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ORIGINAL ARTICLE

Intra-articular lentivirus-mediated delivery of galectin-3 shRNA and galectin-1 gene ameliorates collagen-induced arthritis

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Different members of the galectin family may have inhibitory or stimulatory roles in controlling immune responses and regulating inflammatory reactions in autoimmune diseases such as rheumatoid arthritis (RA). A hypothetical model of a cross talk between galectin-1 and galectin-3 has been established in the circumstance of rheumatoid joints. As galectin-3 is a positive regulator and galectin-1 is a negative regulator of inflammation and autoimmune responses, in this study we evaluated the effects of local knockdown of galectin-3 or overexpression of galectin-1 on ameliorating collagen-induced arthritis (CIA) in rats. Lentiviral vectors encoding galectin-3 small hairpin RNA (shRNA) and galectin-1, as well as two control vectors expressing luciferase shRNA and green fluorescent protein, were individually injected intra-articularly into the ankle joints of rats with CIA, and their treatment responses were monitored by measuring the clinical, radiological and histological changes. Our results show that both knockdown of galectin-3 and overexpression of galectin-1 induced higher percentages of antigen-induced T-cell death in the lymph node cells from arthritic rats. Furthermore, these treatments significantly reduced articular index scores, radiographic scores and histological scores, accompanied with decreased T-cell infiltrates and reduced microvessel density in the ankle joints. Our findings implicate galectin-3 and galectin-1 as potential therapeutic targets for the treatment of RA.
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Keywords: *galectin-3; galectin-1; collagen-induced arthritis; T-cell death; angiogenesis; rheumatoid arthritis*

Introduction

Rheumatoid arthritis (RA) has a worldwide distribution, and is associated with significant morbidity and mortality.[1,2] Greater understanding of the etiology of RA has brought about a large number of potential molecular targets for the development of therapeutic candidates.[1,3,4] Experimental animal models of RA, including collagen-induced arthritis (CIA), have provided a valuable tool to dissect underlying mechanisms involved in the disease process.[5] Genes of interest can be delivered locally and efficiently at the joint by intra-articular (i.a.) injection into ankle joints of rats with CIA.[6–9] Such proof of concept in animal models of RA provides important validation of the pre-clinical miniature, and has led to the elucidation of putative roles for certain molecules in the pathogenesis of inflamed joints.

Galectins are a family of endogenous lectins with affinity for β-galactoside-containing oligosaccharides, and their different members may have inhibitory or stimulatory role in controlling immune responses and regulating inflammatory reactions in autoimmune diseases.[10] In RA, the galectin-3-binding protein was particularly expressed at sites of bone destruction and, in contrast, the expression of galectin-1 was never found at sites of joint invasion.[11] Furthermore, defective mononuclear cell apoptosis in infiltrates of synovial tissues from juvenile RA correlated with upregulation of galectin-3 and downregulation of galectin-1.[12] In addition, not only being involved in inflammation, galectin-3 was reported to contribute to the activation of RA synovial fibroblasts.[13] Therefore, a hypothetical model of a cross talk between galectin-1 and galectin-3 has been established in the inflammatory circumstance of rheumatoid joints.[14,15] Recently, galectin-1 was shown to induce the production of interleukin-10 and attenuate the secretion of interferon-γ in activated T cells, and galectin-3 was shown to promote the production of proinflammatory cytokines, such as interleukin-6 and

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tumor necrosis factor-α, in synovial fibroblasts.[16,17] A single injection of syngenic DBA/1 fibroblasts engineered to secret galectin-1 at the onset of disease abrogated experimental arthritis in mice.[18] Furthermore, microarray analysis of peripheral mononuclear cells has identified galectin-3 as a blood biomarker in the rat CIA model.[19]

Local gene transfer into the rheumatoid joints has been evaluated to bypass the inherited obstacle associated with delivery of therapeutic compounds.[20–22] Lentivirus-mediated gene transfer has been shown to result in efficient expression of transgenes in synovial tissues.[21–22] As galectin-3 and galectin-1 appear to be positive and negative regulators, respectively, of inflammation and autoimmune responses, in this study we investigated the effects of galectin-3 gene silencing or galectin-1 overexpression mediated by lentiviral gene transfer in rats with CIA. Our findings implicate galectin-3 and galectin-1 as potential therapeutic targets for treating RA.

Results

Expression of galectins in the synovial tissue of rats with CIA

We first examined the expression of galectin-1 and galectin-3 in the synovium of rats with CIA. Significant upregulations of galectin-3 were detected from day 11 onward, with a greater than threefold increase compared with normal rats, in the joint extracts from the synovial tissue of rats after immunization with collagen on days 0 and 7 (Figure 1a). By contrast, the level of galectin-1 was increased approximately fourfold on day 8, but declined rapidly afterward (Figure 1a). These observations suggest the involvement of galectins in experimental arthritis, and provide a rationale to explore whether silencing galectin-3 expression or overexpressing galectin-1 would ameliorate arthritic symptoms in rats.

Selection of shRNA targeting galectin-3

Four small hairpin RNA (shRNA) sequences were designed to specifically target rat galectin-3. We co-transfected 293 cells with pEGFP-N1/Gal3 plasmid, which encodes green fluorescent protein (GFP)-fused galectin-3, and various pSuper-based shRNA expression vectors and analyzed the expression of GFP by flow cytometry. The level of GFP expression was inversely proportional to the efficacy of shRNA in knocking down galectin-3 expression. We found that the percentage of GFP-positive cells was less in cells transfected with sh537 or sh44 than those transfected with sh597 or sh654 (Figure 1b). Furthermore, sh537 or sh44 exhibited similar activity to the GFP shRNA. Figure 1c shows the representative histogram of 293 cells expressing GFP after transfection with sh537, sh44, shGFP and control

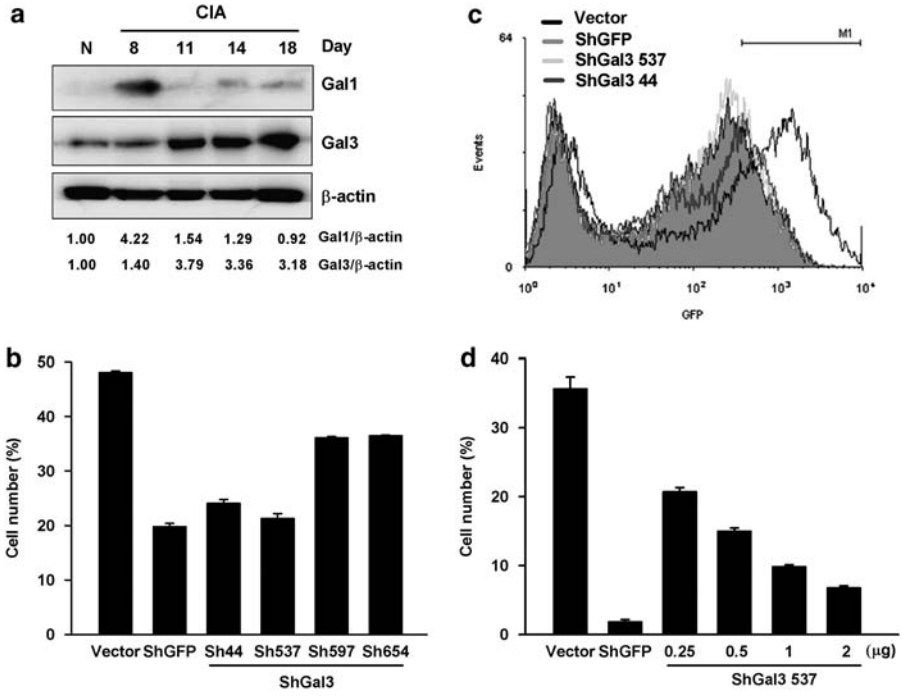


Figure 1 Levels of galectins in the ankle joints of rats with CIA and selection of shRNA targeting galectin-3. (**a**) Decreased galectin-1 (Gal1) and increased galectin-3 (Gal3) expressions during the time course of CIA, as determined by immunoblot analysis. N, normal. Expression of β-actin served as the loading control. The levels of the intensity of the bands corresponding to Gal1, Gal3 and β-actin were determined by densitometry. Ratios between the intensity of the bands corresponding to Gal3 or Gal1 and those corresponding to β-actin were calculated. Values shown below the blots are the fold increase in the ratio in each lane relative to the ratio obtained for the control lane from the sample collected from a normal rat. (**b**, **c**) Screening of effective Gal3 shRNA by a fusion green fluorescent protein (GFP) system. 293 cells were co-transfected with 2 μg of pSuper, pSuper/shGFP or pSuper/shGal3 plasmid and 2 μg of pEGFP-N1/Gal3 plasmid. (**b**) The percentage of GFP-positive cells was determined by flow cytometric analysis 48 h after transfection. (**c**) The representative histogram of 293 cells expressing GFP after transfection with different shRNA or control vectors. (**d**) Knockdown of GFP expression by shGal3 537 in a dose-dependent manner. 293 cells were co-transfected with various amounts of shGal3 537 plasmid and pEGFP-N1/Gal3 plasmid. The percentage of GFP-positive cells was determined by flow cytometric analysis 48 h after transfection. Values are the mean ± s.e.m. of the mean ($n = 3$).

constructs. To further confirm that sh537 can be a candidate shRNA specific to galectin-3, we co-transfected 293 cells with various amounts of sh537 plasmid and pEGFP-N1/Gal3 plasmid. We found that sh537 dose-responsively reduced the percentage of GFP-positive cells (Figure 1d). Therefore, sh537 was chosen and cloned into the lentiviral vector to generate pLVTHM/shGal3 plasmid.

Production of recombinant lentiviruses

Various recombinant lentiviruses, including Lt.shGal3, Lt.shLuc, Lt.Gal1 and Lt.GFP were generated. Immunoblot analysis validates that treatment of Lt.shGAL3, but not Lt.GFP, significantly reduced the expression of rat galectin-3–GFP fusion protein, but not human galectin-3 (Figure 2a). Expressions of galectin-1 and GFP transgenes in 293 cells transduced with Lt.Gal1 and Lt.GFP were verified by immunoblotting, respectively (Figure 2b). *In vivo* GFP expression was also detectable in the Lt.GFP-injected ankle joint of rats with CIA, whereas the Lt.Gal1-injected counterpart showed only background fluorescence (Figure 2c).

Clinical and radiographic features of rat with CIA after treatment with Lt.shGal3 or Lt.Gal1

We assessed whether galectin-3 knockdown or galectin-1 overexpression mediated by lentiviral gene transfer on days 7 and 8 in the ankle joints has beneficial effects on the arthritis symptoms. Our results revealed that the articular index scores both in the rats treated with Lt.shGal3 (Figure 3a) and Lt.Gal1 (Figure 3b) were significantly lower than those in their control counterparts, indicating reduced joint inflammation and pathological changes in the treated rats. Furthermore, 5×10^6 transducing units (TUs) of Lt.shGal3 exerted higher activity in suppressing arthritis development than 5×10^5 TU of Lt.shGal3 ($P < 0.05$), whereas the effect induced by Lt.Gal1 did not differ between the two doses. Notably, Lt.shGal3 was more effective than Lt.Gal1 in ameliorating the arthritis symptoms ($P < 0.05$). In another set of the experiment, rats with CIA were treated with 5×10^6 TU of recombinant lentiviruses. The plain radiography on day 17 revealed that rats treated with Lt.shGal3 or Lt.Gal1 had more intact joint structure, as compared with their two control counterparts (Figure 3c). The radiological score, based on joint space width, degree of bony destruction and soft tissue sweling, was also significantly lower in the rats treated with Lt.shGal3 or Lt.Gal1 (Figure 3d). However, the combination treatment regimen with Lt.Gal1 and Lt.shGal3 did not significantly enhance the therapeutic efficacy as compared with either Lt.Gal1 or Lt.shGal3 treatment alone (data not shown).

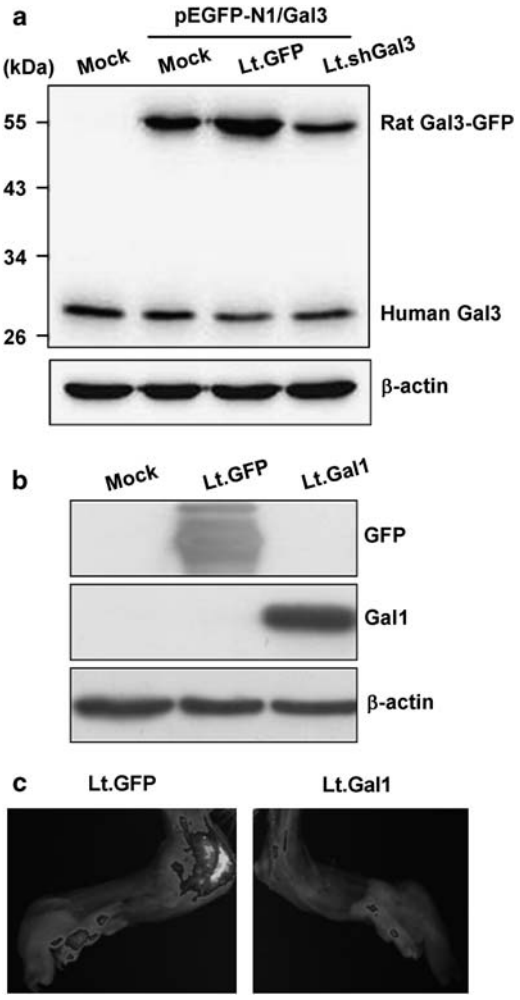


Figure 2 Characterization of different recombinant lentiviruses. (**a**) Knockdown of the expression of rat galectin-3–GFP fusion protein by Lt.shGal3 but not by Lt.GFP in cells transfected with pEGFP-N1/Gal3 plasmid. 293 cells that had been transduced with Lt.shGal3 or Lt.GFP at a multiplicity of infection of 10 for 2 days were transfected with pEGFP-N1/Gal3 (1 μg). After 3 days, cell lysates were collected, and expression of galectin-3 was examined by immunoblotting. (**b**) Detection of galectin-1 and GFP expression in 293 cells transduced with Lt.Gal1 and Lt.GFP, respectively. Cells were infected with Lt.Gal1 or Lt.GFP at an multiplicity of infection of 10 for 48 h, and their lysates were harvested for detecting the expression of galectin-1 and GFP by immunoblotting. (**c**) *In vivo* detection of GFP expression in the ankle joint from an arthritic rat following Lt.GFP injection. On day 7, Lt.GFP and Lt.Gal1 (5×10^6 transducing units (TUs)) were injected into the left and right ankle joints, respectively, of a rat that had been immunized with collagen on days 0 and 7. Expression of GFP was visualized after 3 days with a Berthold Night Owl CCD camera equipped with 480 nm excitation and 520 nm emission filters.

Histopathological features and angiogenesis in rat with CIA after treatment with Lt.shGal3 or Lt.Gal1

Histopathological examination of the joint tissues from Lt.GFP- or saline-treated ankles revealed pannus formation, wherein synovial tissue containing more cell infiltrates erodes cartilage and bone (Figure 4a). However, in either the Lt.shGal3- or Lt.Gal1-treated ankles, joint tissues showed relatively milder changes with no erosion on cartilages. Moreover, there were fewer microvessels in the synovium from the Lt.shGal3- or Lt.Gal1-treated rats than those from the Lt.GFP- or saline-treated rats, as identified by immunohistochemical staining for von Willebrand factor (Figure 4b). The histological score of synovial hyperplasia, cartilage erosion and leukocyte infiltration was significantly lower in the Lt.shGal3- or Lt.Gal1-treated rats compared with their two control counterparts (Figure 4c). Quantitative data revealed reduced microvessel density in the Lt.shGal3- or Lt.Gal1-treated synovium (Figure 4d). Of note, Lt.shGal3 was more effective than Lt.Gal1 in reducing angiogenesis in the synovium.

Effects of Lt.shGal3 or Lt.Gal1 treatment on T cells in the ankle joints of rat with CIA

Immunohitochemical staining showed a significant decrease in CD3-positive cells that infiltrated to the synovium in the Lt.shGal3- or Lt.Gal1-treated groups (Figure 4e). Quantitative analysis also confirmed significant lower numbers of T cells in the synovium of Lt.shGal3- and Lt.Gal1-treated groups compared with those from the two control groups (Figure 4f). Lymph node cells from rats treated with Lt.shGal3 or Lt.Gal1 were assessed *in vitro* for cell

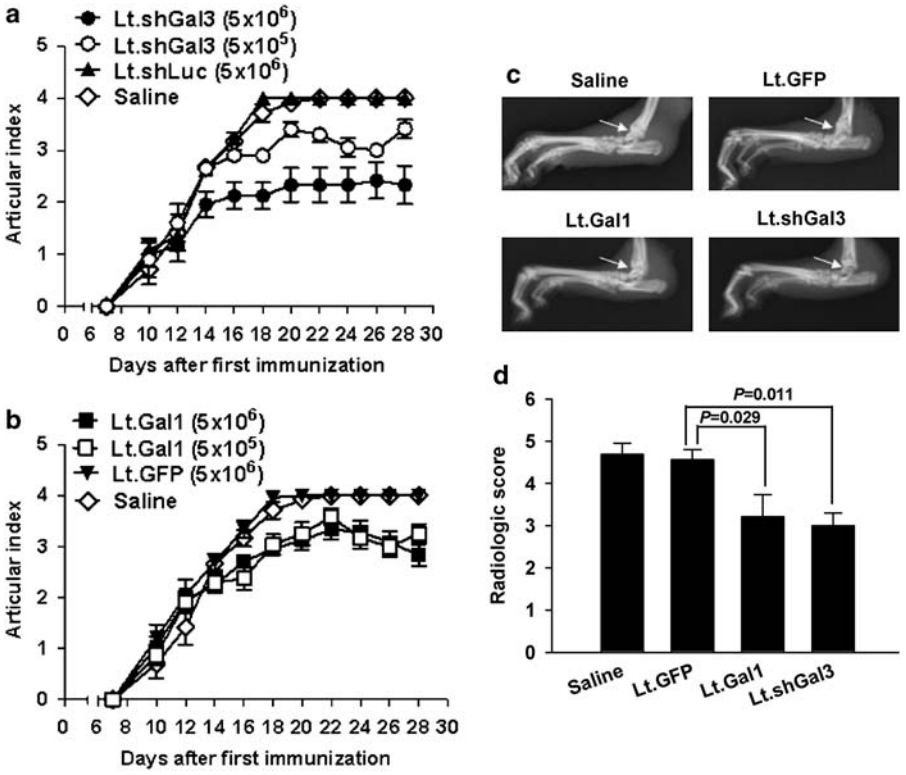


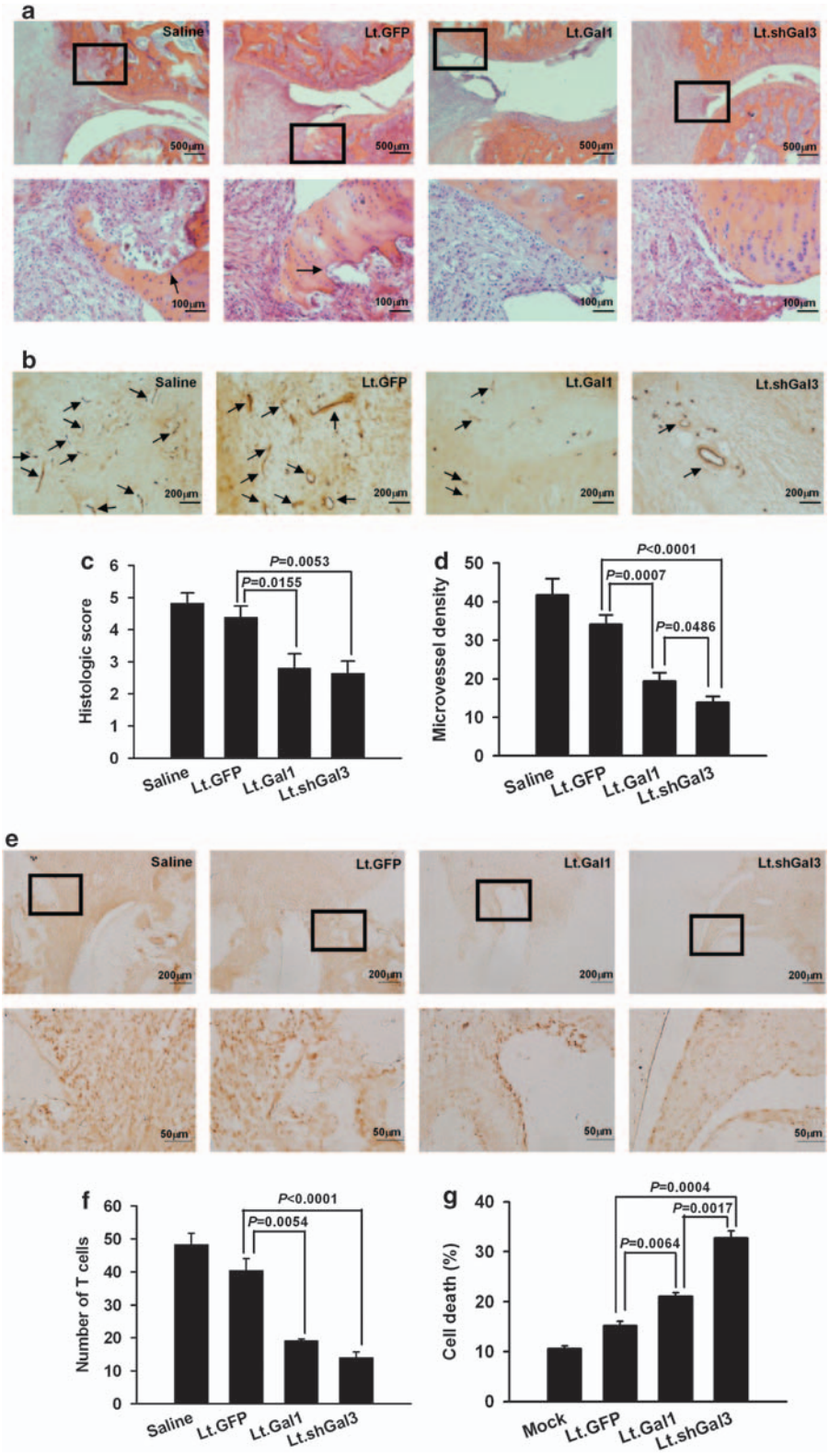
Figure 3 Amelioration of clinical symptoms of rats with CIA by treatment with Lt.shGal3 or Lt.Gal1. (**a**, **b**) Groups of six rats were intradermally injected with collagen on days 0 and 7 followed by intra-articular (i.a.) injection of 5×10^6 or 5×10^5 TU of different recombinant lentiviruses or saline on days 7 and 8. Reduction of the articular index score in rats treated with Lt.shGal3 (**a**) and Lt.Gal1 (**b**). Values are the mean ± s.e.m. of the mean ($n = 12$ ankle joints of 6 rats). $P < 0.0001$ for Lt.shGal3 or Lt.Gal1 (5×10^6 or 5×10^5)-treated rats versus saline-treated rats and Lt.shGal3 (5×10^6 or 5×10^5)-treated rats versus Lt.shLuc-treated rats; $P = 0.001$ and $P = 0.0112$ for Lt.Gal1 (5×10^6)- and Lt.Gal1 (5×10^5)-treated rats versus Lt.GFP-treated rats, respectively; $P = 0.0258$ and $P = 0.0174$ for Lt.shGal3 (5×10^6)-treated rats versus Lt.shGal3 (5×10^5)- and Lt.Gal1 (5×10^6)-treated rats, respectively. (**c**) Representative photographs of the rat ankles. Groups of 7–8 rats were intradermally injected with collagen on days 0 and 7 followed by i.a. injection of 5×10^6 TU of different recombinant lentiviruses or saline on days 7 and 8. Plain radiography revealed that rats treated with Lt.shGal3 or Lt.Gal1 had more intact joint structure, as compared with their two control counterparts. Arrows indicate obvious differences in the degree of joint damage between groups. (**d**) Reduction of the radiologic score on day 18 in rats treated with Lt.shGal3 or Lt.Gal1. Values are the mean ± s.e.m. of the mean ($n = 7$–8 ankle joints of 7–8 rats).

Figure 4 Histopathological and immunohistochemical analysis in the joint tissue from rats with CIA receiving different recombinant lentiviruses. Groups of 7–8 rats were treated as described in Figure 3c. (**a**) Representative joint sections from rats treated with saline or Lt.GFP on day 18 were characterized by inflammatory cell infiltration with cartilage damage and underlying bone erosion, whereas those from rats treated with Lt.shGal3 or Lt.Gal1 showed less inflammatory cell infiltration and relatively intact joint surface. Bottom panels are higher-magnification views (original magnification × 200) of the respective boxed areas in the top panels (original magnification × 40). Synovial tissue is stained violet, whereas bone and cartilage are stained dark red and light red, respectively. Arrows indicate pannus formation, where synovial tissue erodes the cartilage and bone. Scale bars shown on the top and bottom panels represent 500 and 100 μm, respectively. (**b**) Representative Lt.Gal1- or Lt.shGal3-treated joint sections collected on day 18, showing reduced angiogenesis, as determined by immunohistochemical staining with anti-von Willebrand factor antibody (original magnification × 100). Arrows denote vWF-positive microvessels. Scale bars represent 200 μm. (**c**) Histologic joint scores. Values are the mean ± s.e.m. of the mean ($n = 7$–8 ankle joints of 7–8 rats). (**d**) Microvessel density in the joint tissue of rats receiving different treatments, as determined by averaging the number of vWF-positive microvessels in three fields (× 100 magnification) of the highest vessel density in each section. Values are the mean ± s.e.m. of the mean ($n = 7$–8 ankle joints of 7–8 rats). (**e**) Representative Lt.Gal1- or Lt.shGal3-treated joint sections showing reduced T cell infiltrates compared with those treated with Lt.GFP or saline, as identified by staining for CD3. Bottom panels are higher-magnification views (original magnification × 400) of the respective boxed areas in the top panels (original magnification × 100). Scale bars shown on the top and bottom panels represent 200 and 50 μm, respectively. (**f**) Number of CD3-positive T cells, as determined from three fields (× 400 magnification) of the highest density of positively stained cells in each section ($n = 3$–6). (**g**) Higher percentages of antigen-induced T-cell death from rats treated with Lt.Gal1 or Lt.shGal3 compared with their two control counterparts. Cells obtained from the inguinal lymph nodes were infected with Lt.Gal1, Lt.GFP or Lt.shGal3 at a multiplicity of infection of 10 and cultured for 72 h. The cells were then treated with denatured collagen for 24 h. They were stained with propidium iodide and analyzed for the proportions of dead cells by flow cytometry ($n = 3$).

death after being treated with type II collagen. As shown in Figure 4g, treatment of Lt.Gal1 and, in particular, Lt.shGal3 increased the percentage of cell death compared with their two control counterparts. These results suggest that the reductions of T-cell density *in vivo* by Lt.shGal3 and Lt.Gal1 might have been attributed to the higher antigen-induced T-cell death in the synovium.

Discussion

The galectins have recently received increasing attention as the regulator of immune homeostasis and inflammatory response.[10,23] Galectins bind to and crosslink multiple glycoconjugates found on the cell surface, and can further trigger transmembrane signaling transductions



through which diverse cellular responses are modulated.[10] A number of intracellular functions have been reported for galectin-3, including positive growth regulatory ability on T cells.[24] While galectin-1 induces cell death, endogenous galectin-3 has anti-apoptotic function.[25] Recent studies suggest that endogenous galectin-3 brings about apoptosis resistance by engaging apoptosis regulation pathways or modulating homeostasis of mitochondria.[25–27] Galectin-3 is secreted by cultured cells and detectable in extracellular fluid under inflammatory conditions and, through its multivalent lectin activity, galctin-3 induces interleukin-2 production by T cells.[28] Galectin-3 binds to the extracellular matrix in the carbohydrate-dependent manner and promotes cell adhesion by activating adhesion molecules.[10,23] Furthermore, our experiments showed significant reduction of proliferation and impairment of adhesion to fibronectin-coated plates of Lt.shGal-3-transfected synovial fibroblasts (data not shown). The emerging data from studies using galectin-3-deficient mice support the role of this molecule in promoting inflammatory responses.[29]

In this study, we have shown that i.a. administration of lentiviral vectors expressing either galectin-3 shRNA or galectin-1 shortly before the disease onset can ameliorate arthritis in rats with CIA. Our gene therapy results with Lt.Gal1 confirmed and extended earlier findings showing that administration of galectin-1 protein or fibroblasts engineered to secrete galectin-1 on the day of the disease onset abrogated the clinical and histopathological manifestations of CIA in DBA/1 mice.[18] Moreover, lymph node cells from galectin-1-treated mice were more susceptible to antigen-induced T-cell death than the control mice. We explored i.a. delivery of galectin-1 and shRNA targeting galectin-3 through lentivirus-mediated gene transfer. Our results show that treatment of Lt.Gal1 or Lt.shGal3 ameliorates arthritic symptoms in rats with CIA. We applied only two doses of 5×10^6 or 5×10^5 TU lentiviral vectors on days 7 and 8 in rats after being immunized with collagen on days 0 and 7. Even after using such low viral titers, we still observed significant improvement of arthritis symptoms in the clinical, histological and radiological aspects. To enhance the therapeutic efficacy, treatment regimens, such as dosing and schedule, can be further optimized. Of note, our animal studies have failed to show any superiority of combination therapy of Lt.Gal1 and Lt.shGal3 over monotherapy (data not shown). The cause of lacking additive or synergistic effect on suppressing arthritis by the combination therapy remains to be determined.

I.a. gene delivery strategies target the synovium. It is well documented that lentiviruses are capable of infecting efficiently and stably transduing non-dividing and terminally differentiated cells.[30–32] Lentiviral vector can efficiently transduce synovial lining cells in the knees of rats without eliciting an inflammatory response.[21,33] It is expected to provide long-term expression of transgenes as it can integrate into host chromosomes. The integration of lentiviral vectors into the host cells within the ankle joint in our study is unknown, but it is presumed to occur *in vivo*. As treatment of Lt.shGal3 or Lt.Gal1 has a beneficial effect on the amelioration of arthritis symptoms, it is reasonable to speculate that T cells and macrophages as well as fibroblasts may be targeted by lentiviral vectors. Gouze *et al*.[34] identified two distinct sub-populations of genetically modified cells within the joint of rats. Contrary to what was originally thought, the transient sub-population was composed almost exclusively of synovial fibroblasts, which have a high rate of cell turnover in normal joints, whereas the stable transduced cells consist of fibroblasts resident in fibrous articular tissues, such as the ligaments, tendons and capsule.

Gouze *et al*. also showed that persistent, i.a. transgene expression can be achieved only with an immunologically compatible transgene and vector in immunocompetent animals.[34] Expression of human transgene rapidly diminished in immunocompetent rats and was below the detection limit after 21 days. In marked contrast, transgenic expression could persist for the lifetime in immunodeficient nude rats. Their results suggest that cells within joint tissues are capable of sustained expression of transgenes. Furthermore, long-term expression of transgene is mainly dependent on the ability of the transduced cell to avoid immune recognition, and vector integration is not a major consideration. In our study, the galectin-1 cDNA used to construct lentiviral vector was of human origin. Moreover, in addition to expressing galectin-3 shRNA, Lt.shGal3 also expresses GFP encoded from the backbone of lentiviral plasmid, pLVTHM. Therefore, it would be reasonable to speculate that the transgene expression by Lt.Gal1 and Lt.shGal3 may not be persistent for a long time, as a human transgene and a lentiviral vector that contains additional coding sequences were used. We presume that cellular immune responses may function to avoid persistent transgene expression. Based on the same reasons, re-administered viral vectors might not be efficacious. Because lentiviral vector is thought to be less immunogenic than other viral vectors, such as adenovirus, neutralizing antibodies induced by the first administration of lentiviral vector may be minimal. However, in our system, as galectin-1 and GFP expressed in the rat ankle joints are not of rat origin, immune responses elicited by the host may lead to loss of cells expressing these transgenes. For clinical applications, human transgenes should be used for gene transfer.

It is well documented that galectin-1 inhibits cell growth, induces cell-cycle arrest and promotes apoptosis of activated, but not resting immune cells.[35] In contrast, galectin-3 can act in a dual fashion; while extracellular galectin-3 has apoptotic activity, intracellular galectin-3 can protect cells from apoptosis.[10,23] In the present study, we monitored the expression of galectin-1 and galectin-3 in the ankle joint during disease progression in rats with CIA. The levels of galectin-3 were elevated over time, whereas galectin-1 expression was dramatically suppressed after the onset of arthritis. This result is consistent with clinical studies in patients with RA and juvenile idiopathic arthritis.[11,12] Furthermore, we demonstrated reduced T-cell infiltration and decreased angiogenesis in the synovial tissues of arthritic rats that received gene therapy. Consistently, the lymph node cells from rats treated with Lt.shGal3 or Lt.Gal1 had increased susceptibility to antigen-induced T-cell death.

Accumulating studies have identified an important role for galectins in tumor angiogenesis that contributes to tumor progression and metastasis.[23] Galectin-3 stimulates neovascularization, and is involved in tumor-related angiogenesis.[36] Similarly, galectin-1 is crucial for tumor angiogenesis. In the galectin-1-null mouse, tumor growth is markedly impaired because of insufficient

tumor angiogenesis.[37] Synovial angiogenesis also has an important role in the inflamed joints of patient with RA. In this study, the Lt.shGal3-treated rats with CIA had reduced microvessel density within the synovial tissues, thus supporting the angiogenic role for galectin-3. It was shown that cleavage of galectin-3 by activated matrix metalloproteases in tumors is an active process during tumor progression.[38] In our immunoblot analysis using monoclonal anti-galectin-3 antibody for detecting galectin-3 in the ankle joints of rats during the time course of CIA, apart from the main 32-kDa band representing galectin-3 (Figure 1a), the presence of additional bands with smaller molecular sizes was evident from day 11 onward, which might be the galectin-3 fragments presumably cleaved by matrix metalloprotease (data not shown). Thus, the late occurrence of galectin-3 during the late stage of the disease progression in the arthritic rats suggests that the appearance of galectin-3 in the joint tissues may have been due to the activation of matrix metalloproteases in the synovial tissues. We have also shown that gene delivery of galectin-1 decreased angiogenesis in the synovial tissues. This result contradicted the angiogenic role for galectin-1 in tumor angiogenesis. As activated T cells have an important role in angiogenesis through their ability to secrete vascular endothelial growth factor into the inflammatory sites, increased antigen-activated T-cell death may have contributed to the reduced microvessel density in the synovial tissues of the Lt.Gal1-treated rats.[39]

Collectively, our results provide a proof of principle that i.a. administration of lentiviral vectors expressing galectin-3 shRNA or galectin-1 can ameliorate arthritis symptoms in rats with CIA. Our animal study shows the feasibility of manipulating the expression of galectin-1 and galectin-3 as a potential therapeutic strategy for treating patients with RA.

Materials and methods

Construction of plasmids

The lentiviral vector pWPXL/hGal1 encoding human galectin-1 was constructed by replacing the coding region of GFP of pWPXL with human galectin-1 cDNA fragment (452 bp) at the *Bam*HI/*Sma*I sites.[40] Rat galectin-3 cDNA fragment (~900 bp) with the stop codon TAA mutated to CAA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of mRNA from rat spleen with sense primer 5′-AACGGATCCAATGGCAGACGG-3′ and antisense primer 5′-CTTACCGGTTG GATCATGGCGTG-3′, in which a *Bam*HI site and an *Age*I site were introduced onto the 5′-end and the 3′-end, respectively. The resulting PCR product was digested with *Bam*HI and *Age*I, and fused inframe and upstream to the coding region of GFP in pEGFP-N1 (Clontech, Palo Alto, CA, USA) at the *Bam*HI/*Age*I sites, yielding pEGFP-N1/Gal3. To construct shRNA targeting galectin-3, we selected four heteroduplexes (shRNA), sh44, sh537, sh597 and sh654, which contain different 19-bp target sequences. The number following 'sh' indicates the position of the oligonucleotide at the 5′-end of galectin-3 cDNA (GenBank accession no. NM031832). The heteroduplexes supplied as 60-nucleotide oligomers were annealed and inserted downstream of the H1 promoter into pSuper (Oligoengine, Seattle, WA, USA) at the *Bgl*II and *Hin*dIII sites, resulting in pSuper/shGal3 44, 537, 597 and 654. The shRNA expression cassette containing the sequences for H1 promoter and shGal3 537 was further recovered from pSuper/shGal3 537 plasmid by *Eco*RI and *Cla*I digestion and ligated into the same sites of lentiviral transfer plasmid pLVTHM, resulting in pLVTHM/shGal3 plasmid that simultaneously expresses galectin-3 shRNA and GFP.

Screening of galectin-3 shRNA and production of lentiviral particles

Sub-confluent 293 cells (2×10^5) were co-transfected with 2 μg of pSuper control plasmid, pSuper/shGFP or pSuper/shGal3 and 2 μg of pEGFP-N1/Gal3 by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Flow cytometric analysis of GFP expression was performed 48 h later to evaluate the degree of suppression of GFP expression by different pSuper/shGal3 plasmids. Recombinant lentiviruses were produced by transient transfection of 293T cells with pLVTHM/shGal3, pWPXL/hGal1, pWPT or pLKO.1-shLuc along with the packaging plasmid psPAX2 and the VSV expression plasmid pMD2.G using the calcium phosphate precipitation method as described previously.[41] Luciferase shRNA-expressing plasmid pLKO.1-shLuc (TRCN 0000072246) was obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. After 48 h, lentiviral particles were collected and concentrated from the supernatant using ultracentrifugation. Thus, Lt.shGal3, Lt.Gal1, Lt.GFP and Lt.shLuc lentiviruses were produced. Titers in TU of Lt.GFP were determined in human rhabdomyosarcoma TE671 cells based on the GFP expression by flow cytometric analysis. Physical titers of lentiviruses expressed as viral particles (VP) were also determined by analysis of the virus-associated p24 core protein (QuickTiter Lentivirus titer kit, Cell Biolabs, San Diego, CA, USA). Based on the titers of TU and viral particle for Lt.GFP, we estimated that 2000 viral particle of our recombinant lentiviruses equals to approximately 1 TU.

Induction of CIA and lentivirus-mediated gene transfer

Groups of 6–8 male Sprague-Dawley rats were intradermally injected with bovine type II collagen on days 0 and 7.[42,43] Both ankles of the rats were injected with 5×10^6 or 5×10^5 TU of various recombinant lentiviruses or with saline on days 7 and 8. In a separate experiment, Lt.GFP and Lt.Gal1 (5×10^6 TU) were injected into the left and right ankle joints of a rat with CIA on day 7, respectively, and the expression of GFP *in vivo* was visualized after 3 days with a Night Owl CCD camera (model LB 981, Berthold Technologies, Bad Wildbad, Germany) equipped with 480 nm excitation and 520 nm emission filters (Chroma Technology, Rockingham, VT, USA). Exposure time of 1 s was sufficient to visualize GFP fluorescence in the ankle joint. Images were generated using WinLight software (Berthold Technologies). All animal experiments were carried out in accordance with the guidelines approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University.

Clinical and radiological evaluation of lentivirus-mediated gene transfer

The articular index scores of the rats after immunization with type II collagen and subsequent treatment with

lentiviral vectors or saline were measured as described previously.[44] Upon sacrifice on day 18, rat ankles were removed and immediately put on ice, and then radiographic examinations were performed. Radiographs were scored on the joint space change, degree of bony destruction and the soft tissue swelling, and all scores were summed up with a total scale from 0 to 10.[44]

Immunoblot, histological and immunohistochemical analyses

The ankle homogenates from rats with CIA at different time points were monitored for the expression of galectin-1 and galectin-3 by immunoblotting. Furthermore, 293 cells were infected with Lt.Gal1 or Lt.GFP at a multiplicity of infection of 10 for 48 h, and their lysates were harvested for detecting the expression of galectin-1 and GFP by immunoblotting. They were also infected with Lt.shGal3 or Lt.GFP at an multiplicity of infection of 10 for 2 days followed by transfection with pEGFP-N1/Gal3 (1 μg). After 3 days, cell lysates were examined for the expression of galectin-3 by immunoblotting. Antibodies against human galectin-1 obtained from rabbit immune serum,[40] rat galectin-3 (Santa Cruz, Santa Cruz, CA, USA), GFP (Santa Cruz) and β-actin (Sigma-Aldrich, St Louis, MO, USA) were used. Signals were detected by chemiluminescence, and intensity was quantitated by densitometry in some blots. Sections of paraffin-embedded joint specimens were stained with hematoxylin and eosin and immunostained with rabbit anti-von Willebrand factor (Dako, Carpinteria, CA, USA) or anti-CD3 (BD Biosciences, San Diego, CA, USA) antibodies as described previously.[6–9]

Assay of antigen-mediated activation-induced T-cell death in the lymph node cells transduced with lentiviral vectors

Rats with CIA were sacrificed on day 18. Cells obtained from the inguinal lymph nodes were infected with Lt.Gal1, Lt.GFP or Lt.shGal3 at an multiplicity of infection of 10 in the presence of polybrene (8 μg ml^{-1}) and cultured for 72 h. Cells were then treated with denatured type II collagen (100 μg ml^{-1}) for 24 h. They were stained with propidium iodide and analyzed for the proportions of dead cells by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data are expressed as the mean ± s.e.m. of the mean. Differences in articular index scores between two treatment groups were compared by repeated-measures analysis of variance (ANOVA) using SAS software version 9.1 (GLM program; SAS Institute, Cary, NC, USA).[9] Differences in the remaining data between two groups were compared with Student's *t*-test. *P*-values less than 0.05 were considered significant.

Conflict of interest

The authors declare no conflict of interest.

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