GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Effects of an Interleukin-15 Antagonist on Systemic and Skeletal Alterations in Mice with DSS-Induced Colitis

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Accepted for publication
February 12, 2013.

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Inflammatory bowel diseases are commonly complicated by weight and bone loss. We hypothesized that IL-15, a pro-inflammatory cytokine expressed in colitis and an osteoclastogenic factor, could play a central role in systemic and skeletal complications of inflammatory bowel diseases. We evaluated the effects of an IL-15 antagonist, CRB-15, in mice with chronic colitis induced by oral 2% dextran sulfate sodium for 1 week, followed by another 1% for 2 weeks. During the last 2 weeks, mice were treated daily with CRB-15 or an IgG2a control antibody. Intestinal inflammation, disease severity, and bone parameters were evaluated at days 14 and 21. CRB-15 improved survival, early weight loss, and colitis clinical score, although colon damage and inflammation were prevented in only half the survivors. CRB-15 also delayed loss of femur bone mineral density and trabecular microarchitecture. Bone loss was characterized by decreased bone formation, but increased bone marrow osteoclast progenitors and osteoclast numbers on bone surfaces. CRB-15 prevented the suppression of osteoblastic markers of bone formation, and reduced osteoclast progenitors at day 14, but not later. However, by day 21, CRB-15 decreased tumor necrosis factor α and increased IL-10 expression in bone, paralleling a reduction of osteoclasts. These results delineate the role of IL-15 on the systemic and skeletal manifestations of chronic colitis and provide a proof-of-concept for future therapeutic developments. (*Am J Pathol* 2013, 182: 2155–2167; <http://dx.doi.org/10.1016/j.ajpath.2013.02.033>)

Although the etiology of inflammatory bowel diseases (IBDs), notably Crohn's disease and ulcerative colitis remain unclear, accumulating data suggest that altered innate and adaptive immune responses to enteric antigens play a pivotal role in intestinal inflammation.¹ Patients with IBD also have low areal bone mineral density (BMD), deficits in trabecular volumetric bone mineral density, and an increased incidence of osteoporotic fractures, particularly at the spine and in women with Crohn's disease.^{2,3} The pathogenesis of osteoporosis in IBD involves both an increase in bone resorption and an inhibition of bone formation, resulting in a negative bone mineral balance.⁴ In addition to inflammation, these alterations in bone turnover are thought to be due to malnutrition, vitamin D and calcium deficiency, and corticosteroid treatment.⁵

Several mouse colitis models have been reported.^{6–8} Among them, dextran sulfate sodium (DSS) administration for 7 days is capable of inducing colitis even in severe

combined immunodeficient mice, arguing for an early T-cell-independent mechanism of intestinal inflammation in this model.⁹ In contrast, T cells may play a more important role in worsening or perpetuating the disease when chronic colitis is induced by a prolonged period of DSS administration.¹⁰ In this case, bone mineral density and volume were reported to decrease in parallel to increased levels of tumor necrosis factor α (TNF α), and to recover on cessation of DSS administration and with colitis improvement.¹¹

Several pro-inflammatory cytokines produced by activated T cells in IBD actually contribute to bone loss. They include TNF α ,^{12–14} IL-1 β ,¹⁵ IL-6,^{16,17} and interferon (IFN)- γ ,¹⁸

Supported by a Crohn's and Colitis Foundation of America grant (S.L.F. and S.F.-L.), a Hans Wilsdorf Foundation grant (S.F.-L.), and Novartis Consumer Health Foundation, Novartis Foundation, and European Crohn's and Colitis Organisation fellowships (D.D.P. and B.B.-L.R.).

which have been shown to enhance osteoclast differentiation and bone resorption and/or to inhibit osteoblast functions and bone formation. In addition, receptor activator of nuclear factor-kappa B ligand (RANKL) and its antagonist, osteoprotegerin (OPG), play a key role in the regulation of osteoclastogenesis and bone loss in inflammatory disorders, including IBD.^{19–21}

IL-15, a major pro-inflammatory cytokine and T-cell growth factor²² is produced by epithelial and endothelial cells and monocytes/macrophages.^{23,24} High levels of IL-15 have been found in blood and inflamed mucosa of patients with IBD.^{25,26} IL-15 regulates both innate and adaptive immune responses, is involved in the differentiation and proliferation of natural killer (NK) cells and T cells, and contributes to hematopoietic cell homeostasis.²⁷ The trimeric receptor for IL-15 (IL-15R) is expressed on T cells, and it includes the constitutively expressed IL-2R β and IL-2R γ c chains, and a unique IL-15R α chain only expressed on activated mononuclear cells.²⁸ In IBD, increased levels of IL-15 in the inflamed intestine favor the expansion and activation of intraepithelial NK T cells and CD8⁺ T cells.^{29,30} Hence, IL-15 knockout (*IL-15*^{−/−}) mice are less sensitive to both acute and chronic colitis after DSS administration.³¹ This attenuation of colitis severity in *IL-15*^{−/−} mice is associated with a decrease of several T-cell subpopulations and of the production of TNF α , IL-12, and IFN γ .

IL-15 not only induces proliferation and activation of T cells, but also directly acts on bone remodeling by stimulating pre-osteoclast differentiation.³² Indeed, osteoclasts express the high affinity IL-15R α chain and IL-15 influences osteoclastogenesis both directly, by co-stimulating RANKL-induced differentiation and activation processes in osteoclasts, and indirectly, by inducing proliferation and activation of T cells and their production of IFN γ and RANKL.³³ In turn, IL-15R α deficient (*IL-15R α* ^{−/−}) mice present a high bone mass phenotype and improved bone microarchitecture in both the cancellous and cortical bone compartments.³³

The potential therapeutic effect of an IL-15 mutant/Fc2a fusion protein (CRB-15) antagonist of IL-15R has been reported in several immune and inflammatory disorders. On the one hand, CRB-15 improves allograft survival due in part to the inhibition of activation and proliferation of CD8⁺ alloreactive T cells.³⁴ On the other hand, CRB-15 prevents the development of arthritis and blocks disease progression in a mouse model of collagen-induced arthritis. This effect is mainly associated with a decrease in synovitis in the joints and lymphocytic infiltration and reduced *in situ* production of pro-inflammatory cytokines, TNF α , IL-1 β , IL-6, and IL-17.^{35,36}

Accordingly, we hypothesized that IL-15 could play a central role in the systemic and skeletal complications of IBD. For this purpose, we studied the effects of CRB-15 in a mouse model of DSS-induced chronic colitis. We show herein that inhibition of IL-15 has distinct effects on bone formation and osteoclasts, and at least transiently improves

bone loss, colitis clinical manifestations, and survival in mice exposed to DSS for 3 weeks.

Materials and Methods

Experimental Procedures

Female C57BL/6 mice, obtained from the animal core of our institution (Centre Médical Universitaire, Faculty of Medicine, Geneva) were used for this study. All mice were kept under conventional housing conditions with free access to a standard diet (RM3; Special Diets Services, Essex, UK).

Chronic colitis was induced in 10-week-old female mice by adding 2% of DSS (TdB Consultancy AB, Uppsala, Sweden) to the drinking water for 1 week, followed by 1% DSS for 2 weeks. Mice were treated daily with an antagonist of IL-15, CRB-15 (5 μ g/100 μ L per mouse i.p., as previously described³⁵) from day 7 to day 21 after DSS administration to evaluate the effects of CRB-15 in established disease (ie, during the chronic phase of the colitis after reduction of DSS administration). As CRB-15 is a recombinant fusion protein consisting of a point-mutated IL-15 and the constant region of murine IgG2a,^{35,37} an intact IgG2a (5 μ g/100 μ L per mouse, i.p.) was used as a control to confirm that the modulatory effect observed with CRB-15 was not due to the immunoglobulin part of the molecule but to the antagonist of IL-15. The experiment was repeated three times with similar results, totaling 7 to 27 mice per group. Water intake was monitored twice a week in each cage and related to the consumed quantity per mouse and per day. To assess the severity of colitis, mice were weighed and followed for the presence of rectal bleeding (Colo-rectal test; Axon Lab AG, Baden, Switzerland) three times per week. Femoral BMD (mg/cm²) was determined at baseline, 7 days, 14 days, and 21 days after DSS administration. Mice were euthanized by injection with 120 mg/kg ketamine/16 mg/kg xylazine, and cervical dislocation 7 days (only for isolation of intraepithelial lymphocytes), 14 days, or 21 days after DSS administration. The fifth lumbar vertebra and femurs were collected for skeletal analyses, colons were removed for histological evaluation, RNA isolation, and myeloperoxidase activity determination, and spleen, lymph nodes, and bone marrow were collected for immunological analyses. The mice that died during the experiment were also evaluated for bone parameters and integrated in the different groups for analyses according to their time of death. All of the animal procedures were approved by the ethical committee of University of Geneva and the state of Geneva Veterinarian Office.

A disease activity index (DAI) score based on body weight loss and detection of fecal blood was determined. For each parameter, a score of 0 to 4 was attributed, giving rise to a maximal DAI score of 8. The score was evaluated as follows: weight loss scores were: 0 = no loss; 1 = 5% to 10%; 2 = 10% to 15%; 3 = 15% to 20%; and 4 = >20%. Fecal blood scores were: 0 = negative colorectal test; 2 = positive colorectal test; 4 = macroscopic blood in stools.

Bone Mineral Density and Microarchitecture

Femoral BMD (mg/cm^2) was measured *in vivo* using dual energy X-ray absorptiometry (pDXA, PIXImus; GE-Lunar Corporation, Madison, WI). Quantitative microcomputed tomography (μCT , UCT40; Scanco Medical AG, Basserdorf, Switzerland) was used as previously described.³⁸ Specifically, the trabecular bone volume fraction (percentage), trabecular thickness (μm), trabecular number (mm^{-1}), and trabecular separation (μm) were evaluated at the fifth lumbar vertebra and distal femoral metaphysis, whereas for the cortical bone, the average total volume (mm^3), bone volume (mm^3), and cortical thickness (μm) were evaluated at the femoral midshaft.

Histomorphometry

To measure dynamic indices of bone formation, mice received 20 mg/kg subcutaneous injections of calcein (Sigma-Aldrich, Buchs, Switzerland) at 9 and 2 days before euthanasia. Femurs were dehydrated in graded ethanol and embedded in methyl methacrylate (Merck, Darmstadt, Germany). Transversal sections (8 μm thick) of the midshaft and sagittal sections (5 μm thick) were cut with a Leica Polycut E microtome (Leica Corporation Microsystems AG, Glattpburg, Switzerland) and mounted unstained for evaluation of fluorescence. Histomorphometric measurements were performed on the secondary spongiosa of the distal femoral metaphysis and on the endocortical and periosteal bone surfaces in the middle of the femur by using a Q image analyzer (Leica Corporation) at $\times 40$ magnification. All parameters were calculated and expressed according to standard formulas and nomenclatures³⁹: mineral apposition rate ($\mu\text{m}/\text{day}$), single-labeled surface per bone surface (percentage), and double-labeled surface per bone surface (percentage). Mineralizing surface per bone surface (percentage) was calculated by adding double-labeled surface per bone surface and one-half single-labeled surface per bone surface. Bone formation rate ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$) was calculated as the product of mineralizing surface per bone surface and mineral apposition rate. Only mineral apposition rate and bone formation rate obtained on the endocortical bone surfaces were presented in the *Results*. Sagittal sections (5 μm thick) are stained with modified Goldner's trichrome to evaluate resorption parameters on the secondary spongiosa of the distal femoral metaphysis: number of osteoclasts per bone area, percentage of bone surface occupied by osteoclasts, and number of osteoclasts per bone perimeter. Only the number of osteoclasts per bone area is presented in the *Results*.

Serum Biochemistry, Bone Turnover, and Cytokine Determination

At baseline, 7 days, 14 days, and 21 days after DSS administration, mice were kept fasting for 6 hours and blood was collected by retro-orbital bleeding for biochemical evaluation. Sera were tested by ELISA to evaluate

C-terminal telopeptides of type I collagen (CTX, RatLaps EIA; Immunodiagnostic Systems, Frankfurt, Germany), by RIA to measure osteocalcin (Tecommedical, Sissach, Switzerland) and by a mouse cytokine 20-plex antibody bead kit (Invitrogen, Basel, Switzerland) for the determination of inflammatory cytokines levels. Data were analyzed on a Bio-Plex 200 System (Bio-Rad, Luminex xMAP Technology, Reinach, Switzerland).

RNA Isolation from Bone and Real-Time PCR

The whole femurs were excised, both femoral extremities were removed, and the shaft was flushed with cold phosphate-buffered saline to remove the bone marrow. Femoral diaphysis and extremities were immediately pulverized into a fine powder and homogenized in peqGold Trifast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) using a FastPrep system apparatus (QBiogene, Illkirch, France) to achieve quantitative RNA extraction. Total RNA was extracted and then purified on miniature columns (RNeasy minikit; Qiagen, Basel, Switzerland). Single-stranded cDNA templates for quantitative real-time PCR analyses were performed using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR was performed using predesigned TaqMan gene expression assays (Table 1) consisting of two unlabeled primers and a FAM dye-labeled TaqMan MGB probe and the correspondent buffer TaqMan universal PCR master mix (Applied Biosystems, Rotkreuz, Switzerland). A Biomek 2000 robot (Beckman Coulter, Nyon, Switzerland) was used for liquid handling in 384-well plates with three replicates per sample. The cDNA was PCR-amplified in a 7900HT SDS System, and raw threshold cycle (*Ct*) values were obtained from SDS software version 2.0 (Applied Biosystems). For each gene, the highest quantity was arbitrarily designated a value of 1.0. The mean quantity was calculated from triplicates for each sample, and this quantity was normalized to the similarly measured

Table 1 Taqman Gene Expression Assays and Primers for SYBR Green PCR

Gene	TaqMan gene expression assays reference
<i>IL-6</i>	Mm00446190
<i>IL-10</i>	Mm00439616
<i>TNFα</i>	Mm99999068
<i>OPG</i>	Mm00435452
<i>RANKL</i>	Mm00441908
<i>ALP</i>	Mm00475834
<i>Col1A1</i>	Mm00801666
Gene	Primers for SYBR Green qPCR
<i>GAPDH</i>	Sense: 5'-GCTAAGCAGTTGGTGGTGCA-3' Antisense: 5'-TCACCACCATGGAGAAGGC-3'
<i>IL-1β</i>	Sense: 5'-CAACCAACAAGTGATATTCTCCATG-3' Antisense: 5'-GATCCACACTCTCCAGCTGCA-3'
<i>IL-6</i>	Sense: 5'-ACAACCACGGCCTTCCTACTT-3' Antisense: 5'-CACGATTTCCTCAGAGAACATG TG-3'

mean quantity of the glyceraldehyde-3-phosphate dehydrogenase normalization gene. mRNAs obtained from different times of sacrifice were analyzed separately.

Colon Histology and Immunohistochemistry

At sacrifice, a segment of distal colon was excised and fixed in 10% buffered formalin. Paraffin sections were stained with H&E according to standard protocols. Histological scoring was performed by a pathologist as previously described.⁴⁰ Briefly, score consists of an inflammatory component (0 = rare inflammatory cells in the lamina propria; 1 = increased numbers of inflammatory cells in the lamina propria; 2 = confluence of inflammatory cells; extending into the submucosa; and 3 = transmural extension of the infiltrate) and a tissue damage component (0 = no mucosal damage; 1 = discrete lymphoepithelial lesions; 2 = surface mucosal erosion; 3 = extensive mucosal damage and extension through deeper structures of the bowel wall). The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). For immunohistochemistry, the sections were deparaffinized in xylene, rehydrated in a descending series of ethanol. Deparaffinized slides were pretreated in 3% hydrogen peroxide in methanol and rinsed in tap water, followed by nonspecific avidin/biotin blocking (avidin/biotin Blocking Kit; Reactolab S.A., Servion, Switzerland) according to the manufacturer's instructions. The slides were incubated with the primary antibody, the biotin anti-mouse $\gamma\delta$ T-cell receptor GL3 (BD Pharmingen, Basel, Switzerland) for 2 hours at 37°C and then with the fluorescein isothiocyanate-conjugated avidin reagent (BD Pharmingen) for 30 minutes at room temperature. Finally, the slides were washed and dehydrated for mounting. Immunohistology staining was analyzed using Axioskop 2 Plus and AxioVision Release software version 4.8 from Zeiss (Göttingen, Germany).

MPO Activity

Total proteins were extracted from colon samples using 0.5% Cetavlon, 10 mmol/mL morpholine-3-propanesulfonic acid (MPS, pH 7), extraction buffer, and were quantified using bicinchoninic acid protein assay (Bio-Rad, Reinach, Switzerland). Myeloperoxidase (MPO) activity was determined on 10 μ g of colon proteins by spectrophotometry (405 nm/550 nm) as previously described.⁴¹

RNA Isolation from Colon and Real-Time PCR

RNA extraction was performed on a piece of colon using the Rneasy Mini kit (Qiagen). Single-stranded cDNA templates for quantitative real-time PCR analyses were performed using ThermoSript RT-PCR System (Invitrogen) following the manufacturer's instructions. Then PCR reaction was performed in duplicate on a MyiQ iCycler (Bio-Rad, Hercules, CA) using 96-well microtiter plates (Bio-Rad, Basel, Switzerland). The primers used in this experiment

were described in Table 1. Quantification of input cDNA was performed using a standard curve as previously described.⁴²

Immune Cell Isolation and Analysis

Peripheral lymph node and spleen cells from mice were harvested, and cell suspensions were prepared in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. T cells were enriched using Dynal Mouse T Cell Negative Isolation Kit (Invitrogen) and processed for analysis.

To collect bone marrow cells, femurs were excised, and both femoral extremities were cut to remove the bone marrow from the diaphysis by flushing with cold phosphate-buffered saline.

At sacrifice, the colon was excised to isolate intraepithelial lymphocytes (IELs) but the number of isolated cells was not enough to perform subpopulations analysis. Consequently, the small intestine was excised and IELs were isolated using modified methods as previously described.⁴³ Briefly, fat tissue was removed, Peyer's patches were mechanically excised and the gut was extensively flushed with Hank's Buffered Salt Solution (Sigma-Aldrich). Intestinal pieces were longitudinally opened and cut into 5-mm pieces. Tissue pieces were incubated in calcium- and magnesium-free Hank's Buffered Salt Solution (Sigma-Aldrich) containing 2 mmol/L EDTA and 1 mmol/L dithiothreitol (Sigma-Aldrich) for 60 minutes at 37°C with magnetic stirring before being vigorously vortexed and filtered through 250- μ m nylon gauze. Intestinal IELs were obtained by a second filtration procedure through a nylon wool column. Cell population was washed twice and lymphocytes were enriched by discontinuous 15/30/44% Percoll (Bioscience, Uppsala, Sweden) on a Lympholyte M gradient (Cedarlane, Hornby, ON, Canada) for 20 minutes at 600 g at room temperature. Lymphocytes were harvested from the Percoll 44% Lympholyte M interface.

The following rabbit anti-mouse monoclonal antibodies fluorochrome-conjugated and isotype-matched control monoclonal antibodies: CD3, CD4, CD8, CD8 $\alpha\alpha$, CD8 $\alpha\beta$, TCR $\alpha\beta$, TCR $\gamma\delta$, CD11b, CD44, NK1.1 CD117, and CD115 were purchased from BD Pharmingen or eBioscience (Vienna, Austria). Three-to-five color immunofluorescence was performed to assess surface marker expression on T cells isolated from treated mice. Cells were washed twice with PBS (completed with 2% serum and 1% NaN₃) and treated successively with fluorochrome-conjugated monoclonal antibodies on ice for 30 minutes and washed with completed PBS. Cell staining was analyzed using a flow cytometer (FACSCalibur or FACS Aria, BD Biosciences, Basel, Switzerland) and Cell Quest Pro software version 5.2.1 or FACS DIVA 6 software (BD Pharmingen). Gating CD3⁺ cells was performed to analyze T-lymphocyte subpopulations and CD8 β ⁺ cells were excluded to analyze the CD8 $\alpha\alpha$ ⁺ subpopulation. To study osteoclastogenic population in bone marrow,⁴⁴ analysis of CD115 and/or CD117 expression was done on CD3[−] CD45[−] CD11b[−] BM cell population.

Statistical Analysis

Data were analyzed using a one-factor or two-factor analysis of variance to assess the effects of DSS administration and CRB-15 treatment. As appropriate, post hoc testing was performed using Fisher's Protected Least Significant

Difference (PLSD). The Kaplan—Meier method was used to analyze survival, and the differences between each individual curve with each other were calculated using a log-rank test. Differences were considered significant at $P \leq 0.05$. Data are presented as means $\pm$ SEM unless otherwise noted.

Results

Improved Survival and Disease Activity Index by CRB-15

In the absence of other treatments, administration of 2% DSS for 1 week followed by 1% DSS for the next 2 weeks induced a severe colitis (as evaluated by body weight loss and bloody stools) starting from day 3. Hence mice exposed to DSS experienced 15% weight loss during the first week compared to their initial body weight (Figure 1A). In parallel, based on body weight loss and the presence of fecal blood, DSS rapidly increased the DAI from days 2 to 7 (Figure 1B). After DSS dose reduction to 1% and the beginning of treatments, weight loss continued at the same rate in mice receiving the IgG2a control antibody, but was attenuated in those receiving CRB-15 (-7.7% in DSS-CRB-15 versus -14.6% in DSS-IgG2a at day 11 compared to baseline). Importantly, water (containing DSS) intake was similar in IgG2a and CRB-15 groups (data not shown) indicating that weight differences between groups could not be explained by different DSS intakes. After 3 weeks, body weight had partially recovered in DSS-exposed mice and was no more different between IgG2a and CRB-15 treatment groups, although it remained significantly lower than controls (H₂O) (Figure 1A). After the beginning of treatments, the DAI continued to rise until 14 days in DSS-exposed mice treated with IgG2a, but then stabilized as the most severely affected mice died. In contrast, CRB-15 rapidly and significantly decreased DAI during the first week of treatment (Figure 1B). Consequently, CRB-15 significantly improved the survival rate in mice exposed to DSS at 14 and 21 days (63.0% in DSS-CRB-15 versus 33.3% in DSS-IgG2a; $P < 0.05$ at 14 days; and 55.6% in DSS-CRB-15 versus 25.9% in DSS-IgG2a; $P < 0.05$ at 21 days) (Figure 1C).

Effects of CRB-15 on Colonic and Systemic Inflammation

Next we examined whether the effects of CRB-15 on morbidity and mortality were related to an improvement of

Figure 1 Body weight, disease activity index, and survival rate in IgG2a- and CRB15-treated mice. Mice received 2% DSS in drinking water for 1 week followed by 1% DSS for 2 weeks. I.P. treatment with 5 μ g/100 μ L per mouse CRB-15 or IgG2a began after 7 days of DSS administration. Four groups of mice were analyzed: H₂O-IgG2a, H₂O-CRB15, DSS-IgG2a, and DSS-CRB15. **A:** Body weight was measured three times per week. **B:** Disease activity index was based on body weight and hemocultures. **C:** Survival rates of mice were monitored every day. Values are the means $\pm$ SEM of three independent experiments ($n = 7$ to 27 mice per group). **Arrows** indicate the beginning of treatment. * $P < 0.05$ versus H₂O, $^{\dagger}P < 0.05$ versus H₂O-IgG2a, $^{\ddagger}P < 0.05$ versus H₂O-CRB15, and $^{\S}P < 0.05$ versus DSS-CRB15.

Figure 2 Analysis of intestinal inflammation in IgG2a- and CRB15-treated mice. **A:** Colon length was measured at time of sacrifice. Values are the means $\pm$ SEM of two independent experiments ($n = 5$ to 16 mice per group). **B:** Representative images of colon stained with H&E were obtained after 21 days of DSS (original magnification, $\times 40$). **C:** Histological scores of colon, based on inflammation and mucosal damage, were evaluated 14 days and 21 days after DSS administration. Each value was represented by a **black dot** and mean by a **black line** ($n = 4$ to 8 mice per group). **D:** MPO activity was measured 14 days after DSS administration. Each value was represented by a **black dot** and mean by a **black line** ($n = 6$ mice per group). **E:** Expression of mRNA for *IL1 β* and *IL6* genes was determined as a ratio of mRNA to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. mRNA expression was analyzed 14 days after DSS administration. Values are the means $\pm$ SEM ($n = 6$ mice per group). **F:** The percentage of TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ among CD3⁺ cells of intestinal epithelium was analyzed by flow cytometry 7 days, 14 days, and 21 days after DSS administration. Values are means $\pm$ SEM of three experiments with a pool of two mice per group. **Arrows** indicate the beginning of treatment. * $P < 0.05$ versus H₂O, † $P < 0.05$ versus H₂O-IgG2a, and ‡ $P < 0.05$ versus H₂O-CRB15.

inflammatory parameters both in the colon and systemically. The colon was significantly shortened by DSS administration at days 14 and 21, consistent with the presence of colitis (Figure 2A). Moreover, histological analysis indicated a massive destruction of the colonic epithelium associated with a transmural infiltration of inflammatory cells in DSS-IgG2a-treated mice compared to controls (H₂O) (Figure 2B) and an elevated histological score of colon based on infiltration of inflammatory cells and mucosal damage at days

14 and 21 (Figure 2C). Average levels of MPO activity, a marker of inflammatory response directly related to neutrophil infiltration, were also elevated in mice exposed to DSS (Figure 2D). Eventually, gene expression level of two pro-inflammatory cytokines, IL-1 β and IL-6, were increased after 14 days and 21 days of DSS administration in the colon (Figure 2E). After 2 weeks of treatments, colon length did not significantly differ between IgG2a and CRB-15 groups, which could be explained by a survival bias

(ie, a less severe degree of colitis among the fewer survivors in the IgG2a group) (Figure 2A). However, in some but not all DSS-CRB-15-treated mice, colonic epithelium was partially preserved despite the presence of edema and inflammatory infiltrates (Figure 2B). The heterogeneity of the colonic response to CRB-15 was further illustrated by the histological score of colon, which on average was elevated in both treatment groups (CRB-15 and IgG2a) but remained ≤ 3 in 4 of 6 of the CRB-15-treated mice after 14 days (Figure 2C). Similarly to the histological score, average levels of MPO activity were not different between IgG2a and CRB-15, whereas 4 of 6 CRB-15-treated mice had MPO activity < 20 units/L and not different from H₂O controls (Figure 2D). Finally, CRB-15 did not prevent the increase in gene expression level of IL-1 β and IL-6 in the colon of DSS-exposed mice (Figure 2E and data not shown).

Next we examined the proportion of small intestinal $\alpha\beta$ T and $\gamma\delta$ T cells, the latter accounting for $\leq 40\%$ to $\leq 50\%$ of intraepithelial lymphocytes IELs⁴⁵ and exerting anti-inflammatory properties in various mouse colitis models.^{46,47} DSS administration induced an increased proportion of $\gamma\delta$ T cells, and respectively, decreased $\alpha\beta$ T cells at day 7 (Figure 2F). After 1 week and more than 2 weeks of treatment, CRB-15 further increased the proportion of $\gamma\delta$ T cells (77.1% versus 49.5% in IgG2a), and decreased $\alpha\beta$ T cells, in DSS-exposed mice compared to IgG2a (Figure 2F). Among $\gamma\delta$ T cells, no treatment-related differences were noted in the various subpopulations (CD4, CD8 $\alpha\alpha$, CD8 $\alpha\beta$, CD4, CD8 $\alpha\alpha$, and CD4 CD8 $\alpha\beta$; Supplemental Table S1). Immunohistochemistry of colon allowed confirmation that $\gamma\delta$ T cells were present in the colon of mice and seemed to be increased in DSS-CRB-15-treated mice (Supplemental Figure S1).

In contrast to IELs, we found no significant effects of IL-15 blockade on T-cell populations in spleen and lymph nodes. Moreover, levels of pro-inflammatory cytokines, such as IL-2, IL-17, or IFN γ , and the anti-inflammatory cytokine IL-10 in blood were not significantly modulated in the presence versus absence of colitis (data not shown). These results suggested that in this model of chronic colitis, inflammation was not systemic.

CRB-15 Delays Colitis-Associated Bone Loss

Femoral BMD increased over the course of three weeks, in control (H₂O) mice, whereas BMD remained stable or decreased in mice exposed to DSS (Figure 3A). However, CRB-15 initially attenuated BMD loss in these animals, to the point that in mice treated with CRB-15, femur BMD did not differ between DSS-CRB-15 and the control (H₂O-IgG2a or H₂O-CRB-15) groups at 14 days. At 21 days, however, femoral BMD was similar in CRB-15- and IgG2a-treated mice from the DSS groups (Figure 3A) and significantly lower than in the control groups.

Trabecular microarchitecture was analyzed *ex vivo* in vertebra and distal femur at 14 and 21 days. A significant

decrease in trabecular bone volume fraction and trabecular thickness occurred in DSS-IgG2a-treated mice compared to control mice at 14 days and 21 days after DSS administration. We noted that control mice had a low femur trabecular bone volume fraction at 21 days without which we can explain it and consequently no difference appeared between DSS-exposed mice and controls. CRB-15 prevented these alterations in bone microarchitecture at 14 days. However, 21 days after DSS administration, bone volume fraction and trabecular thickness were not significantly different in DSS mice treated with CRB-15 compared to IgG2a (Figure 3B). Lower cortical bone volume and cortical thickness were also observed in mice exposed to DSS for 21 days without significant difference between CRB-15 and IgG2a (Supplemental Table S2).

CRB-15 Decreases Osteoclast Numbers

To understand the mechanisms by which CRB-15 delayed bone loss in this model, we next analyzed its effects on bone

Figure 3 BMD and bone microarchitecture in IgG2a- and CRB15-treated mice. **A:** BMD was measured at the femur at baseline, and at 7, 14, and 21 days after DSS administration. Values are the mean of three independent experiments ($n = 7$ to 27 mice per group). **Arrow** indicates the beginning of treatment. **B:** Distal femur and vertebral trabecular bone volume fraction and trabecular thickness were determined by microcomputed tomography 14 days and 21 days after DSS administration. Values are the means $\pm$ SEM of three independent experiments ($n = 5$ to 18 mice per group). $^{\dagger}P < 0.05$ versus H₂O-IgG2a, $^{\ddagger}P < 0.05$ versus H₂O-CRB-15, and $^{\S}P < 0.05$ versus DSS CRB 15.

Figure 4 Indices of bone turnover in IgG2a- and CRB15-treated mice. **A:** Level of osteocalcin was measured in sera at baseline, and at 7, 14, and 21 days after DSS administration. **Arrow** indicates the beginning of treatment. **B:** Mineral apposition rate and bone formation rate were determined 21 days after DSS administration on bone surfaces at the femoral midshaft. **C:** Number of osteoclasts per bone area was measured 21 days after DSS administration on the secondary spongiosa of the proximal femur metaphysis. **A–C:** Values are means $\pm$ SEM of two independent experiments ($n = 5$ to 12 mice per group). **D:** CD117- and CD117/CD115-positive cells were evaluated in bone marrow at 14 and 21 days after DSS administration. **Black dot** represents CD3⁺ CD45⁺ CD11b⁺ CD117⁺ cell population on the **left panel**, and CD3⁺ CD45⁺ CD11b⁺ CD117⁺ CD115⁺ cells on the **right panel**. Each value was represented by a **black dot** and mean by a **black line** ($n = 3$ mice per group). Data were analyzed using a 2F-analysis of variance and post hoc testing was then performed using Fisher's Protected Least Significant Difference to compare IgG2a and CRB-15 in the DSS subgroup of mice. * $P < 0.05$ versus H₂O, † $P < 0.05$ versus H₂O-IgG2a, ‡ $P < 0.05$ versus H₂O-CRB-15, and § $P < 0.05$ versus DSS-CRB-15.

turnover. Serum levels of osteocalcin were modestly but significantly decreased after 7 days of DSS administration, and remained lower in IgG2a-treated mice compared to controls (H₂O) until 21 days (Figure 4A). Furthermore, dynamic histomorphometrical indices of bone formation (mineral apposition rate and bone formation rate), as evaluated at the end of the experiment, were decreased in mice exposed to DSS (Figure 4B). CRB-15 first induced a low but significant increase in osteocalcin levels in both DSS and H₂O mice, which then remained stable. Hence, after 21 days, osteocalcin levels did not significantly differ between DSS mice treated with CRB-15 and mice without colitis (H₂O-IgG2a) (Figure 4A). However, mineral apposition rate and bone formation rate were not significantly better after 2 weeks of CRB-15 treatment (Figure 4B).

Mice exposed to DSS had a markedly increased osteoclasts number, as evaluated at the end of the experiment (Figure 4C), and CD117⁺ and CD117/CD115⁺ osteoclast progenitors⁴⁴ in the bone marrow at 14 and 21 days (Figure 4D). The osteoclasts number was significantly attenuated after 2 weeks of CRB-15 treatment (Figure 4C). Moreover, CRB-15 delayed the increase in osteoclastogenic

progenitors in the bone marrow until 21 days (Figure 4D). Compared to IgG2a, CRB-15 also prevented the increase in CD44⁺ leukocytes and in particular of CD11b⁺ monocytes/macrophages in mice exposed to DSS (Table 2). These results suggested that in addition to its direct effects on osteoclasts,³³ CRB-15 did prevent the recruitment and/or expansion of immune cells in the bone marrow.

CRB-15 Reverses the Pro-Inflammatory Cytokine Profile in the Bone Marrow

We next analyzed expression levels of genes implicated in bone formation and resorption, namely alkaline phosphatase (ALP), Collagen 1A1 (Col1A1), RANKL, and OPG, as well as pro- and anti-inflammatory cytokines, such as TNF α , IL-6, and IL-10 in the bone. In keeping with the decrease of serum osteocalcin (previously described), ALP and Col1A1 expression was profoundly inhibited in (DSS) colitis mice (Figure 5A). CRB-15 prevented the decrease of ALP and Col1A1 at 14 days, but not later (Figure 5A).

Tnf α gene expression more than doubled in DSS exposed mice, and CRB-15 tended to prevent this increase from 14

Table 2 Analysis of Immune Cell Populations in Bone Marrow of IgG2a- and CRB15-Treated Mice

Immune cell subset (%)	Days after DSS	H ₂ O		DSS	
		IgG2a	CRB-15	IgG2a	CRB-15
CD44	14 days	22.90 ± 1.91	12.04 ± 0.60	27.00 ± 4.90*	20.29 ± 5.78
	21 days	21.38 ± 8.30	ND	43.62 ± 3.28 [†]	37.29 ± 1.18
CD4	14 days	1.85 ± 0.51	1.73 ± 0.37	1.75 ± 0.140	1.55 ± 0.05
	21 days	1.48 ± 0.34	ND	0.79 ± 0.39	0.75 ± 0.29
CD8	14 days	3.65 ± 1.29	2.51 ± 0.34	3.82 ± 0.26	3.6 ± 0.04
	21 days	1.01 ± 0.25	ND	0.85 ± 0.33	1.49 ± 0.22
NK1.1	14 days	2.07 ± 1.02	1.19 ± 0.17	1.75 ± 0.19	1.5 ± 0.35
	21 days	1.36 ± 0.12	ND	0.92 ± 0.21	1.25 ± 0.28
CD11b	14 days	23.14 ± 1.13	22.48 ± 1.47	41.59 ± 1.44* [†]	30.78 ± 8.78
	21 days	10.12 ± 1.69	ND	40.09 ± 5.11 ^{†§}	23.96 ± 1.47 ^{†‡}
CD117	14 days	1.17 ± 0.13	1.0 ± 0.11	2.33 ± 0.18* ^{†§}	1.2 ± 0.31 [†]
	21 days	1.30 ± 0.11	ND	3.2 ± 0.44 [†]	3.93 ± 0.47 [†]
CD115	14 days	1.4 ± 0.21	0.93 ± 0.23	1.23 ± 0.14	1.0 ± 0.29
	21 days	3.13 ± 0.14	ND	3.4 ± 1.11	4.07 ± 0.67
CD117/CD115	14 days	0.77 ± 0.09	0.60 ± 0.06	1.57 ± 0.145* ^{†§}	0.73 ± 0.14 [‡]
	21 days	0.57 ± 0.03	ND	0.93 ± 0.23	1.43 ± 0.29 [†]

Values are the means ± SEM of two independent experiments ($n = 3$ mice per group).

* $P < 0.05$ versus H₂O-CRB-15.

[†] $P < 0.05$ versus H₂O-IgG2a.

[‡] $P < 0.05$ versus DSS-IgG2a.

[§] $P < 0.05$ versus DSS-CRB-15.

ND, not determined.

days, but this effect only reached the significance at 21 days (Figure 5C), and *Il6* gene expression levels remained unchanged (Figure 5C). CRB-15 also enhanced the expression of the anti-inflammatory cytokine IL-10 in DSS mice at day 21 (Figure 5C). In contrast, both RANKL and OPG were reduced 14 days after DSS administration, and OPG remained low at 21 days compared to H₂O controls, without significant CRB-15 effects (Figure 5B).

Discussion

A DSS mouse colitis model associated with bone loss has previously been described.⁴⁸ Using this model and a selective inhibitor of IL-15, CRB-15,³⁷ we have now delineated the role of this major T cell growth factor and inflammatory cytokine on the systemic and skeletal complications of colitis and characterized the mechanisms by which IL-15 contributes to alter bone remodeling in IBD. Consistent with the profile of bone turnover markers in humans^{49–51} and other mouse colitis models,^{48,52,53} bone loss in DSS-treated mice was characterized by suppressed bone formation but an increased number of osteoclast progenitors (CD117⁺) in the bone marrow and osteoclasts on bone surfaces. These alterations result from the dual effects of inflammatory cytokines that inhibit osteoblasts while activating osteoclastogenesis.^{12–18} Hence, the inhibition of *Col1a1* and *ALP* gene expression, as well as the increase of osteoclast numbers after DSS administration were paralleled by a marked stimulation of *Tnfα* gene expression in bone. This explains why treatment of IBD patients with infliximab, a TNFα inhibitor, is associated with

some improvement of bone mineral density and an increase of bone ALP.^{54,55}

IL-15 has been implicated in both colonic inflammation^{25,26} and osteoclast activation.³³ In turn, CRB-15 prevented the increase of CD117⁺ cells at day 14 and reduced osteoclast numbers at 21 days. These effects occurred without a significant improvement of the OPG/RANKL ratio, which was markedly lower at the end of the experiment, and may therefore be at least partly attributed to the inhibition of IL-15 itself. Indeed, IL-15 is able to enhance osteoclastogenesis both directly, by co-stimulating RANKL-induced differentiation and function in osteoclasts, and indirectly, by inducing proliferation and activation of T cells and their production of IFNγ and RANKL.³³ Moreover, CRB-15 decreased *Tnfα* and increased *Il10* gene expression after 21 days, which could contribute to the significant decrease of osteoclast numbers observed at the end of the experiment. Indeed, IL-10 deficient mice develop colitis with osteopenia,⁵² whereas IL-10 inhibits osteoclastogenesis.⁵⁶ On another hand, CRB-15 did not influence *IL-6* gene expression in bone, another pro-inflammatory cytokine implicated in IBD and known to inhibit bone formation¹⁶ while increasing bone resorption.⁵⁷

Although CRB-15 significantly reduced *Tnfα* gene expression only by day 21, it fully prevented the inhibition of ALP and *Col1A1* expression and partially restored serum osteocalcin levels early on (day 14). It is noted that CRB-15 tended to increase osteocalcin levels and bone mineral density even in control mice (ie, in the absence of inflammation). Taken together with our previous observations of an increased bone mass in *IL-15Rα*^{−/−} mice³³, these results

Figure 5 Gene expression in bone of IgG2a- and CRB15-treated mice. In each experiment, mRNA extraction was performed on the femur at 14 or 21 days after DSS administration. Gene expression was determined as a ratio of mRNA to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. **A:** Expression of mRNA for Col1A1 and ALP genes. **B:** Expression of mRNA for receptor activator of NF- κ B ligand (RANKL) and OPG genes. **C:** Expression of mRNA for IL-6, TNF α , and IL-10 genes. Values are means $\pm$ SEM ($n = 4$ mice per group). $^{\dagger}P < 0.05$ versus H₂O-IgG2a, $^{\ddagger}P < 0.05$ versus H₂O-CRB-15, and $^{\S}P < 0.05$ versus DSS-CRB-15.

suggest that IL-15 could further inhibit bone formation independently of TNF α . As a result, CRB-15 initially restored femur BMD gain and delayed the alterations of trabecular microarchitecture associated with colitis. However, these protective effects were not maintained over the entire length of the experiment. Hence, we propose that IL-15 and TNF α could both be implicated in bone loss in the early stages of IBD, whereas decreased OPG expression may become the predominant mechanism of bone loss over time. This hypothesis is further supported by prior evidence showing that recombinant OPG inhibits bone resorption and restores bone mass in the adoptive transfer mouse colitis model, however, without improving bone formation.⁵⁸ Similarly, exogenous recombinant OPG reverses bone alterations in IL-2 deficient mice. Moreover, in this model, OPG exerts beneficial effect on the development of colitis and inflammation by decreasing dendritic cells.⁵³

In colitis mice, the number of leukocytes and in particular, CD11b positive cells in the bone marrow were two to four times higher than controls. In contrast, the various T-cell populations in the bone marrow, lymph nodes, and spleen apparently remained unaffected. Thus, monocytes/

macrophages seem to be the predominant vector of inflammation within the bone marrow secondary to DSS-induced colitis. Inflammatory cell infiltrates associated with elevated level of TNF α have been previously reported in bone marrow of mice that develop colitis after transfer of CD4⁺CD45RB^{Hi} T cells, although the subpopulations of inflammatory cells were not specifically evaluated in that study.⁵⁸ An increased number of inflammatory cells, CD11b positive monocytes, and CD4 positive T cells has also been observed in the bone marrow of arthritic rats.⁵⁹ Monocytes and macrophages are major sources of IL-15, which in turn exerts an autocrine activity on macrophages, stimulating their production of TNF α .²⁴ Hence, elevated TNF α expression in the bone marrow of colitis mice could be the result of infiltrating macrophages responding to IL-15, and likely additional stimuli. This could explain how CRB-15 was capable to prevent the recruitment and/or expansion of CD11b⁺ cells in the bone marrow and to eventually decrease TNF α , thereby limiting a TNF α -mediated inflammatory loop in the bone microenvironment.

DSS administration induced early weight loss and a marked increase in DAI and mortality. Nevertheless, in

conditions of chronic colitis (1% DSS), body weight partly recovered and the DAI eventually stabilized in IgG2a-treated mice. Inhibition of IL-15 prompted weight recovery, quickly decreased DAI, and ultimately improved survival. By the end of the experiment, however, the beneficial effects of CRB-15 on the systemic manifestations of the disease were no more apparent. This could be due to a survivors effects (ie, survival of only less severely affected control mice at the end of the experiment) but could also be explained by a progressive loss of function of IL-15, and/or its inhibitor, during the phase of chronic inflammation (although IL-15 receptor remained expressed in colon 21 days after DSS administration, data not shown). Moreover, and as previously described,⁶⁰ the effects of CRB-15 on the clinical manifestations of colitis did not necessarily reflect the severity of inflammation and mucosal destruction in the colon, which on average did not significantly differ between DSS-IgG2a and DSS-CRB-15-treated mice. Nevertheless, the effects of CRB-15 on colon damage and inflammation appeared to be heterogeneous, with approximately 50% of mice showing a marked reduction of inflammatory markers (such as MPO activity), whereas the other half did not. These observations might be explained by the dual role of IL-15 on intestinal inflammation. On the one hand, IL-15 is highly expressed in the inflamed mucosa of IBD patients and stimulates proliferation and activation of T cells and their production of inflammatory cytokines such as TNF α and IFN γ .⁶¹ Hence, IL-15 deficient mice are less sensitive to both acute and chronic colitis after DSS administration.³¹ On the other hand, it has been shown that IL-15 protects intestinal epithelial cells from apoptosis and consequently, neutralization of IL-15 could aggravate epithelial damage, cell death, and the severity of the disease.⁶² In our model, no modifications of peripheral subpopulations of T cells and cytokines levels in blood were observed after DSS administration or CRB-15 treatment. In contrast, CRB-15 enhanced the proportion of $\gamma\delta$ T cells in the small intestinal epithelium. Previous reports indicate that large numbers of $\gamma\delta$ T cells are present in the intestinal epithelium after DSS administration to improve tissue repair, whereas $\gamma\delta$ T cells deficient mice are more sensitive to mucosal damage induced by DSS.⁶³ Although $\gamma\delta$ T cells were also present in the colon epithelium, conclusions from our work are limited by the absence of analysis of T-cell subpopulations directly in the colon.

In conclusion, these results highlight the functions of IL-15 on the inhibition of bone formation and stimulation of osteoclastogenesis in IBD. In turn, they provide evidence that IL-15 blockade by CRB-15 delays bone loss, whereas improves the clinical manifestations and survival on DSS-induced chronic colitis. However, the systemic and skeletal benefits of CRB-15 were limited and did not entirely correlate with its effects on colon inflammation and damage, which were heterogeneous. These observations confirm and extend the role of IL-15 on bone remodeling and may support the development of IL-15 antagonists for treatment in IBD.

Acknowledgments

We thank the excellent technical work of Juliette Cicchini, Madeleine Lachize, Casimir de Rham, Joël Pimenta, Delphine Mouron, and Catherine Pythoud.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.02.033>.

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