Lentiviral Small Hairpin RNA Knockdown of Macrophage Inflammatory Protein-1 γ Ameliorates Experimentally Induced Osteoarthritis in Mice

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Abstract

Immune cells are involved in the pathogenesis of osteoarthritis (OA). CD4⁺ T cells were activated during the onset of OA and induced macrophage inflammatory protein (MIP)-1 γ expression and subsequent osteoclast formation. We evaluated the effects of local knockdown of MIP-1 γ in a mouse OA model induced by anterior cruciate ligament transection. The mouse macrophage cell lines and osteoclast-like cells generated from immature hematopoietic monocyte/macrophage progenitors of murine bone marrow were cocultured with either receptor activator of NF κ B ligand (RANKL) or CD4⁺ T cells. The levels of MIP-1 γ and RANKL in cells and mice were examined by enzyme-linked immunosorbent assay (ELISA). The osteoclastogenesis was evaluated using tartrate-resistant acid phosphatase and cathepsin K staining. OA was induced in one hind-leg knee joint of B6 mice. Lentiviral vector encoding MIP-1 γ small hairpin RNA (shRNA) and control vector were individually injected intra-articularly into the knee joints, which were histologically assessed for manifestations of OA. The expression of MIP-1 γ and matrix metalloproteinase (MMP)-13 and the infiltration of CD4⁺ T cells, macrophages, and osteoclastogenesis in tissues were examined using immunohistochemistry. CD4⁺ T cells were involved in OA by inducing MIP-1 γ expression in osteoclast progenitors and the subsequent osteoclast formation. Neutralizing MIP-1 γ with a specific antibody abolishes RANKL-stimulated and CD4⁺ T-cell-stimulated osteoclast formation. MIP-1 γ levels were significantly higher in synovium and the chondro-osseous junction of joints 90 days postsurgery. The number of infiltrated CD4⁺ T cells and macrophages and IL-1 β expression were reduced in the synovial tissues of mice treated with MIP-1 γ shRNA. Histopathological examinations revealed that mice treated with MIP-1 γ shRNA had less severe OA than control mice had, as well as decreased osteoclast formation and MMP-13 expression. Locally inhibiting MIP-1 γ expression may ameliorate disease progression and provide a new OA therapy.

Introduction

OSTEOARTHRITIS (OA), A CHRONIC and progressive disorder of the joints that is part of the aging process, is one of the most prevalent diseases in humans. The etiology of OA is not fully understood. However, it is believed that OA is predominantly a disease in which structural changes occur in articular cartilage (Pelletier *et al.*, 2000). Current treatments are only symptom modifying, not curative. Although they suppress the pain and inflammation associated with the disease, conventional OA therapeutics do not inhibit the underlying tissue catabolism, which allows the disease to progress into

irreversible extracellular matrix loss and chronic disability. Severe OA is treated with total hip or knee replacement. Greater understanding of the etiology of OA has brought about a large number of potential molecular targets for the development of candidate therapies (Mix *et al.*, 2001; Chen *et al.*, 2010; Ashraf *et al.*, 2011; Gege *et al.*, 2012). Genes of interest can be delivered locally and efficiently to the joints of mice with OA (Hsieh *et al.*, 2009, 2010; Shen *et al.*, 2011a).

Inflammation is pivotal in the pathogenesis of OA. In the synovium of joints with OA, the inflammatory changes include synovial hypertrophy and hyperplasia with an increased number of lining cells, and the infiltration of a mixed

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population of inflammatory cells within the sublining tissue. Lymphocyte populations included T lymphocytes expressing CD4 and CD8 surface markers (Muir *et al.*, 2011). In patients with severe OA, the extent of inflammation reached that observed in patients with clinically diagnosed rheumatoid arthritis (Farahat *et al.*, 1993). Activated CD4⁺ T cells contribute to the development of OA through direct cell–cell contact or through soluble mediators (Wagner *et al.*, 1994; Aarvak *et al.*, 1999). We previously showed that CD4⁺ T cells promoted OA progression by inducing macrophage inflammatory protein-1 γ (MIP-1 γ) (Shen *et al.*, 2011b), a member of a C-C chemokine family containing MIP-1 α , MIP-1 β , MIP-1 γ , RANTES, and so on. MIP-1 γ is produced by monocytes and other types of leukocytes. By binding to chemokine receptor 1 (CCR1), a specific receptor on neutrophils, MIP-1 γ acts as a chemoattractant that induces the chemotaxis of CD4⁺ T cells, CD8⁺ T cells, and monocytes (Poltorak *et al.*, 1995; Mohamadzadeh *et al.*, 1996). MIP-1 γ promoted osteoclast formation and survival through the receptor activator of the nuclear factor (NF)- κ B ligand (RANKL) pathway (Kong *et al.*, 1999; Okamatsu *et al.*, 2004). Osteoclast activation leads to the resorption and subsequent destruction of bone. We previously showed that in synovium infiltrated by CD4⁺ T cells, MIP-1 γ expression was higher, which activated osteoclast formation and exacerbated OA (Shen *et al.*, 2011b). On the basis of these findings, MIP-1 γ knockdown may be a promising therapeutic strategy for OA.

Lentivirus-mediated gene transfer causes the efficient expression of transgenes in chondrocytes and synovial tissue (Coughlan *et al.*, 2010; Chen *et al.*, 2012). In this study, we examined (i) how CD4⁺ T cells induce osteoclast formation and (ii) the effects of MIP-1 γ gene silencing mediated by lentiviral gene transfer in mice with OA.

Materials and Methods

Quantitative reverse transcription–polymerase chain reaction assay

Total RNA was extracted from RAW264.7 cells using TRIzol (Invitrogen, Carlsbad, CA) and complementary DNA (cDNA) was synthesized using a cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA) according to the manufacturer's directions. The quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed on an StepOnePlus real-time PCR system (Applied Biosystems) using SYBR Premix Ex Taq (Tli RNase H Plus) (Takara Bio Inc., Shiga, Japan). The 5' and 3' mouse *CCR1* gene-specific primers were designed as previously described (Okamatsu *et al.*, 2004). The 5' and 3' mouse *GAPDH* gene-specific primers were 5'-GTT GTC TCC TGC GAC TTC AAC A-3' (sense) and 5'-TTG CTG TAG CCG TAT TCA TTG TC-3' (antisense). The qRT-PCR data were analyzed by the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). The fluorescence signal intensities were quantified as a C_t (threshold cycle) value with the StepOne Software v2.2 (Applied Biosystems). Normalization was performed using mouse *GAPDH* as an internal control, and relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_t}$ method. The $2^{-\Delta\Delta C_t}$ values of the treated groups were compared with the control groups and presented as fold changes as compared with the control condition.

The tartrate-resistant acid phosphatase assay

Mouse macrophage RAW264.7 cells were cultured in complete medium consisting of Dulbecco's modified Eagle's medium, 10% cosmic calf serum (Hyclone, Logan, UT), 2 mM L-glutamine, and 50 μ g/ml of gentamicin at 37°C in 5% CO₂. We treated cells with RANKL (50 ng/ml; Peprotech, Rocky Hill, NJ) to stimulate osteoclast formation. Osteoclasts in culture were identified by histochemically staining tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St. Louis, MO) with or without MIP-1 γ neutralization antibody (R&D Systems, Minneapolis, MN). To quantify TRAP signals, areas containing the largest number of osteoclasts were identified by scanning the sections at $\times 100$ magnification. After the fields with the largest number of osteoclasts were determined, individual osteoclasts were counted at $\times 400$ magnification. The number of osteoclasts was determined by averaging the number of osteoclasts in five areas at $\times 400$ magnification.

In vitro coculture system

For osteoclast-like generation, mouse bone marrow cells were isolated and incubated in α -minimum essential medium with 50 ng/ml of mouse M-CSF (R&D Systems) and 100 ng/ml of RANKL to stimulate osteoclast formation. The medium was refreshed on day 3. To generate CD4⁺ T cells, total splenocytes were prepared from mice after the red blood cells had been lysed. Splenocytes were incubated with mouse CD4 (L3T4) microbeads (Macs; Miltenyi Biotec, Auburn, CA), after which CD4⁺ T cells were purified by passing them through a mass spectrometry column to enrich CD4⁺ T cells (Macs; Miltenyi Biotec). The yielded CD4⁺ T cells were at least 95–97% pure (confirmed using flow cytometry). The bone marrow cells (2.5×10^5 /well) were then incubated with either purified CD4⁺ T cells (5×10^5 /well) or with CD4⁺ T cell-conditioned medium (CM; 30%/well).

Enzyme-linked immunosorbent assay

To quantify the MIP-1 γ , RANKL, and interferon- γ (IFN- γ), the levels of these cytokines and chemokines in the cell lysate and tissue homogenates were determined using an ELISA kit (R&D Systems).

Screening MIP-1 γ small hairpin RNA and producing lentiviral particles

Three heteroduplexes (small hairpin RNA, shRNA) containing different 19 bp RNA segments to target different locations of the *MIP-1 γ* gene were selected (National RNAi Core Facility, Taipei, Taiwan). The number following "sh" indicates the position of the oligonucleotide at the 5'-end of *MIP-1 γ* cDNA (GenBank accession no. NM 011338). The heteroduplexes, supplied as 60-nucleotide oligomers, were annealed and inserted downstream of the U6 promoter into pLKO.1 plasmid, resulting in pLKO.1/sh240, pLKO.1/sh346, and pLKO.1/sh935. The three recombinant lentiviruses, accompanied by two control lentivirus, were produced by transiently transfecting pLKO.1/sh240, pLKO.1/sh346, pLKO.1/sh935, pLKO.1/shLuc, or pWPT, respectively, into 293T cells along with the packaging plasmid psPAX2 and envelope plasmid pMD2.G, using the calcium phosphate precipitation method as previously described

FIG. 1. The effect of MIP-1 γ antibody on murine osteoclast differentiation stimulated by receptor activator of nuclear factor (NF)- κ B ligand (RANKL). **(A)** The levels of MIP-1 γ in RANKL-treated RAW264.7 cells. MIP-1 γ expression was determined by enzyme-linked immunosorbent assay (ELISA). **(B)** The expression of chemokine receptor 1 (CCR1) in RANKL-treated RAW264.7 cells was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). GAPDH was used as an internal control. Values shown are relative expression levels with the level in control cells arbitrarily set to 1. **(C)** Photomicrograph of osteoclastogenic RAW264.7 cell cultures with α -minimum essential medium (MEM; control), stimulated by RANKL (50 ng/ml) or by RANKL plus MIP-1 γ antibody at different concentrations (0.5 and 2.5 μ g/ml). Cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP; $\times 100$ magnification, scale bar = 200 μ m). **(D)** TRAP-positive multinucleated cells were quantified. Data are means $\pm$ SD. * p < 0.05, ** p < 0.01, and *** p < 0.001. Color images available online at www.liebertpub.com/hum

(Salmon and Trono, 2007; Wang *et al.*, 2010). After 48 hr, lentiviral particles (VPs) were collected and concentrated from the supernatant using ultracentrifugation. Physical titers of lentiviruses expressed as VPs were determined by analyzing the virus-associated p24 core protein (Lenti-X p24 Rapid titer Kit; Clontech, Mountain View, CA). Effective lentiviral shRNA was screened by infecting these viruses with mouse fibroblast NIH3T3 cells and RAW264.7 cells and, using ELISA, analyzing their inhibitory effect on MIP-1 γ expression. The lentivirus containing the most effective *shMIP-1 γ* RNA target sequences (sh935), lentivirus (Lt.) *shMIP-1 γ* , as well as two control viruses, Lt.shLuc and Lt.GFP, was used for the animal study.

Animal models

Seven-week-old male B6 mice were purchased from the Laboratory Animal Center of National Cheng Kung University. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the university's Laboratory Animal Care and Use Committee. To

induce experimental OA, each mouse was anesthetized with Zoletil 50 (10 mg/kg; Virbac, Carros, France) and then subjected to a previously described (Kamekura *et al.*, 2005; Shen *et al.*, 2011b) modified anterior cruciate ligament transection (ACLT) procedure. Immediately after surgery (day 0), 28 mice were randomly divided into 4 groups (n = 7 each). They were intra-articularly injected in the ACLT knees with either Lt.shMIP-1 γ or Lt.shLuc (5.79×10^8 VPs) on day 60 and day 75 postsurgery. Mice in the normal saline (NS) group were injected with 15 μ l of NS using the same schedule as described above. The sham-operated group received no treatment. Ninety days after surgery, the mice were euthanized for a histological examination.

Histological assessment and immunohistochemistry

To test virus targeting *in vivo*, Lt.GFP, a lentiviral vector expressing green fluorescent protein (GFP) was intra-articularly injected (2×10^8 VPs) and the mice were killed 72 hr later (n = 5 per group). To evaluate the histopathologic changes in the joints of the mice, ACLT was used to induce OA (n = 7 per

FIG. 2. The effect of MIP-1 γ antibody on murine osteoclast differentiation after stimulation by CD4⁺ T cells. **(A)** The levels of MIP-1 γ , soluble RANKL and IFN- γ secreted by CD4⁺ T cells 7 days after concanavalin A (ConA) induction. **(B)** The levels of MIP-1 γ in osteoclastogenic nonadherent bone marrow cell cultures with medium (Control), stimulated by RANKL (50 ng/ml), by activated CD4⁺ T cells (5×10^5 per well, ConA pulsed), and by corresponding CD4⁺ T cell-conditioned medium (CM) (30% per well). **(C)** Photomicrograph of osteoclastogenic cell cultures with medium, stimulated by RANKL, by activated CD4⁺ T cells, and by CM with or without MIP-1 γ antibody (2.5 μ g/ml), respectively. Cells were fixed and stained with TRAP (TRAP stain, $\times 400$ magnification, scale bar = 50 μ m). **(D)** TRAP-positive multinucleated cells were quantified. Data are means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Color images available online at www.liebertpub.com/hum

group). These mice were killed 90 days later. Their cartilage and synovial membranes were removed, fixed, and embedded in paraffin. Serial sections (5 μ m thick) were cut and stained with Safranin-O/fast green and hematoxylin and eosin. The histologic change in cartilage was scored using Mankin's histologic grading method (Mankin *et al.*, 1971). The histologic changes in synovial surface tissue and subsynovial tissue were evaluated and scored (Yoshimi *et al.*, 1994). Briefly, the grading system assigns separate scores based on two categories: (a) three subcategories of the synovial lining layer—(i) hyperplasia of the synovial lining cells (0–3 points), (ii) hypertrophy of the synovial lining layer (0–3 points), and (iii) the infiltration of inflammatory cells (0–3 points)—and (b) three subcategories of the subsynovial tissue—(i) the proliferation of granulation tissue (0–3 points), (ii) vascularization (0–3 points), and (iii) the infiltration of inflammatory cells (0–3 points). Total scores in each category were calculated; the

maximum score was 18 points. To test specific protein expression, the samples from joints were prepared from mice with different treatment. Serial sections of cartilage (and synovium) were stained with rat antimouse CD4 (L3T4; 1:100; H129.19; BD Biosciences Pharmingen, San Diego, CA), Mac-3 (a macrophage marker; 1:100; M3/84; BD Biosciences Pharmingen), GFP (B-2; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), IL-1 β (H-153; 1:100; Santa Cruz Biotechnology), MIP-1 γ antibody (1:100; Peprotech), matrix metalloproteinase (MMP-13; 1:50; Santa Cruz Biotechnology), and cathepsin K (an osteoclast marker; Proteintech, Chicago, IL) at 4°C overnight. After they had been sequentially incubated with the appropriate secondary antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hr at room temperature with aminoethyl carbazole as the substrate chromogen (Invitrogen Zymed Laboratories, Camarillo, CA), the slides were counterstained with hematoxylin.

FIG. 3. The expression of MIP-1 γ in the knee joints of mice with OA. (A) Anterior cruciate ligament transection (ACLT) was used to induce OA in mice ($n=5-7$) on day 0. Levels of MIP-1 γ in the joint extracts were determined by ELISA on days 30 and 90. Data are means $\pm$ SD. *** $p < 0.001$. (B) MIP-1 γ -positive cells (arrows) are visible in the lower metaphysis (immunohistochemical [IHC] stain, upper panels, $\times 100$ magnification, scale bar = 200 μ m) and in the chondro-osseous (C-O) junction (middle panels, $\times 200$ magnification, scale bar = 100 μ m). MIP-1 γ expression was higher in the synovium (lower panels) in the ACLT-group mice than in the Sham-group mice 90 days postsurgery ($\times 200$ magnification, scale bar = 100 μ m). Color images available online at www.liebertpub.com/hum

Statistical analysis

All data are means $\pm$ standard deviation. The quantification of the number of osteoclasts and MIP-1 γ , RANKL, and IFN- γ expression was determined using the Student's *t*-test. Significance was set at $p < 0.05$. JMP 8.0 (SAS Institute, Cary, NC) was used to analyze the histological data. Significance between groups was estimated using one-way analysis of variance. To evaluate the differences between groups, we used Tukey's Honestly Significant Difference test set at $p < 0.05$.

Results

MIP-1 γ antibody inhibited *in vitro* osteoclast formation

Our results showed that there was endogenous expression of MIP-1 γ in RAW264.7 cells (782.48 ± 59.96 pg/ml) (Fig. 1A). However, the levels of MIP-1 γ were significantly increased in RANKL-treated RAW264.7 cells ($4,168.43 \pm 1,343.50$ pg/ml; $p = 0.012$). The expression of CCR1 in cells induced by RANKL is

more than five times as that in controls (5.76 ± 2.76 vs. 1.07 ± 0.51 ; $p = 0.0445$; Fig. 1B). *In vitro* osteoclast differentiation was significantly and dose dependently (52.20 ± 13.33 , 0.5 μ g/ml, $p = 0.0033$; 18.21 ± 6.69 , 2.5 μ g/ml, $p = 0.000024$) inhibited in RAW264.7 cells treated with MIP-1 γ antibody plus RANKL (50 ng/ml), but not in those treated with RANKL only (94.22 ± 18.39) (Fig. 1C and D). The levels of MIP-1 γ and soluble RANKL secreted by concanavalin A (ConA)-activated CD4 $^{+}$ T cells were significantly increased when compared with the controls (MIP-1 γ : 338.52 ± 115.71 pg/ml vs. 86.67 ± 63.29 pg/ml, $p = 0.03$; RANKL: 488.10 ± 35.82 pg/ml vs. 261.45 ± 102.35 pg/ml, $p = 0.0224$, respectively) (Fig. 2A). Nevertheless, there was no significant difference in the levels of IFN- γ 7 days after ConA induction (337.23 ± 7.73 pg/ml vs. 327.02 ± 4.52 pg/ml; $p = 0.119$). Furthermore, we isolated osteoclast-like cells from mice and cocultured them with activated CD4 $^{+}$ T cells. The levels of MIP-1 γ in CD4 $^{+}$ T cell-activated cells were increased compared with controls (107.22 ± 7.01 ng/ml vs. 0.76 ± 0.04 ng/ml; $p = 0.000012$) (Fig. 2B). Since the level of MIP-1 γ secreted by activated CD4 $^{+}$ T cells was 338.52 ± 115.71 pg/ml (Fig. 2A), most increased MIP-1 γ should come from

activated osteoclast-like cells. Although at lower levels in CD4⁺ T cell-CM-activated osteoclast-like cells, significant increase of MIP-1 γ expression was seen (0.96 ± 0.11 ng/ml; $p = 0.0478$). CD4⁺ T cells activated osteoclast differentiation. This was true for co-cultures of osteoclast progenitors with CD4⁺ T cells and for those progenitors treated with CD4⁺ T cell-CM (Fig. 2C). However, osteoclast differentiation was inhibited after the MIP-1 γ antibody was added. The number of osteoclasts fell in CD4⁺ T-cell-treated TRAP-positive multinucleated cells (from 19.20 ± 5.45 to 7.20 ± 1.09 ; $p = 0.0013$) and CM-treated TRAP-positive multinucleated cells (from 11.20 ± 2.59 to 3.40 ± 1.14 ; $p = 0.00026$) (Fig. 2D).

MIP-1 γ expression during the progression of OA

MIP-1 γ levels in the knee joints of mice with OA in the ACLT and Sham groups were not significantly different 30 days postsurgery (422.24 ± 163.01 pg/ml vs. 441.61 ± 103.06 pg/ml); however, they were significantly higher in the ACLT group 90 days postsurgery (Sham group: 464.75 ± 70.24 pg/ml vs. ACLT group: 729.62 ± 68.26 pg/ml; $p = 0.0007$) (Fig. 3A). MIP-1 γ expression was detected only in the lower metaphysis in the Sham group (Fig. 3B), whereas it was detected in the chondro-osseous junction of the ACLT group, and in the synovium of mice 90 days postsurgery.

Selecting shRNA sequences to target MIP-1 γ

Three shRNA sequences, sh935, sh346, and sh240, were designed to specifically target mouse MIP-1 γ . NIH-3T3 and RAW264.7 cells were infected with viruses that coded the three different shRNAs; then, the expression of MIP-1 γ proteins was analyzed. The inhibitory efficacy of sh935 (73.32%; $p = 0.0139$) and sh346 (72.47%; $p = 0.016$) on MIP-1 γ expression was similar in NIH3T3 cells (Fig. 4A) compared with the shLuc, a control virus encoding luciferase shRNA. In RAW264.7 cells, the inhibitory efficacy of sh935 was 78.25% ($p = 0.005$) and of sh346 was 54.9% ($p = 0.011$) compared with shLuc (Fig. 4A). We then named the recombinant virus carrying sh935-targeted sequence as Lt.shMIP-1 γ , and used it for the subsequent studies. When induced with RANKL, the levels of MIP-1 γ in Lt. shLuc-treated cells increased ($2,037.76 \pm 491.41$ pg/ml), and its expression could be significantly inhibited (Fig. 4B) by treating cells with Lt.shMIP-1 γ ($1,136.44 \pm 74.84$ pg/ml, $p = 0.0348$).

In vivo histopathologic evaluation in mice with OA after recombinant lentivirus shMIP-1 γ treatment

To test the virus targeting specificity *in vivo*, Lt.GFP, a lentiviral vector expressing GFP, was intra-articularly injected (2.0×10^8 VPs) into mice. The GFP could be detected in most of the superficial and deep zones of cartilage as well as synovia 72 hr after virus injection (Fig. 5A). After surgery, we intra-articularly injected in the ACLT knees with either Lt.shMIP-1 γ or Lt.shLuc (5.79×10^8 VPs) on day 60 and day 75 postsurgery. Ninety days after surgery, more CD4⁺ T cells were infiltrated in the synovial membranes of NS and Lt.shLuc group mice than in those of Lt.shMIP-1 γ group mice (Fig. 5B). Many macrophages were localized in the synovial membranes of NS and Lt.shLuc group mice, but only occasionally found in those of Lt.shMIP-1 γ group mice. Abundant expression of IL-1 β was also observed in the synovia of NS and Lt.shLuc group mice compared with that of Lt.shMIP-1 γ group ones. The levels of

FIG. 4. Targeting MIP-1 γ using shRNA. Screening of effective MIP-1 γ shRNA by infecting (A) NIH3T3 and RAW264.7 cells with three lentiviruses carrying different MIP-1 γ shRNA (sh935, sh346, and sh240). (B) The inhibitory efficacy of Lt.shMIP-1 γ in RANKL-treated RAW264.7 cells. Knockdown of MIP-1 γ expression was determined using ELISA. Data are means $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$.

RANKL in mice treated with Lt.shMIP-1 γ were significantly reduced compared with those treated with Lt.shLuc and NS (Lt.shMIP-1 γ group: 876.35 ± 35.13 pg/ml vs. Lt.shLuc group: 998.00 ± 79.94 pg/ml, $p = 0.0143$; Lt.shMIP-1 γ group: 876.35 ± 35.13 pg/ml vs. NS group: $1,024.67 \pm 120.97$ pg/ml, $p = 0.0331$; Fig. 5C). Nevertheless, there was no significant difference in levels of IFN- γ among the four groups. No change has been observed either in the levels of MIP-1 α (data not shown). These results indicated that MIP-1 γ was the predominant C-C chemokine produced by CD4⁺ T cell-stimulated cells at the late stage of OA. The blocking of MIP-1 γ may inhibit the recruitment of CD4⁺ T cells and macrophages into the synovial membranes and the subsequent inflammatory responses.

FIG. 5. Inflammatory responses in the knee joints in *Lt.shMIP-1 γ* -treated mice. **(A)** The tissue distribution of lentivirus after intra-articular injection. *Lt.GFP* was intra-articularly injected (2×10^8 VPs) into mice. Mice in the PBS group were injected with $15 \mu\text{l}$ of PBS ($n=5$ each group) (IHC stain, $\times 600$ magnification, scale bar = $20 \mu\text{m}$, cartilage; $\times 400$ magnification, scale bar = $50 \mu\text{m}$, synovium). **(B)** After ACLT, either *Lt.shMIP-1 γ* or *Lt.shLuc* (5.79×10^8 VPs) were intra-articularly injected in the ACLT knees on day 60 and day 75 postsurgery ($n=7$ each group). Mice in the normal saline (NS) group were injected with $15 \mu\text{l}$ of normal saline. The sham-operated group received no treatment. The expression of infiltrated CD4^+ T cells, macrophages (Mac-3-positive cells; indicated with arrows), and IL-1 β in the ACLT knees was detected (IHC stain, $\times 200$ magnification, scale bar = $100 \mu\text{m}$). **(C)** The levels of soluble RANKL and IFN- γ in mice after *Lt.shMIP-1 γ* treatment. Data are means $\pm$ SD. * $p < 0.05$. Color images available online at www.liebertpub.com/hum

Histopathologic analysis showed a severe reduction of cartilage thickness and loss of chondrocytes both in the NS group and in the *Lt.shLuc* group (Fig. 6A). However, in the *Lt.shMIP-1 γ* group, the severity of the lesions was reduced, and some fibrillation and irregularities were seen on the surface of the cartilage. In the sham-operated knee joints, most of the articular cartilage had a smooth surface. Synovium in the NS and *Lt.shLuc* groups showed hyperplasia and hypertrophy of the lining cells and greater cellular infiltration (Fig. 6B). In the *Lt.shMIP-1 γ* group, only mild hyperplasia and mononuclear infiltration were seen compared with the Sham group. The osteoarthritic score in the *Lt.shMIP-1 γ* group was significantly lower than that in the NS and *Lt.shLuc* groups ($p < 0.001$; Table 1). The synovitis score in the *Lt.shMIP-1 γ* group was also significantly lower than those in the NS and *Lt.shLuc* groups ($p < 0.001$). *MIP-1 γ* expression was higher in the metaphysis (Fig. 6C) and synovium of the NS and *Lt.shLuc* groups, but lower in the *Lt.MIP-1 γ* group. Furthermore, osteoclast immunoreactivity was commonly detected in the proximal tibial growth plate.

More cathepsin K-positive cells were detected in the NS and *Lt.shLuc* groups than in the *Lt.shMIP-1 γ* group (Fig. 6D). Chondrocytes positive for the expression of MMP-13, a key regulator of cartilage degradation, were localized to all zones (superficial, deep, and subchondral) of articular cartilage in the NS and *Lt.shLuc* groups, but only occasionally found in the deeper zones of cartilage in the *Lt.shMIP-1 γ* group (Fig. 6E). Taken together, these results suggested that inhibiting the *MIP-1 γ* gene had modulated the formation of osteoclasts in the knee joints of mice after the induction of OA and that it had attenuated the development of OA.

Discussion

The dynamic interplay of the immune and skeletal systems is thought to influence homeostasis and contribute to degenerative diseases (Nakashima and Takayanagi, 2009). CD4^+ T cell regulation of osteoclastogenesis causes subchondral bone remodeling and initiates OA (Kotake *et al.*, 2001; Crema *et al.*, 2010). Patients with progressive OA

FIG. 6. Histological analysis in the knee joints in Lt.shMIP-1 γ -treated mice ($n=7$ per group). Cartilage on the posterior tibia condyle is shown. **(A)** The NS specimen shows remarkably decreased Safranin-O staining and chondrocyte hypocellularity (Safranin-O/fast green stain, $\times 100$ magnification, scale bar = $200\ \mu\text{m}$, upper panels; the insets represent the magnified area, $\times 400$ magnification, scale bar = $50\ \mu\text{m}$, lower panels). Cartilage surfaces showed fibrillation and clefts in the Lt.shLuc-treated mice. The Lt.shMIP-1 γ specimen had an irregular superficial layer of cartilage. The surface layer of cartilage in the Sham specimen was smooth and showed no significant changes. **(B)** Synovia in the NS- and Lt.shLuc-treated mice showed hyperplasia and hypertrophy of synovial-lining cells (hematoxylin and eosin stain, $\times 200$ magnification, scale bar = $100\ \mu\text{m}$, upper panels; the insets represent the magnified area, $\times 600$ magnification, scale bar = $20\ \mu\text{m}$, lower panels). The Lt.shMIP-1 γ specimen showed slightly more cell proliferation in the synovial lining and cell infiltration than did the sham-operated specimen. **(C)** MIP-1 γ expression was lower in the joints of Lt.shMIP-1 γ -treated mice than in those of the NS- and Lt.shLuc-treated mice 90 days postsurgery (IHC stain, $\times 40$ magnification, scale bar = $500\ \mu\text{m}$, upper panels; the insets represent the magnified area, $\times 200$ magnification, scale bar = $100\ \mu\text{m}$, lower panels). **(D)** Fewer cathepsin K-positive cells (osteoclasts: arrows) were detected in the tibial metaphysis of Lt.shMIP-1 γ -treated mice than in NS- and Lt.shLuc-treated mice (IHC stain, $\times 200$ magnification, scale bar = $100\ \mu\text{m}$). **(E)** There were fewer MMP-13-positive chondrocytes in the Lt.shMIP-1 γ -treated mice than in the NS- and Lt.shLuc-treated mice (IHC stain, $\times 200$ magnification, scale bar = $100\ \mu\text{m}$). NS, normal saline.

TABLE 1. COMPARISON OF THE ARTICULAR CARTILAGE CHANGE AFTER TREATMENT

Group	Osteoarthritic score	Synovitis score
NS ($n=7$)	9.29 $\pm$ 1.78	11.86 $\pm$ 0.94
Lt.shLuc ($n=7$)	8.57 $\pm$ 1.88	9.36 $\pm$ 1.44
Lt.shMIP-1 γ ($n=7$)	4.34 $\pm$ 0.89 ^a	5.64 $\pm$ 0.75 ^a
Sham ($n=7$)	2.00 $\pm$ 0.65	2.50 $\pm$ 0.58

Lt.shLuc, lentivirus encoding luciferase small hairpin RNA; Lt.shMIP-1 γ , lentivirus encoding macrophage inflammatory protein-1 γ small hairpin RNA; NS, normal saline.

Data are means $\pm$ SD and analyzed using one-way analysis of variance and Tukey's Honestly Significant Difference tests.

^a $p<0.001$ compared with the NS and Lt.shLuc groups.

usually have high subchondral bone turnover (Buckland-Wright *et al.*, 2007). Osteoclast activation causes excessive bone resorption and subsequent bone destruction. We previously (Shen *et al.*, 2011b) showed that CD4⁺ T cells induced osteoclast formation through the expression of MIP-1 γ . In this study, we showed that activated CD4⁺ T cells could increase the production of MIP-1 γ and RANKL (Fig. 2A). Both of these factors were able to induce osteoclast formation. When cocultured with activated CD4⁺ T cells, osteoclast progenitors were able to express high levels of MIP-1 γ protein and differentiate into osteoclasts. Similar results were found in those osteoclast progenitors cocultured with CD4⁺ T cells CM. T cells themselves may produce MIP-1 γ or stimulate other cells, such as multinucleated osteoclast-like cells, to produce MIP-1 γ through the RANKL signaling pathway. The effects could be blocked by the addition of MIP-1 γ neutralization antibody. These results suggested that through direct or indirect contact with a macrophage cell line or mouse osteoclast-like cells, activated CD4⁺ T cells may induce osteoclastogenesis.

When setting up OA animal models, we classified the disease stages based on the radiologic, macroscopic, and histopathologic evaluation. Moderate cartilage damage and synovia inflammation were observed 60 days after surgery, and it continued to get more severe till 90 days at sacrifice. We did not detect a significant increase of MIP-1 γ in the joints of mice until 90 days after OA induction, which corresponded to severe OA. On the basis of this evidence, we therefore gave the mice two doses of lentiviral vectors (5.79×10^8 VPs) on days 60 and 75 postsurgery, which corresponded to moderate to severe OA. Our results showed that the severity of disease could be attenuated by intra-articular injections of shMIP-1 γ using this regimen. This treatment is more close to a delayed intervention. Intra-articular administration of Lt.shMIP-1 γ could both inhibit an abnormal cartilage turnover and inflammation.

Although cartilage lesions are the main feature of OA, subchondral bone destruction can trigger further cartilage degradation and contribute to the progression of disease. Lesions occur in subchondral bone are accompanied with a decrease of bone volume, suggesting that the bone resorption might impact cartilage remodeling and trigger OA. Accumulating evidence suggests that modifying subchondral bone provides alternative therapy for OA (Strassle *et al.*, 2010). Bisphosphonates, commonly used to prevent and treat osteopenia and osteoporosis, were recently used for treating

OA (Muehleman *et al.*, 2002; Hayami *et al.*, 2004; Funck-Brentano *et al.*, 2012). Although promising results showed in animals, clinical trials using bisphosphonates to target osteoclasts have mixed and disappointing therapeutic effects (Bingham *et al.*, 2006; Ramnaraine *et al.*, 2012). When using this kind of drug, better results were seen in patients taking protective chronic doses. As a matter of fact, patients came to hospitals often have heterogeneous or advanced cases of OA, or both. To develop a therapeutic agent for symptomatic OA would be more desirable than using a preventive agent. The effects of shMIP-1 γ on a later stage of OA have potentials for treatment of this disease.

Instead of using the MIP-1 γ -neutralizing antibody, we chose lentiviral vector-encoding MIP-1 γ shRNA to inhibit osteoclast formation based on the following reasons: First, according to the results from clinical trials, utilizing neutralizing antibody such as anti-TNF agents required long-term continuing treatment to control chronic diseases such as rheumatoid arthritis (Maini *et al.*, 1999). However, such treatments used for patients are extremely expensive and time-consuming (Thrall, 2005). In contrast, using recombinant viral vectors for gene delivery to patients with chronic disease would benefit from both cost and therapeutic efficiency. Lentiviral vectors are able to infect a wide variety of dividing and nondividing cells, integrate stably into the host chromosomes, and result in long-term expression of the transgene (Singer and Verma, 2008). Upon one or two shots, the therapeutic levels of transgene can be usually achieved. Although there are safety concerns regarding to insertional mutagenesis, their integration profile seems more favorable than that of retroviral vectors. Several clinical trials are ongoing using lentiviruses for the treatment of congenital and acquired diseases and showing promising results (Mátraí *et al.*, 2010). Additionally, we and other laboratories (Wazen *et al.*, 2006; Buckland-Wright *et al.*, 2007; Chen *et al.*, 2011, 2012; Marzaioli *et al.*, 2012; Ramnaraine *et al.*, 2012) showed that lentiviral vector can efficiently transduce the synovial-lining cells, cartilage, and even calcified tissue, including osteoclasts, in the knees of animals without eliciting an inflammatory response. An efficient and stable gene transduction using lentiviral vectors in human osteoclast could be obtained for 4 weeks at a multiplicity of infection of 10 (Chu *et al.*, 2008). Furthermore, the lentiviral transduction did not affect the function of osteoclast. We used Lt.GFP to test the virus targeting specificity. The GFP expression could be detected in the chondrocytes and synoviocytes, demonstrating that the lentiviral vectors were able to transduce genes efficiently into the cartilage and synovia.

Previously, we showed that 30 days after ACLT induction, both total CD4⁺ T cells and active CD4⁺ T cells increased in spleens (Shen *et al.*, 2011b). In this study, our results revealed a more significant elevation of MIP-1 γ levels in the joints at the late stage than the early stage of OA (Fig. 3). It seemed to have different dynamics between the CD4⁺ T cells infiltration and the MIP-1 γ expression in joints. In fact, the CD4⁺ T cells infiltration, although decreased along with disease progression, occurred in the synovium at all time from OA initiation to 90 days at sacrifice. We were thus able to detect a colocalization of CD4⁺ T cells and MIP-1 γ expression in synovial tissues at the late stage of OA (Shen *et al.*, 2011b). Although the activation of CD4⁺ T cells may occur during the onset of OA, the effects caused by activated CD4⁺ T cells on the

composition of extracellular matrix can be more persistent. The other possibility is that different factors, such as IFN- γ and RANK secreted by different Th cell subsets, Th1 and Th17, respectively, may act at different time-points during OA progression. At the early stage of OA, when a large number of Th cells are activated, Th1 cells and the cytokines they produce, such as IFN- γ , play essential roles in initiating OA. At a later stage of OA, despite a decrease in the total number of Th cells, Th17 cells may activate now and continuously induce RANK production, resulting in subsequent disease exacerbation. However, this hypothesis required further investigation. IFN- γ produced by CD4⁺ T cells (Th1 and $\gamma\delta$ T) had functions in macrophage activation, inflammation, T cell differentiation, and tissue remodeling (Hu and Ivashkiv, 2009; Pappalardo and Thompson, 2013). During OA progression, it was believed that IFN- γ exerted an anti-osteoclastogenic effect on arthritis (Rho *et al.*, 2004). It was recently reported that IFN- γ was able to suppress the collagen-induced arthritis through the regulation of Th17 (Lee *et al.*, 2013). Others demonstrated that IFN- γ tended to express in the early inflammatory arthritis or even at the pre-arthritic state (Adarichev *et al.*, 2005; Raza *et al.*, 2005). Our previous study showed that more substantial expression of IFN- γ was observed in synovium 30 days after OA induction than 90 days at sacrifice (Shen *et al.*, 2011b). It appeared that IFN- γ expressed and affected the disease in the initiation of OA. In the current study, our *in vitro* data showed that 7 days after ConA induction, the RANKL and MIP-1 γ were both increased in activated CD4⁺ T cells except for IFN-1 γ . In addition, the *in vivo* data showed that at a later stage of OA, no significant change of IFN- γ expression was detected among the four groups (Fig. 5C). The temporal expression difference of IFN- γ and MIP-1 γ is probably the reasons why more osteoclasts and severe tissue damage were seen at the late stage of OA.

Several mediators, such as RANKL and IL-17 induced by activated Th cells, can stimulate osteoclast differentiation and formation. Like MIP-1 γ , targeting these factors could inhibit the osteoclastogenesis and the following bone sorption (Dimitrova *et al.*, 2012; Park *et al.*, 2012). Using antibodies that specifically targeted to RANKL, clinical trials were conducted to treat osteoporosis and rheumatism arthritis, and showed promising results (Cohen *et al.*, 2008; Cummings *et al.*, 2009; Dore *et al.*, 2010). Okamoto *et al.* (2004) reported that among all the mediators screened, MIP-1 γ was the most strongly upregulated cytokines in osteoclasts derived from RANKL-stimulated monocytes/macrophages. The pro-survival effect of RANKL was in part depends on its ability to induce MIP-1 γ . In consistent with the previous reports, our data showed that MIP-1 γ -neutralizing antibody could block the RANKL-stimulated osteoclastogenesis *in vitro*. Not only CD4⁺ T cells, but also most infiltrated cells (such as monocytes, neutrophils, and osteoclasts) in joints could produce MIP-1 γ . Since MIP-1 γ chemottracts these cells into synovium, it may therefore affect the subsequent cell differentiation, survival, and activation likely via an autocrine way. An amplified therapeutic effect would be achieved when knocking down MIP-1 γ . This might be the reason that inhibiting MIP-1 γ expression in joint tissues would also reduce the RANKL expression in joints (Fig. 5C). Although there is no known human homolog, using shRNA to target *MIP-1 γ* proves that effectively modulating

the osteoclast and subchondral bone can significantly attenuate the OA.

In conclusion, we have provided evidence that CD4⁺ T cells directly and indirectly induce the formation of osteoclasts. The lentivirus that encoded *MIP-1 γ* shRNA was used to treat OA. Our data showed that locally inhibiting MIP-1 γ expression reduced the formation of osteoclasts and cartilage damage and led to the amelioration of OA.

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No competing financial interests exist.

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