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Attenuation of Murine Collagen-Induced Arthritis by Targeting CD6

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Objective. CD6 is an important regulator of T cell function that interacts with the ligands CD166 and CD318. To further clarify the significance of CD6 in rheumatoid arthritis (RA), we examined the effects of targeting CD6 in the mouse model of collagen-induced arthritis (CIA), using CD6-knockout (CD6-KO) mice and CD6-humanized mice that express human CD6 in lieu of mouse CD6 on their T cells.

Methods. We immunized wild-type (WT) and CD6 gene–KO mice with a collagen emulsion to induce CIA. For treatment studies using CD6-humanized mice, mice were immunized similarly and a mouse anti-human CD6 IgG (UMCD6) or control IgG was injected on days 7, 14, and 21. Joint tissues were evaluated for tissue damage, leukocyte infiltration, and local inflammatory cytokine production. Collagen-specific Th1, Th9, and Th17 responses and serum levels of collagen-specific IgG subclasses were also evaluated in WT and CD6-KO mice with CIA.

Results. The absence of CD6 reduced 1) collagen-specific Th9 and Th17, but not Th1 responses, 2) the levels of many proinflammatory joint cytokines, and 3) serum levels of collagen-reactive total IgG and IgG1, but not IgG2a and IgG3. Joint homogenate hemoglobin content was significantly reduced in CD6-KO mice with CIA compared to WT mice with CIA ($P < 0.05$) (reduced angiogenesis). Moreover, treating CD6-humanized mice with mouse anti-human CD6 monoclonal antibody was similarly effective in reducing joint inflammation in CIA.

Conclusion. Taken together, these data suggest that interaction of CD6 with its ligands is important for the perpetuation of CIA and other inflammatory arthritides that are T cell driven.

INTRODUCTION

CD6 is a 105–130-kd cell surface glycoprotein that is expressed by almost all mature T lymphocytes, many natural killer cells, and a small subset of B lymphocytes. Evidence of a role of CD6 in T cell activation includes the effects of anti-CD6 monoclonal antibodies (mAb) on the proliferation of freshly isolated T cells and recall responses of T cell clones (1,2), the influence of CD6 on T cell receptor–associated signaling events (2–6), altered activation and differentiation of T cells from CD6-knockout (CD6-KO) mice (7,8), and the effects of CD6 on thymic selection of developing T cells (7,9). Recent data also suggest that CD6 may be very important in T cell–driven autoimmune diseases both in humans and in animal models. Loci associated with CD6 affect susceptibility to multiple sclerosis (MS) (10) and Behçet's syndrome, and the severity of psoriasis (11). Notably, an anti-CD6 mAb is effective in the treatment of psoriasis (12).

Although CD6 was one of the earliest "CD antigens" to be described, it is only recently that attention has refocused on the potential for CD6 as a treatment target in immune-mediated diseases. CD166, also known as activated leukocyte cell adhesion molecule (ALCAM), was the first endogenous ligand of CD6 to be identified (13), and is expressed on the surface of numerous types of hematopoietic and nonhematopoietic cells, including activated lymphocytes and antigen-presenting cells (13). Recently, CD318, a cell surface molecule expressed on various mesenchymal and epithelial cell types (but not on mature lymphoid or myeloid cells), was also shown to be a ligand of CD6 (14). CD166 and CD318 bind to different domains of CD6 and appear to

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have distinct functions in autoimmune conditions. For instance, CD166-KO mice developed worse experimental autoimmune encephalomyelitis (EAE) (15), while CD318-KO mice, similar to CD6-KO mice, were protected against EAE (14). Interestingly, CD6, CD166, and CD318 are all strongly expressed in the joints of patients with rheumatoid arthritis (RA) (14,16–18).

In RA, both CD166 and CD318 are expressed by fibroblast-like synoviocytes (FLS), and these ligands participate in T cell binding to FLS (14,18,19). FLS also shed some of their surface CD318 into RA synovial fluid, and we have shown that soluble CD318 (sCD318) is chemotactic for T cells at concentrations equivalent to the gradient between RA synovial fluid and serum (14). These findings imply a role for the CD6–CD6 ligand axis in the pathogenesis of RA. To test the potential effectiveness of CD6-targeted therapeutics in the treatment of inflammatory arthritis, we used 2 mouse models of collagen-induced arthritis (CIA): genetically altered mice that lack CD6, and mice in which human CD6 has been substituted for mouse CD6 to allow in vivo testing of anti-human CD6 mAb.

MATERIALS AND METHODS

Animals. Wild-type (WT) mice, CD6-KO mice, and CD6-humanized mice (on a DBA/1J background) (8) were maintained under pathogen-free conditions in the animal facility of the Lerner Research Institute, Cleveland Clinic. All procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Cleveland Clinic, and all were done in accordance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and institutional guidelines.

Induction of murine CIA. We immunized age- and sex-matched WT and CD6-KO mice with a collagen emulsion to induce CIA as previously described (20). Mice were immunized at the base of the tail with bovine type II collagen (Chondrex) emulsified (2 mg/ml) at 1:1 ratio in a custom-made Freund's complete adjuvant containing 4 mg/ml *M tuberculosis* (BD Biosciences) in a total volume of 50 μl.

Clinical assessment of CIA. We used a scoring system identical to that used in previously published studies of CIA (21). Briefly, each joint was inspected and scored for the severity of swelling on a scale of 0–3, where 0 = normal appearance, 1 = mild, 2 = moderate, and 3 = severe, yielding a maximum score of 12 for each mouse. Swollen digits were noted, but paws were only considered arthritic when the entire paw was inflamed for 2 consecutive days. The day of onset of arthritis was recorded for each mouse. During the chronic phase of the arthritis, paws and digits were inspected for distortion and manipulated to identify loss of flexion (ankylosis). Severity scores were obtained by summing the inflammation, distortion, and ankylosis scores. Scores for each mouse were used to derive a mean arthritis severity score for the groups at each time point examined. Development of CIA in the CD6 gene–KO mice and WT control mice was monitored by visual scoring every other day from 21 days after immunization until day 28.

For treatment studies using CD6-humanized mice, we immunized mice as described above, and mice were randomly separated into 2 groups 7 days after immunization. One group was injected intraperitoneally (IP) with 0.4 mg of a mouse anti-human CD6 IgG (UMCD6) (22) on days 7, 14, and 21, and the other group received the same amount of purified mouse IgG (Jackson ImmunoResearch) as a control. Mice were then monitored, and clinical scores were recorded 3 times a week in a blinded manner. At the end of the experiments, mice were euthanized, and peripheral blood lymphocytes, splenocytes, and draining lymph nodes were analyzed for percentages of CD4+ and CD6+ T cells. Splenocytes were used to carry out antigen-specific Th1, Th9, and Th17 recall assays, and paws/joints were analyzed using histologic and histochemical assays.

Histologic analysis of mouse joint tissue. Some of the mouse ankles were embedded in OCT for sectioning. Briefly, serial synovial tissue sections (5–8 μm) were cut frozen and stored at –80°C for future hematoxylin and eosin and immunofluorescence staining. Frozen slides were allowed to thaw and dry at room temperature and subsequently fixed in acetone for 10 minutes at 4°C. For immunohistochemistry, tissue slides were blocked with 5% donkey serum and 20% fetal bovine serum (FBS) in phosphate buffered saline (PBS) at 37°C for 1 hour. After blocking, fluorescein isothiocyanate (FITC)–labeled anti-mouse CD3 antibody was added and incubated for 2 hours at room temperature. FITC-tagged mouse IgG was used as a control. DAPI was used at a 1:5,000 dilution in PBS for 5 minutes. Slides were then mounted in Fluoromount-G and visualized under a fluorescence microscope.

Mouse joint homogenate preparation and multiplex assays. Mice were euthanized and joints and sera were collected. Front paws and hind joints were used in the study. Joints were removed directly below the hairline and snap-frozen in liquid nitrogen. All joints were stored at –80°C until processed. Each joint was thawed on ice and quickly homogenized on ice in 1–2 ml PBS containing a tablet of proteinase inhibitors (10 ml PBS/tablet; Boehringer Mannheim). Homogenized tissues were centrifuged at 2,000*g* at 4°C for 10 minutes, and then filtered, aliquotted, and stored at –80°C until analyzed by enzyme-linked immunosorbent assay (ELISA). Protein concentrations were determined using a BCA Protein Assay kit (ThermoFisher), and the concentrations of 32 different mouse cytokines/chemokines in the homogenates were measured by a multiplex assay (Eve Technologies) and normalized against the total protein of each tissue lysate.

ELISA for murine CD318. We used a direct ELISA to measure the levels of murine CDCP1 (CD318) in mouse joint tissue homogenates (R&D Systems).

Serum collagen-specific IgG measurement. To measure type II collagen–specific IgG levels in the serum, blood samples were collected from the tail vein of each mouse and analyzed by ELISA. Briefly, sera were diluted (1:1,000) and then incubated in wells of a 96-well ELISA plate coated with 10 μg/ml purified type II collagen. After washing, horseradish peroxidase (HRP)–conjugated rabbit anti-mouse IgG (1:4,000; Southern Biotechnology) was added into each well and the levels of type II collagen–specific IgGs in the sera were determined by measuring optical density (OD) at 450 nm after color development using a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (ThermoFisher Scientific). To determine the levels of different type II collagen–specific IgG subclasses, similar ELISAs were performed with IgGs specific for mouse IgG1, IgG2a, IgG2b, and IgG3 (Sigma-Aldrich) as the detecting antibodies. All samples were run in duplicate.

Type II collagen–specific Th1, Th9, and Th17 cell recall assays. Spleens were collected and processed from WT and CD6-KO mice with CIA, or from CD6-humanized mice treated with UMCD6 or control mouse IgG after CIA induction. Splenocytes (0.4×10^6) were incubated with or without 10 μg/ml type II collagen or the same concentration of an irrelevant peptide (ovalbumin [OVA] 323–339) in RPMI 1640 complete medium with 10% (volume/volume) FBS in each well of a 96-well plate. After 72 hours, interferon-γ (IFNγ), interleukin-9 (IL-9), and IL-17A levels in the culture supernatants were measured by ELISA following standard protocols. Briefly, ELISA plates were coated with 4 μg/ml anti-mouse IFNγ IgG (BioLegend), anti-mouse IL-9 IgG (BD Biosciences), or anti-mouse IL-17A IgG (BioLegend) overnight and then blocked with 1 mg/ml bovine serum albumin. Next, culture supernatants were added to each well and incubated for 2 hours. After washing, 1 μg/ml biotinylated anti-mouse IFNγ, IL-9, or IL-17A IgG was added into each well and incubated for an additional 1 hour. The plates were then developed after incubation with streptavidin–HRP (1:4,000; BioLegend) and TMB substrate. The concentrations of IFNγ, IL-9, and IL-17A in the supernatants were calculated by measuring OD at 450 nm. All samples were run in duplicate.

Hemoglobin (Hgb) measurement on hind joint homogenates. For measurement of Hgb concentration, a reflection of vascularity of the tissue, mouse joints were homogenized as previously described. Hgb levels were measured by adding 25 μl of homogenate mixed with 25 μl of TMB reagent to 96-well plates. Samples were incubated for 5 minutes at room temperature, and absorbance was measured using a Microplate Manager ELISA reader (BioTek Instruments) at 450 nm. Hgb concentrations were determined by comparison with a standard curve. Values were normalized by dividing the Hgb concentration by the amount of total protein in the homogenate.

Statistical analysis. Experiments were repeated at least twice. To determine whether significant differences existed between groups, clinical scores were analyzed using analysis of variance, and other results were compared using Student's *t*-test. *P* values less than 0.05 were considered significant. Data are shown as the mean ± SEM except where indicated otherwise.

RESULTS

Reduced clinical CIA severity in CD6-KO mice. We immunized age- and sex-matched CD6-KO mice and WT control mice with collagen emulsion to induce CIA. Beginning on day 21 after immunization, CIA clinical scores were recorded by an investigator who was blinded with regard to group until day 28, when the WT mice developed severe arthritis and had to be euthanized according to the IACUC-approved study protocol. CIA severity was scored in each joint (front and hind) from WT and CD6-KO mice. A reduction in CIA severity in the CD6-KO mice was evident on day 23 and later (Figure 1), as differences were

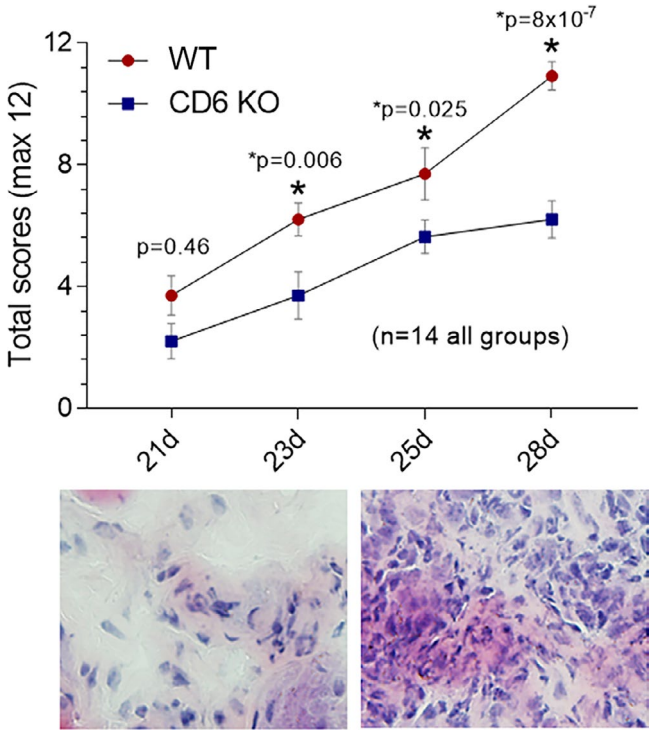

Figure 1. Reduction in the severity of collagen-induced arthritis (CIA) in CD6-knockout (CD6-KO) mice compared to wild-type (WT) mice. Top, Clinical score of CIA severity in WT and CD6-KO mice. CIA severity was measured in each paw and ankle (front and hind joints). A total reduction in CIA severity in the CD6-KO mice was evident on day 23 after induction of CIA and later. Values are the mean ± SEM (n = 14 mice per group). Bottom, Synovial inflammatory response on day 28 in a CD6-KO mouse (left) and a WT mouse (right). WT mice had a robust synovial inflammatory response that was not evident in the CD6-KO mice. Results are representative of 14 mice per group.

significant at all time points except early on day 21. WT mice had a robust inflammatory response to collagen on day 28 that was not evident in the CD6-KO mice. This finding indicates that CD6 deficiency partially protected mice against CIA.

Attenuated clinical CIA severity in CD6-humanized mice treated with UMCD6. To further assess CD6 as a potential target in the treatment of CIA, we induced arthritis in CD6-humanized DBA/1 mice and randomly divided these mice into 2 groups. The first group was injected IP with a mouse anti-human CD6 mAb (UMCD6), and a second group was injected similarly with purified mouse IgG, on days 7, 14, and 21. To monitor CIA development, clinical scores were recorded in a blinded manner from day 21 to day 35. UMCD6 effectively mitigated the severity of joint swelling after day 21 (Figure 2). Moreover, mice treated with IgG had demonstrable synovial lymphocyte staining (fluorescent green cells) on day 35 that was absent in the UMCD6-treated mice.

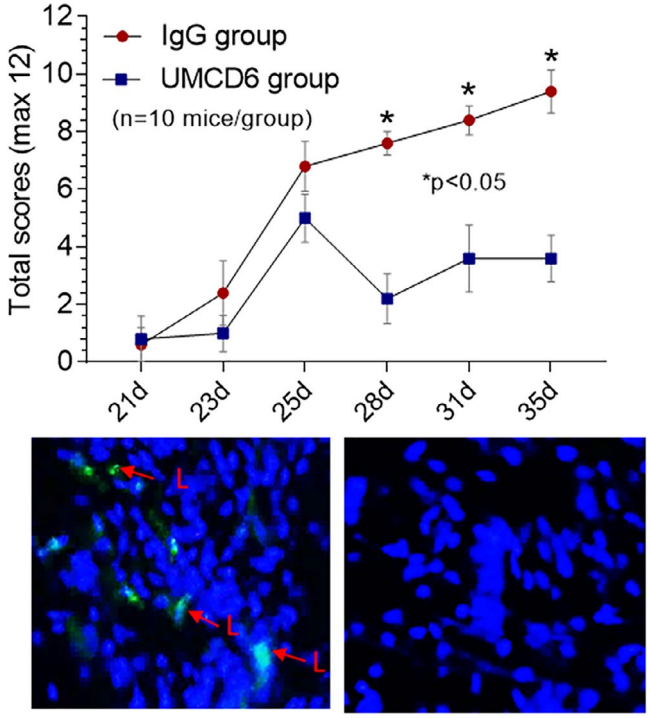

Figure 2. Reduction in the severity of collagen-induced arthritis (CIA) in CD6-humanized mice treated with a mouse anti-human CD6 IgG (UMCD6). Top, Clinical score of CIA severity in CD6-humanized mice treated with IgG and CD6-humanized mice treated with UMCD6. CIA severity was measured in each paw and ankle (front and hind joints) from CD6-humanized mice with CIA that had been treated with either human IgG or UMCD6 antibody on days 7, 14, and 21. A total reduction in CIA severity in the UMCD6-treated mice was evident on day 28 after induction of CIA and later. Values are the mean ± SEM (n = 14 mice per group). Bottom, Synovial CD3+ lymphocyte staining on day 35 in a mouse treated with IgG (left) and a mouse treated with UMCD6 (right). Mice treated with IgG had demonstrable synovial CD3+ lymphocyte (**L**) staining (fluorescent green cells; **arrows**) that was not evident in mice treated with UMCD6. Results are representative of 14 mice per group.

Reduced collagen-specific Th1/Th9/Th17 responses in CD6-humanized mice treated with UMCD6. Collagen-specific Th1//Th9/Th17 recall assays using splenocytes from the UMCD6-treated CD6-humanized mice 35 days after CIA induction were used to evaluate pathogenic T cell responses. These experiments showed that levels of IFNγ, IL-9, and IL-17A in the recall assays were significantly reduced in UMCD6-treated mice with CIA compared to IgG-treated control mice with CIA (Figure 3). This finding demonstrates that UMCD6 profoundly impaired the pathogenic T cell responses in the CD6-humanized mice after CIA induction, which could be a mechanism underlying the reduced clinical severity of CIA observed in the UMCD6-treated mice.

Reduced collagen-specific Th9 and Th17 responses in CD6-KO mice following CIA induction. We collected splenocytes from WT and CD6-KO mice with CIA on day 28 to assess collagen-specific Th1, Th9, and Th17 responses. The corresponding measurements of IFNγ, IL-9, and IL-17A levels in the culture supernatants from recall assays indicated that collagen-specific Th9 and Th17 responses in CD6-KO mice were significantly reduced compared to those in WT mice. Although CD6-KO mice tended to also exhibit reduced collagen-specific Th1 responses, this difference was not significant (Figure 3).

Reduced levels of collagen-specific total IgG and IgG1, but not IgG2a and IgG3, in CD6-KO mice. Previous studies have implicated collagen-specific IgGs in the pathogenesis of CIA. Therefore, we used ELISAs to measure the levels of collagen-specific total IgG and collagen-specific IgG subclasses in sera collected on day 28. Notably, levels of collagen-specific IgGs were significantly reduced in sera from CD6-KO mice compared to WT mice. In the IgG subclass analyses, we observed reduced levels of collagen-specific IgG1 in CD6-KO mice compared to WT mice (Figure 4), while the levels of IgG2a or IgG3 were not significantly affected (data not shown).

Altered cytokine production profiles in the joints of CD6-KO mice after CIA development. Using a cytokine multiplex assay, the levels of 32 inflammatory cytokines from homogenates of collected paw and ankle joint tissues were measured and normalized against the concentrations of total proteins. The paws and hind joints of CD6-KO mice exhibited attenuated tissue damage and leukocyte infiltration, consistent with the significantly reduced clinical scores in CD6-KO mice (Figure 5). In addition, the cytokine multiplex assays revealed that the levels of eotaxin, granulocyte colony-stimulating factor, IFNγ, IL-1β, IL-2, IL-5, IL-9, IL-12 (p70), IL-17, IFNγ-inducible 10-kd protein, keratinocyte

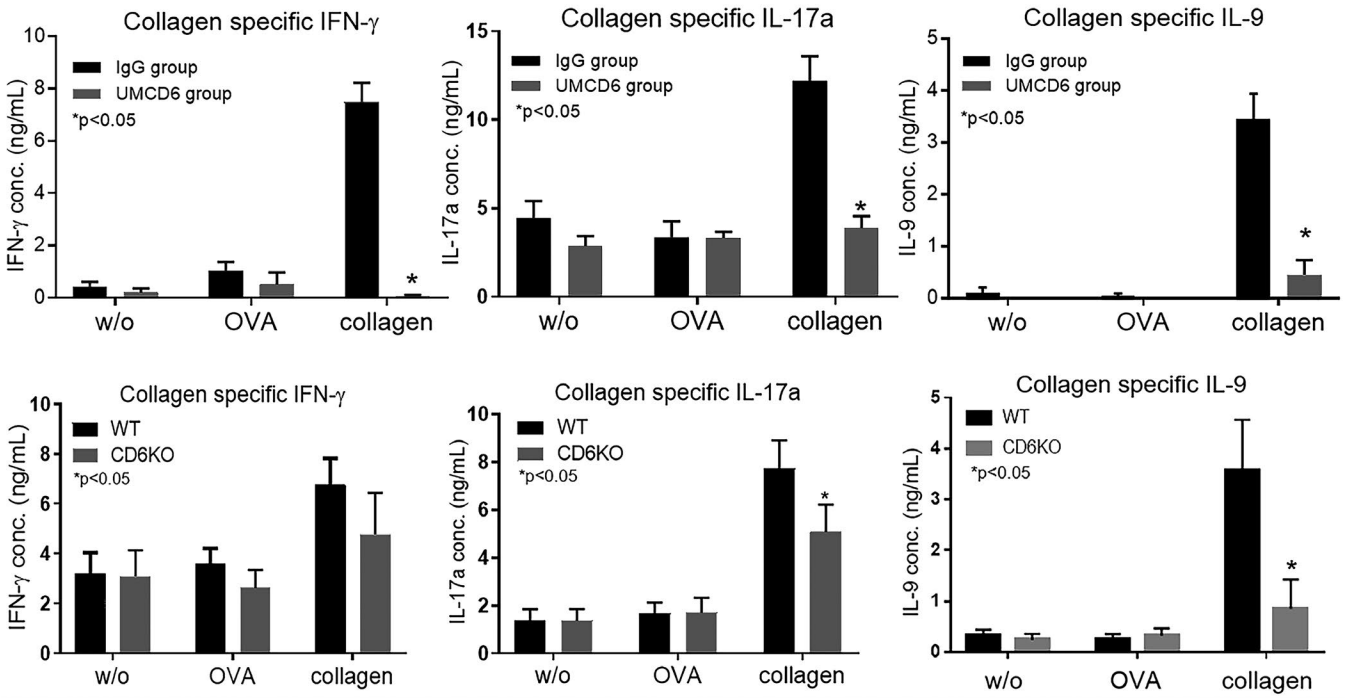

Figure 3. Reduced T cell cytokine production in response to collagen due to blocking or absence of CD6 in mice. Top, Reactivity to collagen in vitro in splenocytes from mice with collagen-induced arthritis (CIA) treated with IgG, but not mice with CIA treated with a mouse anti-human CD6 IgG (UMCD6), in Th1//Th17/Th9 recall assays. Secretion of interferon-γ (IFNγ), interleukin-17A (IL-17A), and IL-9 was significantly lower in UMCD6-treated mice. Controls included no antigen (w/o) and an irrelevant antigen (ovalbumin [OVA] 323–329 peptide). Bottom, Significantly reduced collagen-specific Th17 and Th9, but not Th1, responses in splenocytes from CD6-knockout (CD6-KO) mice compared to wild-type (WT) mice after the induction of CIA. For each cytokine tested, the enzyme-linked immunosorbent assays have sensitivities in the picogram range. Bars show the mean ± SEM (n = 14 mice per group).

chemoattractant, monocyte chemotactic protein 1, macrophage colony-stimulating factor, macrophage inflammatory protein 1α (MIP-1α), MIP-2, RANTES, tumor necrosis factor, and vascular endothelial growth factor (VEGF) were significantly lower in the CD6-KO group compared to the WT group. In contrast, the levels of granulocyte–macrophage colony-stimulating factor, IL-1α, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12 (p40), IL-15, leukemia inhibitory factor, lipopolysaccharide-induced CXC chemokine, monokine induced by IFNγ, and MIP-1β were comparable between the two groups of mice (Figure 5).

UMCD6 modulation of CD6 expression on CD4+ T cells without depletion of this T cell population in treated mice. On day 35 after CIA induction, we obtained blood and draining lymph nodes from CD6-humanized mice treated with UMCD6 or with mouse IgG and analyzed the

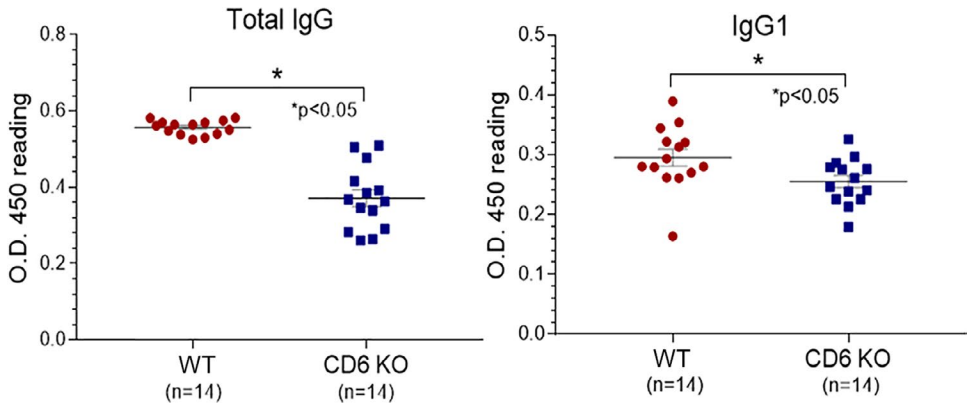

Figure 4. Reduced total serum levels of IgG and IgG1 against type II collagen in CD6-knockout (CD6-KO) mice with collagen-induced arthritis (CIA). Total serum levels of IgG anti–type II collagen (anti-CII) and IgG1 anti-CII in wild-type (WT) mice with CIA and CD6-KO mice with CIA were measured by enzyme-linked immunosorbent assay. Symbols represent individual mice; horizontal lines and bars show the mean ± SEM.

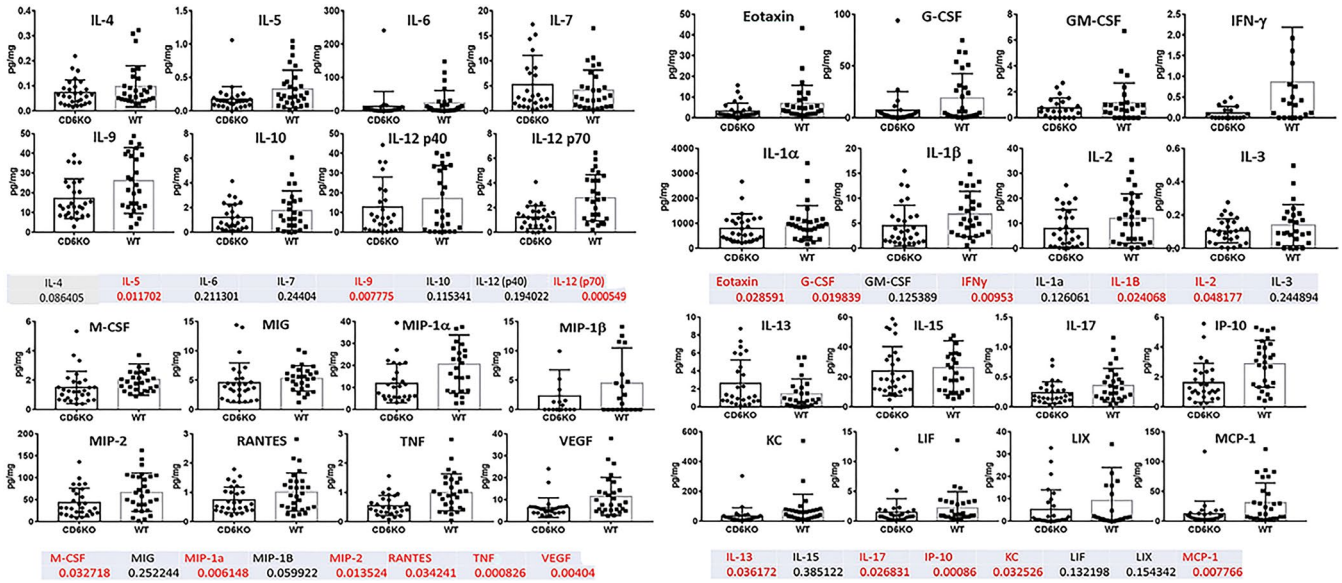

Figure 5. Cytokine panels from joint homogenates of CD6-knockout (CD6-KO) mice with collagen-induced arthritis (CIA) and wild-type (WT) mice with CIA. Joints from WT mice and CD6-KO mice were homogenized and protein concentrations were measured. A cytokine multiplex assay was used to measure the levels of 32 cytokines in homogenates of each of the paw and ankle joint tissues. Cytokine levels were normalized to protein concentration for each joint tissue homogenate (pg cytokine/mg protein). The levels of many proinflammatory cytokines were reduced in CD6-KO mice with CIA compared to WT mice with CIA. *P* values are shown for each cytokine. Significant *P* values are shown in red. Symbols represent individual mouse hind joints; bars show the mean ± SEM. IL-4 = interleukin-4; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte–macrophage colony-stimulating factor; IFNγ = interferon-γ; M-CSF = macrophage colony-stimulating factor; MIG = monokine induced by interferon-γ; MIP-1α = macrophage inflammatory protein 1α; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor; IP-10 = interferon-γ–inducible 10-kd protein; KC = keratinocyte chemoattractant; LIF = leukemia inhibitory factor; LIX = lipopolysaccharide-induced CXC chemokine; MCP-1 = monocyte chemotactic protein 1.

percentages of CD4+ T cells and their CD6 expression levels by flow cytometry. The UMCD6-treated and mock-treated samples contained similar percentages of total CD4+ T cells. However, the percentage of CD4+CD6+ T cells was significantly reduced in the UMCD6-treated mice, and this reduction was even more profound in the draining lymph nodes than in the peripheral blood (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41288/abstract). A further analysis of CD4+ T cells from UMCD6-treated mice revealed that most T cells did not express detectable levels of CD6. Thus, UMCD6 treatment modulated the expression of CD6 on CD4+ T cells rather than depleting these T cells in CD6-humanized mice with CIA.

Reduced Hgb levels in the joint tissue homogenates of CD6-KO mice with CIA. Measurement of Hgb in joint tissue homogenates revealed a significant reduction in Hgb content in CD6-KO mice with CIA compared to WT control mice with CIA, signifying attenuated angiogenic activity in the CD6-KO mice (Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41288/abstract). Reductions in Hgb content were not observed in CD6-humanized mice with CIA treated with UMCD6 compared to control mice (Supplementary Figure 2).

DISCUSSION

CD6 is an important regulator of T cell function that mediates the interactions of T cells with a wide variety of other cell types through engagement of its ligands CD166 and CD318. We previously examined CD318 expression in synovial tissue and sCD318 concentrations in the synovial fluid of RA patients and demonstrated a potential role for CD318 in the recruitment and retention of T cells in the RA joints. To further clarify the significance of CD6 in RA, in this study we examined the effects of targeting CD6 in the mouse model of CIA using CD6-KO mice and CD6-humanized mice that express human CD6 in lieu of mouse CD6 on their T cells. Using these models, we analyzed the joints of the ankles and paws for tissue damage, leukocyte infiltration, and local inflammatory cytokine production. We also compared collagen-specific Th1, Th9, and Th17 responses and serum levels of collagen-specific IgG subclasses and used cytokine multiplex assays to measure the levels of 32 cytokines in homogenates of joint tissues.

We found that CD6-KO mice had significantly lower clinical arthritis scores than WT mice, suggesting that CD6 acts to

attenuate the severity of joint inflammation in CIA. The absence of CD6 reduced 1) collagen-specific Th9 and Th17 responses, 2) the levels of many proinflammatory joint cytokines and chemokines, 3) serum levels of collagen-reactive total IgG and IgG1, but not IgG2a and IgG3, and 4) synovial angiogenesis in CD6-KO mice. Recall assays using collagen, or OVA 323–339 peptide as an irrelevant antigen, showed significant reductions in the production of IFNγ, IL-17, and IL-9 in mice with CIA when CD6 was targeted. These findings are consistent with our previously published data showing that T cells from CD6-KO mice had enhanced proliferation, but diminished Th1 and Th17 cell differentiation in response to anti-CD28 and anti-CD3 antibodies (8). Moreover, treating CD6-humanized mice with a mouse anti-human CD6 mAb was similarly effective in reducing joint inflammation in CIA. The reduction in inflammatory cytokine levels occurred without significant depletion of T cells and was even more profound in UMCD6-treated mice than in CD6-KO mice. Furthermore, we showed that there were more synovial CD3+ lymphocytes in the tissue sections prepared from IgG-treated mice with CIA than in those from UMCD6-treated mice with CIA, likely due to an overall reduction in T cell recruitment to inflamed synovium in response to therapy.

The previously identified ligand of CD6 is ALCAM (CD166), a member of the immunoglobulin gene superfamily that is widely expressed on cells of the immune system, including activated T and B lymphocytes, and on many types of mesenchymal, epithelial, and endothelial cells in various organs and tissues. Indications that a second ligand of CD6 might exist arose initially from functional studies in which T cell adhesion to keratinocytes and synovial fibroblasts was found to be dependent on CD6 but not on CD166 (19). Moreover, these interactions involved a CD6 ligand whose expression was increased by IFNγ, a cytokine that does not up-regulate CD166 (17,19). Subsequently, an mAb was generated that identified a 130-kd molecule that also bound to a CD6–Ig fusion protein (18); we recently identified this CD6 ligand as CD318 (14). CD318 is expressed by a variety of tissue cell types with which T cells interact in organ-targeted autoimmune diseases, but it is not expressed on mature cells of the immune system such as lymphocytes and myeloid cells (2,18). CD166 and CD318 bind to distinct domains of CD6, and it appears possible that CD6 can bind both ligands concurrently (14).

Recent work suggests that the appearance of sCD318 in synovial fluid is a potential biomarker for RA and closely related conditions, and also highlights the importance of T cell interactions with FLS and with molecules shed from the FLS surface (14). UMCD6 treatment of mice with CIA reduced lesional levels of sCD318, possibly through down-regulation of the expression of proinflammatory cytokines such as IFNγ, which in turn up-regulate CD318 expression. This effect on sCD318 levels was not observed in CD6-KO mice (Supplementary Figure 2). On the other hand, CD6-KO mice with CIA showed reduced synovial angiogenesis compared to WT mice, but this effect was not seen in UMCD6-treated CD6-humanized mice. These differences suggest that, notwithstanding similar effects on T effector cell subset differentiation, the genetic absence of CD6 versus the use of anti-CD6 antibody impacts CIA by mechanisms that differ in subtle ways at the target organ, the synovium. Elucidation of the mechanistic basis for these differences will require additional experimental approaches, possibly including tissue-specific manipulation of the expression of CD6 ligands.

While no animal model is an exact replica of RA, the murine model of CIA has proven to be very useful in studies of the pathogenesis of RA (20). CIA is typically induced in mice or rats by immunization with heterologous type II collagen, the principal type of collagen that is present in articular cartilage. In susceptible strains of rodents, an autoreactive response against type II collagen is induced, leading to synovitis and joint destruction over an interval of several weeks. As in human RA, multiple immune and inflammatory events and pathways are involved, including autoreactive Th1 and Th17 T cells, autoantibodies, proinflammatory cytokines, and activated, tissue-destructive synovial cells (20). Moreover, synovial enrichment of Th9 cells shows a positive correlation with disease activity in RA, and synovial IL-9 prolongs the survival of neutrophils, increases their matrix metalloproteinase 9 production, and facilitates Th17 cell differentiation (23).

Since Hgb levels have been correlated with joint inflammation and up-regulated angiogenesis in RA, and in animal models of RA (24–26), we examined Hgb levels as a measure of tissue vascularity in the joint tissue homogenates of the WT and CD6-KO mice with CIA. Our data show significant reductions in joint Hgb levels in the CD6-KO mice with CIA compared to WT mice with CIA, and this corresponds to reductions in several key angiogenic factors that we measured in our cytokine array, including VEGF.

In studies using CD6-KO mice, Orta-Mascaro and colleagues observed heightened autoimmunity and exacerbated disease in the CIA model (7). These experiments were performed in the C57BL/6 mouse strain in which the severity of CIA is modest. In view of the apparent contradiction between our previous findings in EAE and these results in CIA, we performed experiments to assess the role of CD6 in CIA, using the DBA mouse strain, in which CIA is considerably more severe than in C57BL/6 mice. We found that, as in EAE, the absence of CD6 is indeed protective in CIA in the DBA strain, and that treatment with anti-CD6 has therapeutic effects.

CD6-humanized mice provide a useful preclinical in vivo model for demonstrating the effectiveness of anti-CD6 antibodies in the treatment of autoimmune conditions. UMCD6 significantly reduced inflammatory responses in the target organs of CIA, EAE, and experimental autoimmune uveitis in these mice (2,27). Further, UMCD6 is an effective agent in suppressing differentiation of autoreactive lymphocytes into pathogenic, cytokine-producing Th1 and Th17 cells. Similar alteration in the differentiation of Th1 and Th17 cells occurs in CD6-KO and CD318-KO mice (2).

Although there is some evidence that CD6 can augment signal transduction through the T cell receptor complex (3,28), our recent studies in CD6-deficient mice indicate that CD6 attenuates the early stages of T cell activation and also enhances differentiation of Th1 and Th17 CD4 effector cells (8). These Th1 and Th17 CD4+ effector cells then drive autoimmune disease(s), including RA. Precisely how CD6 signals upon engagement of its natural ligands is unknown, but the cytoplasmic region of CD6 can be phosphorylated by multiple kinases (29). Many details of how CD6 participates in T cell signaling remain unclear, including the positioning of CD6 with respect to the "immunological synapse" formed through contact of the T cell with antigen-presenting cells. Thus, the role of CD6 in fomenting organ-targeted autoimmunity may specifically reflect CD6 interaction with CD318, which is expressed on key tissue cells in these diseases including neurons and endothelial cells in the brain, synovial fibroblasts in the joint, and keratinocytes in the skin (2,18). In the 1980s, an IgM anti-CD6 was tested in clinical trials on MS (30) and allograft rejection (31), but the small size and uncontrolled design of those studies precluded determination of therapeutic efficacy. More recently, an anti-CD6 mAb termed itolizumab has been extensively studied in humans with psoriasis and was demonstrated to be of significant clinical benefit, leading to approval for treatment of psoriasis in India (12).

UMCD6 is an mAb that has very high affinity for CD6 (16) without depleting T cells in CD6-humanized mice. UMCD6 instead appears to strip CD6 from the T cell surface, likely through internalization. If anti-CD6 mAb were similarly non–lymphocyte-depleting in humans, host defenses against infection might be relatively well preserved compared to other immunosuppressive strategies, and could lead to a safety advantage for CD6-directed mAb (2,14). The findings of the present study, taken together with what is already known about the role of CD6 in other mouse models of autoimmune diseases, strongly suggest that CD6 is required for the development and perpetuation of inflammatory arthritis and other autoimmune disorders that are T cell driven, and that attenuation of such disorders is achievable through targeting of CD6.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ruth had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Li, Ruth, Fox, Lin.

Acquisition of data. Li, Ruth, Rasmussen, Athukorala, Weber, Amin, Campbell.

Analysis and interpretation of data. Li, Ruth, Singer, Fox, Lin.

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Errata

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In the article by Gilis et al in the December 2019 issue of *Arthritis and Rheumatology* (Deletion of Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein 1 in Mouse T Cells Protects Against Development of Autoimmune Arthritis but Leads to Spontaneous Osteoporosis [pages 2005–2015]), a statement regarding shared senior authorship by two of the authors was inadvertently omitted from the title-page footnotes. The statement that should have appeared was "Drs. Beyaert and Elewaut contributed equally to this work."

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In the article by Kwon et al in the May 2020 issue of *Arthritis and Rheumatology* (Negative Regulation of Osteoclast Commitment by Intracellular Protein Phosphatase Magnesium-Dependent 1A [pages 750–760]), there was an error in the grant number shown for one of the grants that supported the study. The paragraph in the title-page footnotes should have read "Supported by grants from the Basic Science and Engineering Research Program (2018R1A2B2001867) and the National Research Foundation of Korea Medical Research Council (funded by the Korean government's Ministry of Science, ICT, and Future Planning) to Drs. Chang and Y.-G. Kim (2018R1A5A2020732 and NRF-2019-R1F1A1059736, respectively)."

We regret the errors.