Prevention of collagen-induced arthritis by an anti-glycan monoclonal antibody reactive with 6-sulfo sialyl Lewis x in mice

マウスコラーゲン誘導関節炎モデルにおける
抗 6-スルホシアリルルイス X 糖鎖抗体の予防効果の解析

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Abbreviation

RA	Rheumatoid arthritis
sLex	Sialyl Lewis x
CIA	Collagen-induced arthritis
CII	Type II collagen
NSAIDs	Nonsteroidal anti-inflammatory drugs
DMARDs	Disease-modifying anti-rheumatic drugs
Th1	T helper 1 cytokine
Th17	T helper 17 cytokine
RF	Rheumatoid factor
ACPAs	Anti-citrullinated protein antibodies
mAb	Monoclonal antibody
CAIA	Collagen antibody-induced arthritis model
AA	Adjuvant-induced arthritis
COMP	Cartilage Oligomeric Matrix Protein
PIA	Pristane-induced arthritis
SCWA	Streptococcal cell wall-induced arthritis
AIA	Albumin-induced arthritis
GPI	glucose-6-phosphate isomerase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HEVs	High endothelial venules
PLNs	Peripheral lymph nodes
PBS	Phosphate buffered saline

CFA	Complete Freund's adjuvant
IFA	Incomplete Freund's adjuvant
ELISA	Enzyme-linked immunosorbent assay
ALNs	Axillary lymph nodes
ILNs	Inguinal lymph nodes
RT-qPCR	Reverse transcription-quantitative PCR
mRNA	Messenger RNA
MLNs	Mesenteric lymph nodes
IFN-γ	Interferon-γ
IL-17	Interleukin-17
TNF-α	Tumor necrosis factor-α
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
EDTA	Ethylene diamine tetraacetate
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
APC	Allophycocyanin
PE-CY7	Phycoerythrin-Cyanine7
PerCP-Cy5.5	Peridinin chlorophyll protein-Cyanine5.5
7-AAD	7-Aminoactinomycin D

Abstract

Rheumatoid arthritis, known as RA, is an inflammatory disease characterized by inflammation of the synovial tissue, which has a substantial impact on the quality of life of sufferers. The interaction between L-selectin and its glycoprotein ligands, which have been modified with 6-sulfo sialyl Lewis x (6-sulfo sLex), is essential for facilitating the migration of lymphocytes to trigger immunological responses. This process holds promise as a potential treatment target for RA. At first, we effectively created RA models using DBA/1 and C57BL/6 mice. subsequently, our primary focus was to investigate the preventative effects of an anti-6-sulfo sLex monoclonal antibody, SF1, on collagen-induced arthritis (CIA) in DBA/1 mice. Mice were given mAb SF1 starting on day 21 after the second immunization with type II collagen (CII). Subsequently, we investigated the impact of SF1 on both the clinical and histological aspects of disease advancement. Surprisingly, SF1 effectively reduced the clinical symptoms and histological markers related to the severity of arthritis. The results of the enzyme-linked immunosorbent test consistently showed that SF1 suppressed the generation of CII-specific IgG2a antibodies. Furthermore, based on reverse transcription-quantitative PCR analysis, SF1 suppressed the expression of interferon-γ (IFN-γ), T helper 1 cytokine (Th1), T helper 17 cytokine (Th17), as well as inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) and interleukin-1β (IL-1β) in axillary lymph nodes and inguinal lymph nodes. The flow cytometry results demonstrated that SF1 effectively suppressed the growth of B cells and CD4+ T cells, as well as the development of central effector and central memory CD4+ T cells, in the lymph nodes of the CIA model mice. Safety

experiments verified that SF1 did not induce harm to the vital organs of the mice studied during dosing. Overall, these findings suggest that SF1, a monoclonal antibody that targets sulfated glycans, has the potential to effectively prevent CIA in mice and could serve as a promising new treatment for rheumatoid arthritis.

Keywords:

Rheumatoid arthritis, Lymphocyte homing, L-selectin, 6-sulfo sialyl Lewis x, Anti-glycan antibody

1. Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a progressive, long-lasting inflammatory disease that affects several systemic systems as well as the joints. Though continuous inflammation of the synovial joints is the hallmark of RA, the disease frequently has far-reaching systemic repercussions that may affect several organ systems and have a significant negative impact on the overall quality of life of patients[1]. Chronic inflammation causes symptoms like stiffness, swelling, and discomfort in the joints, which makes it very difficult to go about everyday tasks, go to work, and connect with people.

The pathogenesis of RA involves a complex network of interactions among abnormal immune responses, genetic predisposition, and environmental triggers[2]. These factors collectively drive the progression of the disease, making it a multifaceted condition that is challenging to understand and treat fully. Approximately 1% of the global population is affected by RA, but this prevalence is not evenly distributed[3]. It varies significantly across different regions and populations, likely due to differences in environmental factors, dietary habits, and genetic backgrounds. For instance, studies have shown higher prevalence rates in certain ethnic groups, indicating a genetic component to the development[4].

A closer examination of clinical manifestations reveals a wide range of symptoms beyond joint discomfort. Patients may experience systemic symptoms such as fever, fatigue, anemia, osteoporosis, and muscle weakness, all of which seriously threaten their physical health. More critically, RA patients face increased risks of

cardiovascular diseases and certain cancers[5]. These complications exacerbate the physical burden on patients and may negatively impact their mental health. The persistent pain, discomfort, and uncertainty about the future can lead to significant psychological issues, including anxiety and depression[6]. The chronic nature of RA often requires patients to adapt to a lifetime of fluctuating symptoms and varying levels of disability, which can be mentally exhausting and socially isolating.

In terms of treatment, various medications are available, including nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids, and biologics[7]. While these treatments primarily aim to control disease progression and alleviate symptoms, they do not cure the disease. Additionally, long-term use of these medications can lead to a series of side effects, such as gastrointestinal reactions, liver and kidney damage, and increased susceptibility to infections due to immunosuppression. This highlights the need for a balanced approach to managing RA, where the benefits of symptom relief and disease control must be weighed against the potential risks of treatment side effects[8].

Despite the advances in RA treatment, the need for in-depth research into the pathogenesis remains pressing. Identifying new therapeutic targets is crucial for developing more effective and safer treatments. Recent research has focused on understanding the molecular and cellular mechanisms underlying RA, including the roles of specific immune cells, cytokines, and genetic factors. For example, identifying specific biomarkers associated with RA progression has opened new avenues for early diagnosis and targeted therapy[9].

Moreover, developing personalized medicine approaches tailored to the genetic

and molecular profiles of individual patients holds promise for improving treatment outcomes. Such approaches could lead to more precise targeting of the underlying disease mechanisms, potentially reducing the risk of side effects and improving the quality of life for patients. Additionally, exploring the role of environmental factors, such as diet, lifestyle, and exposure to certain infections, could provide insights into preventive strategies for RA[10]. The future of RA treatment may increasingly rely on precision medicine, where treatment plans are tailored to the genetic, immunological, and other biological characteristics of individual patients. Immunotherapies, stem cell treatments, and gene-editing technologies hold promise for RA patients[11]. Additionally, a key direction in drug development is the search for new molecules that can offer both high anti-inflammatory efficacy and low side effects[12].

To summarize, RA is a multifaceted autoimmune disorder that has significant impacts on the entire body. Although existing treatments provide relief from symptoms and help manage the disease, they do not provide a cure and may have associated adverse effects[13]. Further investigation into the pathophysiology of rheumatoid arthritis (RA) is crucial in order to devise novel therapeutic approaches that can better and more safely address this incapacitating condition. By acquiring a more profound comprehension of the fundamental mechanisms and pinpointing novel treatment targets, we can aspire to enhance the quality of life for patients with rheumatoid arthritis and propel the progress of autoimmune disease therapy[14].

1.2 Rheumatoid arthritis animal model in mice

Mouse models are indispensable tools in RA research, playing a crucial role in understanding the pathogenesis of the disease and testing potential treatments. By

establishing mouse models that closely mimic the pathological mechanisms of human RA, researchers can simulate and study the progression of the disease in detail. These models provide a platform for observing the development of arthritis and serve as essential tools for testing the efficacy and safety of new drugs[15].

1.2.1 Collagen-induced arthritis model

Trentham developed the collagen-induced arthritis model (CIA) for animal arthritis research[16]. Type II collagen found in various animals can cause arthritis. Autoantibodies against type II collagen have been found in RA patients' serum and synovial fluid, indicating that type II collagen may play a role in the disease's pathogenesis[17]. The CIA, an endogenous self-antigen-mediated animal model, is primarily regulated by two types of T cells, Th1 and Th2, which reach equilibrium following immunization[18]. When the Th1/Th2 subgroups in the blood are imbalanced, serum levels of IL-1β and tumor necrosis factor (TNF)-α increase due to excessive immune responses mediated by glycan-dependent lymphocyte homing to lymph nodes [19].

Type II collagen protein (CII) was combined with complete Freund's adjuvant in equal parts to make an emulsion for the first immunization induction. After 21 days, an incomplete Freund's adjuvant was used for booster immunization. Alternatively, booster immunization can be performed using a mixture of emulsions containing equal amounts of type II collagen protein and incomplete Freund's adjuvant. The CIA animal model is more stable over time, but its onset is slower[20]. The disease's peak incidence occurs between days 26 and 35 following the first immunization. DBA/1 mice are commonly used because they respond to type II collagen protein in chickens,

cattle, and pigs[21]. According to studies using the CIA model, patients with RA have a high concentration of nuclear rod bacteria, which is positively correlated with the severity of the disease[22]. In this model, baricitinib was found to reduce the severity of arthritis symptoms while also improving neuropsychological symptoms like depression and fatigue[23].

1.2.2 Collagen antibody-induced arthritis model

The collagen antibody-induced arthritis model (CAIA) extends the CIA model. This model induces severe arthritis in mice using monoclonal antibodies against a specific binding site within type II collagen combined with lipopolysaccharide administration[24]. The clinical and pathological features of the disease are like those of the CIA. However, the disease is primarily defined by the infiltration of macrophages, multinucleated cells, and inflammatory cells rather than by T and B cell responses. This model is ideal for investigating individual gene products and cytokines and screening and testing anti-inflammatory and immunomodulatory drugs[25].

The method involves injecting monoclonal type II collagen antibodies into mice's peritoneal cavity on day 0, followed by LPS injections from Escherichia coli on day 3. After 2-4 days of initial immunization, signs of inflammation appear, followed by severe arthritis on days 6-8. CAIA has a high incidence, reproducibility, and stability, and it can cause arthritis in nearly all mouse strains, including those insensitive to the CIA model[26-27]. Studies have used the CAIA model to investigate the role of LPA signaling[28]. The relationship between changes in tryptophan metabolism and inflammatory diseases was investigated by combining the CAIA

model and quantitative tryptophan metabolomics[29].

1.2.3 Adjuvant-induced arthritis model

The adjuvant-induced arthritis (AA) model is a traditional RA modeling method that relies primarily on Freund's adjuvant, which includes both complete Freund's adjuvant (CFA) containing heat-killed Mycobacterium tuberculosis and incomplete Freund's adjuvant (IFA) lacking bacterial components[30]. A protein in Mycobacterium tuberculosis has a structure similar to a glycoprotein molecule found on the synovial membranes of joints. Freund's adjuvant injection activates the immune system, which can trigger T cells to mistakenly attack the joints, resulting in an immune response against the joints[31]. This model resembles human RA regarding disease symptoms, pathological manifestations, and extra-articular symptoms.

The procedure entails injecting Freund's adjuvant into the ankles and surrounding joints of mice. Inflammation symptoms appear 48 hours after the initial immunization, with secondary lesions appearing approximately 10-12 days later. C5BL/6 mice, susceptible to unilateral joint symptoms, are commonly used. The AA model was used to investigate coronary endothelial dysfunction associated with myocardial hypertrophy and decreased tolerance to ischemia[32]. The effects of paeoniflorin on RA and neutrophils were studied using an AA mouse model[33].

1.2.4 Cartilage oligomeric matrix protein-induced arthritis model

The Cartilage Oligomeric Matrix Protein-Induced Arthritis Model (COMP) is a method for generating arthritis. COMP, or thrombin-sensitive protein, is a non-collagenous glycoprotein[34]. It is found primarily in cartilage, tendons, and synovium, where it catalyzes the formation of collagen I and collagen II fibers and is

critical for collagen assembly and extracellular matrix stability[35]. COMP mutations can cause chondrocyte apoptosis, resulting in skeletal system disorders. The pathological mechanism is linked to the immune response of T cells to COMP[36].

The procedure entails injecting a 1:1 mixture of COMP and CFA/IFA into the base of the mouse skin. On day 35, COMP is added to the IFA to boost immunity[37]. C57BL/6 mice are used in the model. COMP can cause severe arthritis by triggering a strong autoantibody response. However, this model lacks stability and does not cause permanent joint damage[38-39].

1.2.5 Pristane-induced Arthritis Model

T cells and MHC play a role in the chronic arthritis known as pristane-induced arthritis (PIA). Pristane is a non-immunogenic chemical that functions similarly to adjuvants, inducing inflammation and exacerbating immune responses[40]. PIA relies primarily on the activation of $CD4^+$ T lymphocytes to cause inflammation, which is caused by the activation of self-reactive T lymphocytes due to non-specific immune system stimulation[41].

PIA has high model reproducibility and causes pathological changes in joint tissue like human RA. In mice, PIA can be associated with elevated levels of various autoantibodies[42]. The procedure entails injecting pristane into the abdominal cavity of mice, followed by an additional booster in week 8. Swelling and erythema in mouse feet can be seen after week 17, but severe symptoms appear after week 29. BALB/c mice exhibit chronic development and are commonly used to establish the PIA model. The effect of the Nramp-1 allele on phagocyte activation in the arthritis process is currently being studied using the PIA mouse model[43]. Studies on the

genetic background of AIRmin mice revealed varying levels of susceptibility to PIA[44].

1.2.6 Streptococcal cell wall-induced arthritis model

Streptococcal cell wall-induced arthritis (SCWA) is an animal model of arthritis caused by streptococcal cell wall peptidoglycan components with pro-inflammatory properties[45]. Streptococci first activate specific B and T lymphocytes, then activate adaptive immune responses, resulting in the development of arthritis in animals[46]. This model shows synovial proliferation, inflammatory cell infiltration, and symmetric joint damage, similar to RA in humans. However, this model lacks high rheumatoid factor (RF) titers and does not produce rheumatoid nodules[47].

Acute joint swelling appears 24 hours after injection of a streptococcal cell wall peptidoglycan polysaccharide components suspension and tends to resolve by week 4. BALB/c and DBA/1 mice can be used to induce unilateral acute arthritis, which, with repeated injections, can progress to chronic arthritis. Studies have found that the mouse CSWA model has a lower success rate than the rat SCWA model[48].

1.2.7 Methylated bovine serum albumin-induced arthritis model

The methylated bovine serum albumin-induced arthritis model (AIA) is classified as an antigen-induced arthritis model. Initially, chronic synovitis in rabbits was caused by injecting ovalbumin into the joint cavity[49]. It is a T-cell-dependent arthritis model marked by increased synovial fluid, inflammatory cell infiltration, and vascular opacity formation[50]. This model is appropriate for investigating larger joints with lesions, such as those in rabbits and sheep[51]. The experimental method was later improved and applied to mice[52-53]. The method involves inducing arthritis in mice

using methylated bovine serum albumin (mBSA), CFA, and IFA[54-55].

Other antigen-induced mouse models of arthritis include those induced by ovalbumin[56], zymosan[57], and glucose-6-phosphate isomerase (GPI) fragment, which has recently been used in experiments[58]. Many of the antigens in these models need to be modified, which can lead to single-joint onset.

1.2.8 The tumor necrosis factor (TNF-α) model

Activated macrophages mainly secrete TNF-α, and a small amount is secreted by activated T cells, natural killer cells, and mast cells[59]. TNF-α is an immune regulator of normal and chronic inflammation. In the TNF-α model, the mouse TNF-α gene is replaced with the human TNF-α (hTNF-α) gene. The mouse endogenous promoter regulates the expression of hTNF-α, inducing arthritis like human RA[60-61].

From week 3, the model mouse begins to show arthritis symptoms, which gradually worsens over time and cannot heal. The model is accompanied by swelling in the ankle from weeks 3 to 4 and peaks from weeks 17 to 21[62]. Therefore, compared with the induced arthritis model, the TNF-α transgenic mouse model has a stable phenotype. The lesions, including joint cartilage damage and fibrous tissue generation, are symmetrical bilateral polyarthritis and these features are very similar to human RA, making it very useful for studying TNF-α-related interventions and can also be used to explore cartilage destruction[63].

1.2.9 K/BxN model

The K/BxN model is generated by crossing R28TCR transgenic male mice with non-obese diabetic female mice susceptible to autoimmunity[64]. This model has a large amount of autoantibodies in its serum. The serum of K/BxN mice is transferred

to B cell-deficient mice, which results in severe swelling in the joints and other inflammatory symptoms within two days of serum transfer[65]. This model is also known as the K/BxN serum transfer arthritis (K/BxN STA) model[66]. The serum of hybrid offspring mice can induce various mouse models of arthritis. The symptoms of arthritis appear acutely in the model mice from 3-4 weeks of their age, similar to clinical signs of RA[67]. The success of this model is determined by symptoms such as elevated levels of inflammatory factors, narrowing of the joint cavity, and inflammatory cell infiltration[68].

This model has a high incidence rate, with a rapid onset of disease, and is mainly used to study the mechanisms of activation of different innate immune cells, such as neutrophils, macrophages, and mast cells, driven by autoantibodies that cause the formation of immune complex[69-70].

1.2.10 SKG model

SKG mice, a new genetic model of RA, which depends on the BALB/c background, develop chronic arthritis spontaneously after being injected with zymosan under specific conditions[71]. This model shares standard features with human RA in terms of female susceptibility, the presence of rheumatoid factors and type II collagen antibodies[72]. It is also a spontaneous arthritis model with a gene harboring hidden mutation that is highly dependent on the expression of IL-17 and other cytokines. The model also presents extra-articular lesions, leading to pneumonia and dermatitis[73-74].

1.2.11 IL-1 receptor antagonist knockout model

In the IL-1 receptor antagonist knockout model, the IL-1 receptor antagonist

(IL-1Rα) gene is knocked out mice genome, thereby removing the restriction on the activation of its receptor by pro-inflammatory cytokine IL-1, subsequently leading to inflammation[75]. The IL-1Rα gene-deficient mutant mice and their offspring upon crossing with BALB/c mice develop spontaneous arthritis[76]. This model is currently applicable only to BALB/c mice. The pathological phenotype of the IL-1Rα gene knockout mouse model is similar to that observed in human RA and can be used to study the role of cytokines in the onset of RA[77].

1.3 6-sulfo sialyl Lewis x glycans

The abnormal migration and localization of immune cells are particularly critical in the complex pathogenesis of RA. This process involves a series of intricate molecular interactions, among which the interaction between L-selectin and 6-sulfo sialyl Lewis x (6-sulfo sLex) is a core component. Understanding these interactions can provide significant insights into potential therapeutic targets for RA and other autoimmune diseases[78].

L-selectin, a unique form of cell adhesion molecule, is only present on the surface of leukocytes and has a vital function in the movement and localization of immune cells. The distinctive architecture of this entity allows it to attach to particular glycoproteins found on the surface of vascular endothelial cells[79]. Out of these glycoproteins, 6-sulfo sLex holds particular significance. 6-sulfo sLex is a sulfated carbohydrate structure that is present on glycoproteins located on high endothelial venules in lymph nodes. These glycoproteins serve as molecules that bind to L-selectin, enabling leukocytes to attach and then move along the endothelium. This process is crucial for leukocytes to migrate to areas of inflammation[80].

An aberrant activation of the interaction between L-selectin and 6-sulfo sLex has been reported in the pathogenic setting of RA. This anomaly results in a significant increase in leukocytes entering the synovial tissue of the joints, which then initiates a prolonged and harmful inflammatory reaction[81]. The excessive buildup of immune cells in the joints worsens local joint pain and swelling and leads to gradual joint deterioration and reduced function. The ongoing recruitment of immune cells, facilitated by the interactions between L-selectin and 6-sulfo sLex, sustains the inflammatory cycle, resulting in chronic inflammation and the distinctive joint destruction observed in RA[82].

Thorough investigation of the interaction mechanism between L-selectin and 6-sulfo sLex is essential for comprehending the development of rheumatoid arthritis (RA) and discovering novel therapeutic approaches. Scientists are working hard to create new medications that can disrupt this connection, such as small-molecule inhibitors or particular antibodies[83]. The objective of these medications is to inhibit the anomalous movement of immune cells, so diminishing localized joint inflammation and safeguarding the joints against more harm. An effective method involves utilizing monoclonal antibodies that specifically target L-selectin or 6-sulfo sLex, thereby blocking their binding and subsequently reducing the migration of leukocytes[84].

Another potential therapeutic strategy is the development of small-molecule inhibitors that can disrupt the L-selectin/6-sulfo sLex interaction. These inhibitors could be designed to block the binding sites on L-selectin or 6-sulfo sLex, thereby preventing leukocyte adhesion and migration (Fig. 1). The advantage of

small-molecule inhibitors is their potential for oral administration and ability to penetrate tissues more effectively than large antibody molecules[85].

Investigation into these treatment approaches is now in its nascent phase, but, initially studies demonstrate significant promise. Animal studies have demonstrated that interfering with the interaction between L-selectin and 6-sulfo sLex can effectively decrease inflammation and minimize joint damage in rheumatoid arthritis models. These findings indicate that focusing on this pathway could be an effective treatment for RA and potentially other autoimmune disorders that are characterized by aberrant movement of white blood cells [86].

Moreover, comprehending the wider consequences of L-selectin and 6-sulfo sLex interactions in the movement of immune cells could potentially result in novel strategies for controlling additional inflammatory and autoimmune disorders. Similar mechanisms of leukocyte migration and adhesion are implicated in disorders such as multiple sclerosis, inflammatory bowel disease, and psoriasis. Potential therapeutics for many autoimmune and inflammatory illnesses could be developed by focusing on the L-selectin/6-sulfo sLex pathway[87].

This research direction provides a promising outlook for the treatment of RA and presents novel strategies for addressing other autoimmune illnesses. By gaining a more profound comprehension of this mechanism, our aim is to create more accurate and efficient treatments in the future, thereby enhancing patients' quality of life and propelling the field of autoimmune disease therapy forward. As scientific investigation advances, the examination of L-selectin and 6-sulfo sLex interactions is expected to uncover new therapeutic targets and techniques. This will lead to the

development of more efficient and precise treatments for RA and other related conditions[88].

In previous investigations, we successfully generated and characterized a unique anti-glycan monoclonal antibody (mAb), designated as SF1, which demonstrates remarkable specificity towards 6-sulfo sLex glycans[89]. The profound suppressive effects of SF1 on lymphocyte homing and allergic rhinitis in murine models have been documented, hinting at its potential broader application in the prophylaxis of various immune-related pathologies. Building upon these promising findings, the present study undertakes a comprehensive exploration of the prophylactic efficacy of SF1 in CIA using DBA/1 mice. This investigation aims to elucidate the immunomodulatory properties of SF1 and assess its therapeutic value in mitigating autoimmune disorders, thereby paving the way for novel treatment strategies in clinical settings.

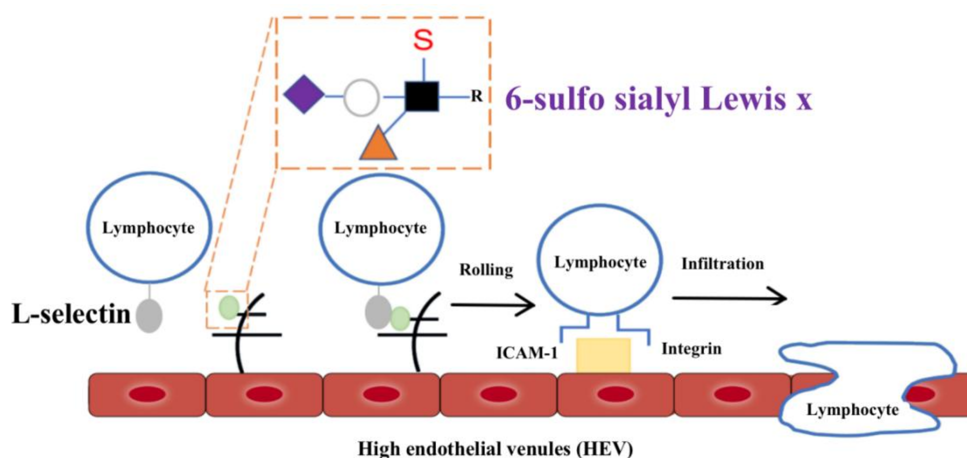


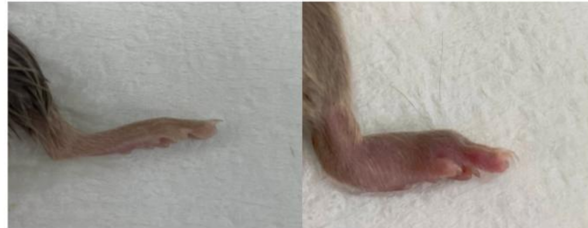
Figure 1. Antibody SF1 specifically binds to L-selectin on the surface of HEVs in secondary lymphoid organs. HEV, high endothelial venules.

2. Results

2.1 Collagen-induced arthritis mouse models

Collagen-induced rheumatoid arthritis models were established using DBA/1 and C57BL/6J mice. DBA/1 mice received a booster immunization on day 21, with clinical scores indicating the onset of paw swelling on the fourth day post-booster. The disease severity progressively increased over time, and by day 49, paw thickness (Fig. 2A) and the average clinical score of the paws was close to 14 (Fig. 2B). In C57BL/6J mice, a secondary immunization was performed on day 14, with paw swelling observed as early as the second day post-immunization. By day 49, paw thickness (Fig. 3A) and the average clinical score of the mice's paws exceeded 12 (Fig. 3B). The experimental results preliminarily confirmed the successful induction of rheumatoid arthritis models in different mouse strains using collagen. Compared with the pre-experimental results, B6 mice showed less stability especially in the first month and weaker sensitivity in disease development. Therefore, DBA/1 mice were used for the following research.

A



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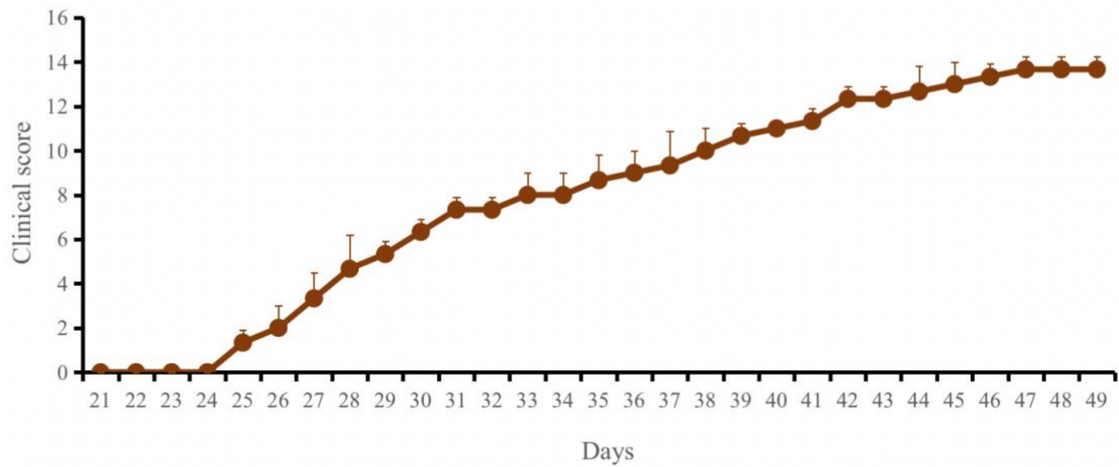

Figure 2. Collagen-induced rheumatoid arthritis model in DBA/1 mice

(A) Representative picture of hind limbs collected on day 0 and 49. (B) Arthritic scores after the second immunization (n=3).

A

B

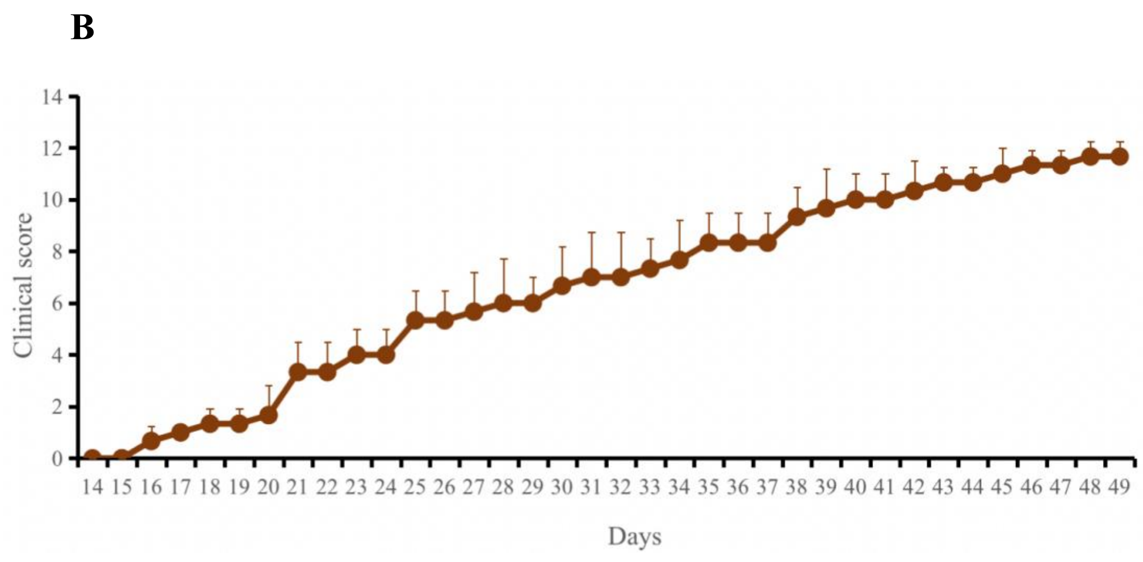

Figure 3. Collagen-induced rheumatoid arthritis model in C57BL/6J mice

(A) Representative picture of hind limbs collected on day 0 and 49. (B) Arthritic scores after the second immunization (n=3).

2.2 Purification of SF1 antibody

Nude mice are a mutated breed of laboratory mice with a degenerate or dysfunctional thymus. These mice are invaluable for generating large quantities of highly pure antibodies, crucial for in vivo experiments. Previous research in our laboratory successfully cultivated hybridomas that produce a highly targeted anti-sLex mAb, SF1. To obtain a substantial quantity of mAb SF1, we injected Pristane intraperitoneally into Charles River BALB/c nude mice and housed them for two weeks. Subsequently, we injected 1×10^7 SF1 hybridoma cells suspended in sterile PBS intraperitoneally.

After another two weeks, once the abdomens of the nude mice had noticeably expanded, we collected the ascites fluid. We treated the recovered fluid with n-octanoic acid and concentrated it using ammonium sulfate. We verified the purity of the sample by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 4). As a result, we obtained an adequate quantity of purified SF1 monoclonal antibody, paving the way for further studies.

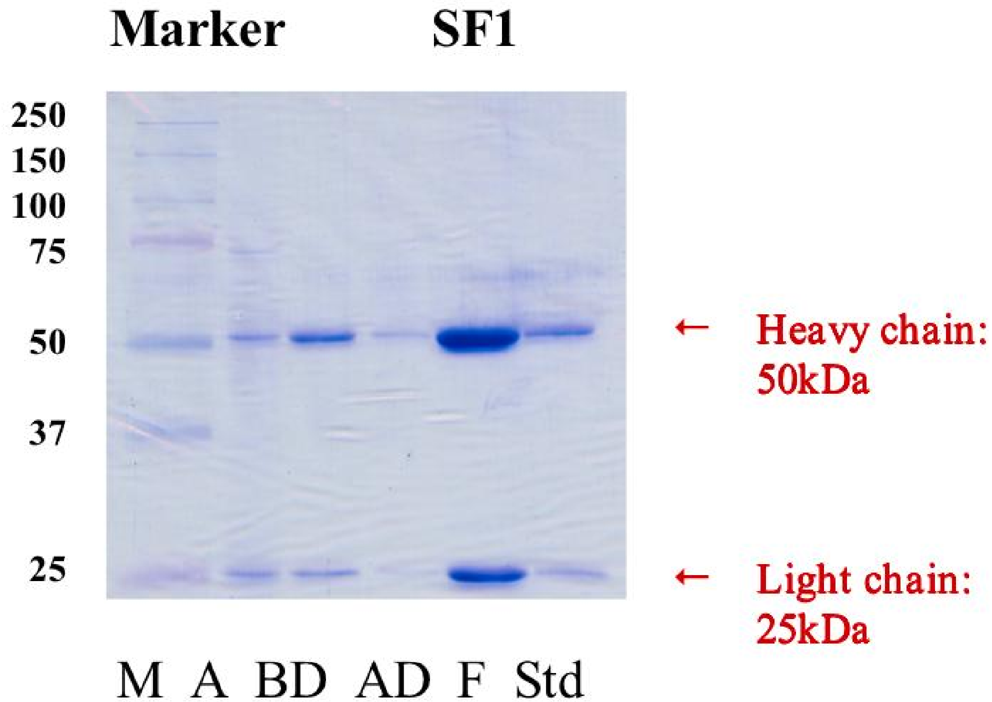

Figure 4. Monoclonal antibody SF1 purification

SDS-PAGE purification of SF1 antibody. SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis. M, marker; A, ascites; BD, before dilysis; AD, after dilysis; F, final product; Std, standard.

2.3 SF1 binds HEVs in lymph nodes of DBA/1 mice

We have previously reported that SF1 selectively binds to HEVs in the PLNs and mesenteric lymph nodes (MLNs) in C57BL/6J mice depending on sulfate, fucose, and sialic acid modifications, thereby indicating that 6-sulfo sLex glycans (Fig. 5) are expressed in the HEVs of these mice. To examine whether these results could also be applied to DBA/1 mice, we performed immunofluorescent staining of PLNs,

including ALNs, ILNs and MLNs, from DBA/1 mice using SF1. Herein, the examined lymph nodes from DBA/1 mice exhibited SF1-stained HEVs, similar to those identified in C57BL/6J mice, confirming that 6-sulfo sLex glycans were expressed in tissues from DBA/1 mice. Accordingly, SF1 can be used in a CIA model established in DBA/1 mice.

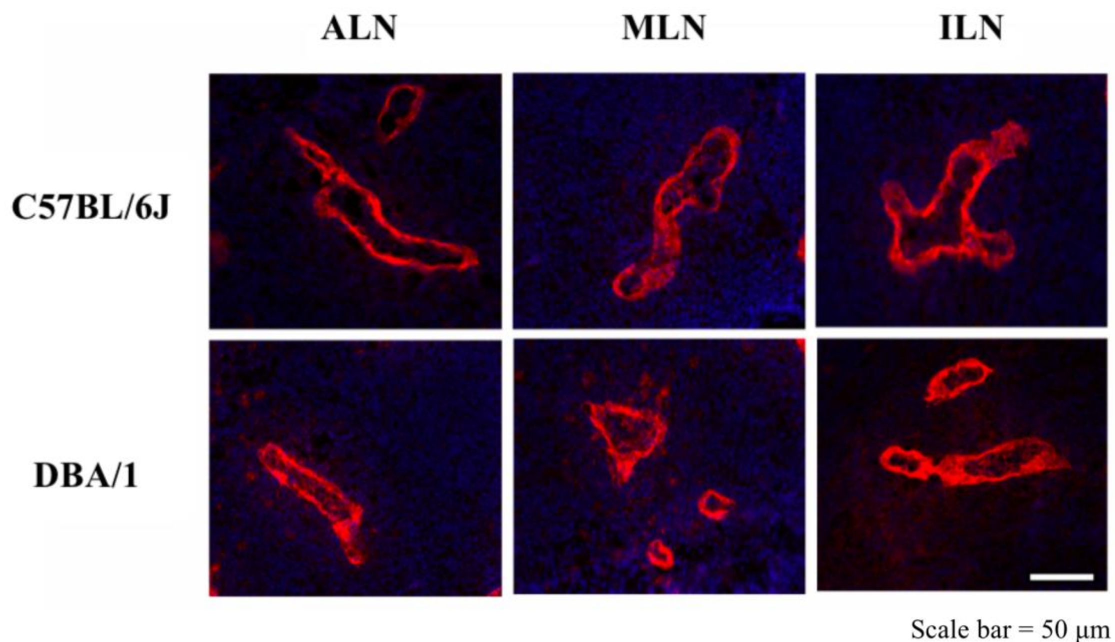

Figure 5. Immunofluorescence of PLN tissue sections from C57BL/6J and DBA/1 mice treated with SF1. Frozen sections of PLNs (ALNs and ILNs) and MLNs from C57BL/6J and DBA/1 mice were incubated with biotinylated SF1, followed by incubation with Alexa Fluor 594-streptavidin (red) and DAPI (blue). PLNs, peripheral lymph nodes; ALNs, axillary lymph nodes; ILNs, inguinal lymph nodes; MLNs, mesenteric lymph nodes.
Scale bar = 50 μm.

2.4 SF1 ameliorates CIA in DBA/1 mice

To determine the preventive effects of SF1 against autoimmune disorders, we examined its effects on CIA in DBA/1 mice. As shown in (Fig. 6A), SF1 was administered intraperitoneally to mice from day 21 after the second immunization with CII, and its effects on the clinical score, disease incidence, and histology of the synovium were examined. The clinical scores for arthritis were significantly reduced on days 30 and 31 after treatment with SF1 (Fig. 6B). The SF1-treated group showed delayed disease onset and reduced frequency of disease incidence. Conversely, more than 80% of the control group mice developed CIA by day 31 (Fig. 6C).

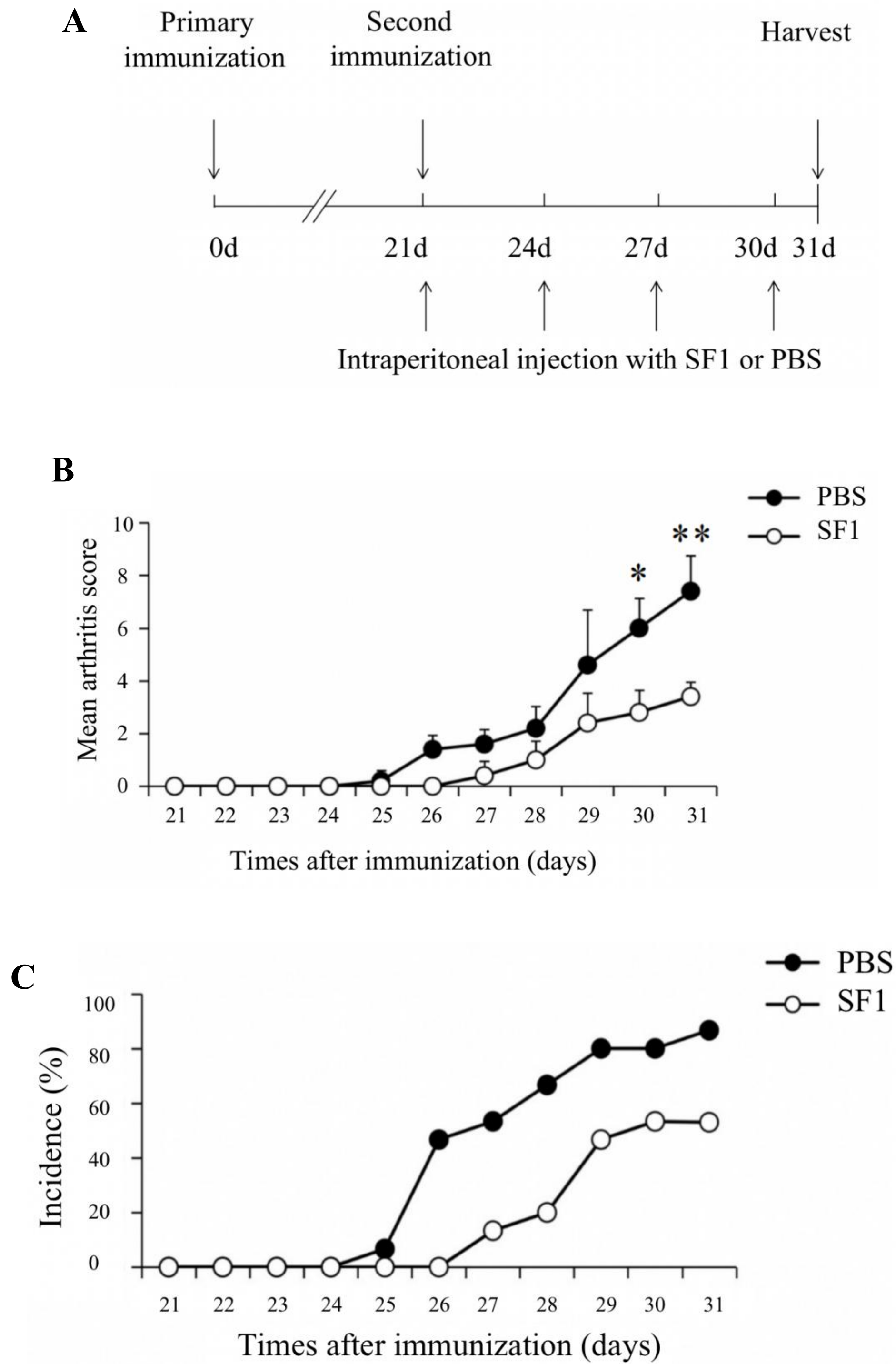

Figure 6. Effects of SF1 on CIA in DBA/1 mice

(A) Scheme illustrating the experimental setup. (B) Arthritic scores of DBA/1 mice after the second immunization (n=15). *p < 0.05, **p < 0.01, compared with the PBS-treated group. (C) The incidence of arthritis in DBA/1 mice after the second immunization (n=15). CIA, collagen-induced arthritis; PBS, phosphate-buffered saline.

2.5 SF1 attenuates symptoms of CIA in DBA/1 mice

We investigated whether SF1 affects the symptoms of CIA mice (PBS or SF1 group n=15). Hematoxylin and eosin (H&E) analyses showed that SF1 attenuated inflammatory infiltration and fibroblast proliferation in the knee joint (Fig. 7A) or synovium (Fig. 7B). In addition, SF1 treatment alleviated the destruction and edema of cartilage in the ankle joint (Fig. 7C) and intertarsal joint (Fig. 7D) of mice. These results suggest that injection of SF1 decreased joint inflammation and bone destruction in CIA.

2.6 SF1 suppresses CII-specific serum IgG levels

CIA is an autoimmune disease characterized by the production of CII-specific IgG, which forms a pathogenic immune complex in the joints. Therefore, we next performed ELISA using CII-coated plates to determine CII-specific IgG levels in the sera of mice treated with or without SF1. Compared with the PBS-treated control group, the SF1-treated group showed a significant reduction in total IgG against CII in the serum (Fig. 8A). Furthermore, ELISA using IgG class-specific secondary antibodies revealed that CII-specific IgG2a, but not CII-specific IgG1, was reduced in the sera of SF1-treated mice (Fig. 8B and C).

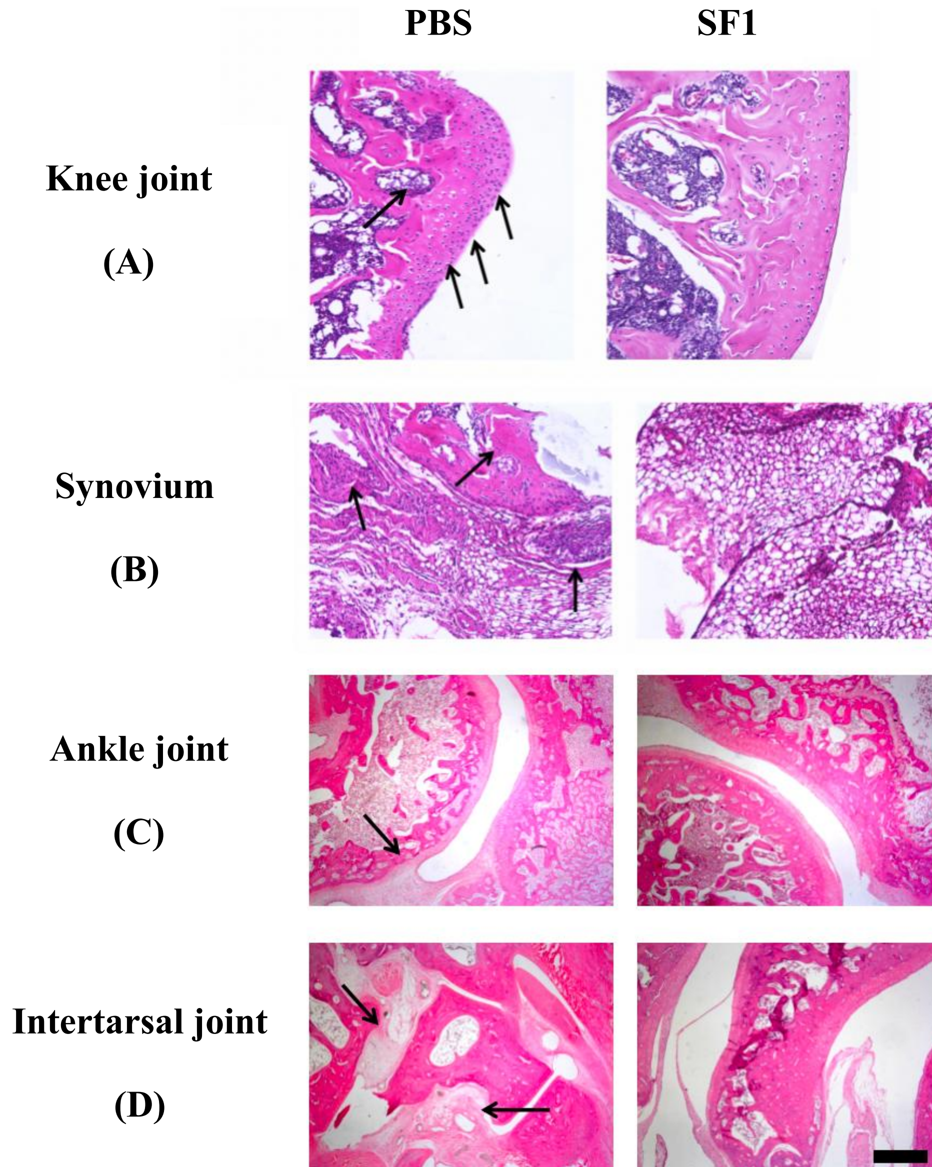

Figure 7. SF1 mitigates CIA in DBA/1 mice

(A) Representative H&E staining of knee joint sections collected from PBS or SF1 group (n=15). (B) Representative H&E staining of synovium sections collected from PBS and SF1 group (n=15). (C) Representative H&E staining of ankle joint collected from PBS and SF1 group (n=15). (D) Representative H&E staining of intertarsal joint collected from PBS and SF1 group (n=15). H&E, hematoxylin and eosin. Scale bar = 50 μm.

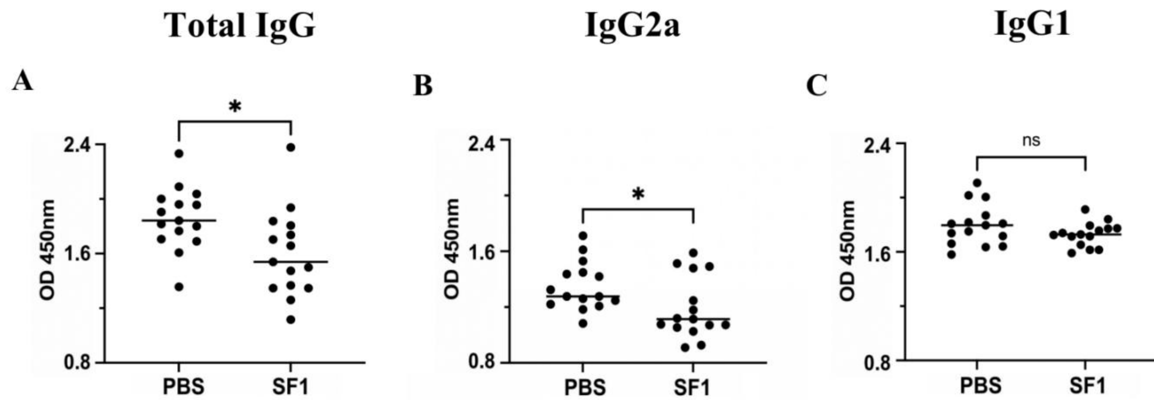

Figure 8. Administration of mAb SF1 suppresses the protein level in CIA
The amount of type II collagen (CII)-specific antibodies in the serum of CIA-induced DBA/1 mice was determined using ELISA. Serum levels of IgG, IgG1, and IgG2a were measured using ELISA (n=15). ELISA, enzyme-linked immunosorbent assay; ns, not significant, *p < 0.05.

2.7 SF1 inhibits cytokine expression in lymph nodes

IgG2a and IgG1 are mainly produced by T helper 1 (Th1) and Th2 immune responses, respectively. Based on the above results, SF1 could suppress Th1 immune responses against CII in the draining lymph nodes of CIA-induced DBA/1 mice. Given that interferon (IFN)-γ is primarily secreted by Th1 cells; IL-17 is primarily secreted by Th17 cells, we next examined the expression of IFN-γ by RT-qPCR using total RNA prepared from the draining lymph nodes of the PBS- and SF1-treated groups. IFN-γ expression was significantly suppressed in the SF1-treated group. The SF1-treated group exhibited significantly lower levels of IL-17, TNF-α, IL-1α, IL-1β expression than the PBS-treated group (Fig. 9). Collectively, these results suggest that

SF1 suppresses Th1, Th17 and inflammatory immune responses that lead to the production of pathogenic CII-specific IgG2a in lymph nodes.

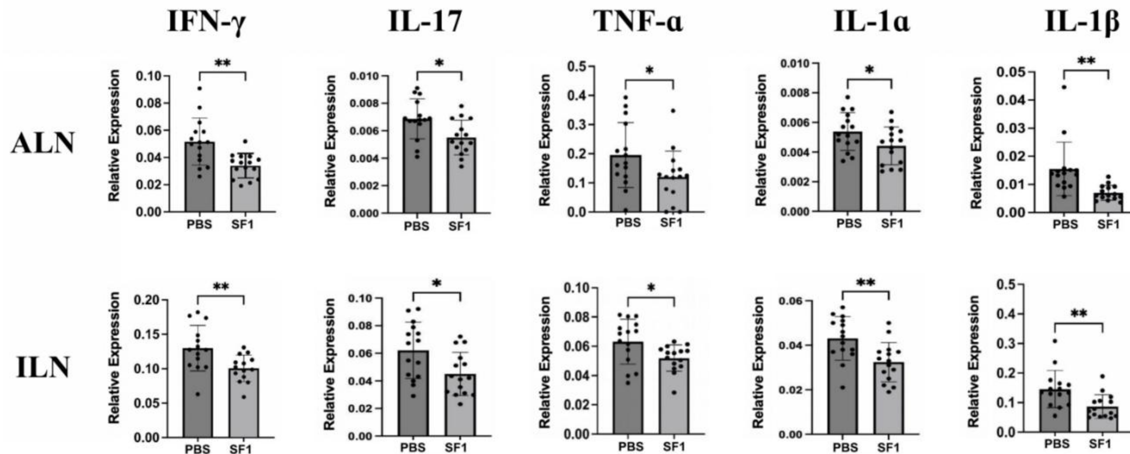

Figure 9. Administration of mAb SF1 suppresses the nucleic acid level in CIA

The mRNA expression levels of IFN-γ, IL-17, TNF-α, IL-1α and IL-1β in ALNs and ILNs in the CIA-induced DBA/1 mice were determined by RT-qPCR (n=15). *p < 0.05, **p < 0.01. IFN-γ, interferon-γ; IL-17, interleukin-17; TNF-α, tumor necrosis factor-α; IL-1α, interleukin-1α; IL-1β, interleukin-1β. ALNs, axillary lymph nodes; ILN, inguinal lymph nodes; RT-qPCR, reverse transcription-quantitative PCR.

2.8 SF1 reduces cell numbers in lymph nodes

B cells are instrumental in the pathogenesis of RA as they are activated to produce rheumatoid factor, which, in addition to a number of other autoantibodies, is associated with RA initiation and pathogenesis. To investigate a potential role of B cells involvement in the pathogenesis of CIA, we examined the relative and absolute numbers of B cells

(CD19+CD4−) on day 31. We observed a significant increase in the proportion of B cells in ALN and ILN, which lasted throughout the study time (Fig 10A and B).

$CD4^+$ T cells are indispensable for CIA induced inflammation and paw swelling. $CD4^+$ T cells play a crucial role in regulating the immune response, particularly in autoimmune diseases. Their ability to activate and direct other immune cells makes them a key component of the inflammatory process. Because of this, we sought to quantify the level of $CD4^+$ T cells in ALN and ILN, which serve as representative paw-draining lymph nodes, on day 31. Our findings revealed a significant reduction in the proportions of $CD4^+$ T cells in both ALN and ILN, affecting both their relative and absolute numbers. This decrease suggests a potential downregulation of the immune response in these specific lymph nodes (Fig. 11A and B).

In the inflammatory synovium of rheumatoid arthritis patients, changes in the level of memory $CD4^+$ T cells are critical to the progression of the disease. Memory $CD4^+$ T cells can be subdivided into central memory and effector memory $CD4^+$ T cells. The results indicate that CD44 expression is enhanced in all memory T cells, while differential expression of CD62L is commonly utilized to distinguish between central memory and effector memory T cells. We stained for CD44 and CD62L and observed a significant reduction in both the relative and absolute numbers of central memory (CD4+CD62L+CD44+) $CD4^+$ T cells isolated from ALN (Fig. 12A and B) and ILN (Fig. 13A and B) after SF1 treatment. Among the effector memory (CD4+CD62L-CD44+) $CD4^+$ T cells, the comparison of absolute numbers in ALN showed differences. Both the relative and absolute numbers of effector memory $CD4^+$ T cells in ILN exhibited significant decreases.

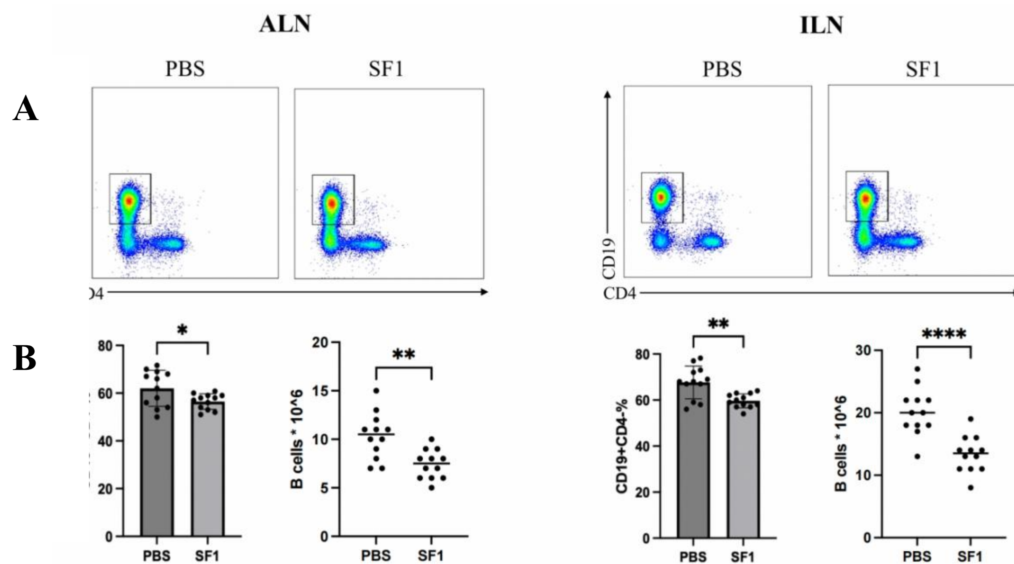

Figure 10. SF1 reduces B cell numbers in lymph nodes in CIA mice

Cells were prepared from ALN and ILN on day 31 and analyzed by flow cytometry. (A) Representative flow cytometry dot plot of B cells. (B) Relative number and absolute number.

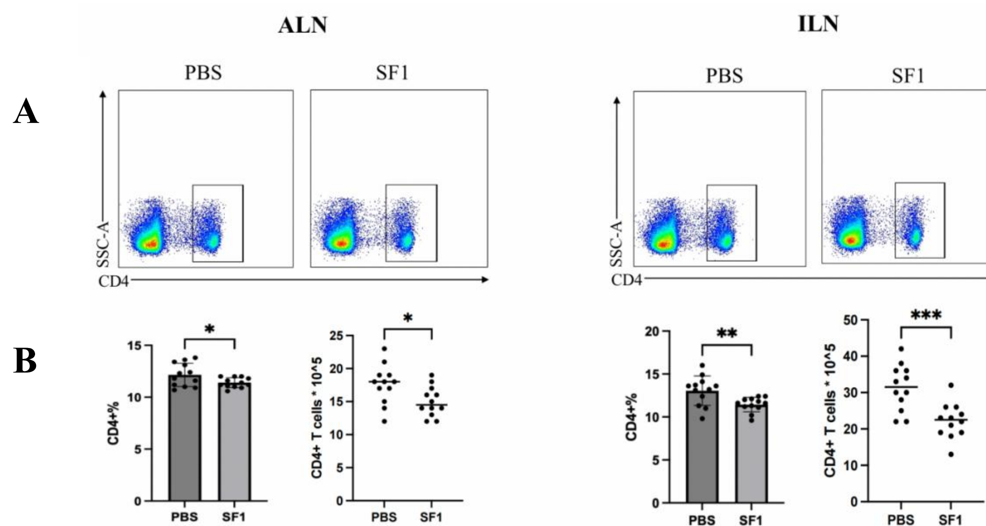

Figure 11. SF1 reduces CD4^{+} T cell numbers in lymph nodes in CIA mice

Cells were prepared from ALN and ILN on day 31 and analyzed by flow cytometry. (A) Representative flow cytometry dot plot of CD4^{+} T cells. (B) Relative number and absolute number.

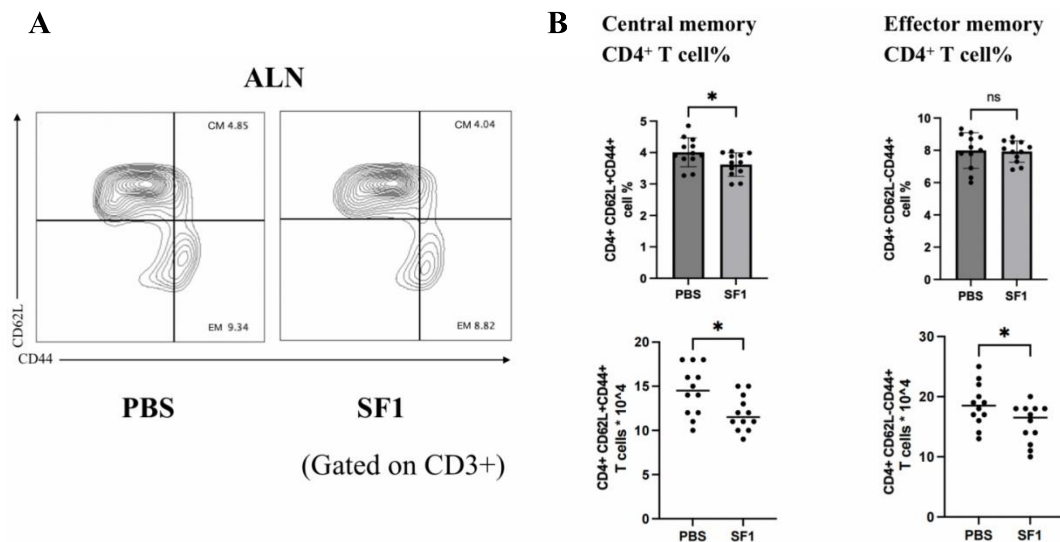

Figure 12. Central/effector memory CD4$^+$ T cells were isolated from ALN of CIA mice on day 31 and analyzed by flow cytometry

(A) Representative flow cytometry dot plot of central/effector memory CD4$^+$ T cells from ALN. (B) Relative number and absolute number.

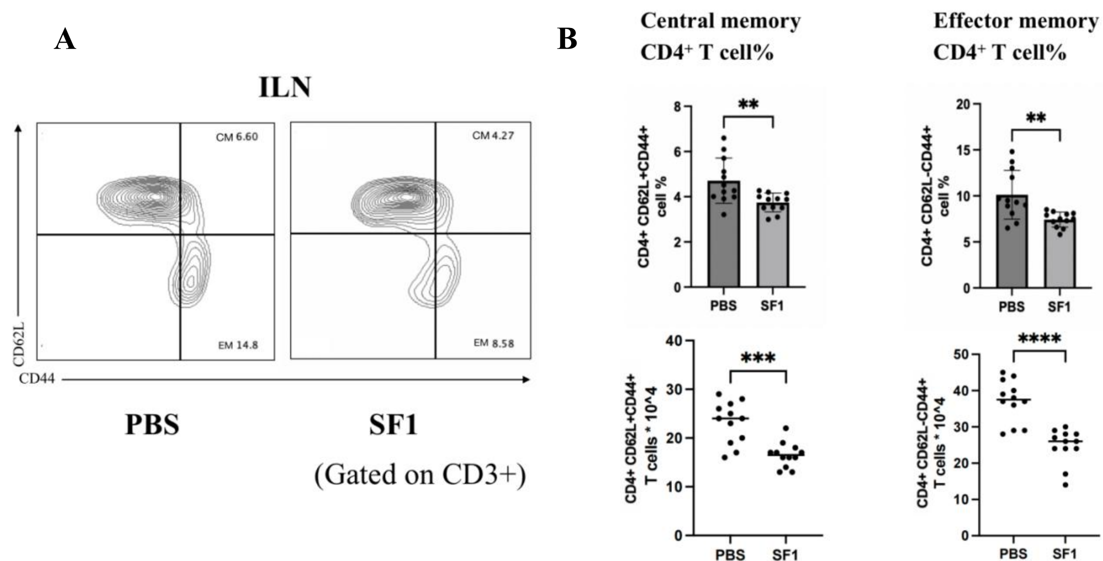

Figure 13. Central/effector memory CD4$^+$ T cells were isolated from ILN of CIA mice on day 31 and analyzed by flow cytometry

(B) Representative flow cytometry dot plot of central/effector memory CD4$^+$ T cells from ILN. (B) Relative number and absolute number.

2.9 SF1 does not cause tissue damage in various organs

To assess the side effects of SF1, specifically its potential impact on vital organs, we examined histological sections of key organs from mice treated with SF1. Through histological analysis using H&E staining, we aimed to detect any signs of tissue damage or adverse reactions. We examined the heart, liver, spleen, lungs, and kidneys from CIA induced DBA/1 mice treated with or without SF1. The morphology of examined tissues appeared normal when compared with that of untreated DBA/1 mice (Fig. 14). These results suggest that SF1 does not cause tissue damage in these organs, providing preliminary evidence for its safety profile.

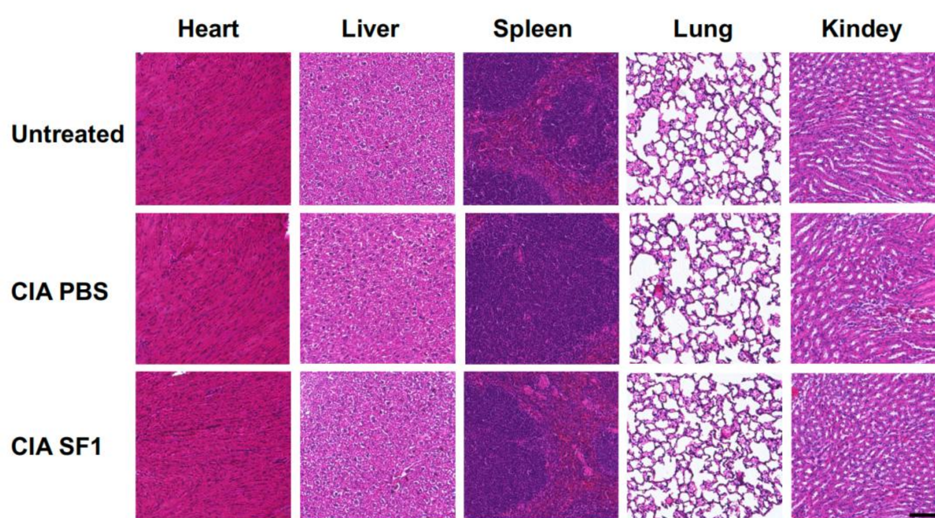

Figure 14. Hematoxylin-eosin-stained sections of various tissues from CIA-induced mice H&E staining of the heart, liver, spleen, lung, and kidney from untreated control DBA/1 mice and CIA induced DBA/1 mice administered PBS (CIA PBS) or SF1 (CIA SF1). Scale bar = 50 μm. H&E, hematoxylin and eosin.

3. Discussion

L-selectin, a constituent of the selectin group of cell-cell adhesion proteins, has a vital function in guiding lymphocytes to peripheral lymph nodes, so enabling future immunological responses. The protein is present on the outer surface of lymphocytes and acts as a receptor that guides the cells to certain locations by attaching to particular molecules called ligands. One example of such ligands is 6-sulfo sLex glycans, which are found on high endothelial venules (HEVs). This interaction is crucial for the movement and placement of lymphocytes within the lymphoid tissues[90]. Based on this mechanism, we postulated that targeting the interaction between selectin and its ligand could be a viable approach for developing drugs to treat immune-related disorders, such as autoimmune and allergy problems. In order to investigate this theory, we analyzed the impact of a new anti-glycan antibody called SF1, which specifically targets 6-sulfo sLex glycans, in a murine CIA model. The findings of our study indicate that SF1 has the ability to improve CIA in DBA/1 mice by inhibiting the generation of antibodies specific to antigens and the release of cytokines in the lymph nodes that drain the affected area. This leads to a decrease in the inflammatory response and the concomitant tissue damage commonly observed in rheumatoid arthritis.

RA is a chronic autoimmune condition that typically necessitates lifelong treatment and care. Significant improvements have been achieved in comprehending and managing RA, although it continues to be a disabling condition marked by enduring inflammation, joint deterioration, and systemic involvement[91]. During the development of RA, there is the occurrence of small areas of tissue death (necrosis),

the formation of adhesive tissue (granulation adhesions), and the development of fibrous tissue on the surface of the joints. At advanced stages, these alterations result in gradual joint stiffness, destruction, abnormalities, and eventual loss of physical function. Current therapy for rheumatoid arthritis, while they work well, can be excessively costly and are frequently not included in healthcare systems, resulting in variable worldwide management and outcomes[92]. These treatments mostly focus on the advanced stage of the disease, where there is already substantial joint damage. Nevertheless, it is possible to identify autoantibodies and other biomarkers several years before the appearance of clinical symptoms, indicating a potential opportunity for early intervention[93]. Our investigation utilizing SF1 reveals that addressing specific molecular interactions, such as the interaction between L-selectin and 6-sulfo sLex glycan, in early-stage treatment can effectively delay the evolution of clinical symptoms and provide relief from pain in affected patients. Implementing early intervention measures, as exemplified by the effectiveness of SF1 in the CIA model, has the potential to transform the management of RA by averting significant joint injury and enhancing long-term results.

The CIA model is a widely utilized and thoroughly defined animal model for researching RA. The condition closely mimics multiple features of human rheumatoid arthritis, such as synovitis, pannus development, cartilage deterioration, and bone erosion[94]. This paradigm involves the immunization of DBA/1 mice with type II collagen emulsified in CFA, resulting in an autoimmune response specifically directed towards the joint tissues. This model is very valuable for assessing the effectiveness of potential therapy drugs and comprehending the fundamental

principles of RA pathogenesis[95]. In our investigation, we employed this model to evaluate the therapeutic efficacy of SF1, uncovering its capacity to regulate the immune response and mitigate the severity of the disease. The CIA model in DBA/1 mice has played a crucial role in discovering important inflammatory mediators and evaluating new therapies, including the encouraging benefits reported with SF1. By specifically focusing on the initial phases of the immune response, SF1 exhibits promise as a therapeutic agent capable of modifying the progression of the disease and enhancing the overall well-being of individuals with rheumatoid arthritis.

The findings of the current study revealed that SF1 could significantly reduce the clinical score and pathology of CIA and inhibit elevated CII-specific IgG2a levels in mouse serum. Furthermore, SF1 suppressed the mRNA expression of various cytokines in the draining lymph nodes, including IFN-γ produced by Th1 cells and inflammatory cytokines. Accordingly, the mode of action of SF1 can be summarized as follows: (1) SF1 inhibits lymphocyte homing to draining lymph nodes, where self-antigen CII-specific immune responses occur. (2) Blockade of lymphocyte homing suppresses Th1 immune responses that lead to the production of pathogenic CII-specific IgG2a in the draining lymph nodes. (3) SF1-mediated blockade of lymphocyte homing also suppresses the expression of inflammatory cytokines, including IFN-γ, IL-17, TNF-α, IL-1α, IL-1β extending inflammatory responses to the joints of CIA-induced DBA/1 mice.

In summary, the results of the current study indicate that SF1 can prevent CIA by suppressing Th1 and inflammatory immune responses in draining lymph nodes. Previously, we have reported that SF1 inhibits lymphocyte homing to nasal-associated

lymphoid tissues and Th2 cell-mediated murine allergic rhinitis[89]. It is well-established that lymphocyte homing is fundamental for both Th1, Th17 and Th2 immune responses; hence, we believe that SF1 can suppress immune responses, irrespective of their types. Collectively, the results of the current study suggest that SF1 could serve as a novel therapeutic agent against various immune-related diseases.

4. Materials and methods

4.1 Animals

Animal care and experiments were conducted in accordance with the guidelines of the Chiba University Animal Care and Use Committee. Six-week-old male DBA/1 mice (18–20 g) were purchased from Charles River Laboratories, Japan, and subsequently housed in an animal facility with controlled humidity and temperature under a 12-h light/dark cycle.

4.2 SF1 antibody purification

Culture SF1 hybridoma cells to about 10*7 cells. BALB/c nude mice were sensitized for two weeks using Pristane. About 10*7 cultured SF1 hybridoma cells were resuspended in sterile PBS and injected intraperitoneally into sensitized BALB/c nude mice. Two weeks later, the ascites of BALB/c nude mice was recovered. Add 0.033 time the volume of ascites of n-octanoic acid to the ascitic fluid and stir at room temperature for 30 minutes. The obtained liquid was centrifuged at 4 degrees Celsius for 20 minutes using 9000 rpm. The supernatant was dialyzed against PBS using a 0.8 µm filter. The obtained liquid was slowly added with Ammonium sulfate at 4 °C. After stirring for 6 hour, the liquid was collected and centrifuged at 4 degrees Celsius for 20 minutes using 9000 rpm. The supernatant was dialyzed against PBS using a 0.8um filter.

4.3 Immunofluorescence analysis

Acetone-fixed frozen sections (7-µm) from C57BL/6J and DBA/1 mice were incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate-buffered saline (PBS) to block nonspecific binding sites. The sections were

then incubated with 5 μg/mL biotinylated SF1 overnight at 4 °C. After washing, the sections were incubated for an additional 1 h in the dark with 1.0 μg/mL Alexa Fluor 594-streptavidin (Thermo Fisher Scientific, USA) and 0.5 μg/mL 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Daido, Japan). After re-washing, sections were mounted using Mount Quick (Hokkaido, Japan). Images were obtained using a fluorescence microscope (BZ-X800; KEYENCE).

4.4 Induction of arthritis

On day 0, chick type II collagen (CII; 2 mg/mL, Chondrex, USA), emulsified in an equal volume of complete Freund's adjuvant (CFA; Chondrex, USA) containing 4% heat-killed *Mycobacterium Tuberculosis* H37 RA, was intradermally administered to DBA/1 mice (100 μL per mouse) at the tail base. On day 21, a second immunization was performed (50 μL per mouse) using incomplete Freund's adjuvant (Chondrex, USA) instead of CFA. And the clinical arthritis scores were evaluated as described previously[96] as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot, and digits or ankylosis of the limb. The mice were observed daily for 31 days, and each paw was examined; a maximum arthritis score of 16 was established for the four paws.

4.5 Study groups and SF1 administration

On day 21 post-first immunization with CII/CFA emulsion, DBA/1 mice were randomly divided into two groups (n=5/group): the SF1-treated group was

intraperitoneally administered 200 μL SF1 (0.5 mg/mL in sterile PBS, 100 μg per mouse), while the PBS-treated group received sterile PBS, on days 21, 24, 27, and 30. All mice were sacrificed on day 31.

4.6 Histological analysis

The DBA/1 mice were sacrificed via anesthesia and knee joint, synovium, ankle joint and intertarsal joint were collected on day 31 following second immunization. All the tissues were dissected, fixed in 4% paraformaldehyde solution for 24 h. Among them, knee joint, ankle joint and intertarsal joint need to be decalcified in 10% ethylene diamine tetraacetate for a week, with the solution renewed every two days, and then embedded in paraffin. Standard frontal sections of 3 μm were prepared and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, USA) to examine tissue damage. Images were obtained using a fluorescence microscope (BZ-X800; KEYENCE).

4.7 Enzyme-linked immunosorbent assay (ELISA)

Antigen-specific antibody levels were measured using ELISA. To detect antigen-specific IgG, IgG1, and IgG2a, CII was used as the coating antigen. Serum was prepared from DBA/1 mouse peripheral blood on day 31 post-first immunization with CII/CFA emulsion by centrifugation at 5,000 rpm for 10 min. The wells of 96-well ELISA plates (Corning, USA) were overnight coated with 5.0 μg/mL CII in PBS, and nonspecific binding sites were blocked with 3% BSA in PBS for 2 h. Diluted serum samples (1:125 dilution) were added to wells and incubated for 1 h. After washing with 0.05% Tween 20 in PBS, 1.0 μg/mL HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2a (SouthernBiotech, USA) were added and incubated

for 30 min. After washing, 1-step Ultra ELISA substrate (Thermo Fisher Scientific, USA) was added to each well. The reaction was stopped by adding 2 M H_2SO_4, and the optical density was measured at 450 nm using a 96-well spectrophotometer (Rainbow Thermo, Switzerland).

4.8 Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from axillary lymph nodes (ALNs) and inguinal lymph nodes (ILNs) using TRIzol (Thermo Fisher Scientific, USA), and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Japan). NanoDrop One (Thermo Fisher Scientific, USA) was used to determine cDNA concentration. THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) was used for RT-qPCR, and mRNA expression in each sample was normalized to that of β-actin with the ΔΔCt method using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The following primers were used in the present study:

β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′
and 5′- ATGGAGCCACCGATCCACA-3′;
IFN-γ, 5′-TCAAGTGGCATAGATGTGGAAGAA-3′
and 5′-TGGCTCTGCAGGATTTTCATG-3′;
IL-17, 5´-GCAAACATGAGTCCAGGGAGA-3´
and 5´-CACGCTGAGCTTTGAGGGAT-3´;
TNF-α, 5′-GCCACCACGCTCTTCTGTCTAC-3′
and 5′- GGGTCTGGGCCATAGAACTGAT-3′;
IL-1α , 5´-ACGTCAACAACGGGAAGAT-3´
and 5´-AAGGTGCTGATCTGGGTTGG-3´;
IL-1β, 5′-TGCCACCTTTTGACAGTGATG-3′
and 5′-TGATGTGCTGCGAGATT-3′.

4.9 Flow cytometric analysis

ALN and ILN were filtered and centrifuged at 4℃, 440×g for 5 min. The supernatant was then removed and cells in the pellet were suspendend in 0.1% BSA-PBS incubated with light avoidance at 4℃ for 20 min with the following antibodies: CD19-Brilliant Violet 510TM anti-mouse (BioLegend, USA, Lot: B366808), CD3-APC anti-mouse (BioLegend, USA, Lot: B304830), CD4-APC/Cyanine7 anti-mouse (BioLegend, USA, Lot: B382616), CD44-FITC anti-mouse/human (BioLegend, USA, Lot: B378953), CD62L-PE-CY7 anti-mouse (BioLegend, USA, Lot: B103081), 7-AAD-Viability Staining Solution (BioLegend, USA, Lot: B347569). The samples were washed with PBS containing 0.1% BSA and centrifuged at 440×g for 5 min at 4℃, resuspended in 250 μL filtered-PBS. Subsequently, all antibodies were washed by PBS. After that, the samples were detected using flow cytometry (Beckman Coulter CytoFlex, USA) and data analysis was performed by FlowJo software (BD Biosciences, USA). Data processing and analysis were done with FlowJo software 10.6.1. (BD Biosciences).

4.10 Statistical analysis

Student's t-test was used to determine statistical significance between experimental groups. All results were analyzed using one-way analysis of variance (ANOVA). Data with p-values <0.05 were deemed statistically significant (*$p < 0.05$, **$p < 0.01$, ***$P<0.001$, ****$P<0.0001$, ns, not significant).

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During my Ph.D., I also experienced pain. This period of my life taught me a truly important lesson about pain: I could not escape it, could not avoid it, I just had to go through it and feel it. And while I was feeling it, I had to be so patient, so kind, and so loving to myself. Then, I was surprised to find that I had become stronger because of this.

I want to reiterate my heartfelt thanks to Prof. Kawashima. I am committed to upholding the high standards he has set and strive to become a responsible scientist who can make meaningful contributions to society.

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Publications

1. Prevention of Collagen-Induced Arthritis by an Anti-Glycan Monoclonal Antibody Reactive with 6-Sulfo Sialyl Lewis x in DBA/1 Mice. **Zihong Wei**, Hiroto Kawashima. Monoclon Antib Immunodiagn Immunother. 2024 Feb;43(1):3-9. doi: 10.1089/mab.2023.0019.

2. Rheumatoid Arthritis Animal Model in Mice. **Zihong Wei**, Jianfeng Wang, Hiroto Kawashima. Animals and Zoonoses. (In press)

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