

Downregulation of Monocyte Chemoattractant Protein-1 Involving Short Interfering RNA Attenuates Hapten-induced Contact Hypersensitivity

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Contact hypersensitivity (CHS) is a common skin disease, presenting clinically as allergic contact dermatitis. At inflammatory sites in a typical CHS model in the mouse ear, elevated expression of monocyte chemoattractant protein-1 (MCP-1) has been reported. MCP-1 is a potent chemotactic factor for many types of leukocytes including monocytes/macrophages and T cells. In this study, we aimed at developing a therapy for CHS involving RNA interference targeting MCP-1. A short interfering RNA (siRNA) to mouse MCP-1 successfully inhibited the secretion of MCP-1 by a fibroblastic cell line, L929, and RAW 264.7 cells derived from macrophages, and strikingly suppressed ear swelling in a CHS model. The siRNA systemically administered inhibited the infiltration of both monocytes/macrophages and T cells in the CHS model. Atelocollagen was used in this therapy as a delivery reagent for siRNA into the animal body. Atelocollagen facilitated the incorporation of the siRNA into macrophages/monocytes and fibroblasts, which vigorously secrete MCP-1 protein at inflammatory sites in CHS. This therapy had no adverse effects such as induction of interferon, or liver or renal damage. Our data indicate that the systemic delivery of siRNA targeting MCP-1 is a potent therapeutic strategy for CHS treatment.

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INTRODUCTION

Chemokines play pivotal roles in the chemotaxis and activation of different subsets of leukocytes.¹ Human monocyte chemoattractant protein-1 (MCP-1), which is a prototype chemokine, was originally purified and cloned from tumor cell lines as a monocyte-specific chemoattractant.^{2,3} MCP-1 was found to be a homolog of the product of the JE gene, a platelet-derived growth factor inducible gene cloned from murine fibroblasts.⁴ MCP-1 attracts not only monocytes but also T cells^{5,6} and NK cells,⁷ and induces histamine release from basophils.⁸ Various types of cells such as

monocytes, T cells, mast cells, endothelial cells, fibroblasts, and keratinocytes secrete MCP-1 upon inflammatory stimuli.⁹ MCP-1 is overexpressed in a variety of pathological conditions including atherosclerosis,¹⁰ rheumatoid arthritis,¹¹ glomerulonephritis,¹² psoriasis,¹³ and allergic contact dermatitis.^{14,15}

Hapten-induced contact hypersensitivity (CHS) is one of the most common inflammatory diseases of the skin, presenting clinically as allergic contact dermatitis. The CHS response consists of two distinct phases. In the sensitization phase, haptens applied to the skin are captured by skin dendritic cells including epidermal Langerhans cells. Activated Langerhans cells migrate to regional lymph nodes and activate antigen-specific T-cell precursors. The elicitation phase of CHS is induced by re-exposure to the same hapten. In the elicitation phase, epicutaneously applied haptens activate resident skin cells, such as keratinocytes, fibroblasts, endothelial cells and mast cells; these cells release proinflammatory substances including cytokines and chemokines.^{16,17} This leads to the rapid recruitment of monocytes and T cells, which are additional sources of chemokines, and to the constitution of a local inflammatory response which peaks at 24–48 hours after hapten challenge. The expression of MCP-1 has been detected preceding infiltration of monocytes and lymphocytes in the early stage of the elicitation phase, and is continuous during the elicitation phase.^{14,15} Neutralizing antibodies to MCP-1 results in inhibition of T-cell and monocyte recruitment and inflammation in cutaneous delayed-type hypersensitivity.¹⁸ MCP-1 transgenic mice show enhanced inflammatory responses on CHS together with an increase in infiltrating dendritic cells.^{19,20} Judging from these results, MCP-1 is a suitable molecular target for treating CHS.

RNA interference has revealed highly sequence-specific gene silencing in which short pieces of double-stranded RNA, *i.e.*, short interfering RNA (siRNA), suppress the expression of genes exhibiting sequence homology.^{21,22} There is currently an intense research effort aimed at developing siRNAs as therapeutics for many kinds of diseases.²³ In this study, we adopted the RNA interference strategy to downregulate the MCP-1 gene, aiming to develop a novel therapy for CHS. We used the atelocollagen-mediated systemic

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delivery method²⁴ for siRNA. Atelocollagen stabilizes administered siRNAs in both the bloodstream and tissues,^{25–27} and efficiently introduces them to an inflammatory region in the animal body. Here we show a striking therapeutic effect of systemically delivered MCP-1 siRNA on CHS.

RESULTS

Effects of synthetic siRNAs on the expression of MCP-1 from L929 and RAW 264.7 cells

We examined five chemically synthesized siRNAs for targeting murine MCP-1, as described in **Supplementary Figure S1**. Of the five siRNAs, MCP-1 siRNA no.5 most potently suppressed the secretion of MCP-1 by mouse fibroblasts; L929 cells, after transfection with LipofectAMINE-PLUS reagent. Enzyme-linked immunosorbent assay (ELISA) for MCP-1 revealed that MCP-1 siRNA no. 5 suppressed MCP-1 production to 1% of the level in the control cultures (**Figure 1a**). Based on the results, we selected MCP-1 siRNA no. 5 (termed MCP-1 siRNA) as the most highly functional one, and subjected it to further study. Scrambled siRNA (termed MCP-1 siRNA-SCR) as a control showed no effect (**Figure 1b**). The RNA interference effect of MCP-1 siRNA was further examined in a murine macrophage cell line, RAW 264.7, after nucleofection with an Amaxa electroporation system, as described under Materials and Methods. MCP-1 siRNA inhibited MCP-1 production to 30% of the level in the control cultures electronically pulsed but without siRNA (**Figure 1b**). An assay involving a fluorescein isothiocyanate-labeled siRNA and fluorescence-activated cell sorting analysis revealed that the transfection efficiency for RAW 264.7 cells was 30% with lipofection and 70% with the Amaxa electroporation system. L929 cells exhibited over 95% transfection efficiency with lipofection (data not shown). Therefore, the lower efficiency of MCP-1 suppression in RAW 264.7 cells may reflect the

low transfection efficiency. MCP-1 siRNA decreased the MCP-1 messenger RNA level when compared with MCP-1 siRNA-SCR in both L929 and RAW 264.7 cells, whereas the expression of genes related to MCP-1 (MCP-2, MCP-3 and MCP-5, ref. 9) did not change upon transfection of MCP-1 siRNA (**Figure 1c**).

Elevated MCP-1 expression in ears with CHS

2,4-Dinitrofluorobenzene (DNFB) induced ear swelling in sensitized mice, whereas naive mice did not exhibit ear swelling 24 hours after the ear challenge (**Figure 2a**, left panel). ELISA for MCP-1 revealed a DNFB-induced expression of MCP-1 in the ears of sensitized and naive mice 4 hours after the ear challenge. MCP-1 production significantly increased in a time-dependent manner in the challenged ears of sensitized mice ($P < 0.001$), whereas only a small amount of MCP-1 was observed in those of naive mice at 24 hours (**Figure 2a**, right panel). Olive oil/acetone without DNFB did not induce ear swelling or MCP-1 production in sensitized mice (control in **Figure 2a**). We confirmed the expression profile of MCP-1 messenger RNA in the CHS model by reverse transcriptase polymerase chain reaction (RT-PCR) (**Figure 2b**). Histological examination of sensitized mice demonstrated edema and severe inflammatory cell infiltration in the challenged ear skin (**Figure 2c**). Immunohistochemical analysis showed marked increases in MCP-1 expression by the cells with fibroblastic morphology and mononuclear cells in the dermis of the challenged ears (**Figure 2c**). These pathological changes progressed in a time-dependent manner with a peak at 24 hours. We observed that keratinocytes and microvessels showed only weak expression of MCP-1 at each time point (**Figure 2c**) and immunohistochemically confirmed the secretion of MCP-1 by fibroblasts with two antibodies to fibroblast-specific protein-1 (ref. 28) and MCP-1 (data not shown), in accordance with the previous report.²⁹

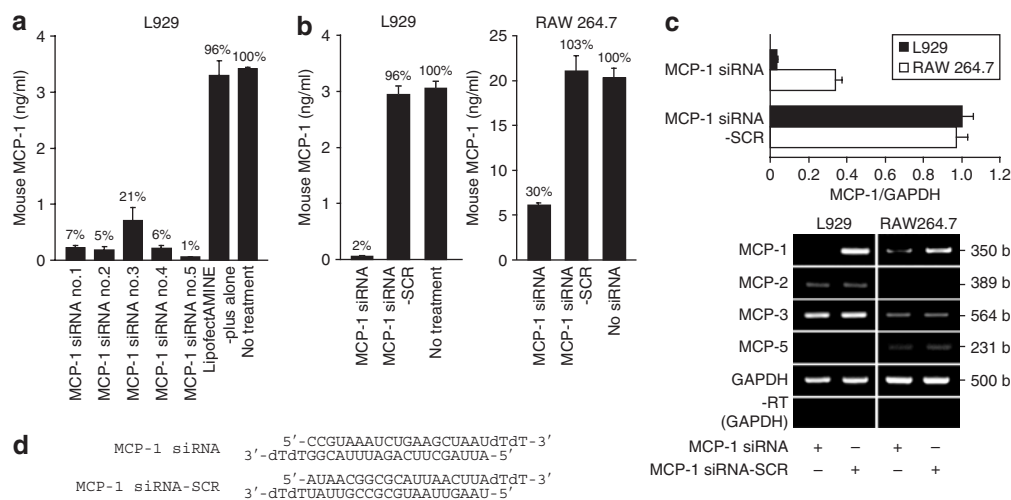


Figure 1 Decrease in monocyte chemoattractant protein-1 (MCP-1) expression in L929 and RAW 264.7 cells transfected with various short interfering RNAs (siRNAs). **(a)** L929 cells were transfected with MCP-1 siRNA nos. 1, 2, 3, 4, and 5, LipofectAMINE-PLUS alone, or no siRNA, respectively. **(b)** L929 and RAW 264.7 cells were transfected with MCP-1 siRNA, MCP-1 siRNA-SCR, or no siRNA. In both **a**, **b**, the MCP-1 concentrations in the conditioned media were determined by enzyme-linked immunosorbent assay. The results represent the means $\pm$ SD ($n = 4$ dishes). **(c)** Total RNA was extracted from L929 and RAW 264.7 cells transfected with MCP-1 siRNA or MCP-1 siRNA-SCR, and processed for quantitative real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) to determine MCP-1 expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The expression of MCP-1, MCP-2, MCP-3, or MCP-5 was also examined using semiquantitative RT-PCR. –RT, no reverse transcriptase. The predicted size of each PCR product is shown. **(d)** Structures of MCP-1 siRNA and its scrambled control (MCP-1 siRNA-SCR) are shown.

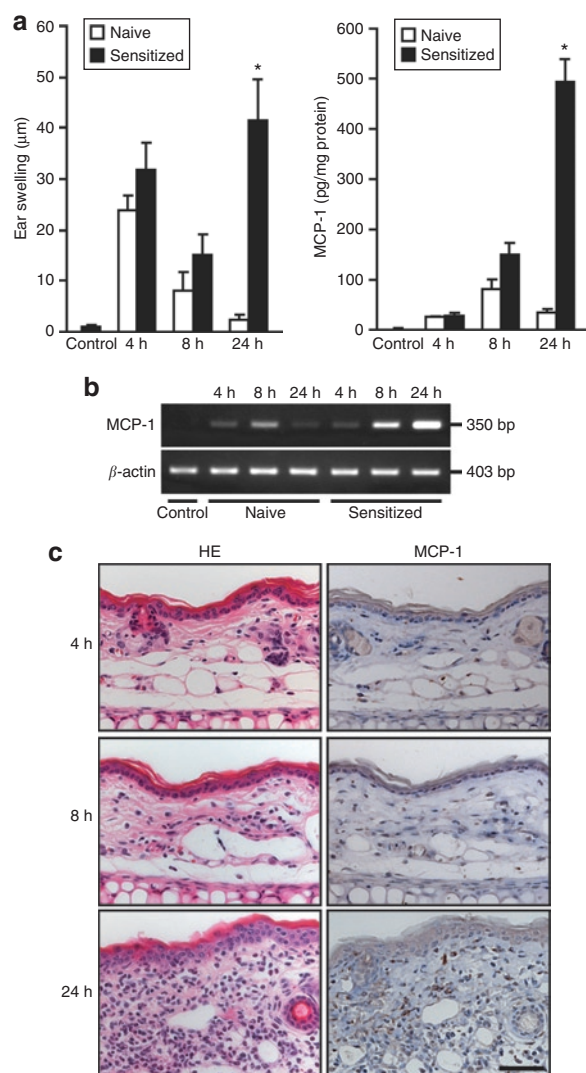


Figure 2 Inflammatory response and monocyte chemoattractant protein-1 (MCP-1) expression in ears on contact hypersensitivity induced by 2,4-dinitrofluorobenzene (DNFB). **(a)** Sensitized and naive mice were challenged with 0.2% DNFB [white bars, non-sensitized mice (naive); black bars, sensitized mice]. Sensitized mice challenged with olive oil/acetone without DNFB were used as a control. Left panel, the thickness of the challenged ears was measured 4, 8, and 24 hours after the ear challenge. The results represent the means $\pm$ SE ($n = 6$). Right panel, amount of MCP-1 measured by enzyme-linked immunosorbent assay. The results represent the means $\pm$ SE ($n = 5$). *, $P < 0.001$ versus naive mice at the same time point. **(b)** Experiments were performed as in **a**. Total RNA was extracted from the excised ears (at 4, 8, and 24 hours) and the expression of MCP-1 was assessed by semiquantitative reverse transcriptase polymerase chain reaction (reverse transcriptase PCR). β -actin was used as a control. The predicted size of each PCR product is shown. **(c)** Histological sections of the challenged ears (at 4, 8, and 24 hours) of sensitized mice were stained with hematoxylin and eosin (HE) and anti-mouse MCP-1 antibody. Bar, 50 μ m.

Therapeutic effect of MCP-1 siRNA mixed with atelocollagen on CHS

To determine the therapeutic effectiveness of MCP-1 siRNA on the CHS response, treatment with MCP-1 siRNA mixed with atelocollagen was examined, as summarized in **Figure 3a**. We performed systemic treatment involving a single injection or two

injections of MCP-1 siRNA with atelocollagen, because the MCP-1 expression in the challenged ears increased in a time-dependent manner (**Figure 2**). Mice with ear challenge were intravenously injected with 6.25, 25 or 50 μ g MCP-1 siRNA or MCP-1 siRNA-SCR mixed with atelocollagen. The mice in the single-injection group were injected 30 minutes after the ear challenge, and those in the two-injection group, 30 minutes and 8 hours later (**Figure 3a**). We measured the ear thickness, and excised the ears 24 hours after the ear challenge. MCP-1 siRNA with atelocollagen significantly suppressed the ear swelling in both groups, *i.e.*, with one and two injections, whereas MCP-1 siRNA-SCR, atelocollagen alone and phosphate-buffered saline did not show such an effect at all ($P < 0.01$, **Figure 3b**). The inhibitory effect was dependent on the dose of MCP-1 siRNA and the injection time(s). The decrease in the MCP-1 content in the ears treated with MCP-1 siRNA was correlated with the injected dose of MCP-1 siRNA and the injection time(s) (**Figure 3c**). The decreased messenger RNA level of MCP-1 in the treated ears was shown by quantitative real-time RT-PCR (**Figure 3d**). Histological examination revealed that ear swelling and inflammatory cell infiltration were inhibited in the challenged ears treated with MCP-1 siRNA compared with those treated with MCP-1 siRNA-SCR (**Figure 3e**). Immunohistochemical analysis proved that MCP-1 expression in the challenged ears treated with MCP-1 siRNA was less than in those treated with MCP-1 siRNA-SCR (**Figure 3e**). Furthermore, mice were injected once 8 hours after the ear challenge with 50 μ g of MCP-1 siRNA or MCP-1 siRNA-SCR mixed with atelocollagen as shown in **Supplementary Figure S2a**. Although the single injection at 8 hours after the challenge with MCP-1 siRNA along with atelocollagen suppressed the ear swelling ($P < 0.05$, **Supplementary Figure S2b**), the therapeutic effect was weaker than that of the single injection at 30 minutes after the challenge. Another functional siRNA (MCP-1 siRNA no. 2) with atelocollagen gave us similar therapeutic effects, showing that our therapeutic effects are specific ones and not due to off target effects. Thus, two injections of MCP-1 siRNA no. 2 (50 μ g $\times$ 2) with atelocollagen significantly suppressed both the ear swelling and MCP-1 expression ($P < 0.05$), whereas MCP-1 siRNA no. 2-SCR with atelocollagen did not (**Supplementary Figure S3**).

Infiltration of macrophages and T cells was reduced in the ears treated with MCP-1 siRNA

The infiltration of macrophages in the challenged ears treated with MCP-1 siRNA (50 μ g $\times$ 2) was significantly less than in those treated with MCP-1 siRNA-SCR (**Figure 4a** and **b**). In the elicitation phase of CHS, the recruitment and activation of antigen-specific T cells was considered to be essential.¹⁶ Therefore, we also stained T cells in the challenged ears with anti-CD3 antibody. We observed a decrease in the recruitment of T cells in the ears treated with MCP-1 siRNA compared with those treated with MCP-1 siRNA-SCR (**Figure 4c** and **d**).

Atelocollagen is indispensable for delivery of MCP-1 siRNA in CHS

To examine the advantage of atelocollagen in the systemic delivery of siRNA, 50 μ g of MCP-1 siRNA without atelocollagen was injected twice. No therapeutic effect of MCP-1 siRNA without

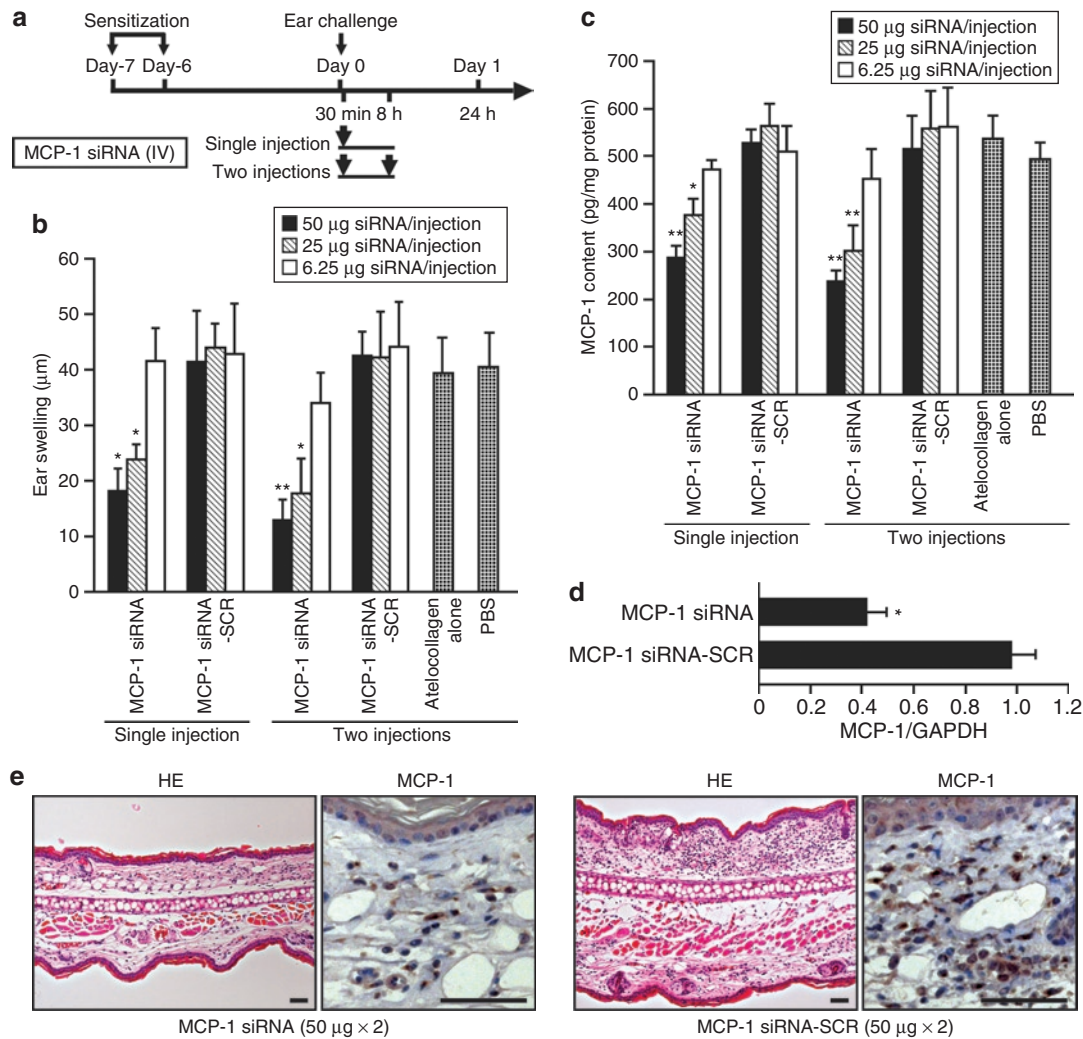


Figure 3 Therapeutic effect of monocyte chemoattractant protein-1 (MCP-1) short interfering RNAs (siRNA) mixed with atelocollagen on contact hypersensitivity. **(a)** Design of the therapy. Sensitized mice were intravenously (IV) injected with 6.25, 25, or 50 μg of MCP-1 siRNA or MCP-1 siRNA-SCR mixed with atelocollagen, atelocollagen alone, or phosphate-buffered saline (PBS). Mice were treated with a single injection (30 minutes) or two injections (30 minutes and 8 hours) after the ear challenge. **(b)** Ear swelling was determined 24 hours after the ear challenge. The results represent the means $\pm$ SE ($n = 6$). *, $P < 0.05$; **, $P < 0.01$ versus the same amount of MCP-1 siRNA-SCR. **(c)** The excised ears (at 24 hours after the ear challenge) were homogenized and then centrifuged. The amount of MCP-1 in each supernatant was measured by enzyme-linked immunosorbent assay. The results represent the means $\pm$ SE ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ versus the same amount of MCP-1 siRNA-SCR. **(d)** Sensitized mice were injected 30 minutes and 8 hours after the ear challenge with 50 μg of MCP-1 siRNA or MCP-1 siRNA-SCR mixed with atelocollagen. Total RNA was extracted from the excised ears (at 24 hours after the ear challenge). The expression of MCP-1 was assessed by quantitative real-time reverse transcriptase polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The results represent the means $\pm$ SE ($n = 5$). *, $P < 0.05$ versus MCP-1 siRNA-SCR. **(e)** Sections of the excised ears were stained with hematoxylin and eosin (HE) and anti-mouse MCP-1 antibody, respectively. All bars, 50 μm.

atelocollagen was observed (Figure 5a). A siSTABLE-modified siRNA, which is a highly stabilized one with chemical modification, was synthesized by Dharmacon (Lafayette, CO). We examined whether or not two injections (50 μg × 2) of siSTABLE-modified MCP-1 siRNA without atelocollagen had a therapeutic effect. The suppression of MCP-1 secretion by L929 cells with siSTABLE-modified MCP-1 siRNA was equivalent to that with unmodified siRNA (MCP-1 siRNA) (data not shown). Although siSTABLE-modified MCP-1 siRNA without atelocollagen slightly decreased the ear swelling compared to siSTABLE-modified MCP-1 siRNA-SCR, its therapeutic effect was not significant (Figure 5a). To confirm the efficacy of delivery of the siRNA by

atelocollagen, Cy3-labeled MCP-1 siRNA (termed MCP-1 siRNA-Cy3) mixed with or without atelocollagen was injected into the ear-challenged mice. Strong fluorescence intensity in the challenged ears was observed upon injection of MCP-1 siRNA-Cy3 with atelocollagen (Figure 5b). On the other hand, we observed almost no fluorescence in the challenged ears without atelocollagen. No fluorescence was also observed in the normal ears without elicitation regardless of mixing with atelocollagen (Figure 5b). To examine the locations of delivered MCP-1 siRNA-Cy3 with atelocollagen in the challenged ears, sections were stained for nuclei (4',6-diamidino-2-phenylindole), macrophages (F4/80), endothelium (CD31), and fibroblasts (fibroblast-specific protein-1).

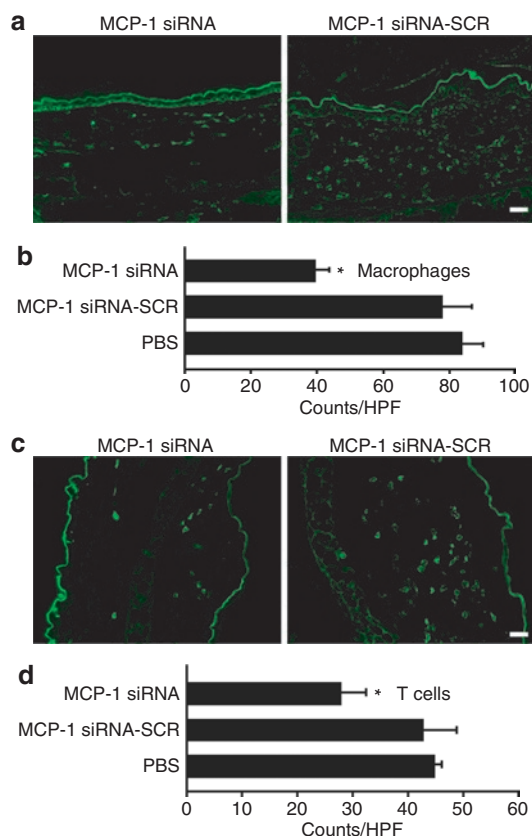


Figure 4 Decreased infiltration of macrophages and T cells in the challenged ears. Sensitized mice were injected 30 minutes and 8 hours after the ear challenge with 50 μ g of monocyte chemoattractant protein-1 (MCP-1) short interfering RNA (siRNA) or MCP-1 siRNA-SCR mixed with atelocollagen, or phosphate-buffered saline (PBS). Cryostat sections of the ears excised at 24 hours were immunostained with anti-mouse F4/80 antibody or (c) anti-mouse CD3 antibody, respectively. The number of cells was determined by examining 10 fields under a microscope at $\times 400$ original magnification (a, b) Macrophages (F4/80)-(c, d) T cells (CD3). The results represent the means $\pm$ SE ($n = 5$). *, $P < 0.05$ versus MCP-1 siRNA-SCR. Bar, 20 μ m.

We found MCP-1 siRNA-Cy3 was co-localized with nuclei. Some spots of red fluorescence were merged with F4/80-, CD31-, or fibroblast-specific protein-1-positive cells (Figure 5d–f).

No adverse effect of MCP-1 siRNA mixed with atelocollagen

MCP-1 siRNA mixed with atelocollagen did not cause interferon- α (IFN- α) induction, whereas poly(I:C), as a positive control, strongly induced it (Figure 6a). We further examined immunostimulatory RNA, which is an siRNA triggering IFN-response,^{30,31} to determine whether or not atelocollagen prevents siRNA-induced IFN-response. A liposome-mediated “immunostimulatory RNA” delivery induced a strong IFN-response, whereas “immunostimulatory RNA” with atelocollagen did not (Y.T. and T.I., unpublished results). Therefore, atelocollagen-mediated delivery system may prevent IFN-response regardless of any sequences of siRNA. No liver damage (aspartate aminotransferase and alanine aminotransferase) or renal damage (blood urea nitrogen and creatinine) was observed in mice injected twice with 50 μ g of MCP-1 siRNA mixed with atelocollagen (Figure 6b).

Comparison between atelocollagen and liposome as a delivery reagent

We examined the systemic treatment with a liposome reagent, DOTAP (*N*-[1(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate) instead of atelocollagen. Sensitized mice were injected 30 minutes and 8 hours after the ear challenge with 50 μ g of MCP-1 siRNA or MCP-1 siRNA-SCR mixed with DOTAP. MCP-1 siRNA with DOTAP decreased the ear swelling when compared with MCP-1 siRNA-SCR, but not significantly (Figure 7). From this result, we can conclude that the atelocollagen-mediated delivery method is superior to the method with liposome.

DISCUSSION

During the elicitation phase of CHS, MCP-1 is released by activated resident skin cells such as fibroblasts, keratinocytes and endothelial cells, and consecutively released by infiltrating monocytes as secondary sources.^{14,17,29,32} Indeed, we observed the secretion of MCP-1 in the challenged ear by keratinocytes, fibroblasts and microvessels at 4 hours, and the vigorous secretion predominantly by fibroblasts and infiltrating mononuclear cells in the dermis at 24 hours (Figure 2). These results and reports^{14,17,29,32} suggested that the expression of MCP-1 by fibroblasts and keratinocytes upon stimulation by DNFB seems to be a trigger of CHS. During the CHS response, the secreted MCP-1 protein recruits monocytes/macrophages and T cells from the circulation, and then the inflammation rapidly grows severe. Once we had these results, we started the therapy 30 minutes after elicitation with DNFB, *i.e.*, as quickly as possible, because we needed to inhibit the initiation phase of MCP-1 expression, and to terminate the chain reaction of the inflammatory response induced by MCP-1. Indeed, the single injection at 8 hours after the ear challenge showed a curative effect, but did not show a potent therapeutic effect like the single injection at 30 minutes (Figure 3b and Supplementary Figure S2b). On the other hand, the two injections (at 30 minutes and 8 hours) showed more curative effects than the single injection at either 30 minutes or 8 hours. For the treatment of CHS, the continuous suppression of MCP-1 by siRNA from the earlier phase was most effective.

Our success in the CHS treatment was greatly owing to the delivery system for siRNA. High molecular weight macromolecules (more than 40 kd) exhibit restricted renal excretion, and prolonged plasma or local half-lives.³³ These characteristics are beneficial for utilizing the enhanced permeability and retention effect of solid tumor tissues. Atelocollagen and DNA or RNA form a macromolecular complex via an ionic interaction.³⁴ Based on this rationale, Ochiya and his group recently succeeded in developing a systemic delivery for siRNA, involving atelocollagen, in order to suppress bone-metastatic tumors.²⁴ It is conceivable that inflammatory tissues also exhibit an enhanced permeability and retention effect. In a recent study, MCP-1 was found to enhance the permeability in the inflammatory region.^{35,36} In fact, MCP-1 siRNA-Cy3 with atelocollagen was taken up by the cells in the inflammatory ears, whereas this was not taken up in the non-inflammatory ears without elicitation (Figure 5b). Furthermore, no fluorescence was observed in blood smears from mice at 4, 8, and 24 hours after the injection of MCP-1 siRNA-Cy3 with atelocollagen (data not shown). These results suggested that MCP-1 siRNA-Cy3 with atelocollagen was

taken up by the cells in the ear with inflammation, but not taken up by the inflammatory cells in the blood stream before migrating to the inflammatory ears. We have demonstrated in this study that siSTABLE-modified siRNA to MCP-1 without atelocollagen does not have a significant effect on CHS. By contrast, unmodified MCP-1 siRNA with atelocollagen had a striking effect. Taking into account that siSTABLE-modified siRNA has an enormously long half-life (190 hours in 50% fetal bovine serum, whereas for unmodified siRNA it is 0.8 hours: Y.T., unpublished results), our data indicate that atelocollagen provides not only stability but also allows preferential delivery to the inflammatory site.

We showed that the siRNA systemically administered with atelocollagen is delivered to the target site and is taken up by target

cells using Cy3-labeled siRNA, consistent with the therapeutic effect. This is the first direct evidence of targeted delivery of systemically administered siRNA with atelocollagen. Takeshita *et al.* previously obtained indirect evidence of this by means of elegant functional image analysis.²⁴ They used siRNA to luciferase and luciferase-expressing bone-metastatic tumors. When the siRNA was injected with atelocollagen via a tail vein, the luciferase activity in tumors captured in an image was dramatically reduced.²⁴ Taken together, our (and their) findings have established efficient systemic siRNA delivery by atelocollagen.

We also examined local treatment for CHS with MCP-1 siRNA. We treated mice with a single painting (8 hours) of MCP-1 siRNA with atelocollagen (siRNA, 1,000 pmol/site; and atelocollagen, 0.5%), however, no therapeutic effects were observed (Supplementary Figure S4). Local therapy for CHS was consequently quite difficult, since the elicitation reagent (DNFB in olive oil) and water-soluble siRNA-atelocollagen complex shed each other on the inflammatory ear. Particularly, since the shedding reaction was remarkable just after the elicitation, we decided not to administer siRNA-atelocollagen complex 30 minutes after elicitation. Liposome-mediated local treatment for CHS was similarly in vain. Upon targeting vaginal^{37,38} and rectal³⁸ "mucosa" unlike skins, some liposome reagents including oligofectamine or lipofectamine 2000 seem to deliver siRNAs effectively.^{37,38}

Anticytokine therapy has been widely introduced for patients with various inflammatory diseases. Monoclonal antibodies against tumor necrosis factor- α , tumor necrosis factor- α receptor and interleukin-6 receptor show the potent effects of reducing the disease activity. However, several adverse effects such as infusion reaction, infection and autoimmunity have been reported.³⁹ Since tumor necrosis factor- α and interleukin-6 are major cytokines in the cytokine/chemokine network and play important roles in the host defence, systemic suppression of these major cytokines might be harmful in the clinical settings.^{39,40} MCP-1, which is the downstream of tumor necrosis factor- α ,^{9,41} might be a much more specific target to treat inflammatory diseases including CHS. Unlike therapy with antibodies, the siRNA molecule hardly exhibits antigenicity upon administration in the animal body,⁴² and, as a delivery reagent, atelocollagen shows neither antigenicity

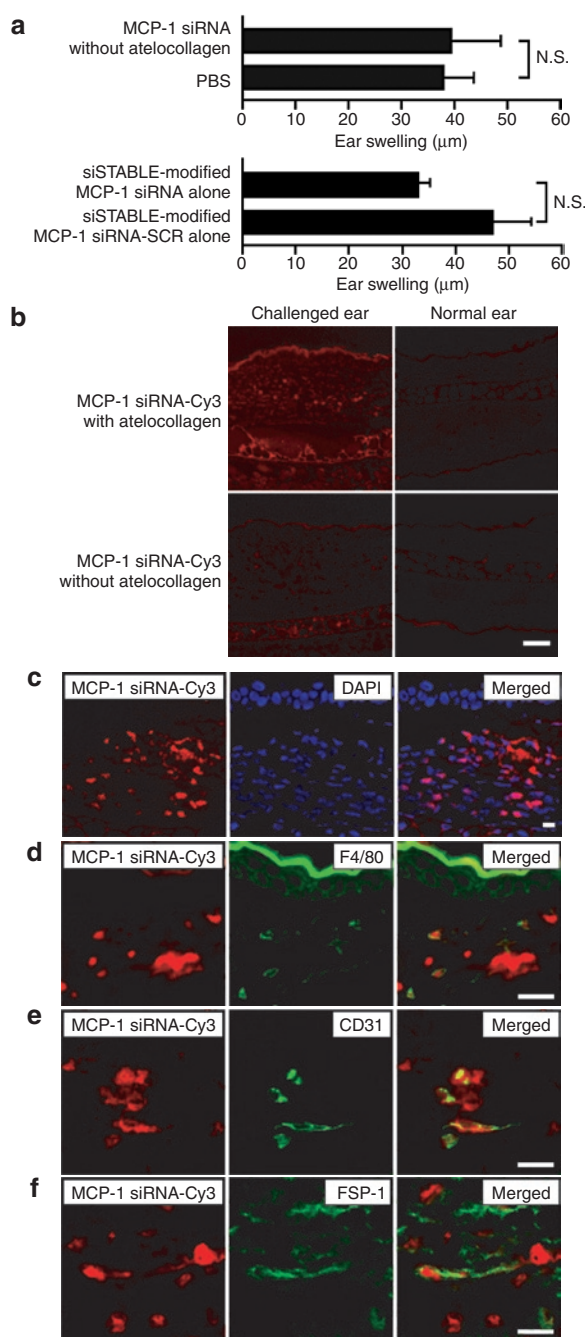


Figure 5 Advantage of atelocollagen as a delivery reagent for short interfering RNA (siRNA) in contact hypersensitivity (**a**) Upper panel, sensitized mice were injected 30 minutes and 8 hours after the ear challenge with 50 μ g of monocyte chemoattractant protein-1 (MCP-1) siRNA without atelocollagen, or phosphate-buffered saline (PBS). Ear swelling was determined 24 hours after the ear challenge. The results represent the means $\pm$ SE ($n = 6$). Lower panel, sensitized mice were injected 30 minutes and 8 hours after the ear challenge with 50 μ g of siSTABLE-modified MCP-1 siRNA or siSTABLE-modified MCP-1 siRNA-SCR without atelocollagen. Ear swelling was determined 24 hours after the ear challenge. The results represent the means $\pm$ SE ($n = 6$). (**b–f**) Sensitized mice were injected with 50 μ g of unlabeled MCP-1 siRNA 30 minutes after the ear challenge, followed by 200 μ g of MCP-1 siRNA-Cy3 with or without atelocollagen 8 hours after the ear challenge. The challenged and normal ears were excised 24 hours after the challenge. (**b**) Frozen sections were made and fluorescence was observed. Bar, 50 μ m. Sections of the challenged ears from mice injected with atelocollagen were stained for nuclei with 4',6-diamidino-2-phenylindole (DAPI) (**c**) and anti-mouse F4/80 anti-body for macrophages (**d**), anti-mouse CD31 antibody for endothelial cells (**e**) and antimouse S100A4/FSP-1 antibody for fibroblasts (**f**), and merged images were obtained. (**c–f**) All bars, 10 μ m. N.S., not significant.

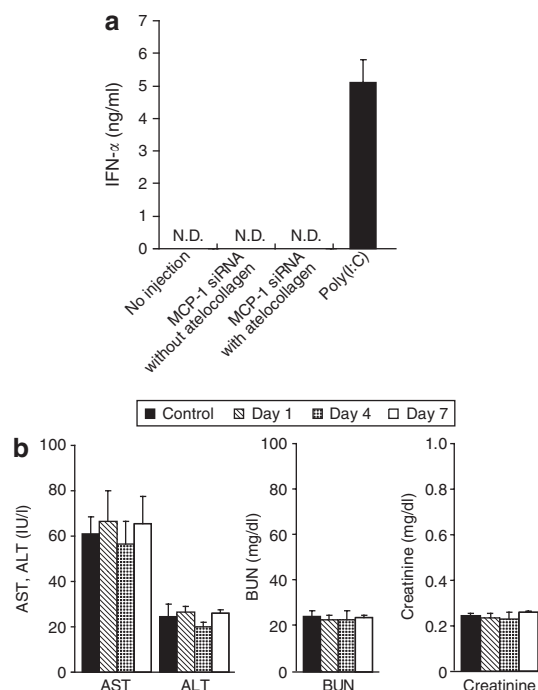


Figure 6 Monocyte chemoattractant protein-1 (MCP-1) short interfering RNA (siRNA) with atelocollagen does not induce interferon- α (IFN- α), or alter the liver enzyme levels or renal function. **(a)** Balb/c mice (8-week-old, female) were intravenously injected with 50 μ g of MCP-1 siRNA with or without atelocollagen. Mice were injected with 50 μ g of poly(I:C) as a positive control. Blood was taken from the inferior vena cava 6 hours postinjection. The IFN- α concentration was determined by enzyme-linked immunosorbent assay. The results represent the means $\pm$ SD ($n = 3$). **(b)** Balb/c mice were injected 30 minutes and 8 hours after the ear challenge with 50 μ g of MCP-1 siRNA mixed with atelocollagen. Serum isolated on days 1, 4, and 7 was used to determine the aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine levels. The results represent the means $\pm$ SD ($n = 3$). N.D., not detected.

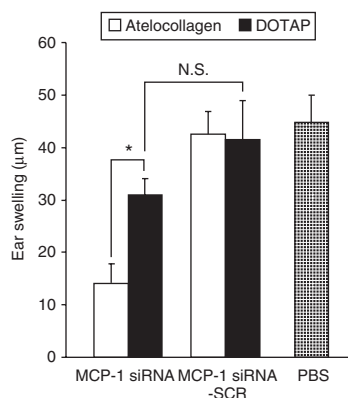


Figure 7 Comparison between atelocollagen and liposome. Sensitized mice were intravenously injected 30 minutes and 8 hours after the ear challenge with 50 μ g of monocyte chemoattractant protein-1 (MCP-1) short interfering RNA (siRNA) or MCP-1 siRNA-SCR mixed with atelocollagen or *N*-[1(2, 3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethylammonium methyl sulfate (DOTAP), or phosphate-buffered saline (PBS) alone. Ear swelling was determined 24 hours after the ear challenge. The results represent the means $\pm$ SE ($n = 6$). *, $P < 0.05$. N.S., not significant.

nor toxicity in animals, since antigenic telopeptides are eliminated through pepsin digestion.⁴³ On the other hand, a siRNA-mediated therapy has one aspect to watch for, *i.e.*, an adverse IFN induction. In this study, we did not find an induction of IFN- α by MCP-1 siRNA during the therapy, when it was intravenously injected (50 μ g) with atelocollagen. Moreover, MCP-1 siRNA showed no liver and renal damages. No gross adverse effects, *i.e.*, loss of body weight, were observed during the therapy (data not shown). Therefore, anti-MCP-1 therapy with the siRNA is safe. This therapy method involving MCP-1 siRNA and the atelocollagen-mediated systemic delivery system is applicable to the treatment of atherosclerosis, rheumatoid arthritis, glomerulonephritis, and psoriasis, all of which involve elevated expression of MCP-1.

MATERIALS AND METHODS

siRNAs. Five siRNAs targeting mouse MCP-1 and one scrambled siRNA (used as a positive control) were designed and examined as to their RNA interference effects. The targeting sequence and the location of each siRNA in mouse MCP-1 complementary DNA are shown in **Supplementary Figure S1**. All siRNAs were synthesized by Dharmacon (Lafayette, CO). We also used MCP-1 siRNA-Cy3 to monitor the delivery and uptake in the ear with CHS upon systemic injection via a mouse tail vein.

Cell culture conditions and transfection of siRNAs. L929 cells (American Type Culture Collection, Manassas, VA) derived from murine fibroblasts were cultured in Eagle's minimum essential medium containing 10% heat-inactivated fetal bovine serum at 37°C under a humidified atmosphere of 5% CO₂. L929 cells were plated at a density of 3×10^5 cells/35-mm tissue culture dish. Cells were transfected with siRNAs in serum-free medium using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA) as described previously.^{25,26} RAW 264.7 cells derived from murine macrophages were cultured in Dulbecco modified Eagle's Medium containing 10% fetal bovine serum. RAW 264.7 cells were electronically transfected with an Amaxa electroporation device and a Nucleofector V kit (Amaxa, Cologne, Germany) according to the manufacturer's instructions. Thus, the cells (2×10^6) in 100 μ l of nucleofector solution V and various siRNAs (100 pmol) were electronically pulsed and then seeded into a 35 mm dish. We used program D-32, as this was recommended by them for transfecting plasmid DNA into RAW 264.7 cells. All conditioned media were collected for ELISA.

CHS model. CHS was induced by the previously described method.^{44,45} Female BALB/c mice (8-week-old, Japan SLC, Hamamatsu, Japan) were sensitized by painting 25 μ l of 0.5% DNFB in acetone/olive oil (4:1) on each animal's shaved abdomen for two consecutive days. Six days after the second treatment, CHS was initiated by painting 15 μ l of 0.2% DNFB in the same solution on the surface of one ear (ear challenge). Mice with ear challenge without sensitization were used as a naive group. In the control group, an ear of each sensitized mouse was painted with 15 μ l of acetone/olive oil without DNFB. Ear thickness was measured with a thickness gauge (Peacock, Tokyo, Japan) before, and 4, 8 and 24 hours after the ear challenge. The ear swelling was determined by subtracting the thickness before ear challenge. The animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

Atelocollagen. Atelocollagen is a highly purified type I collagen from calf dermis.⁴³ Atelocollagen shows neither antigenicity nor toxicity in animals, since antigenic telopeptides are eliminated through pepsin digestion.⁴³ Atelocollagen is commercially available from Koken (Tokyo, Japan).

Treatment of CHS with MCP-1 siRNA with atelocollagen. Sensitized mice were intravenously treated with MCP-1 siRNA or MCP-1 siRNA-SCR mixed with atelocollagen. The final concentration of atelocollagen was

always 0.05%. The dose of siRNA was 6.25, 25, or 50 µg/injection (injection volume: 200 µl). siRNA was diluted with RNase-free phosphate-buffered saline. The therapy procedure is summarized in **Figure 3a**. Mice were treated with a single injection (30 minutes after the ear challenge) or two injections (30 minutes and 8 hours after the ear challenge). The ear swelling was determined 24 hours after the ear challenge, and then the ears were excised. The excised ears were directly frozen in liquid nitrogen for protein analysis or submerged in RNeasy (Qiagen, Hilden, Germany) for total RNA extraction, and then stored at -80°C .

Protein analysis. The excised ears were homogenized in ice-cold CellLytic-MT Mammalian Tissue Lysis/Extraction Reagent containing a protease inhibitor mixture (Sigma Chemical, St Louis, MO), and then centrifuged. The amount of MCP-1 protein in each supernatant was determined by ELISA, and then normalized with protein concentration (Lowry method).

MCP-1 ELISA. The mouse MCP-1 level in the cell culture supernatants and ears were determined using a Quantikine mouse JE/MCP-1 Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Total RNA extraction, semiquantitative RT-PCR and quantitative real-time RT-PCR. Total RNA was extracted from the cells and the excised ears using an RNeasy Mini kit (Qiagen, Hilden, Germany). Semiquantitative RT-PCR was performed with the isolated total RNA (1 µg) using an Omniscript RT kit and a Hot-StarTaq PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sequences of primer and details of PCR conditions to detect mouse MCP-1,⁴⁶ MCP-2,⁴⁷ MCP-3,⁴⁷ MCP-5,⁴⁷ glyceraldehyde-3-phosphate dehydrogenase and β -actin have been reported.^{46,47} For quantitative real-time RT-PCR, total RNA (800 ng) isolated from the ear was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using the TaqMan Gene Expression Assays and 7500 Real-time PCR System (Applied Biosystems, Foster, CA). Results for MCP-1 expression (Assay ID, Mm00441242_m1) were normalized to glyceraldehyde-3-phosphate dehydrogenase expression (Assay ID, Mm99999915_g1).

Histopathology and immunohistochemistry. Excised ears were fixed in 10% formalin, embedded in paraffin and then cut into sections (2 µm thick). The sections were stained with hematoxylin and eosin. For MCP-1 immunostaining, the sections were blocked with normal rabbit serum after boiling in 10 mmol/l citrate buffer, pH 6.0, and then incubated with goat anti-mouse MCP-1 polyclonal antibody (R&D Systems, Minneapolis, MN) overnight at 4°C , followed by with a secondary antibody polymer kit, Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan). The immunoreactivity was visualized with 3-3'-diaminobenzidine tetrahydrochloride. The sections were lightly counterstained with hematoxylin, dehydrated and then examined under a light microscope. Parts of the ear tissues were snap-frozen in liquid nitrogen. Sections (4 µm thick) were cut with a cryostat and fixed in acetone, then incubated with blocking solution of normal goat serum. Macrophages (anti-mouse F4/80 monoclonal antibody: from Serotec, Oxford, UK), T cells (anti-mouse CD3 monoclonal antibody: from Chemicon, Temecula, CA), and endothelial cells (anti-mouse CD31 monoclonal antibody: from BD Pharmingen, San Diego, CA) were immunostained, respectively. Fibroblasts were stained with anti-mouse S100A4/FSP-1 antibody⁴⁸ (LabVision/Neomarker, Fremont, CA). All positive signals were visualized with fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin G, F(ab')₂ (Serotec, Oxford, UK) or fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (Cappel Laboratories, West Chester, PA). F4/80- or CD3-positive leukocytes were counted by examining 10 fields on an ear under a microscope at $\times 400$ original magnification in a blind manner.

Analyses of ears administered Cy3-labeled MCP-1 siRNA. Sensitized mice were intravenously injected with unlabeled MCP-1 siRNA (50 µg) with

atelocollagen 30 minutes after the ear challenge. Successively, the mice were intravenously injected with Cy3-labeled MCP-1 siRNA (200 µg) with or without atelocollagen 8 hours after the ear challenge. Sixteen hours later the second injection, both the challenged and the normal ears were excised and snap frozen in liquid nitrogen, and then sections were cut. Nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma Chemical, St Louis, MO), and macrophages, endothelial cells and fibroblasts were also stained as described in the above section.

Monitoring of IFN induction, liver enzyme and renal function. Balb/c mice were intravenously injected with 50 µg of MCP-1 siRNA with or without atelocollagen, or poly(I:C) (InvivoGen, San Diego, CA) (50 µg) alone. Poly(I:C) was purchased from InvivoGen (San Diego, CA). Blood was taken from the inferior vena cava 6 hours postinjection. The mouse IFN- α concentration was determined with a mouse IFN- α ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). To monitor liver and renal function, sensitized mice were treated with MCP-1 siRNA with atelocollagen (50 µg $\times$ 2). Blood was harvested on days 1, 4, and 7. The levels of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen and creatinine were measured by SRL (Nagoya, Japan).

Preparation of siRNA-liposome complexes. DOTAP was purchased from Roche Diagnostics (Indianapolis, IN). siRNA-liposome complexes were prepared as the previous report⁴⁹: 30 µg of DOTAP was mixed with 70 µl of OptiMEM (Invitrogen, Carlsbad, CA). The solution was combined with 50 µg of various siRNAs resuspended in 100 µl of OptiMEM, and then incubated for 15 minutes. Finally, the mixture (200 µl) was intravenously injected.

Statistical analysis. The data were analyzed using the Mann-Whitney U test, and probability values of less than 0.05 were considered to indicate significant differences.

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SUPPLEMENTARY MATERIAL

Figure S1 Design of five siRNAs to mouse MCP-1.

Figure S2. A single injection of MCP-1 siRNA with atelocollagen at 8 h after ear challenge showed only a weak therapeutical effect.

Figure S3. Therapeutic effect of MCP-1 siRNA no. 2 with atelocollagen.

Figure S4. Local treatment with MCP-1 siRNA with atelocollagen.

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