MUSCULOSKELETAL PATHOLOGY

Transgenic Disruption of Glucocorticoid Signaling in Osteoblasts Attenuates Joint Inflammation in Collagen Antibody–Induced Arthritis

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The role of endogenous glucocorticoids (GCs) in rheumatoid arthritis remains unclear. Herein, we examined the role of osteoblastic GC signaling in collagen antibody–induced arthritis. Intracellular GC signaling was abrogated exclusively in mature osteoblasts via transgenic (tg) expression of 11ß-hydroxysteroid dehydrogenase type 2. Arthritis was induced in 8-week-old male tg mice and their wild-type (WT) littermates. Paw swelling was scored daily from induction to end point (day 14). Inflammation, cartilage degradation, and local bone erosion were assessed at the wrist, knee, and ankle joints. Systemic skeletal changes were determined by microcomputed tomography and histomorphometrical analysis of the tibiae. Both tg and WT mice developed acute arthritis in response to the administration of collagen antibodies. However, compared with WT mice, both clinical and histological indexes of joint inflammation were significantly mitigated in animals with disrupted osteoblastic GC signaling. In WT mice, arthritis was associated with increased bone resorption, decreased bone formation, and significant bone loss. In contrast, bone turnover and bone mass remained unchanged in tg arthritic mice. Disruption of GC signaling in osteoblasts significantly reduces joint inflammation and prevents structural bone and cartilage damage in collagen antibody–induced arthritis. These data corroborate the concept that osteoblasts modulate the inflammatory response in immune-mediated arthritis via a GC-dependent pathway. (Am J Pathol 2016, 186: 1293–1301; http://dx.doi.org/10.1016/j.ajpath.2015.12.025)

Glucocorticoids (GCs) are widely used to treat rheumatoid arthritis and other inflammatory diseases.1,2 Although the pharmacological actions and clinical effects of exogenous GCs are well established,3,4 the functions and mechanisms underlying the effects of endogenous GCs in inflammatory conditions, such as arthritis, are ill defined. We previously reported the effects of osteoblast/osteocyte-specific disruption of GC signaling on the development of inflammation and joint damage in the K/BxN serum-transfer model of arthritis.5 Thus, transgenic (tg) expression of 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) in osteoblasts and osteocytes significantly reduced the extent of K/BxN arthritis, suggesting that cells of the osteoblast lineage are able to regulate local inflammatory processes through a GC-dependent mechanism.5

The K/BxN serum-transfer model mimics the inflammatory phase of spontaneous polyarthritis on the basis of the formation of immune complexes of arthritogenic anti–glucose-6-phosphate isomerase (G6PI) autoantibodies.6 An alternative to this model is on the basis of the effector phase of the classic collagen-induced arthritis model.7 The effector phase is achieved by the passive transfer of immunoglobulins that recognize the well-defined major type II collagen epitopes, and

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therefore is referred to as the collagen antibody–induced arthritis model (CAIA).\(^8\) Evoked by administration of a combination of monoclonal antibodies to type II collagen and lipopolysaccharide,\(^9,10\) the CAIA model is characterized by rapid induction of arthritis, major histocompatibility complex haplotype independence, and high reproducibility.\(^10\) Moreover, it shares similar features with rheumatoid arthritis in humans, including articular inflammation and cellular infiltration in the effector stage, followed by cartilage and bone destruction.\(^11,12\)

Although CAIA and K/BxN serum-induced arthritis share some characteristics, there are significant differences between the two models.\(^11\) Most obviously, the anti-G6PI and anti–type II collagen antibodies have different antigen specificity, which leads to different localization in the joints.\(^11\) In the K/BxN model, inflammation is pronounced in the rear paw joints, with the ankle joints displaying the most consistent and prominent inflammation.\(^5\) In contrast, the CAIA model generates much more widespread arthritis, including both the fore and hind paws and the elbow and knee joints.\(^13\)–\(^16\) Accordingly, the CAIA model is a widely accepted model of human rheumatoid arthritis and a valuable tool to investigate the role of endogenous GC function in inflammatory arthritis.

Herein, we demonstrate that disruption of GC signaling in osteoblasts and osteocytes significantly attenuates joint inflammation and bone and cartilage destruction in the CAIA mouse model. These data corroborate the concept that in certain forms of immune-mediated arthritis, osteoblasts and osteocytes are critically involved in the generation and maintenance of the inflammatory response via a GC-dependent pathway.

**Materials and Methods**

**Tg Mouse Models**

*Col2.3-11βHSD2* tg mice were generated in a CD1 background, as described previously,\(^17\) and were a gift from Dr. Barbara Kream (University of Connecticut Health Center, Farmington, CT). Mice were maintained at the animal facilities of the ANZAC Research Institute (Sydney, NSW, Australia), in accordance with Institutional Animal Welfare Guidelines and according to an approved protocol.

**Initiation of CAIA**

Arthritis was induced in 8-week-old *Col2.3-11βHSD2* tg mice and their wild-type (WT) littermates by i.p. injection of 20 mg/mL ArthitoMab Antibody Cocktail (MD Bioproducts, Zürich, Switzerland) on day 0. Different doses of monoclonal antibodies have been used to induce arthritis in different strains of mice.\(^18,19\) To validate and optimize the CAIA model in the CD1 mouse strain, arthritis was induced using two different doses of antibodies: 3 mg and 8 mg. All of the CAIA mice were boosted with an i.p. injection of lipopolysaccharide on day 3 (1.0 mg/mL, 100 μL per mouse; MD Bioproducts). WT and tg mice injected with phosphate-buffered saline (PBS) served as the control group (CTR). There were no symptoms of inflammation observed during the duration of the study in WT or tg CTR mice injected with PBS. No significant body weight change was observed in WT or tg CAIA mice compared with their control littermates.

**Clinical Assessment of Arthritis**

From the day of arthritis induction, body weight and clinical parameters of arthritis were blindly assessed daily by two independent observers (J.T. and E.W.). As previously described,\(^20\) a clinical scoring system for each limb (fore and hind limbs) was used to evaluate the intensity of arthritis. Score points were assigned as follows: 0 indicates normal; 1 indicates mild to moderate swelling and erythematous ankle and/or one swollen digit; 2 indicates moderate swelling and erythematous ankle or swelling in two digits; and 3 indicates marked swelling along all aspects of the paw or all five digits swollen. The sum of the scores for each limb was determined, with the maximum possible score being 12 points.

Fourteen days after arthritis induction, the mice were harvested and tissues were subjected to microcomputed tomography (micro-CT), histologic, and histomorphologic analysis, as described below.

**Tissue Collection and Specimen Preparation**

After harvesting, the front paw, tibia, and hind paw of each mouse were dissected and fixed in 4% paraformaldehyde/ PBS. After micro-CT analysis, the tibiae and paws were decalcified with 10% EDTA and embedded in paraffin. Serial sections (4 μm thick) were stained with hematoxylin and eosin for general histological evaluation and with toluidine blue for cartilage assessment. To identify osteoclasts, sections were stained for tartrate-resistant acid phosphatase using naphthol-AS-BI phosphate (Sigma, St. Louis, MO) as a substrate and fast red violet LB salt (Sigma) as a detection agent for the reaction product.\(^5\)

**Histopathological Scoring**

Histopathological scoring was performed for inflammatory activity, cartilage degradation, and bone erosion by two independent observers (J.T. and Y.Z.), both being blinded to the genotypes and experimental groups. Using a previously established modified semiquantitative scoring system,\(^5,21\) inflammatory activity was scored on a point scale from 0 to 3, as follows: 0 indicates normal; 1 indicates mild inflammatory infiltration with no soft tissue edema or synovial lining cell hyperplasia; 2 indicates moderate infiltration with surrounding soft tissue edema and some synovial lining cell hyperplasia; and 3 indicates severe infiltration with marked soft tissue edema and synovial lining cell hyperplasia. Cartilage damage was scored as follows: 0 indicates normal; 1 indicates mild loss of toluidine blue staining; 2 indicates moderate loss of toluidine blue staining and cartilage loss; and 3 indicates
marked loss of toluidine blue staining with marked multifocal cartilage loss. Finally, bone erosion was scored as follows: 0 indicates none; 1 indicates mild (some areas of resorption not readily apparent on low magnification with visible osteoclasts); 2 indicates moderate (obvious bone resorption with a few osteoclasts visible); and 3 indicates marked (large erosion areas extending into the bone cortex with numerous osteoclasts visible in all areas).

**Micro-CT**

Micro-CT analyses of tibiae were performed using a SkyScan 1172 scanner (SkyScan, Kontich, Belgium), as previously described.5,22 The tibiae were positioned firmly into place with low-density foam in a sample holder topped with PBS. Scanning was performed at 60 keV, 167 μA, 1475 milliseconds, without using a filter. In total, approximately 1300 projections were collected at a resolution of 7.59 μm per pixel. Reconstruction of sections was performed using GPU Accelerated NRcon software version 1.6.6.0 (SkyScan). After reconstruction, samples were analyzed using CTAn software version 1.8 (SkyScan). A 1-mm-long region of interest starting 0.5 mm proximal to the metaphyseal growth plate for each sample was selected using automated algorithms from the original reconstructed images. Morphological measurements of the region of interest were performed, and the following three-dimensional parameters were obtained: bone volume fraction, trabecular number, trabecular thickness, and trabecular separation.

**Histomorphometric Analysis**

The proximal tibial metaphyses were analyzed using OsteoMeasurexp version 1.01 (OsteoMetrics Inc., Decatur, GA). Bone sections stained for tartrate-resistant acid phosphatase were used to assess bone resorption (osteoclast activity), whereas hematoxylin and eosin—stained sections were analyzed for bone formation activity. The region of interest for the quantitative analysis was a 0.96-mm² area located 0.3 mm lower than the growth plate of the tibia. Osteoblast and osteoclast surfaces were measured relative to bone surface at ×200 magnification.5,22

**Statistical Analysis**

Unless stated otherwise, data are presented as means ± SEM. Comparisons between two groups of normally distributed variables were performed using a t-test. For comparisons between more than two groups, a one-way analysis of variance with subsequent Bonferroni-adjusted post hoc analysis was performed. Significance was accepted where P < 0.05.

**Results**

**Time Course and Degree of Clinical Arthritis**

The CAIA model has not previously been comprehensively characterized in the CD1 mouse strain.23 To validate the model and optimize the antibody dose required, we performed studies using both a low (3 mg) and high (8 mg) antibody concentration.

After induction of arthritis using the low-dose antibody cocktail, both WT and tg mice developed arthritis at a similar rate (Figure 1A). However, in tg CAIA mice, the severity of arthritis plateaued from day 6 onward. In contrast, in WT CAIA mice, the clinical score continued to increase, peaked at day 10, and remained significantly higher than in tg CAIA mice from

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**Figure 1** Development of collagen antibody—induced arthritis (CAIA). CAIA was induced in wild-type (WT) and transgenic (tg) mice by an i.p. injection of 3 mg (A) or 8 mg (C) of the antibody cocktail on day 0, followed by an i.p. injection of lipopolysaccharide on day 3. Control (CTR) mice were injected with phosphate-buffered saline (PBS). Mice were monitored for arthritis development daily for 14 days by scoring the redness and swelling of all four paws. B: Representative images of the front paw of WT and tg CAIA mice 6 days after arthritis induction with 3 mg antibody cocktail and PBS injection (CTR). P values have been generated using t-test comparing WT CAIA with tg CAIA at each time point. Quantitative results are presented as means ± SEM, n = 9 to 10 per group (A, WT and tg CAIA); n = 8 per group (A and C, WT and tg CTR); n = 8 to 9 per group (C, WT and tg CAIA). *P < 0.05, **P < 0.01.
day 9 to 11. **Figure 1B** illustrates the difference in front paw inflammation between CTR, WT CAIA, and tg CAIA mice.

When mice were exposed to the high antibody dose (8 mg), arthritis developed and resolved more rapidly compared with mice induced with the lower dose (Figure 1C). In WT CAIA mice, inflammation did not develop until immediately after the lipopolysaccharide booster injection on day 3, with the clinical scores dramatically increasing and peaking at day 5, before resolving from day 7 onward. Although the tg CAIA mice followed a similar time course of inflammation, the magnitude of the inflammatory changes seen in these mice with disrupted osteoblastic GC signaling was significantly less than that of WT mice (clinical score tg CAIA versus WT CAIA, 4.56 ± 0.85 versus 7.5 ± 0.91; *P* = 0.03). This difference remained until the end of the study at day 14 (clinical score tg CAIA versus WT CAIA, 2.18 ± 0.4 versus 4.86 ± 0.84; *P* = 0.01) (Figure 1C).

**Histopathological Assessment of Arthritis**

Because the higher antibody dose resulted in larger differences in clinical scores when comparing WT with tg CAIA mice, we proceeded to analyze the joints from these animals by histology. Consistent with the clinical scores, inflammatory changes were more pronounced in joints of the front than the rear paws. CTR mice showed no evidence of local inflammation, bone erosion, or cartilage degradation (Figure 2, A—C). In WT CAIA mice, pronounced synovial hyperplasia, cartilage degradation, and bone erosion were present (Figure 2, D—F), whereas these features were attenuated in tg CAIA mice (Figure 2, G—I). Consistent with this, using a semi-quantitative histology scoring method, both synovitis and bone erosion of the front paws were significantly greater in WT CAIA than in tg CAIA mice, although articular cartilage degradation did not differ between the two groups (Figure 2J).

On the other hand, the rear paws of both WT and tg CAIA mice exhibited mild inflammatory infiltration to a similar extent (Figure 3, D, G, and J), and there was no significant difference in bone erosion between the two groups (Figure 3, F, I, and J). However, the degradation seen in the articular cartilage surface of the ankle joints of WT CAIA mice was clearly attenuated in tg CAIA mice (Figure 3, E, H, and J). CTR mice showed no evidence of inflammatory activity, cartilage degradation, or bone erosion (Figure 3, A—C).

**Figure 2** Histological analysis of the front paw joints 14 days after arthritis induction. Representative images of the front paw joints of control (CTR; A–C), wild-type (WT) collagen antibody—induced arthritis (CAIA; D–F), and transgenic (tg) CAIA mice (G–I). Hematoxylin and eosin (H&E) staining to show inflammatory activity, toluidine blue for cartilage degradation, and tartrate-resistant acid phosphatase (TRAP) staining for bone erosion. Arrows indicate sites of synovitis, sites of cartilage degradation, or presence of osteoclasts (red). J: Quantification of the degree of inflammation, cartilage degradation, and bone erosion in the front paws by histological scoring (described in Materials and Methods). *P* values have been generated using *t*-test comparing WT CAIA with tg CAIA mice. Quantitative results are presented as means ± SEM. *P* < 0.05, **P* < 0.01. Scale bar = 200 μm (A–I).
Analysis of the knee joints revealed significantly more pronounced synovitis in WT CAIA mice compared with tg CAIA mice; however, the extent of cartilage degradation did not differ between these two groups (Figure 4). In addition, bone erosion was absent in all WT and tg groups (Figure 4), with the exception of one mouse in the WT CAIA group, which presented with the most severe inflammation.

Skeletal Micro-CT and Histomorphometry

Despite the absence of local bone erosions within the knee joint, micro-CT analysis confirmed systemic bone loss at the proximal tibiae of WT CAIA mice (Figure 5); tibial trabecular bone volume fraction was significantly reduced in WT CAIA mice compared with WT CTR mice. This bone loss resulted from a pronounced decrease in trabecular number, whereas trabecular separation and trabecular thickness remained similar to WT CTR mice. In contrast, there was no difference in bone volume fraction or any other parameter between tg CAIA and CTR mice (Figure 5). These results suggest that tg CAIA mice are protected from inflammation-induced systemic bone loss. However, there was no significant difference observed in any of the four indexes between WT CAIA and tg CAIA mice (Figure 5).

To investigate whether bone loss was because of increased bone resorption or reduced bone formation, the proximal tibiae were analyzed by histomorphometry. WT CAIA mice displayed significantly increased osteoclast surface and number, as well as decreased osteoblast surface compared with WT CTR mice (Figure 6), suggesting that inflammation-induced bone loss in WT CAIA mice resulted from increased bone resorption and decreased bone formation. In contrast, these differences in bone parameters were absent in the tg groups, with bone resorption and formation remaining similar between the tg CAIA and CTR mice (Figure 6). Although no difference was seen in either bone resorption or formation between WT CTR and tg CTR mice, tg CAIA mice were protected from the increase in bone resorption and decrease in bone formation observed in WT CAIA mice (Figure 6).

Discussion

Using the CAIA model, we herein demonstrate that abrogation of endogenous GC signaling in osteoblasts and
osteocytes results in a significant and lasting mitigation of immune-mediated arthritis in mice. Compared with WT CAIA mice, both clinical and histological indexes of inflammatory activity, cartilage degradation, and bone erosion were reduced in tg CAIA mice. In addition, bone turnover and bone volume remained unchanged in tg CAIA mice, whereas WT CAIA mice exhibited stimulated bone resorption, suppressed osteoblast activity, and reduced bone volume, all of which are known sequelae of active inflammation.

The proinflammatory, osteoblast-mediated function of endogenous GCs in arthritis is consistent with the results from our previous studies using K/BxN serum-induced arthritis. Both the previous and present studies demonstrate that functional signaling of endogenous GCs in osteoblasts is necessary to maintain the inflammatory response once the acute immune-mediated arthritis has been established. We have previously demonstrated that murine antigen-induced arthritis is not affected by disruption of GC signaling in osteoblasts/osteocytes. This discrepancy is likely because of the differences in the immunological and inflammatory profile of the three arthritis models. In antibody-mediated models of arthritis (eg, CAIA and K/BxN), arthritis can be induced even if the recipients are devoid of lymphocytes. Immune complexes of arthritogenic antibodies act through neutrophils, macrophages, and mast cells. In contrast, in antigen-induced arthritis, the antibody-mediated inflammatory response is of minor importance, whereas antigen-specific T cells, generated within the adaptive immune response initiated through immunization, play a major role. These findings suggest that the osteoblasts may modulate the innate, rather than adaptive, immune-mediated inflammatory response via a GC-dependent pathway.

Despite the consistency in inflammation outcomes in both tg CAIA and K/BxN mice compared with their WT littermates, the distinct mechanism underlying the difference in inflammation of the CAIA model resulted in its own inflammation phenotype compared with the previously described K/BxN serum-induced arthritis model.

The different antigen specificity of the anti-type II collagen and anti-G6PI antibodies used in the CAIA and K/BxN models, respectively, results in different localization of inflammation in the joints. In the K/BxN mice, most of the inflammation was focused in the rear paw joints, with the ankle joints displaying the most consistent and
prominent inflammation. In contrast, the present study demonstrated that all of the front paw joints, knee joints, and rear paw joints of the CAIA mice become inflamed, with the front paws exhibiting the most obvious inflammation, particularly in the digits. Bone erosion was also observed in smaller joints, including the wrist and ankle joints, but not in the larger joints, such as the knee joints. Because of the preference for inflammation to occur in the interphalangeal and metacarpophalangeal joints rather than the ankle or wrist joints, quantifying inflammation on purely clinical grounds was difficult in this model. To overcome this problem, additional techniques were used to quantify the swelling in CAIA mice, including the measurement of ankle and wrist sizes and digital thickness of swollen paws, as well as an assessment of functional loss of front paw strength caused by swelling via a hanging test. However, none of these methods fully reflected the difference in the degree of inflammation shown by the clinical scores. In addition, some of the measurements resulted in only a modest induction of inflammation. As a result, clinical scoring was determined to be the most suitable method for observing the progression of inflammation in this study.

Despite more joints being affected by inflammation, the overall histological degree of inflammation was much less pronounced in the CAIA mice than in the K/BxN mice. Even in the wrist joints of WT CAIA mice, which displayed the most pronounced inflammation, the severity of inflammation was determined by changes in synovial activity, because inflammatory cells were not observed. This could be because of the difference in arthritogenicity, related to the epitope specificity of the antibodies, with anti-type II collagen antibodies penetrating the cartilage to reach chondrocytes, whereas the anti-G6PI antibodies bind to synovial tissue in addition to the cartilage surface. The degree of arthritis induced by the anti-type II collagen antibody cocktail was also determined by the antibody dose used. Despite comparative peak values in the clinical scores in low- and high-dose CAIA, the latter

Figure 5 Microcomputed tomography analysis of the trabecular bone structure in the proximal tibiae. Bone volume (BV/TV; A), trabecular number (Tb.N; B), trabecular separation (Tb.Sp; C), and trabecular thickness (Tb.Th; D) of a region in the proximal tibia, distant to the site of active inflammation in wild-type (WT) and transgenic (tg) collagen antibody–induced arthritis (CAIA) and control (CTR) mice. P values have been generated using a one-way analysis of variance for multiple groups testing, followed by Bonferroni post hoc comparison. Quantitative results are presented as means ± SEM. *P < 0.05, **P < 0.01.

Figure 6 Histomorphometric quantification of bone turnover 14 days after injection. Representative histological images of tartrate-resistant acid phosphatase staining for osteoclasts (red) at a region distant to the site of inflammation in the proximal tibia of control (CTR; A), wild-type (WT) collagen antibody–induced arthritis (CAIA; B), and transgenic (tg) CAIA mice (C). Quantification of osteoclast surface/bone surface (OcS/BS; D) and osteoclast number/bone surface (Noc/BS; E) as markers of bone resorption and osteoblast surface/bone surface (ObS/BS; F) as a marker of bone formation in WT and tg CAIA and CTR mice. P values have been generated using a one-way analysis of variance for multiple groups testing, followed by Bonferroni post hoc comparison. Quantitative results are presented as means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bar = 500 μm (A–C).
showed abrupt changes of arthritis development and resolution in both WT and tg CAIA mice. The significantly attenuated inflammation in tg CAIA mice compared with WT CAIA mice, induced by the high antibody dose, indicates that sufficient antibody dosing is essential for the effect of endogenous GCs in the regulation of inflammation to become evident. Taken together, disruption of endogenous GC signaling in osteoblasts and osteocytes attenuates inflammation in both WT and tg CAIA mice. The significance of endogenous glucocorticoids in rheumatic diseases. Arthritis Rheum 2011, 63:1–9


