T helper cells promote disease progression of osteoarthritis by inducing macrophage inflammatory protein -1γ

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Running title: T helper cell-induced MIP-1γ promotes OA

SUMMARY

Objective: Immune cells are involved in the pathogenesis of osteoarthritis (OA). We examined the effects of T helper (Th) cells, which induce the expression of macrophage inflammatory protein (MIP-1γ), on the progression of OA.

Design: Using anterior cruciate ligament-transection (ACLT), we induced OA in one hind-leg knee joint of B6 mice. The CD4+ T cells from splenocytes and synovium were flow-cytometrically and immunochemically evaluated, respectively. The knee joints were histologically assessed for manifestations of OA. MIP-1γ levels and NF-κB in the knee joints were measured using enzyme-linked immunosorbent and immunoblotting assays, respectively; inflammatory responses were examined using immunohistochemistry. The osteoclastogenesis was detected by tartrate-resistant acid phosphatase (TRAP) staining.

Results: The number of CD4+ T cells and the expression of IFN-γ increased during OA onset (30 days after ACLT) and then decreased at a later stage of OA (90 days after ACLT). Tissue damage induced by CD4+ T cells was evident at the later stage. The activation of CD4+ T cells induced the expression of MIP-1γ and NF-κB. The expression of MIP-1γ can be detected in synorium which T cells were infiltrated. The increased MIP-1γ expression caused an increase in the number of osteoclasts in joints. The regulation of CD4+ T cell was accompanied by increased macrophage infiltration and matrix metalloproteinase-9 expression. Histopathological examinations revealed that CD4+ T cell knockout (CD4−/−) mice had less expression of MIP-1γ and slower cartilage degeneration than control mice had.

Conclusions: CD4+ T cells were activated during the onset of OA, but cartilage damage was more prominent at a later stage. CD4+ T cells were involved in the pathogenesis of OA: they induced MIP-1γ expression and subsequent osteoclast formation.

Keywords*:* CD4+ T cells, osteoarthritis, anterior cruciate ligament, MIP-1γ, osteoclast

Introduction

Osteoarthritis (OA) is a progressive disorder of articular cartilage. Strong evidence indicates that the structural changes in OA are due to a biomechanical alteration[1]. The factors that cause OA are multiple; they include age, weight, sex, bone density, trauma history, and gene-based susceptibility[2]. Degeneration of joint cartilage, with concomitant changes in synovium and subchondral bone, is the major feature of OA. The proinflammatory mediators induced in OA synovium cause synovial hypertrophy and hyperplasia along with an increased number of lining cells and infiltrating inflammatory cells. We previously[3,4] showed that the intraarticular injection of adenovirus-encoding genes with an anti-inflammatory effect, adenoviral vector encoding kallistatin (AdHKBP) and adenoviral vector encoding thrombospondin-1 (AdTSP-1), significantly reduced the severity of OA in rat models.

Accumulating evidence suggests that T helper (Th) cells are essential in inflammatory responses and cartilage damage[5]. The immune response induced by Th cells is evident from the presence of Th cell infiltrates, the expression of Th cell activation antigens, and the expression of type 1 Th (Th1) cytokines in the synovial membranes of patients with OA. Activated Th cells stimulate monocytes to express several cytokines that contribute to the development of OA through direct cell-cell contact or through soluble mediators such as interferon-γ (IFN-γ) and IL-17[6,7]. Th cells contribute to the pathogenesis of bone destruction under the active control of osteoblasts and osteoclasts. Activated Th cells regulate osteoclastogenesis through the receptor activator of nuclear factor-κB ligand (RANKL) and IFN-γ[8]. Osteoclast activation leads to bone resorption and subsequent destruction. Nevertheless, the mechanism of how Th cells affect osteoclastogenesis remains unclear.

To elucidate the effect of Th cells on OA, we used cytokine array analysis in an anterior cruciate ligament-transection (ACLT) model of OA in wild-type mice and in CD4^{+} T cell knockout (CD4^{−/−}/ACLT) mice.

Methods

ANIMAL MODELS

Seven-week-old male B6 and CD4$^+$ T cell knock out (CD4$^{-/-}$) 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. The mice were divided into groups that were not subjected to ACLT (Sham-group) and that were subjected to ACLT (ACLT-group, CD4$^+$ dep/ACLT group and CD4$^{-/-}$/ACLT group). The mice in ACLT-group are wild-type mice. The mice in CD4$^+$ dep/ACLT group are wild-type mice undergoing temporary CD4$^+$ T cell depletion. The mice in CD4-/-/ACLT group are CD4$^+$ T cell knockout mice. To induce experimental OA, each ACLT-group mouse was anesthetized with tiletamine hydrochloride and zolazepam hydrochloride (10 mg/kg) (Zoletil 50; Virbac, Carros, France) and then subjected to a modified ACLT procedure[9].

FLOW CYTOMETRY

Splenocytes from the Sham and ACLT groups (n = 4 per group) were collected on days 30, 60, and 90 after ACLT. Splenocytes (1 × 10^6 per analysis) were either single-stained with PE-conjugated-anti-CD4 (L3T4; BD Biosciences, San Jose, CA) or double-stained with PE-conjugated-anti-CD4 and FITC-conjugated-anti-CD44 (Pgp-1; BD Biosciences) monoclonal antibodies for an hour at 4°C. The cells were then washed twice with staining buffer and suspended in RPMI-based buffer for flow cytometric analysis.

DEPLETING CD4$^+$ T CELLS IN MICE

To produce mice with a temporary depletion of CD4$^+$ T cells (CD4$^+$ dep), we intraperitoneally injected wild-type mice (n= 5) with anti-mouse CD4 (GK1.5) monoclonal antibody 7 days before ACLT. To efficiently deplete the CD4$^+$ T-cell population, the antibody treatment was repeated at 10-day intervals until the day before the mice were killed.

CD4^{+} T cell depletion (> 90%) from splenocytes was confirmed using flow cytometry with anti-CD4 monoclonal antibodies (L3T4; BD Biosciences).

IMMUNOHISTOCHEMISTRY AND HISTOLOGICAL ASSESSMENT

To analyze CD4^{+} T cell infiltrates in the synovium, ACLT was used to induce OA in wild-type mice (n = 4), which were then killed 30, 60, and 90 days later. Their synovial membranes were removed, fixed, and embedded in paraffin. Serial sections (5 μm thick) were cut and then incubated with rat anti-mouse CD4 (L3T4) (1:100; H129.19; BD Biosciences Pharmingen, San Diego, CA) and IFN-γ (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies at 4°C overnight. To test CD3, MIP-1γ, Mac-3 antibody and matrix metalloproteinase (MMP)-9 expression, the samples from joints were prepared from wild-type and CD4^{−/−} mice (n = 4 per group). Serial sections of cartilage were stained with hamster anti-mouse CD3 antibody (1:100, BD Biosciences Pharmingen), rabbit anti-mouse MIP-1γ antibody (1:100, Peprotech, Rocky Hill, NJ), Mac-3 (1:100; BD Biosciences Pharmingen) and MMP-9 (1:100) (Santa Cruz Biotechnology) at 4°C overnight. After they had been sequentially incubated with the appropriate secondary antibody (1:400) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 hours at room temperature with aminoethyl carbazole as the substrate chromogen (Invitrogen Zymed Laboratories, Camarillo, CA), the slides were counterstained with hematoxylin and eosin.

To evaluate the effect of CD4^{+} T cells at different stages of OA, ACLT was used to induce OA (n = 5 per group) in wild-type mice that CD4^{+} T cells had been temporary depleted (CD4^{+} dep). These mice were killed 30, 60, and 90 days later. Their synovial membranes were removed, fixed, and embedded in paraffin. Serial sections (5 μm thick) were cut and stained with hematoxylin and eosin. To test for the histopathologic changes of cartilage in CD4^{+} T cell knockout mice (CD4^{−/−}), ACLT was used to induce OA (n = 4 per group). On day 45 and 90, the cartilage and synovium were processed for staining with

Safranin-O/fast green and hematoxylin and eosin. The histologic change in cartilage was scored using Mankin's histologic grading method[3,10]. The histologic changes in synovial surface tissue and subsynovial tissue were evaluated and scored[3,11]. 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), [iii] and the infiltration of inflammatory cells (0-3 points). Total scores in each category were calculated; the maximum score was 18 points. On day 90, both groups of mice (n= 6 per group) were killed and their sera collected. The tartrate-resistant acid phosphatase (TRAP) in mouse serum was assayed (MouseTRAP; Immunodiagnostic Systems, Boldon, UK). Osteoclasts in tissues were identified by histochemically staining TRAP using a commercial kit according to the manufacturer's instructions (Sigma, St Louis, MO).

ASSESSING MIP-1γ AND NF-κB EXPRESSION

On day 90, the mice were killed, their joints skinned, and the synovial tissue removed and dissected for homogenization in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail. The homogenates from five mice in each group were pooled. MIP-1γ expression in mice after ACLT was determined using a mouse inflammation antibody array kit (RayBiotech, Atlanta, GA). The intensity of the signals was shown using a digital imaging analysis system (Eastman Kodak Co., Rochester, NY). The relative density was normalized with an internal control and expressed as a ratio of the image density of each group divided by that of the Sham group. To quantify the MIP-1γ in tissue, mouse (n= 6) MIP-1γ levels in the homogenates were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). To test NF-κB activity, the homogenates were subjected

to immunoblot analysis using antibodies against NF-κB p65 antibody (1:1000) (93H1; Cell

Signaling, Danvers, MA). β-actin expression was used as the quantitative control.

STATISTICAL ANALYSIS

All data are represented as means plus 95% confidence intervals (CI). The quantization

of CD4^{+} T cells, MIP-1γ and TRAP proteins in Figures 1A, 3C, and 5B were analyzed using

Student's t test. Significance was set at $P < 0.05$. JMP 8.0 (SAS Institute Inc., Cary, NC) was

used to analyze the histological data in Figures 2B, 6C and supplementary Figure 2B.

Significance between groups was estimated using one-way analysis of variance (ANOVA).

To evaluate the differences between groups, we used Tukey's Honestly Significant

Difference test set at $P < 0.05$.

Results

CD4+ T CELL ACTIVATION DURING THE PROGRESSION OF OA

To examine the alteration of CD4+ T cells in OA, flow-cytometry was used to count the number of CD4+ T cells in splenocytes in the Sham and ACLT groups. The number of CD4+ T cells significantly increased on day 30 in ACLT group [27.6% (25.18-30.02) versus 23.1% (18.42-27.77), $P = 0.017$, Fig. 1A], but the differences on days 60 and 90 were not significant. CD4+ T cell activation was further tested. On day 30, the percentage of CD4+/CD44+ T cells in the ACLT group was 24.5%, higher than the 14.8% in the Sham group (Fig. 1B). The percentages declined in both groups on day 60 (12.8% versus 17.3%), and no difference detected on day 90 (17.0% versus 17.0%). Furthermore, notable increases of CD4+ T cells infiltrated the synovium of mice from the ACLT group on day 30. The numbers of infiltrating cells reduced on day 60 and only a few cells were detected on day 90 (Fig. 1C). No significant change was seen in Sham group at the three time points. IFN-γ was noticeably expressed in synovial tissue from ACLT knee joints on day 30 (Fig. 1D), but barely detectable on days 60 and 90 (data not shown).

In order to assess the effect of CD4+ T cells on OA progression, CD4+ T cells were depleted in wild-type (CD4+ dep) mice and the histopathologic changes analyzed. Synovial membranes from three groups (Sham, CD4+ dep/ACLT, and ACLT) showed no significant alteration during the first two months (Fig. 2A). Specimens from the ACLT group mice showed hyperplasia of the lining cells and increased cell infiltration on day 90. Lesions from the CD4+ dep/ACLT group mice were markedly less severe. The synovitis score in the joints of ACLT group mice was significantly higher than that in CD4+ dep/ACLT on day 90 ($P<$ 0.001) (Fig. 2B). These results suggest that CD4+ T cells may induce inflammation in the early stage of OA, whereas the damage in cartilage caused by inflammation is more prominent in the later stage.

DECREASED MIP-1γ EXPRESSION IN CD4$^{-/-}$ MICE

To identify the proteins affected by CD4$^+$ T cells in cartilage, we induced OA in CD4$^{-/-}$ mice. Cytokine array analysis showed that several cytokines and chemokines—soluble tumor necrosis factor receptors II (sTNF-RII), IL-4, tissue inhibitor of metalloproteinase (TIMP)-1, and MIP-1γ—were altered in the CD4$^{-/-}$/ACLT group mice on day 90 after OA induction (Fig. 3A). The sTNF-RII, IL-4, TIMP-1 expressions were lower, whereas MIP-1γ expression was higher in the ACLT group than in the CD4$^{-/-}$/ACLT groups (Fig. 3B). This result was confirmed using an ELISA assay. MIP-1γ expression was significantly lower in CD4$^{-/-}$/ACLT group mice [7673.26 (5916.08-9430.44) pg/ml] than in ACLT group mice [20321.24 (17260-23382.95) pg/ml, $P < 0.001$] (Fig. 3C). To detect the distribution of T cells and MIP-1γ protein, we performed the IHC on serial sections of joints. The CD3$^+$ T cells were only detected in the bone marrows of the sham and CD4$^{-/-}$/ACLT groups mice whereas lots of CD3$^+$ T cells were infiltrated throughout the synovial membrane in ACLT group mice (Figure 4). Increased expression of MIP-1γ was also detected in ACLT groups. Some locations of MIP-1γ expression were overlapped with those of T cells infiltration. Much less MIP-1γ could be detected in CD4$^{-/-}$/ACLT though some proteins in the synovium of Sham group was observed. The locations where CD4$^+$ T cells infiltrated and MIP-1γ expression were also similar in the synovium of ACLT group mice (Supplementary Fig.1)

DECREASED OSTEOCLASTOGENESIS IN CD4$^{-/-}$ MICE

Because MIP-1γ expression promotes osteoclast survival through NF-κB activation, we next tested the expression of activated NF-κB in mice. Ninety days after ACLT, NF-κB expression in tissue homogenate was markedly lower in CD4$^{-/-}$/ACLT group mice than in Sham and ACLT group mice (Fig. 5A). The levels of the osteoclast marker TRAP were significantly lower in the serum of CD4$^{-/-}$/ACLT group mice (1.18 (1.055-1.305) U/L) than in that of ACLT group mice (2.59 (2.305-2.875) U/L, $P < 0.001$) (Fig. 5B). Large,

multinucleated osteoclasts adjacent to the calcified cartilage were prominent in ACLT group

mice and its reduction was seen in CD4$^{-/-}$/ACLT group mice (Fig. 5C). In addition, more

intense staining of TRAP was seen in the proximal tibial growth plate of ACLT group mice.

These results suggested that the expression of MIP-1γ and activated NF-κB upregulated

osteoclast levels. Increased MIP-1γ expression attracted macrophages into the synovium.

Macrophage infiltration is a major feature of tissue inflammation in OA. Ninety days after

ACLT, the relative number of Mac-3-positive macrophages that had infiltrated the synovium

was higher in ACLT group mice than in CD4$^{-/-}$/ACLT group mice (Fig. 5D). Given that

MMP-9 is essential for recruitment of osteoclast and activated CD4$^+$ T cells stimulate the

degradation of extracellular matrix[5, 12], we measured the expression of MMP-9 in the mice.

MMP-9 expression was higher in the cartilage of ACLT mice than CD4$^{-/-}$/ACLT mice (Fig.

5E).

THE EXPRESSION OF MIP-1γ DURING THE PROGRESSION OF OA IN CD4$^{-/-}$ MICE

        Histopathological examination of the knee joints of ACLT group mice revealed loss of

proteoglycan on day 45, more severe cartilage destruction with disorganized cartilage

contours as well as hypocellularity was seen on day 90. However, the disease progression in

CD4$^{-/-}$/ACLT group was slower. Specimens from CD4$^{-/-}$/ACLT group mice showed a mild

irregularity on the surface on day 45, and decrease in cartilage thickness in the posterior of

the femur on day 90 (Fig. 6A). Most of the articular cartilage in the knee joints of Sham

group mice had a smooth surface. The synovium in the knee joints of ACLT group mice was

thick with moderate cellular infiltration, and the lining cells had proliferated on day 90

(Supplementary Fig. 2). CD4$^{-/-}$/ACLT group mice showed slightly more cell proliferation in

the synovial lining than did Sham group mice. The expression of MIP-1γ was only detected

in the lower metaphysic in Sham and CD4$^{-/-}$/ACLT groups (Fig. 6B). However, the staining

of MIP-1γ can be detected in the synovium of ACLT groups on day 45. On day 90, the

MIP-1γ–positive cells were evident in the chondro-osseous junction. Mankin's scores of joints from ACLT group mice were significantly higher than those of joints from CD4$^{-/-}$/ACLT mice on day 90 (P=0.002) (Fig. 6C). Taken together, these results indicated that the loss of Th cells potently alleviated inflammation and cartilage damage by inhibiting MIP-1γ expression.

Discussion

To determine the potential effect of Th cells on OA development, we induced OA in the knee joints of CD4$^{-/-}$ mice. The levels of several proteins—sTNF-RII, IL-4, TIMP-1, and MIP-1γ—in the knee joints of CD4$^{-/-}$/ACLT mice were changed. The first three proteins showed a mild increase, whereas the MIP-1γ showed a significant reduction. sTNF-RII and IL-4 are both believed to have anti-inflammatory functions during disease progression[13,14]. Four members of the TIMP family—TIMP-1, -2, -3, and -4—have been characterized[15]. TIMP-1 is a dominant cartilage TIMP that inhibits all collagenase, including MMP-1[16]. The equilibrium between MMP-1 and TIMP-1 is controlled by Th type 2 (Th2) cytokines[17]. The absence of CD4⁺ T cells may ameliorate the OA symptoms partially through the suppression of these anti-inflammatory factors. The most obvious alteration in protein level was in MIP-1γ. ELISA showed an approximately 62% reduction of MIP-1γ expression from the cartilage homogenate of CD4$^{-/-}$/ACLT mice when OA was induced. MIP-1γ belongs to a C-C chemokine family containing MIP-1α, MIP-lβ, MIP-1γ, RANTES, etc. MIP-1γ is produced by monocytes and other types of leukocytes. Through binding to chemokine receptor 1 (CCR1), a specific receptor on neutrophils, MIP-1γ acts as a chemoattractant that induces the chemotaxis of CD4⁺ T cells, CD8⁺ T cells, and monocytes[18,19]. These infiltrating immune cells are responsible for orchestrating immune and inflammatory responses in synovium. In fact, the levels of two other members of the MIP-1 chemokine family, MIP-1α and MIP-lβ, have been reported to be increased in the synovial fluid of patients with rheumatoid arthritis[20,21]. The roles of MIP-1γ in patients with arthritic diseases have not yet been clarified. MIP-1γ promoted osteoclast formation and survival through the RANKL pathway[22,23]. Bone-resorbing osteoclasts coordinately interplay with osteoblasts and remodel bone through highly regulated molecular and cellular events. The activity of the osteoclast marker TRAP was remarkably reduced in the serum of CD4$^{-/-}$/ACLT mice. The osteoclast

formation was increased in both subchondral bone and metaphyseal surfaces in the joints of ACLT mice. The staining of MIP-1γ was more evident in the chondro-osseous junction of ACLT groups on day 90. The similarly expressed locations of MIP-1γ and osteoclast markers indicate that bone erosion induced by MIP-1γ may occur here. Our results suggested that CD4^{+} T cells regulate the activity of osteoclasts through MIP-1γ protein expression during the progression of OA.

The RANKL signaling pathway derived from Th cells is responsible for pathologic osteoclastogenesis and focal erosion in arthritis[23]. However, T cells also have a negative regulatory mechanism for inhibiting osteoclastogenesis. IFN-γ and IL-4 produced by the CD4^{+} T cell subsets Th1 and Th2, respectively, showed an anti-osteoclastogenic effect on arthritis[24,25]. We found IFN-γ expression but no apparent cartilage damage 30 days after OA had been induced; however, 90 days after OA had been induced, we found no IFN-γ expression but did find severe cartilage and bone damage. Th cell-induced IFN-γ expression may compromise the activation of osteoclasts and subsequent cartilage damage in the early stage of OA. Th17 cells, a new CD4^{+} T cell subset, have been identified as being primarily responsible for inducing RANK[26], thus causing osteoclast activation. IL-17 produced by Th17 also induced the production of MMP-9 in osteoclasts[27]. Furthermore, the secreted IL-17 induces the expression of three chemokines (monocyte chemotactic protein (MCP)-1, MIP-2, and TIMP-1), and MMP-9 in fibroblasts, and attracts both monocytes and neutrophils to the inflammatory focus[28]. Consistent with these findings, our data showed that monocyte aggregation and MMP-9 staining were more prominent 90 days after OA had been induced. Our results showed that the number of CD4^{+} T cells infiltrating the synovium gradually fell during the disease progression. We suspect that in the early stage of OA, during which a large number of Th cells is activated, Th1 and Th2 cells and the cytokines they induce are important for initiating OA. During the progression of OA, some Th cells (probably Th17

cells) may continuously cause subsequent exacerbation despite a decrease in the number of Th cells. This possibility needs to be examined in additional studies. Our data suggest that temporal activation of CD4$^+$ T cells may affect the way in which OA manifests itself.

Our study had some limitations. First, because of the significant involvement of CD4$^+$ T cells in OA, in this study, their effect on the pathogenesis of OA was focused. Nevertheless, we could not rule out the potential role of CD8$^+$ cytotoxic T cells here, because CD3$^+$ T cells (the common marker of both CD4$^+$ and CD8$^+$ T cells) have been found in the angiocentric infiltrates from the synovial membrane of patients and with OA[5]. Our results also showed that CD3$^+$ T cells were detected in the synovium of mice with OA. In addition, CD8$^+$ T cells specific for common herpes viruses, such as Epstein-Barr virus and cytomegalovirus, have also been nonspecifically trapped, although in fewer patients with OA than in patients with rheumatoid arthritis, at the sites of inflammation in the synovium[29]. Whether these CD8$^+$ T cells recruited in synovium after viral infection are involved in OA is unclear. In a preliminary study (unpublished observations), we found that the number of CD8$^+$ T cells and TIMP-1 expression changed during OA progression in CD8$^+$ T cells knockout mice. How CD8$^+$ T cells affect this gene and the putative functions of the CD8$^+$ T cells involved in OA is under investigation. Second, we used ACLT-induced OA in our study. Based on the histological observation of animal models, we classified day 90 after ACLT treatment as the "late stage" of OA and examined the changes in cytokine expression at this time point. The actual development of OA in humans can persist for several years. Although the pathogenic characteristics of the mice in our OA animal model and in patients with OA are similar, ACLT-induced OA can not be completely translated into the clinical setting. Because cytokines fluctuate during the progression of OA, we could not rule out the possibility that other cytokines affected by CD4$^+$ T cells participate in the development of OA.

In conclusion, we showed that CD4$^+$ T cells were involved in the initiation of OA in a

mouse ACLT model. Cytokine array analysis showed that MIP-1γ protein expression was

significantly lower in CD4$^{-/-}$/ACLT mice. MIP-1γ protein may be responsible for osteoclast

formation and joint destruction in the later stage of OA. We conclude that MIP-1γ protein is a

potential therapeutic target for the treatment of OA.

Conflict of interest

Acknowledgments

Contributions

C.L.W. and I.M.J. have made substantial contributions to the conception, design of the study and interpretation of data. P.C.S. and J.L.H. have made substantial contributions to the acquisition, analysis of data and drafting the article. C.H.L. has made substantial contributions to critical revision of the article for important intellectual content. S.Y.J., P.J.L. and S.H.C. have made substantial contributions to provision of study materials and acquisition of data. J.L.H. (e-mail address:pipi58871053@yahoo.com.tw) integrates the work and makes a final approval of the article for submission.

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Figure Legends

Figure 1. Quantitation of CD4$^+$ T cells in mice subjected to anterior cruciate

ligament-transection (ACLT). (A) Using ACLT, osteoarthritis (OA) was induced in the knee

of one hind leg of mice on day 0. Splenocytes from the Sham and ACLT groups (n = 4) were

collected and stained for surface CD4$^+$ on days 30, 60, and 90. The number of CD4$^+$ T cells

(mean ± 95% confidence intervals) is expressed as the percentage of CD4$^+$ T cells/1×10^6

splenocytes. (B) Splenocytes of four mice per group were pooled and stained for surface CD4

and CD44 on days 30, 60, and 90. Data are expressed as the percentage of CD4$^+$/CD44$^+$ T

cells/1 × 10^6 splenocytes. Data are representative of two independent experiments. (C)

Synovial membranes from the Sham and ACLT groups on days 30, 60, 90 were removed and

stained with antibody against CD4$^+$ (×400 magnification) (D) Immunohistochemical staining

showed higher IFN-γ expression in the synovium of ACLT group mice than of Sham group

mice, which showed no IFN-γ expression on day 30 (×200 magnification).

Figure 2. Evaluation of histological changes in the knee joints after the onset of

ACLT-induced OA. (A) Proliferation of synovial lining cells was mildly greater in CD4$^+$

dep/ACLT group mice than in Sham group mice on day 60. More severe synovitis occurred

in ACLT group mice than in CD4$^+$ dep/ACLT mice on day 90. (Hematoxylin and eosin stain;

×200 magnification). (B) Histological examination showed a significantly higher synovitis

score in the tissues from the knees of ACLT group mice on day 90. Data shown are means ±

95% confidence intervals (n= 5 per group).

Figure 3. Profile of inflammatory cytokines in mice with OA. (A) A cytokine array assay 90

days after ACLT treatment shows a mildly higher level of sTNF-RII, IL-4, and TIMP-1

expression and a markedly lower level of MIP-1γ expression in CD4$^{-/-}$/ACLT group mice

(n= 5 per group) than in ACLT group mice. (B) The relative density of cytokines was

normalized with the internal control and expressed as a ratio of the expression level of

cytokines in each group divided by the expression level in the Sham group. Each value

represents the average of two replicated spots on the membrane. (C) MIP-1γ expression was

significantly higher in ACLT group mice (n= 6) than in Sham group and CD4$^{-/-}$/ACLT group

mice (determined using ELISA). Values are means ± 95% confidence intervals.

Figure 4. The infiltration of T cells and MIP-1γ expression after ACLT in mice.

Representative immunohistochemical images of the cartilage are shown (n = 4 per group). In

the ACLT group mice (middle panel), more T cell infiltration and MIP-1γ expression were

seen in synovium than those of the Sham and CD4$^{-/-}$/ACLT groups mice 90 days

post-surgery (upper and lower panel, ×100 magnification). The overlapped locations of T cell

infiltration and MIP-1γ expression were indicated with arrows in ACLT group mice. The

insets represent the magnified area (× 400 magnification, right panel). The CD3-postive and

MIP-1γ–positive cells were indicated with arrow heads.

Figure 5. The suppression of osteoclastogenesis after ACLT in the absence of CD4$^+$ T cells.

(A) NF-κB expression levels were higher in ACLT group mice than in CD4$^{-/-}$/ACLT group

mice. (B) The serum levels of TRAP were significantly lower in CD4$^{-/-}$/ACLT mice (n= 6

per group) than in ACLT group mice. (C) TRAP staining reveals fewer osteoclasts (arrows)

were formed between the cartilage and subchrondral bone layer (upper panel) and tibial

metaphysis (lower panel) in CD4$^{-/-}$/ACLT group mice (n = 4 per group) as compared to

ACLT group ones (×400 magnification). Cart, cartilage layer; SB, subchrondral bone layer.

(D) The relative number of Mac-3-positive macrophages (×200 magnification) in the

subsynovial tissue of ACLT group mice was higher than that of CD4$^{-/-}$/ACLT group mice.

Synovial membranes from the Sham group mice showed no significant staining. (E)

Immunohistochemical staining showed higher MMP-9 expression in the cartilage from the

ACLT group mice than from the CD4$^{-/-}$/ACLT group mice (×200 magnification).

Figure 6. Cartilage degeneration and MIP-1γ expression on the articular cartilage after

ACLT. Representative histology images of the cartilage on the medial femoral condyle are shown. (A)ACLT group mice showed more aggressive cartilage damage (n= 4 per group). The chondrocyte loss was noted on day 45, and the disease progress to a disappearance of the surface layer cells on day 90 (Safranin-O/fast green stain, ×200 magnification). In the CD4$^{-/-}$/ACLT group mice, the specimen showed a proteoglycan reduction and fissures in the superficial cartilaginous layer (arrow) on day 90. In the sham group, the superficial cartilaginous layer shows a smooth and regular surface. (B) The expression of MIP-1γ was restricted in the lower metaphysis in Sham and CD4$^{-/-}$/ACLT groups mice as indicated with arrows (×400 magnification). The MIP-1γ–positive cells were visible in the synovium and then the chondro-osseous junction when disease progressed. (C) A histological examination showed lower Mankin's scores in the joints from CD4$^{-/-}$/ACLT group mice than in those from ACLT group mice. Data shown are mean ± 95% confidence intervals.