression but failed to express detectable amounts of IL-4 mRNA.

Discussion

It remains controversial whether blockade of the MCP-1 and CCR-2 signaling pathway influences Th cell polarization. MCP-1 can stimulate IL-4 production (17) and its overexpression is associated with defects in cell-mediated immunity (18). Gu *et al*. demonstrated that lymph node cells from MCP-1-deficient mice immunized with trinitrophenol-derivatized ovalbumin (TNP-Ova) synthesized extremely low levels of IL-4, IL-5 and IL-10, but normal amounts of IFN-γ and IL-2. It was also observed that an immunoglobulin subclass switch was not accomplished by MCP-1 deficiency (19). These results indicate that MCP-1 influences Th cell polarization to Th2. On the other hand, CCR2-deficient mice presented a contradictory phenotype to MCP-1-deficient mice. Pulmonary granuloma in presensitized CCR2-deficient mice induced by embolization with beads coupled to purified protein derivative (PPD) of mycobacterium bovis were significantly smaller in size than that of wild-type littermates. CCR2-deficiency in mice was accompanied by a dramatic decrease in the level of IFN-γ in the draining lymph nodes. Production of IFN-γ was also decreased in PPD-sensitized splenocytes from CCR2-deficient mice, and in naïve splenocytes activated by concanavalin A (20). These results indicate that CCR2 influences Th cell polarization to Th1.

In this work several kinds of cytokine gene expressions in the spleen, which reflect immunological responses against systemic inflammation, revealed that mRNA expression of IFN-γ, IL-10 and IL-18 in 7ND-treated mice was comparable with expression in PBS-treated mice or empty plasmid-treated mice. These results indicate that 7ND did not influence systemic helper T cell polarization and autoimmunity. In fact, 7ND did not alter the amount of anti-ds DNA antibody (15). However, Th1 cytokine messages in the localized inflammatory area in the kidney were significantly reduced in 7ND-treated mice. The reduction in the IL-12 message in the glomeruli was reflected the inhibition of macrophage infiltration and activation, and the reduction in both IL-12 and IFN-γ messages in the interstitium reflected the inhibition of Th1 cells and macrophage infiltration and activation. These results are consistent with those documented in MCP-1-deficient MRL-lpr mice (13). Therefore, we suggest that the blockade of MCP-1 has little effect on helper T cell polarization, but acts directly on leukocytes to inhibit their migration and activation in the kidney of MRL lpr mice.

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