

# Decoy Receptor 3 Attenuates Collagen-induced Arthritis by Modulating T Cell Activation and B Cell Expansion

CHIA-PI CHENG, HUEY-KANG SYTWU, and DEH-MING CHANG

**ABSTRACT. Objective.** To investigate the immune-modulated effects of decoy receptor 3 (DCR3) in an experimental model of rheumatoid arthritis (RA).

**Methods.** We delivered DCR3 plasmid into collagen-induced arthritis (CIA) mice using the hydrodynamic method and evaluated the serum level of DCR3 protein by ELISA. After immunization, we assessed disease severity of arthritis incidence, arthritis scores, paw thickness, and means of arthritic limbs, and used hematoxylin and eosin staining to observe synovial hyperplasia. We analyzed numbers of murine splenocytes and inguinal lymphocyte cells, cell populations, and serum proinflammatory cytokines by flow cytometry. We investigated B cell proliferation by carboxyfluorescein succinimidyl ester assay. We evaluated serum levels of total IgG2a and type II collagen-specific IgG and IgG2a using ELISA.

**Results.** DCR3 expression in sera significantly attenuated disease severity in CIA mice. We found that DCR3 inhibited the volume of inguinal lymph nodes, numbers of CD19+ B cells, and populations of interferon- $\gamma$ , interleukin 4 (IL-4), IL-17A, and Foxp3-producing CD4+ T cell *in vivo*. We found that DCR3 inhibited Pam3CSK4 (Toll-like receptor 1/2 ligand)-induced B220+ B cell proliferation *in vitro*. DCR3 treatment reduced the serum level of IL-6, total IgG2a, and CII-specific IgG2a antibody.

**Conclusion.** We postulated that the protective effects of DCR3 in CIA resulted from modulation of the immune system by maintaining the B/T cell balance and decreasing lymphocyte expansion. We suggest DCR3 as a prophylactic and potential therapeutic agent in the treatment of RA. (First Release Sept 1 2011; J Rheumatol 2011;38:2522–35; doi:10.3899/jrheum.110245)

## Key Indexing Terms:

COLLAGEN-INDUCED ARTHRITIS  
T CELL ACTIVATION

DECOY RECEPTOR 3  
B CELL EXPANSION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, leading to severe disability and premature mortality<sup>1</sup>. Although the cause of RA is unknown, an increasing body of evidence has demonstrated that autoimmunity plays a pivotal role in its progression and chronicity. Abnormal B cell-T cell interaction with the phenotypic features of humoral and cellular immunity predominates in RA

and the collagen-induced arthritis (CIA) animal model<sup>2,3,4</sup>. Recently, the IL-17-producing CD4+ T cell (Th17) was shown to play a predominant pathogenic role in RA and CIA<sup>5</sup>. Further, it has been reported that B cell deficiency or treated monoclonal anti-CD3, anti-CD4, or anti-CD20 antibodies in CIA mice were resistant to severe arthritis<sup>6,7,8,9</sup>. Hence, the therapeutic strategy of using immunosuppressive biological agents was given more consideration.

Decoy receptor 3 (DCR3), an immune repressor and immune modulator, has not been well determined in RA and CIA. DCR3 is a soluble protein that belongs to the tumor necrosis factor (TNF) receptor superfamily<sup>10</sup>. DCR3 interacts with its natural ligands, including TNFSF6 (FASLG), TNFSF14 (LIGHT), and TNFSF15 (TL1A)<sup>11,12,13</sup>. Its function is blocking or competing with their ligand-receptor downstream signaling. Studies had shown that DCR3 plays multiple roles in the immune system. DCR3 prevents heart allograft rejection<sup>14</sup>, promotes cancer cell growth by escaping immune surveillance<sup>15,16</sup>, silences the Th1-dominant autoreactive T cell in type 1 diabetes<sup>17</sup>, ameliorates activated B/T cell-induced spontaneous autoimmune crescentic glomerulonephritis (ACGN)<sup>18</sup>, and downregulates Th17 cells in experimental autoimmune encephalomyelitis<sup>19,20</sup>.

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Investigations also found that DCR3 can modulate macrophage and dendritic cell differentiation and maturation<sup>21,22</sup>.

Further, DCR3 has been thought to be capable of regulating immune responses by triggering a “reverse signaling” after binding to membrane-bound ligand(s) on the cell surface. We also found that DCR3 was highly expressed in synovial tissue and synovial fluid in our patients with RA. This was similar to the findings of Hayashi, *et al*<sup>23</sup>. DCR3 was reported to be highly expressed in serum of patients with RA<sup>23,24</sup>. However, mice lacked the DCR3 gene. Also, no study has to date reported the regulatory effects of DCR3 on the immune system in RA and CIA.

We assessed the potential role and functional implications of DCR3 by its overexpression in the murine CIA model. We evaluated the efficacy of DCR3 on clinical symptoms, immune cell populations, proinflammatory cytokines, and autoantibody expression to determine the functions of serum DCR3 in human RA.

## MATERIALS AND METHODS

**Mice.** Male DBA/1J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred under conventional conditions at the Animal Center of the National Defense Medical Center, Taipei, Taiwan; all animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) in Taiwan.

**Induction and assessment of CIA.** CIA was induced as described<sup>25,26</sup>. Briefly, mice at 8–10 weeks old, body weight 19–23 g, were immunized with single-dose intradermal injection of 100 µg bovine type II collagen (CII) emulsified in complete Freund’s adjuvant (Chondrex, Inc.) on Day 0. Disease severity was recorded twice per week with the following scoring system for each limb: score 0: no evidence of erythema and swelling; score 1: erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint; score 2: erythema and mild swelling extending from the ankle to the mid-foot; score 3: erythema and moderate swelling extending from the ankle to the metatarsal joints; and score 4: erythema and severe swelling encompassing the ankle, foot, and digits.

**Plasmid DCR3.** The pCMV-hDCR3 construct was kindly provided by Prof. A. Chen, Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan<sup>18</sup>. The plasmids were amplified with bacteria ECOS-101 (Yeastern Biotech, Taipei, Taiwan), then purified using EndoFree plasmid kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions.

**Hydrodynamics-based gene delivery.** This method was developed by Liu, *et al*<sup>27</sup>. Briefly, naked plasmid DNA administered in saline at 10% body weight into mouse tail vein within 5–10 s could specifically express the gene in the liver and return to basal level in 7 days without causing any damage. DCR3 was a secreted protein. We combined these properties to globally express DCR3 in murine sera. To characterize the expression level and determine the interval at which DCR3 plasmids were to be administered to the animals, we first injected a single dose of 10 µg/g body weight DCR3 plasmid (diluted in 1.8 ml of normal saline) into mice (*n* = 3) by hydrodynamics-based gene delivery through the tail vein as described<sup>27</sup>; mice that received empty vector (pCMV) served as controls. The animals were killed at 0, 12, 24, 36, 48, 72, and 168 h after plasmid administration and serum samples were stored appropriately until analysis by ELISA (Bender MedSystems, Vienna, Austria).

**Cell preparation.** Single-cell suspensions were obtained from spleen and inguinal lymph nodes after passage through 40 µm nylon mesh filters.

Spleen cells were also treated with ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, and 0.1 mM Na<sub>2</sub>EDTA) to lyse red blood cells then washed twice with phosphate buffered saline (PBS). The total number of cells was determined using hemocytometers with microscopic observation of trypan blue-stained cells.

**B cell isolation and CFSE cell-dividing assay.** Splenic B cells were enriched by positive selection of B220-expressing cells with B220 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. B cell samples were routinely enriched to over 96% B220+ cells, as assessed by flow cytometry. Cells were labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE; Invitrogen, Carlsbad, CA, USA) by incubation for 8 min at room temperature with 1 mM CFSE at a density of 2 × 10<sup>7</sup> cells/ml in PBS. Cells were incubated with culture media [RPMI-1640 with 10% fetal bovine serum (FBS)] at 4°C for 5 min and were washed twice with culture media before use. Enriched B cells (2 × 10<sup>5</sup> cells at density 1 × 10<sup>6</sup> cells/ml) were cultured in 96-well plates and were treated with 6 µg/ml F(ab’)<sub>2</sub> goat anti-mouse IgM (no. 115-006-020; Jackson ImmunoResearch), 1 µg/ml lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma-Aldrich) or 50 ng/ml Pam3CSK4 (InvivoGen) and costimulated with 10 µg/ml control human IgG 5 or 10 µg/ml DCR3 protein (R&D Systems). CFSE dilution was assessed by flow cytometry after 72 h of stimulation.

**Flow cytometric analysis.** Splenocytes and lymphocytes from each group of mice were resuspended in RPMI-1640 supplemented with 5% FBS, and 1% penicillin/streptomycin (all from Invitrogen). Surface antigens were stained as described by the manufacturer with the following antibodies: anti-CD3, anti-CD4, anti-CD19, and anti-CD69 (BD Biosciences). For CD4+ cell intracellular cytokine staining [interferon-γ (IFN-γ), interleukin 4 (IL-4), and IL-17A), cells were prestained with CD4-FITC surface marker fixed at 4°C overnight then permeabilized with reagent according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA). For Foxp3 staining, cells were treated with Foxp3 staining buffers (BD Biosciences). Cells were stained with phycoerythrin-conjugated anti-mouse IFN-γ, IL-4, and IL-17A cytokines and Foxp3 transcription factor antibodies (BD Biosciences) for 30 min at room temperature, washed twice with PBS, and protected from light until data collection. All experiments were analyzed by FACSCalibur (BD Biosciences) and CellQuest Pro software.

**DCR3 ELISA and Th1/2 proinflammatory cytokine screening.** Serum levels of hDCR3 were measured using an hDCR3 ELISA kit (Bender MedSystems), according to the manufacturer’s instructions. Briefly, samples, serially diluted standards, dilution buffer, and biotin-conjugate antibody were put into each well of the detection plate, then incubated 2 h at room temperature on a microplate shaker at 100 rpm. After washing with PBS containing 1% Tween 20 six times, 100 µl streptavidin-horseradish peroxidase was added to the wells and incubated at room temperature for 1 h with shaking. After 6 washes, tetramethylbenzidine substrate solution was added to the wells and the color developed in proportion to the amount of bound DCR3. The intensity of the color was measured at absorbance wavelength 450 nm. All the specimens were examined in triplicate and a mean value was obtained for further analysis. For disease progression proinflammatory cytokine detection, sera samples were collected from each group on Days 10 and 26, then analyzed using a Th1/2 FlowCytomix assay kit (Bender MedSystems), according to the manufacturer’s instructions.

**Measurement of serum antibody levels.** For detection of antibody levels of anti-CII IgG, IgG2a, and total IgG2a, sera were collected from each group of mice on Days 10, 26, and 42 after immunization and stored at –80°C until assayed. Antibodies were determined by a commercial ELISA kit (anti-CII IgG and IgG2a from Chondrex; anti-total IgG2a from GenWay). The optical density of the standard serum was diluted according to the manufacturer’s instructions. The relationship of the optical density measured in the standard serum, diluted serially, and the arbitrary units showed good linear correlation in all determinations (*R* = 0.99; data not shown). The anti-CII IgG, IgG2a, and total IgG2a antibody concentrations in the sera, diluted

1:10<sup>4</sup> on Day 10 and 1:10<sup>5</sup> on Days 26 and 42, are presented as relative values (arbitrary units) compared with the optical density of the standard sera.

**Histologic assessment.** Mice were sacrificed by CO<sub>2</sub> on Day 42, and ankle joints were collected, fixed in 10% neutral buffered formalin for 24 h, decalcified in PBS containing 20% EDTA, pH 7.4, and embedded in paraffin. Joint sections (7  $\mu$ m) were then prepared, deparaffinized in xylene, and rehydrated through a graded alcohol series. Routine histology was performed by hematoxylin and eosin (H&E) staining. Then a histological score was evaluated blindly by summing the synovial/cartilage measures (score 0: no inflammatory cell infiltration; score 1: inflammatory cell infiltration to the joint tissues; score 2: synovial lining cell hyperplasia; score 3: pannus formation; score 4: joint cartilage layer destruction) and the bone damage measures (score 0: normal; score 1: minimal loss of cortical bone; score 2: mild loss of cortical trabecular bone; score 3: moderate loss of bone at many sites; score 4: marked loss of bone at many sites; score 5: marked loss of bone at many sites with fragmenting and full-thickness penetration of inflammatory process or pannus into the cortical bone)<sup>28</sup>.

**Statistical analysis.** Data are shown as means  $\pm$  SD and were analyzed using 1-way ANOVA with Newman-Keuls multiple comparisons on posttests. Nonparametric data (mean arthritis score, paw thickness, and means of arthritic limbs) were analyzed by Mann-Whitney U test; p values < 0.05 were considered statistically significant.

## RESULTS

**Serum level of DCR3 protein in treated CIA mice.** Based on the best DCR3 expression pattern in the serum samples of CIA mice, we delivered the DCR3 plasmid to the mice at 7-day intervals. After administrations of DCR3 plasmid, ELISA tests showed that the serum level of DCR3 protein was elevated to a peak at 24 h and returned to the baseline after 1 week in the DCR3-treated CIA mice. However, the CIA mice treated with empty vector did not express DCR3 protein at any timepoint (Figure 1A).

**Effect of DCR3 on the development of CIA.** To examine the role of DCR3 in CIA development, mice were treated with DCR3 plasmid (n = 15) every 7 days for 5 weeks. This procedure began with type II collagen 1 day before immunization. The control group was given the empty vector (n = 12). The first sign of arthritis was observed around 4 weeks after immunization and consisted of erythema and swelling of the feet and ankles. There was no significant difference between experimental and control mice in disease onset or incidence (Figure 1B). However, mean arthritis scores, paw thickness, and mean arthritic limbs were found to be significantly reduced in the DCR3-treated mice compared to the empty vector-treated mice at the late stage (Figure 1C-1E; p < 0.05). Similarly, joints that were examined microscopically demonstrated reduced histopathological severity of arthritis in DCR3-treated mice as compared to empty vector-treated mice (Figure 2).

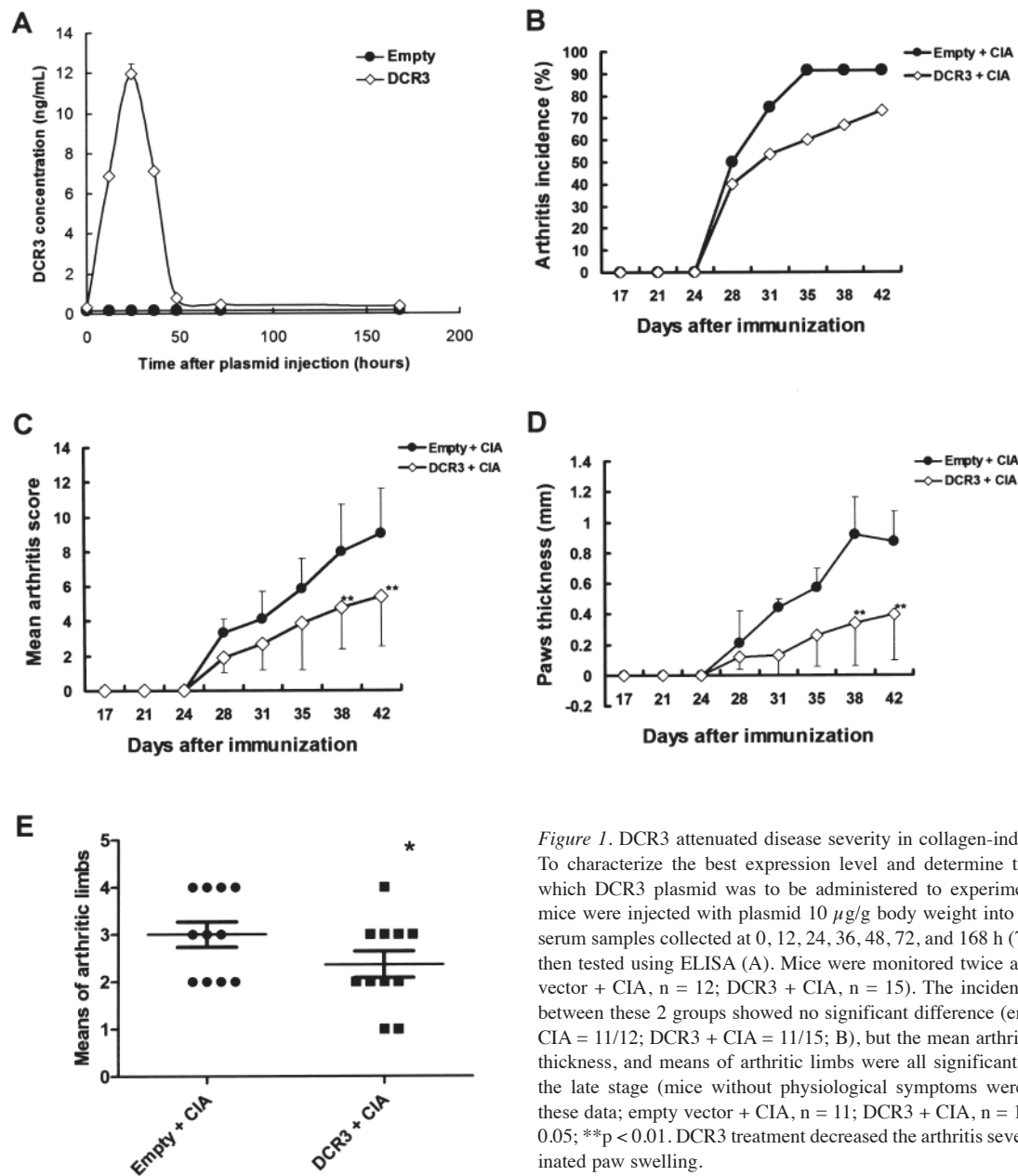
**DCR3 suppressed lymphoproliferation in CIA mice.** Patients with RA exhibited a massive lymphoproliferation in association with the disease incidence and severity<sup>3,29,30</sup>. We investigated the role of DCR3 in the effect of CIA-induced lymphoproliferation. We obtained the lymph nodes and spleens on Days 10 and 26, respectively, after immunization. Surprisingly, the peripheral (inguinal) lymph nodes in

DCR3-treated mice were much smaller than those of the empty vector-treated mice (Figure 3A). Also, the lymph nodes in DCR3-treated mice had lower lymphoproliferative effect on Days 10 and 26 after immunization compared to empty vector-treated mice (n = 4). Normal mice served as negative controls. Counting the total cell numbers of the spleen and lymph nodes, using a hemacytometer with a light microscope, we also found that total cells decreased by about 50% in the lymph nodes of the DCR3-treated mice, compared to empty vector-treated mice, on Days 10 and 26 (Figure 3B; p < 0.01). At these timepoints, the numbers of splenocytes were not significantly different between these groups; however, on Day 26, the DCR3-treated mice had slightly increased numbers of splenocytes (Figure 3C) as compared to the wild-type control.

**DCR3 inhibited the B cell population in CIA mice.** According to previous studies, early-stage CIA is a B cell-dominant autoimmune disease<sup>7,9,31</sup>. We investigated B cells of inguinal lymph nodes and spleen in response to CII immunization. Typically, DBA/1 mice developed signs of arthritis after the first immunization and showed maximum arthritis around Day 35. As shown in Figures 4A and 4C, in DBA/1 mice, immunization once with CII emulsion significantly increased roughly 3-fold the frequency of CD19+CD69<sup>−</sup> B cells in inguinal lymph nodes before the onset of arthritis (Day 10) compared with nonimmunized mice (p < 0.01). The phenomenon decreased only slightly and was sustained to the time of onset of arthritis at Day 26 (Figure 4B and 4C). In the DCR3-treated mice, the CD19+CD69<sup>−</sup> B cell population and absolute B cell numbers were lower than those in the empty vector-treated mice (Figure 4E and 4F; p < 0.01) on Days 10 and 26. However, there was no significant difference in the percentage of CD19+ plus CD69<sup>−</sup> or CD69+ in lymph nodes between the DCR3 and the empty vector-treated groups. Also, the splenic CD19+ B cell population was not influenced on Days 10 and 26 (Figure 4A, 4B, and 4D).

**DCR3 suppressed B220+ B cell division in vitro.** Based on our *in vivo* findings, we investigated whether DCR3 would directly affect B cell proliferation. We stimulated B220+ splenic B cells with IgM (B cell receptor; BCR), LPS (Toll-like receptor-4; TLR4), and Pam3CSK4 (TLR2) to assay the effects of DCR3 on B cell function. We found that DCR3 suppressed Pam3CSK4 (TLR2)-induced B cell proliferation in a dose-dependent manner (Figure 5; p < 0.01). However, DCR3 did not affect BCR-induced and TLR4-induced proliferation (data not shown).

**DCR3 inhibited the activated T cell population in CIA mice.** CIA mice are considered to have a T cell-dependent autoimmune disease<sup>6,8</sup> and DCR3 could target T cell immunity<sup>17</sup>. We investigated T cells from inguinal lymph nodes and spleen in response to CII immunization. As shown in Figures 6A, 6B, and 6C, immunization of DBA/1 mice once

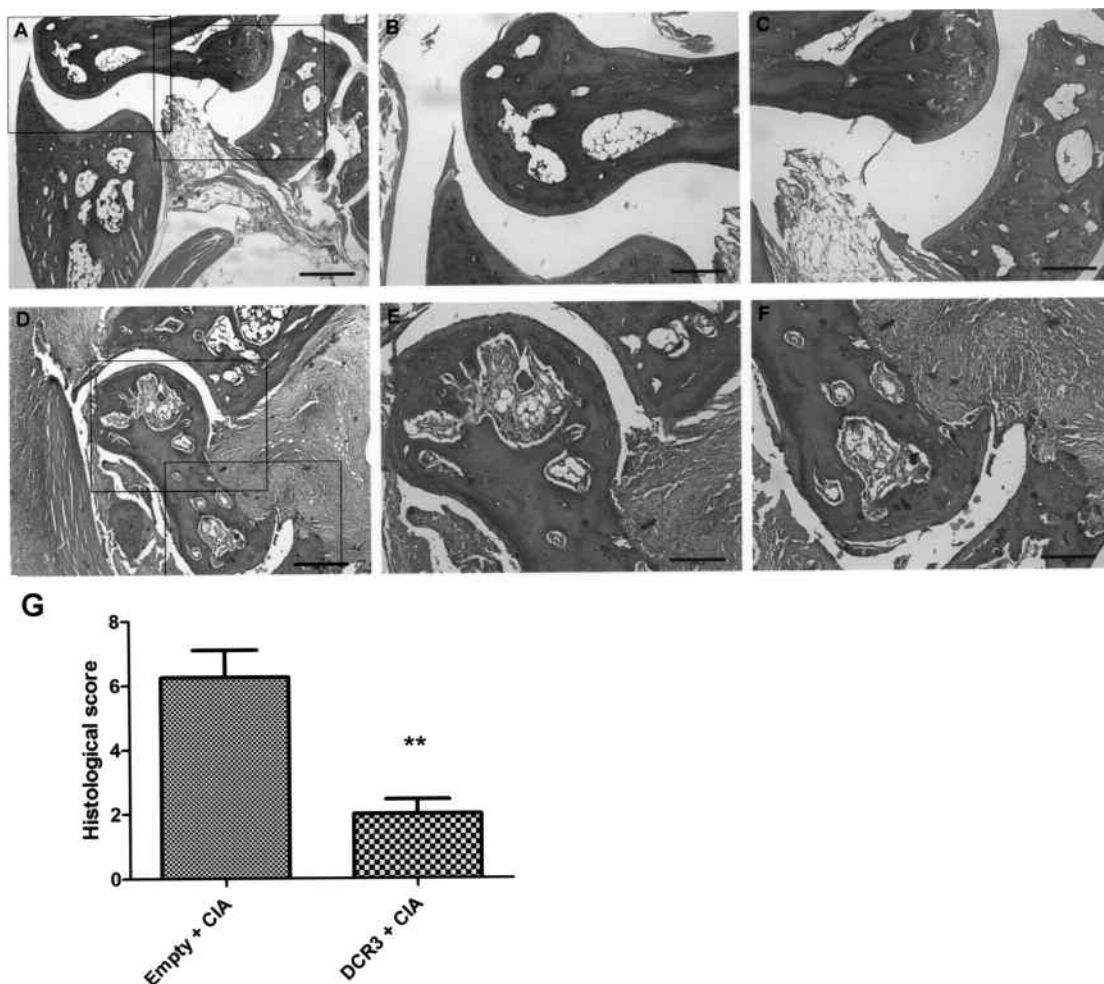


**Figure 1.** DCR3 attenuated disease severity in collagen-induced arthritis. To characterize the best expression level and determine the interval at which DCR3 plasmid was to be administered to experimental animals, mice were injected with plasmid 10  $\mu$ g/g body weight into tail veins and serum samples collected at 0, 12, 24, 36, 48, 72, and 168 h (7 days;  $n = 3$ ), then tested using ELISA (A). Mice were monitored twice a week (empty vector + CIA,  $n = 12$ ; DCR3 + CIA,  $n = 15$ ). The incidence of arthritis between these 2 groups showed no significant difference (empty vector + CIA = 11/12; DCR3 + CIA = 11/15; B), but the mean arthritis score, paw thickness, and means of arthritic limbs were all significantly different at the late stage (mice without physiological symptoms were excluded in these data; empty vector + CIA,  $n = 11$ ; DCR3 + CIA,  $n = 11$ ). C-E: \* $p < 0.05$ ; \*\* $p < 0.01$ . DCR3 treatment decreased the arthritis severity and eliminated paw swelling.

with CII emulsion significantly decreased ~35% of CD3+ T cells in inguinal lymph nodes before the onset of arthritis (Day 10 and Day 26), as compared to nonimmunized mice ( $n = 3$ ). In lymph nodes in the DCR3-treated mice, the CD3+ T cell population was maintained at a higher percentage than in the empty vector-treated mice on Days 10 and 26 (Figure 6C). However, only the Day 26 observation in lymph nodes was statistically significant ( $p < 0.01$ ). Further, we analyzed the populations of CD4 T cells in each group of CIA mice (Figure 6E-6H). We also found that nonactivated CD4+CD69- T cells were maintained at a higher percentage in the total population in the DCR3-treated mice than in the empty vector-treated mice (Figure 6G). Gating the CD4+

cell population (Figure 6I-6L), the portions of activated CD4+CD69+ T cells were less prominent on Day 26 in DCR3-treated mice compared to the empty vector-treated mice (Figure 6K, 6L;  $p < 0.05$ ). When we combined the results of B cells as described above, the B cell/T cell ratio in DCR3-treated mice was close to that of the wild-type control (Table 1). This indicated that DCR3 might affect the early stage of B cell/T cell immune response and dominantly inhibit immunization efficiency on B cell population.

**DCR3 inhibited the Th1, Th2, and Th17 population in CIA mice.** Recently, conventional T helper cells (Th1 and Th2), Th17, and Treg cells were also found to be elevated in CIA mice<sup>5,32</sup>. We further investigated the effect of DCR3 in the



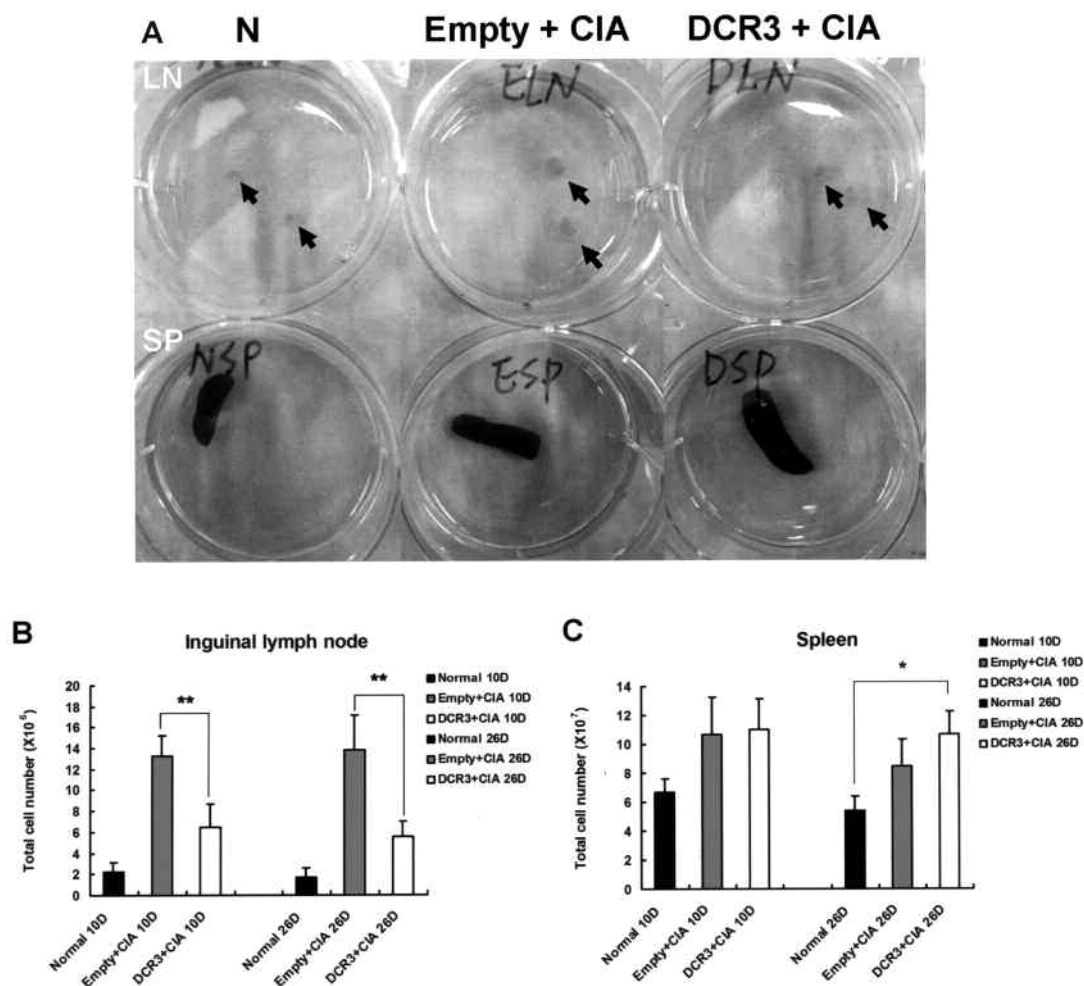
**Figure 2.** Effect of DCR3 on ankle joint histology in mice that were immunized with CII emulsion to induce CIA in the absence or presence of DCR3 plasmid injection. Mice were sacrificed on Day 42 and hind paws sections were stained with H&E. Upper panels: ankle joints from representative DCR3 + CIA mice (n = 11; A, 50× magnification; B, C, 100×); lower panels: ankle joints from empty vector + CIA mice (n = 11; D, 50× magnification; E, F, 100×). Degree of joint synovitis, cartilage destruction, and bone erosion was minor in the DCR3-treated group (G); \*\*p < 0.01.

subtypes of activated T cells in CIA mice. Isolated cells were stained with CD4 cell-surface marker and combined with IFN- $\gamma$ , IL-4, IL-17A lymphokine, or Foxp3 transcription factor. As shown in Figure 7, CIA mice treated with empty vector showed significant increases in IFN- $\gamma$ -, IL-4-, IL-17A-, and Foxp3-producing CD4+ T cells in inguinal lymph nodes and IFN- $\gamma$ -, IL-4-, and IL-17A-producing CD4+ T cells in spleen on Days 10 and 26 compared to non-immunized mice (n = 3). However, in the DCR3-treated mice, the IFN- $\gamma$ -, IL-4-, IL-17A-, and Foxp3-producing CD4+ T cells were decreased in lymph nodes and partially decreased in spleen compared to empty vector-treated mice on Days 10 and 26 (Figure 7E, 7F, 7K, and 7L).

**DCR3 inhibited production of the proinflammatory cytokine IL-6.** To determine the effect of DCR3 in B/T cell interactions in the development of CIA mice, we investigated serum levels of the proinflammatory cytokines IL-1 $\alpha$ , IL-2,

IL-4, IL-5, IL-6, IL-10, IL-17, TNF- $\alpha$ , IFN- $\gamma$ , and granulocyte-macrophage colony-stimulating factor on Days 10 and 26 using a mouse FlowCytomix Th1/2 kit. We found that only IL-6 could be elevated and detected on Days 10 and 26 in CIA mice. Moreover, the IL-6 cytokine level was lower in DCR3-treated CIA mice than empty vector-treated CIA mice on Days 10 and 26 (p < 0.01, n = 3; Figure 8A).

**DCR3 inhibited production of IgG2a and CII-specific IgG2a antibodies.** Previous studies showed that humoral immunity played an important role in the pathogenesis of CIA and RA<sup>33,34</sup>. We next investigated whether DCR3 was involved in regulation of serum levels of CII-specific antibodies. Our results showed that DCR3 dramatically reduced the CII-specific IgG antibodies on Day 26 and IgG2a antibodies on Days 26 and 42 (p < 0.01; n = 4). Also, total IgG2a was significantly decreased in DCR3-treated mice (p < 0.01; n = 3) compared to empty vector-treated mice



**Figure 3.** Spleen and lymph node cells were harvested on Days 10 and 26 from naive or CIA mice given DCR3 or empty vector. Photograph (A) shows 10-day inguinal lymph nodes (upper panel, black arrows) and spleen (lower panel) from each group. Total cell numbers from each group were counted by hemacytometer in 10- and 26-day lymph nodes (B) and 10- and 26-day spleens (C). The total cells decreased by about 50% in lymph nodes of DCR3-treated mice compared to empty-vector mice on Days 10 and 26 (B). Results represent data from 4 independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ .

(Figure 8). However, there were no differences between DCR3-treated and empty vector-treated mice in CII-specific IgG antibodies on Days 10 and 42 (Figure 8C).

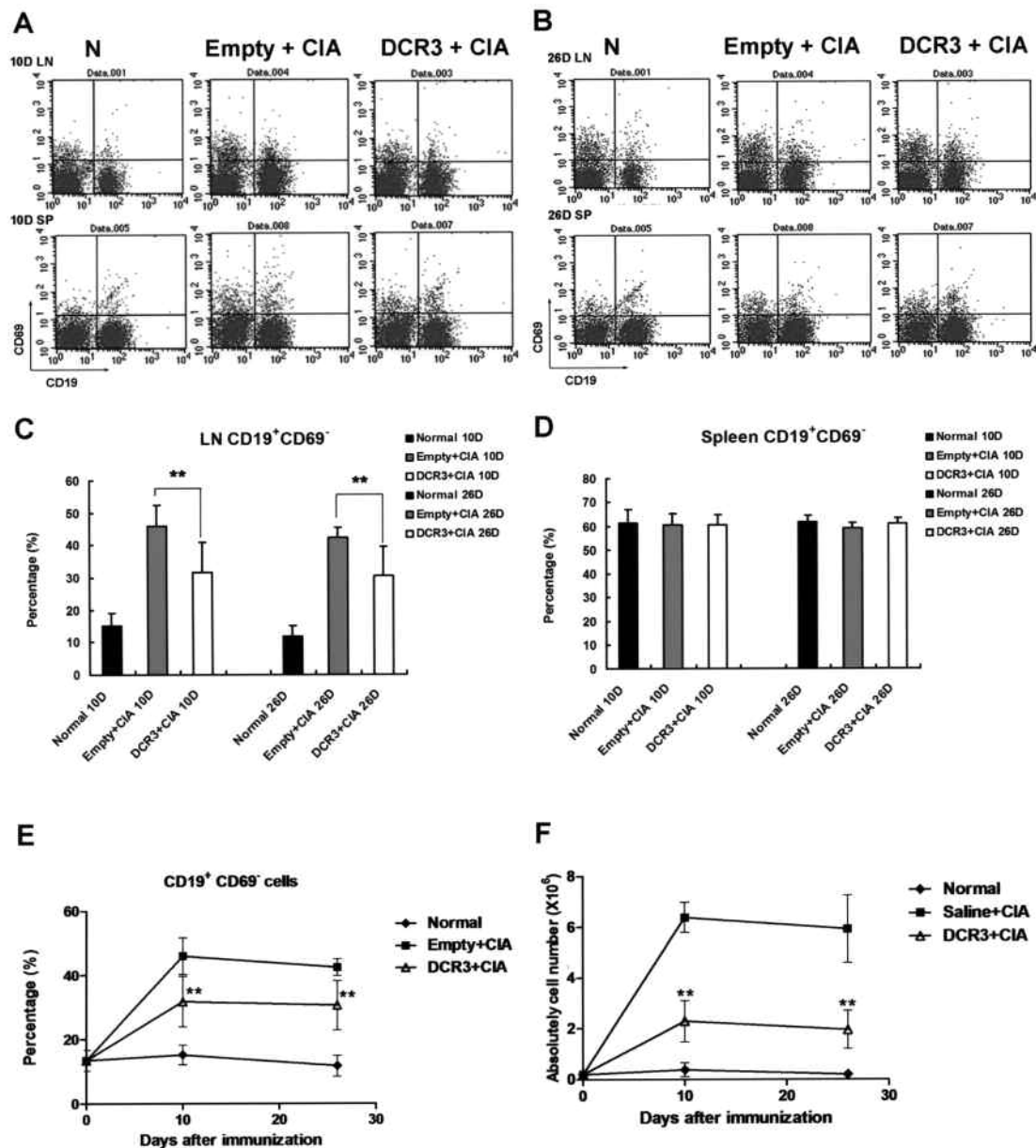
## DISCUSSION

Our data demonstrated that DCR3 can attenuate CIA in mice when administered before the onset of disease. DCR3 inhibited disease development by downregulation of massive lymphoproliferation. DCR3 markedly inhibited the induction of Th1, Th2, Th17, Treg, and CD19+ B cells in inguinal lymph nodes. DCR3 had an inhibitory effect on IL-6 production, B cell proliferation, and antibody production. This might be a consequence of the blockade of effector T cells and B cells, which could synergize with other cytokines for IL-6 production.

Although DCR3 expression was associated with active RA, the physiological role is undefined and remains controversial. Hayashi, *et al* had found that synovial fibroblasts

from patients with RA had high expression of DCR3 and were resistant to Fas ligand-induced apoptosis<sup>23</sup>. However, Takahashi, *et al* found that DCR3 binds to TNF-like cytokine 1A (TL1A) expressed in RA fibroblast-like synoviocytes, resulting in the negative regulation of cell proliferation induced by inflammatory cytokines<sup>35</sup>. Also, FASLG, LIGHT, and TL1A, the natural ligands of DCR3, were highly expressed in patients with RA, and exacerbated disease in CIA mice<sup>36,37,38,39</sup>. Our data showed that DCR3 attenuated arthritis severity by suppressing B cell expansion and the effector T cell activation. This effect was consistent with the modulating effects of DCR3 in blocking B/T cell interaction and costimulation transduced by LIGHT<sup>40</sup>.

Previous studies showed that Fas ligand carries a deficient function in synovial lymphocyte<sup>41</sup>. Also, the level of DCR3 was higher in synovial fluid than in sera. Recently, Cheung, *et al* had shown that LIGHT with polymorphic variants decreased DCR3 binding avidity in RA and DCR3



**Figure 4.** DCR3 plasmid treatment decreased not only the lymphoproliferative effect, but also the CD19<sup>+</sup>CD69<sup>-</sup> B cell population in inguinal lymph nodes (LN; panel C) on Days 10 and 26 (A and B, upper panels) compared to mice given empty vector. The combined trend line (E) and absolute cell numbers (F) also show significant differences. There were no significant differences in spleen CD19<sup>+</sup>CD69<sup>-</sup> B cells (D). Results represent 1 set of data from 4 independent experiments. \*\**p* < 0.01.

was a responsive element in human RA<sup>42</sup>. Therefore, we hypothesized that a high level of DCR3 present in sera was responsive to the autoimmune-driven inflammation and was to naturalize increased soluble ligands TL1A and LIGHT in serum of patients with RA. We took advantage of the fact that mice lack the DCR3 gene; we delivered the plasmid directly into animals of the experimental arthritis model. We found that globally expressed DCR3 protein can attenuate disease severity by modulating peripheral immune response and suppressing immune cell activation. Our finding was similar to those of Ka, *et al*<sup>18</sup>, in which the disease severity

of ACGN mice, an animal model of systemic lupus erythematosus (SLE)-induced nephritis syndrome, was reduced by DCR3, despite high levels of DCR3 in humans with SLE.

In addition, CD69, a common activation marker on leukocytes, has been reported to be functionally impaired in RA<sup>43,44,45</sup>. Also, CD69 plays a negative regulatory role in the development of CIA in mice, which correlates with a diminished local synthesis of transforming growth factor  $\beta$ 1. Anti-CD69 monoclonal antibodies of distinct isotypes could augment or inhibit the severity of CIA<sup>44,46</sup>. Otherwise, T cell activation in rheumatoid synovium is B cell-depend-

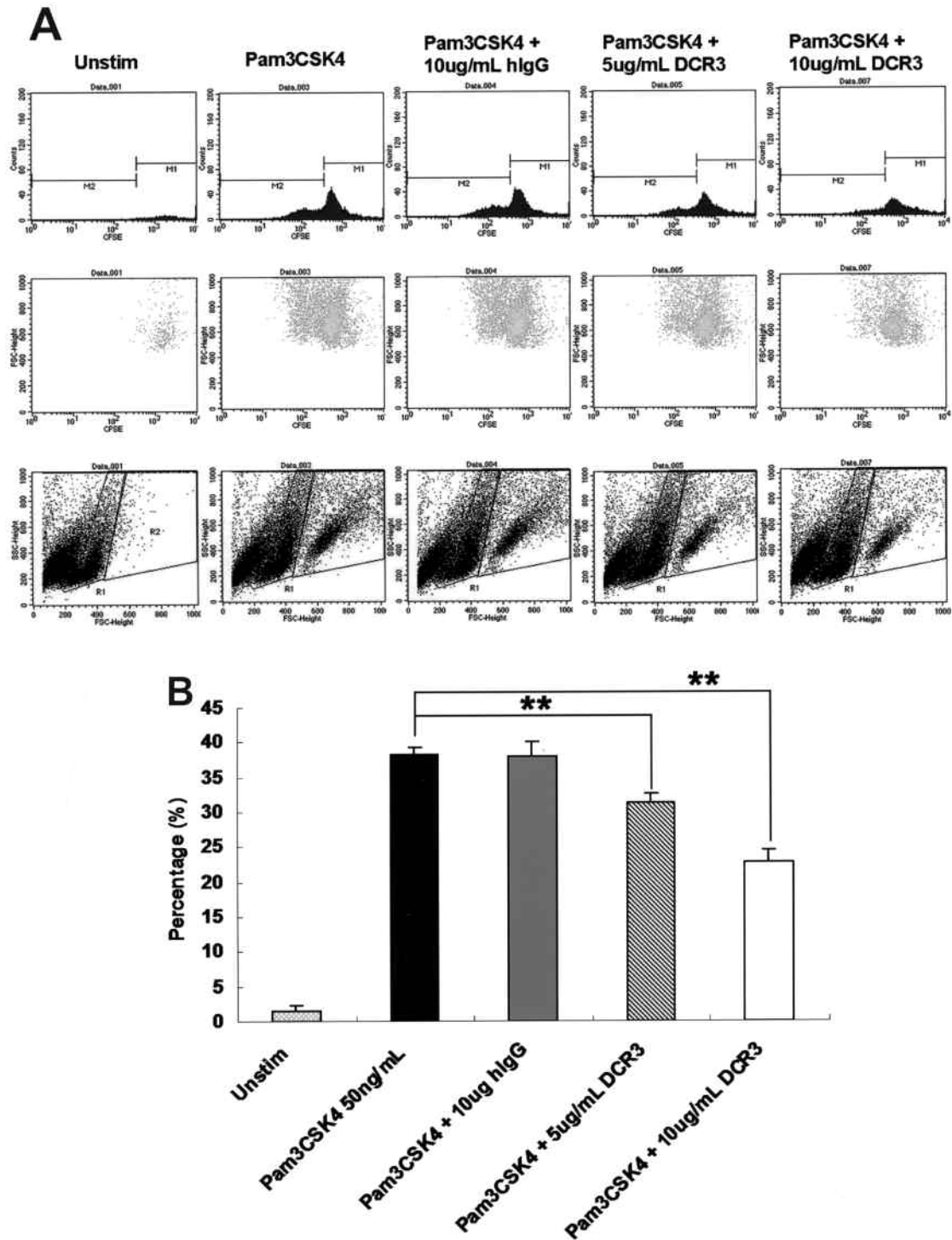


Figure 5. DCR3 suppressed splenic B220+ B cell proliferation. Normal DBA1/J splenic B220+ B cells were labeled with carboxyfluorescein diacetate succinimidyl diester dye and purified by magnetic beads. Cells were treated with 50 ng/ml Pam3CSK4 combined with 10  $\mu$ g/ml control hlgG and 5 or 10  $\mu$ g/ml DCR3. DCR3-treated B220+ B cells showed lower dividing activity than the control group treated with Pam3CSK4 alone or Pam3CSK4 plus hlgG in a dose-dependent manner. Results represent means  $\pm$  SD of 3 independent experiments (\*\* $p < 0.01$ ).

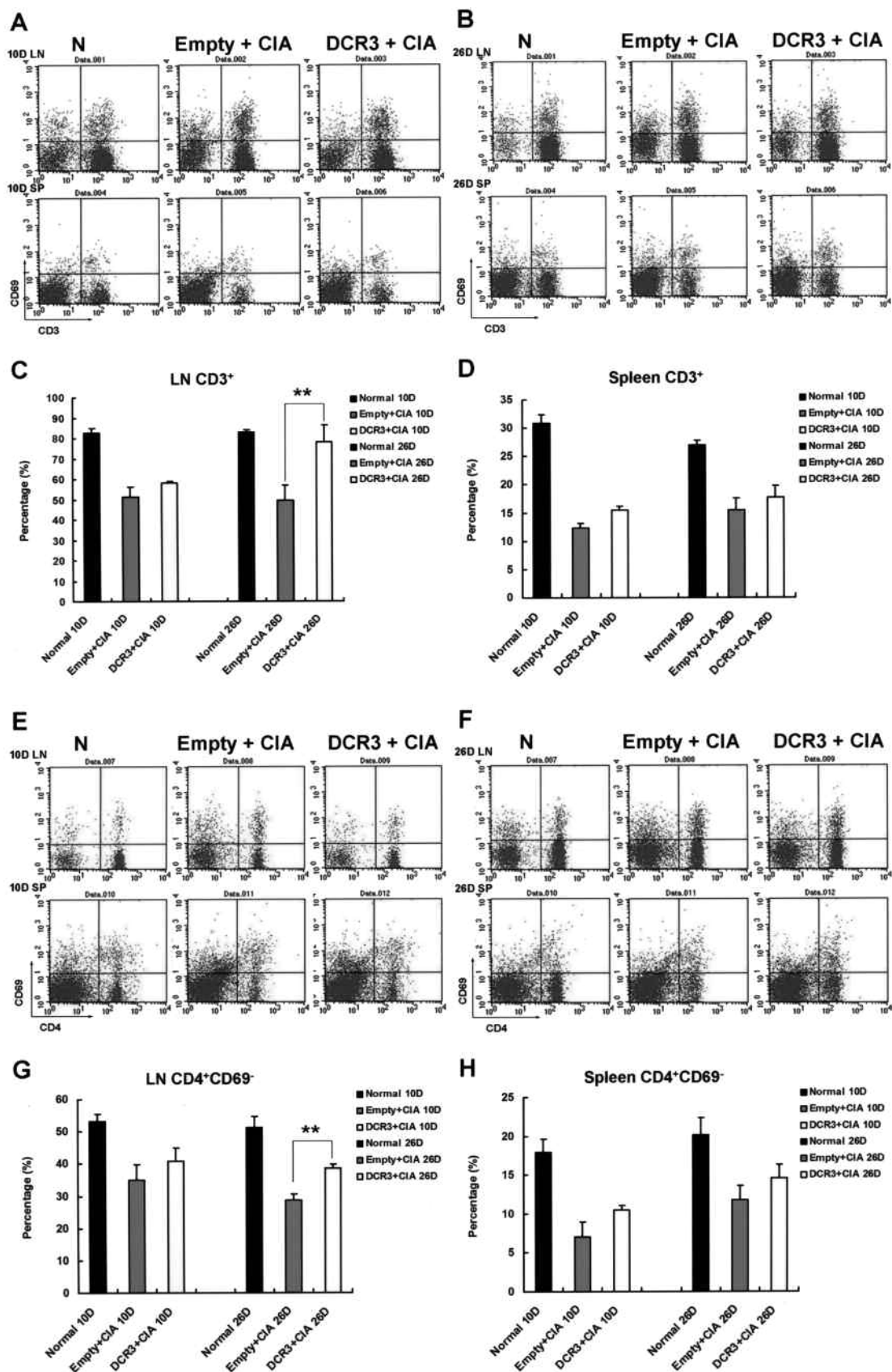


Figure 6.

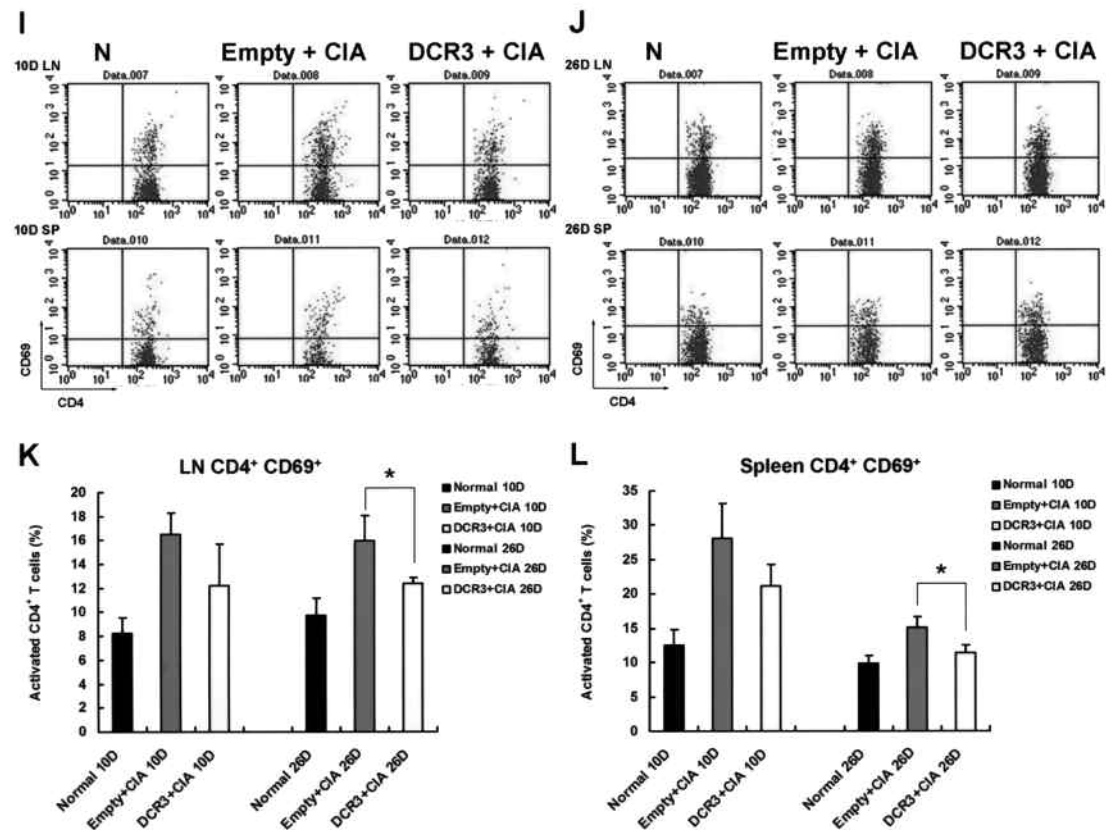


Figure 6. DCR3 maintained a normal B cell/T cell ratio and decreased the effective T cell population in CIA mice. Spleen and lymph node (LN) cells were stained with CD3, CD4, and CD69 cell-surface markers, then analyzed by flow cytometry. CIA mice treated with DCR3 plasmid maintained a normal CD3<sup>+</sup> and CD4<sup>+</sup>CD69<sup>−</sup> T cell population (C and G) in LN on Day 26 compared to mice given empty vector (\*\*p < 0.01). DCR3 decreased the activated CD4<sup>+</sup>CD69<sup>+</sup> T cell population in both lymph node and spleen on Day 26 compared to the empty vector group (\*p < 0.05; K and L). Results represent means ± SD of 3 independent experiments.

Table 1. CD19<sup>+</sup> B cell/CD3<sup>+</sup> T cell ratio of DCR3 treatment in mice with collagen-induced arthritis.

	Normal			Empty Vector + CIA			DCR3 + CIA		
	%CD3 <sup>+</sup>	%CD19 <sup>+</sup>	B/T Ratio	%CD3 <sup>+</sup>	%CD19 <sup>+</sup>	B/T Ratio	%CD3 <sup>+</sup>	%CD19 <sup>+</sup>	B/T Ratio
Inguinal									
Day									
10	82.46 ± 2.3	15.2 ± 3.78	0.18	51.31 ± 5	46 ± 6.57	0.9	58.04 ± 0.64	31.8 ± 9.1	0.54
26	83.02 ± 1.11	11.7 ± 3.49	0.14	49.25 ± 7.85	42.4 ± 3.02	0.86	78.03 ± 8.33	30.6 ± 8.93	0.39
Spleen									
Day									
10	30.49 ± 1.46	61.3 ± 5.95	2.01	12.52 ± 0.92	60.6 ± 4.65	4.84	15.05 ± 0.96	60.6 ± 4.34	4.03
26	27 ± 0.81	61.6 ± 2.83	2.28	15.51 ± 2.09	59.4 ± 2.35	3.83	17.72 ± 2	61.1 ± 2.26	3.45

DCR3: decoy receptor 3.

ent<sup>47</sup>. Our data showed that DCR3 effectively inhibited total CD19<sup>+</sup> B cells by significantly reducing CD19<sup>+</sup>CD69<sup>−</sup> B cell populations in peripheral lymph nodes. We found DCR3 maintained the CD3<sup>+</sup> T cell population after induction of CIA particularly by increasing CD4<sup>+</sup>CD69<sup>−</sup> T cells, which are regarded as memory or suppressive T cells in the lymphatic system. Our data suggested that DCR3 can maintain the B/T cell balance and increase immune tolerance.

Previous studies proved that Th1, Th2, and Th17 cells play distinct roles in CIA development<sup>4,48</sup>. We found that DCR3 can decrease Th1, Th2, and Th17 cell populations in CIA mice. This result is supported by animal studies in other autoimmune diseases<sup>17,18,20</sup>. IL-6 is also required for the development of CIA and Th17 differentiation<sup>49,50</sup>. Also, B cell differentiation was required for IL-6 production<sup>51</sup>. Our data showed that inhibition of proinflammatory IL-6 may

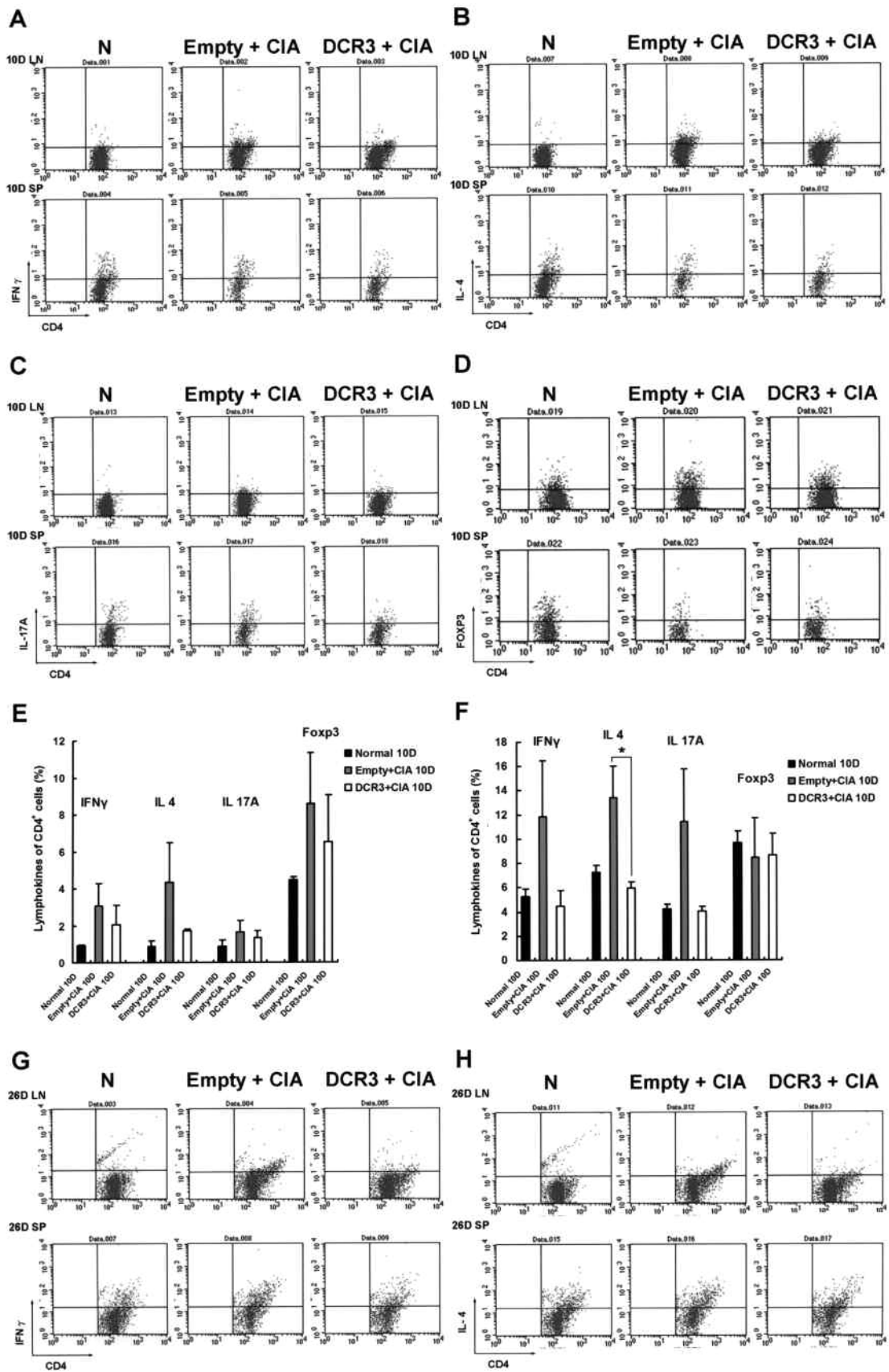
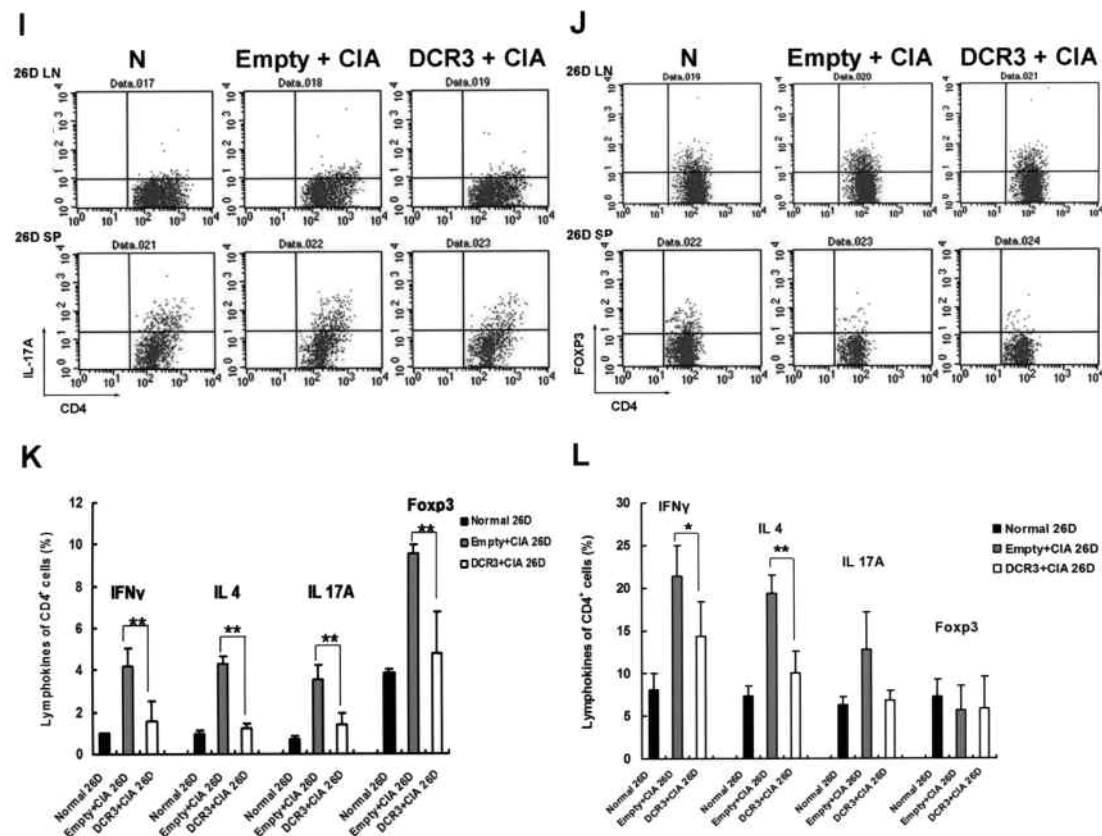


Figure 7.



**Figure 7.** DCR3 suppressed Th1, Th2, and Th17 cell populations in CIA mice. Spleen (SP) and lymph node (LN) cells were stained with IFN- $\gamma$ , IL-4, IL-17A, or Foxp3 combined with CD4 cell-surface marker to assay different CD4 cell populations. DCR3 plasmid suppressed IFN- $\gamma$ -, IL-4-, IL-17A-, and Foxp3-producing CD4+ cell populations in lymph nodes and IFN- $\gamma$ - and IL-4-producing CD4+ cells in spleens on Day 26 compared to mice given empty vector (\*\*p < 0.01; K and L). Except for IL-4-producing CD4+ cells (\*p < 0.05), there were no significant populations on Day 10 (E and F). Results represent means  $\pm$  SD of 3 independent experiments.

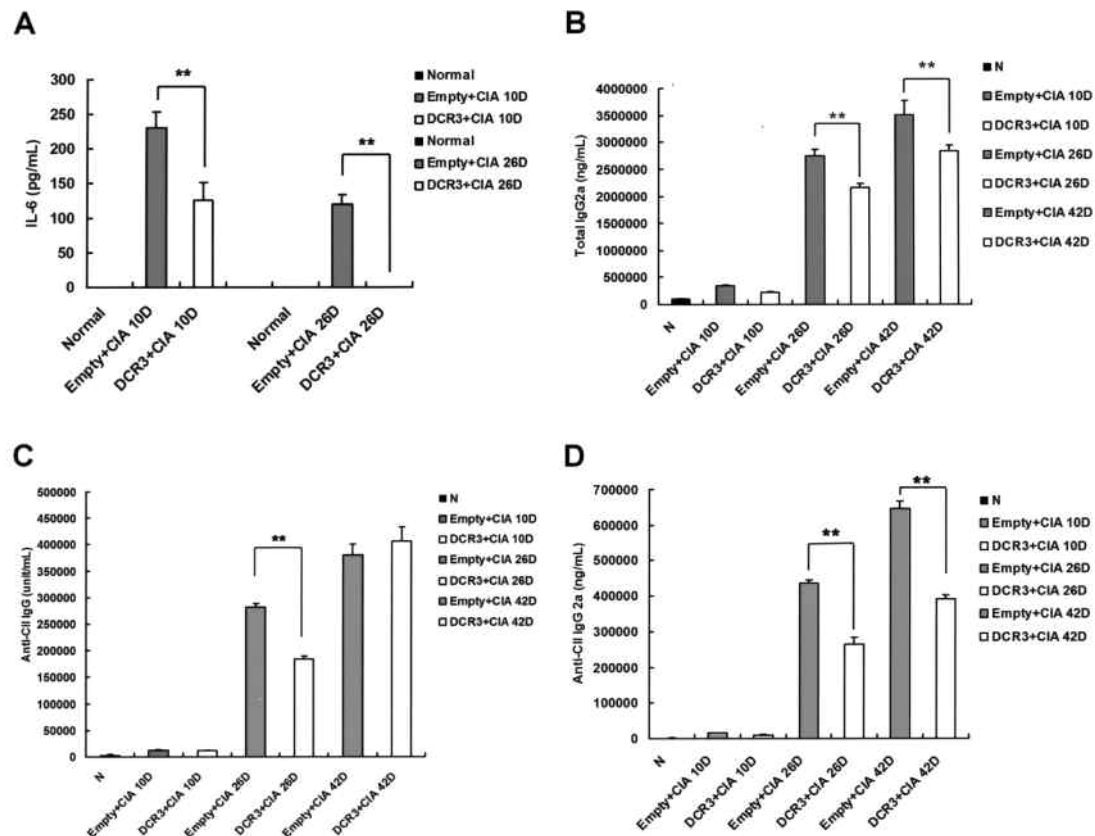
suppress B cell and Th17 cell differentiation and autoantibody production. DCR3-treated mice also had significantly inhibited total IgG2a and anti-CII IgG2a autoantibody production. In mice, it is known that antibodies of IgG2a isotype were the most efficient antibodies to mediate effector functions such as activating complement<sup>52,53,54</sup>. We suggest that DCR3 decreased IgG and IgG2a by inhibiting B cell proliferation. However, we found that DCR3 did not affect the Treg cell population on Day 10. These results suggest that DCR3 did not directly affect peripheral Treg cell expansion and regulatory function at the early stage. From our observations, we deciphered a possible mechanism of how DCR3 decreased the disease severity in CIA mice.

We first proved that direct hydrodynamic delivery and global expression of DCR3 significantly attenuated experimental CIA in mice. We suggest that DCR3 acts directly on modulation of the immune system by inhibiting peripheral inflammation in the lymphoid organs. We found that DCR3 directly inhibited B220+ B cell proliferation after Pam3CSK4 stimulation *in vitro* and decreased lymphoproliferation in peripheral lymphoid organs; decreased total lymphocyte cell numbers, CD19+CD69- cells, and activated T cells; and maintained the B/T cell balance *in vivo*.

Levels of the proinflammatory cytokine IL-6, total IgG2a, CII-specific IgG, and IgG2a in sera were decreased by DCR3 treatment. These were the possible mechanisms by which DCR3 modulated aberrant B/T cell interactions in this arthritis model<sup>55</sup>. We suggest DCR3 as a potential prophylactic and therapeutic agent in RA.

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**Figure 8.** DCR3 treatment decreased production of serum proinflammatory cytokine IL-6 and autoantibody. Serum from normal, empty-vector plus CIA, or DCR3 plus CIA mice was collected on Days 10 and 26 from 3 mice in each group. Only IL-6 was detected and was significantly reduced in the DCR3-treated mice (A). Results represent means  $\pm$  SD of 3 independent experiments (\*\* $p < 0.01$ ). Sera samples were harvested on Days 10, 26, and 42 from naive or CIA mice plus DCR3 or empty vector. CII-specific antibodies of IgG (C), IgG2a (D), and total IgG2a (B) were evaluated by ELISA. There was no significant difference in CII-specific IgG antibodies on Days 10 and 42 (B). There was a significant reduction of CII-specific IgG antibodies on Day 26 and both total IgG2a and CII-specific IgG2a antibodies on Days 26 and 42 in the DCR3-treated mice, as compared to the empty vector controls. Results represent means  $\pm$  SD of 3 independent experiments (\*\* $p < 0.01$ ).

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