Intestinal Inflammation Promotes MDL-1+ Osteoclast Precursor Expansion to Trigger Osteoclastogenesis and Bone Loss

Christopher T. Peek,1 Caleb A. Ford,2 Kara R. Eichelberger,3 Justin Jacobse,1,4 Teresa P. Torres,1 Damian Maseda,1,10 Yvonne L. Latour,1 M. Blanca Piazuelo,4,6 Joshua R. Johnson,9 Mariana X. Byndloss,1,5 Keith T. Wilson,1,4,5,6,7 Jeffrey C. Rathmell,1,5,6,8 Jeremy A. Goettel,1,4,5,6 and James E. Cassat1,2,3,5,6,8,9

1Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee; 2Department of Biomedical Engineering, Vanderbilt University, Nashville, Tennessee; 3Department of Pediatrics, Division of Pediatric Infectious Diseases, Vanderbilt University Medical Center, Nashville, Tennessee; 4Division of Gastroenterology, Hepatology, and Nutrition, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; 5Vanderbilt Institute for Infection, Immunology, and Inflammation, Vanderbilt University Medical Center, Nashville, Tennessee; 6Center for Mucosal Inflammation and Cancer, Vanderbilt University Medical Center, Nashville, Tennessee; 7Veterans Affairs Tennessee Valley Healthcare System, Nashville, Tennessee; 8Vanderbilt Center for Immunobiology, Vanderbilt University Medical Center, Nashville, Tennessee; 9Vanderbilt Center for Bone Biology, Vanderbilt University Medical Center, Nashville, Tennessee; 10Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania

SUMMARY

This work utilizes multiple models of intestinal inflammation to demonstrate a pathologic expansion of osteoclast precursors associated with bone loss during intestinal inflammation. Data from this study highlight the feasibility of targeting a pro-osteoclastogenic co-receptor to ameliorate colitis-associated bone loss.

BACKGROUND & AIMS: Inflammatory bowel disease (IBD) is characterized by severe gastrointestinal inflammation, but many patients experience extra-intestinal disease. Bone loss is one common extra-intestinal manifestation of IBD that occurs through dysregulated interactions between osteoclasts and osteoblasts. Systemic inflammation has been postulated to contribute to bone loss, but the specific pathologic mechanisms have not yet been fully elucidated. We hypothesized that intestinal inflammation leads to bone loss through increased abundance and altered function of osteoclast progenitors.

METHODS: We used chemical, T cell driven, and infectious models of intestinal inflammation to determine the impact of intestinal inflammation on bone volume, the skeletal cytokine environment, and the cellular changes to pre-osteoclast populations within bone marrow. Additionally, we evaluated the potential for monoclonal antibody treatment against an inflammation-induced osteoclast co-receptor, myeloid DNAX activation protein 12-associating lectin-1 (MDL-1) to reduce bone loss during colitis.

RESULTS: We observed significant bone loss across all models of intestinal inflammation. Bone loss was associated with an increase in pro-osteoclastogenic cytokines within the bone and an expansion of a specific Cd11b+/Ly6Chi osteoclast precursor.
Inflammatory bowel disease (IBD) is hallmarked by severe gastrointestinal inflammation that is mediated, in part, by aberrant innate and adaptive immune responses. Additionally, the inflammation that patients with Crohn’s disease and ulcerative colitis experience frequently impacts extra-intestinal sites. Up to 40% of patients with IBD experience extra-intestinal symptoms, which can occur in nearly every tissue type before, concurrent with, or after the onset of colitis.\(^1\) Musculoskeletal disease is among the most common extra-intestinal manifestations of IBD and ultimately confers a 40% increased risk of osteoporotic fracture compared with the general population.\(^4\) Although nutritional status and medication use are potential contributors to colitis-associated bone loss, accumulating evidence supports a crucial role for inflammation in driving bone loss during IBD. For example, patients with normal vitamin D and calcium levels can still experience IBD-associated bone loss, as can patients who have not yet started IBD treatment.\(^5,6\) Moreover, cytokine blocking therapy partially prevents bone loss in patients with IBD and correlates with improved serum markers of bone resorption.\(^7,8\) One potential mechanism by which IBD-associated inflammation triggers bone loss is through the actions of inflammatory cytokines on skeletal cells.

The goal of this study was to further define how gastrointestinal inflammation leads to bone loss through impacts on osteoclasts. Osteoclasts are multi-nucleated, myeloid lineage cells capable of resorbing bone. Osteoclast differentiation and survival require receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) signaling and are further regulated by the RANKL decoy receptor, osteoprotegerin (OPG). Multiple myeloid cell types across various tissue sources can differentiate into osteoclasts in the presence of M-CSF and RANKL. Cells capable of differentiating into osteoclasts include LSK (lineage negative, Sca-1\(^+\), c-Kit\(^+\)) hematopoietic stem cells (HSCs), monocytes, and dendritic cells.\(^9-12\) Patients with IBD-associated bone loss exhibit altered RANKL/OPG levels and elevated serum markers of bone turnover, suggesting that osteoclast differentiation and activity are increased in this patient population.\(^6,13\)

Importantly, the isolated binding of M-CSF and RANKL to their cognate receptors is insufficient to induce osteoclast formation in vivo or in vitro without the coordinated signaling of several osteoclast co-receptors.\(^14\) Activating co-receptors promote osteoclast formation through intracellular immunoreceptor tyrosine-based activation motif (ITAM)-signaling adaptors.\(^14\) Intracellular ITAM-signaling adaptors, such as DNAX activation protein 12 (DAP-12) or FcεR1 gamma chain, pair with receptors capable of binding membrane-bound and extracellular ligands to transduce ITAM activation and trigger calcium fluxes that initiate osteoclast formation.\(^15\) Myeloid DAP-12-associating lectin-1 (MDL-1; also known as C-type lectin domain family 5, member A or CLEC5A) and triggering receptor expressed on myeloid cells 2 (TREM2) are examples of DAP-12-associating osteoclast co-receptors, whereas FcεR1 gamma chain-associated co-receptors include osteoclast activating receptor (OSCAR) and paired-immunoglobulin-like receptor A (PIR-A). Although some ligand-receptor pairs have been identified for these osteoclast co-receptors, many remain poorly described.

Osteoclasts and their precursors are exquisitely sensitive to inflammatory cytokines (eg, interleukin [IL]-1 and tumor necrosis factor-alpha [TNF-\(\alpha\)]), chemokines (eg, spondysine-1-phosphate and CCL-2), and colony stimulating factors (eg, granulocyte colony-stimulating factor [G-CSF]) that can further modulate osteoclast differentiation and function.\(^16-21\) The sensitivity of osteoclasts to the surrounding cytokine milieu is in part driven by cross regulation of ITAM-associated osteoclast co-receptors. G-CSF, IL-23, and TNF-\(\alpha\) have been previously reported to enhance MDL-1 expression on myeloid cells and increase osteoclast formation in vitro and in vivo.\(^12,22-24\) Activation of MDL-1 on myeloid cells, in turn, results in the robust release of RANTES/CCL-5, CXCL-10, TNF-\(\alpha\), and G-CSF.\(^22,23,25,26\) Several of these osteoclastogenic cytokines are crucial to both the intestinal and extra-intestinal pathogenesis of IBD.\(^27-29\)

Previous studies have examined bone loss in murine models of colitis, including chemical injury, genetic, T cell
mediated, and infection models. However, the mechanisms by which gastrointestinal inflammation promotes bone loss through effects on skeletal cells are still being uncovered. We hypothesized that changes in osteoclast progenitor populations during intestinal inflammation contribute to IBD-associated bone loss. Using chemical injury, infectious, and adoptive T cell transfer models of gastrointestinal inflammation, we discovered that an increase in the surface expression of the osteoclast co-receptor MDL-1 on Cd11b<sup>-/-</sup>Ly6Ch<sup>i</sup> osteoclast precursors (OCPs) is associated with enhanced osteoclastogenesis and significant bone loss during colitis. Anti-MDL-1 antibody treatment was sufficient to blunt ex vivo enhanced osteoclast formation in OCPs derived from mice with chemical colitis. Finally, in vivo, anti-MDL-1 was effective in limiting bone loss during chemically induced colitis.

**Results**

**Intestinal Inflammation Results in Trabecular Bone Loss**

To investigate bone loss during colitis, we first induced gastrointestinal inflammation via chemical injury with dextran sulfate sodium (DSS). Mice were given 3% DSS orally for 1 week followed by a 1-week recovery period. As expected, DSS treatment led to weight loss and colonic injury (Figure 1, A–B). We evaluated bone loss using micro-computed tomography (micro CT). DSS-treated mice demonstrated a 37% reduction (P < .0001) in trabecular bone volume over total volume (BV/TV) 14 days following DSS treatment compared with controls (Figure 1, C–D). Trabecular bone loss was associated with a significant reduction in trabecular bone thickness (P < .0001), increased trabecular separation (P < .01), loss of trabeculae (P < .05), and reduced connective density (P < .05) (Figure 1, E–H). To evaluate if this bone loss was preceded by changes in the number of osteoclasts, we performed histomorphometry on tartrate-resistant acid phosphatase (TRAP)-stained femur sections. Histologically, we observed a significant increase in osteoclast number per bone surface (P < .05), but not osteoclast surface per bone surface (P = .06) at day 7 following DSS administration (Figure 1, I–K). These results indicate that trabecular bone loss occurs during a chemical injury model of intestinal inflammation, and that an increase in osteoclast number precedes trabecular bone loss.

Next, we tested whether alternative models of intestinal inflammation are associated with bone loss. First, we utilized an adoptive T cell transfer (ACT) model of colitis where Rag1<sup>−/−</sup> mice are injected with naïve CD4<sup>+</sup>CD25<sup>−</sup>CD45RB<sup>hi</sup> T cells and subsequently develop colitis. Mice subjected to ACT experienced colonic injury and bone loss, with a 65% reduction in trabecular BV/TV (P < .001) (Figure 2, A and Figure 3, A). We subsequently investigated if 2 infectious models of intestinal inflammation were associated with bone loss. Mice subjected to infectious colitis following inoculation of *Citrobacter rodentium* developed intestinal inflammation, bacterial colonization, and significant trabecular bone loss (P < .05) compared with mock infection (Figure 2, B and Figure 3, B). We next infected mice with either wild-type (WT) *Salmonella enterica* subspecies *enterica* serovar Typhimurium (STm), or an attenuated STm mutant, ΔinvA::tetRA ΔspiB::KSAC (invA spiB), that lacks functional type III secretion systems 1 and 2 (T3SS1 and T3SS2) and does not induce robust intestinal inflammation. We observed that mice infected with WT STm developed cecal injury, bacterial colonization, and significant trabecular bone loss (P < .01) compared with infection with invA spiB (Figure 2, C and Figure 3, C). Importantly, both *C. rodentium* and WT STm infection led to bacterial colonization, intestinal inflammation, and bone loss in the absence of significant changes in weight over the course of the infection (Figure 2, B–C). Taken together, these data indicate that trabecular bone loss occurs during T cell driven and infectious models of intestinal inflammation. The data further show that bone loss during STm-infectious colitis is driven by pathogen virulence programs that promote intestinal inflammation.

**Intestinal Inflammation Alters Cytokine Abundance Within the Bone**

We hypothesized that bone loss during intestinal inflammation was mediated, in part, by alterations in the cytokine milieu within the bone microenvironment. To test this hypothesis, we performed multiplexed cytokine analysis on homogenized femurs from mice with or without colitis. We observed significant increases in several chemokines and inflammatory cytokines, including G-CSF, TNF-α, IL-12p40, MCP-1/CCL-2, RANTES/CCL-5, and keratinocyte-derived chemokine/CXCL1 across multiple colitis models (Figure 3, D and Figure 4). Notably, G-CSF, IL-12p40, and TNF-α were among the most significantly increased cytokines across DSS, ACT, *Citrobacter rodentium*, and STm-induced colitis (Figure 3, D). Additional cytokines, including IL-23 and CCL-20, were also significantly elevated during DSS-induced colitis (Figure 4). These data suggest that cells within the murine bone environment experience an altered cytokine milieu in the setting of intestinal inflammation.

**Intestinal Inflammation Results in the Expansion of Pre-osteoclast Populations**

Because we observed that multiple models of intestinal inflammation dramatically increased G-CSF, which potently regulates myelopoiesis, we evaluated changes in myeloid-derived osteoclast progenitor populations during colitis. Specifically, we examined LSK HSCs, Cd11b<sup>−/−</sup>Ly6Ch<sup>i</sup> OCPs, and monocytes. Monocytes and LSK cells were gated according to Figure 5. OCPs were gated as demonstrated in Figure 6, A. We observed a significant increase in the relative abundance of monocytes, LSKs, and OCPs within the bone marrow across chemical and infectious models of intestinal inflammation (Figure 7, A–C). During ACT-induced intestinal inflammation, we observed an increase in LSK and OCPs, but not monocytes (Figure 7, D). Collectively, these data reveal that intestinal inflammation drives an expansion of pre-osteoclast populations.
Figure 1. DSS colitis leads to trabecular bone loss. Mice were administered 3% DSS (closed circles) or water (control, open circles) for 7 days, after which time DSS was switched to water. (A) Percent baseline weight of mice with or without DSS colitis. (B) Histologic injury score of H&E-stained colons on days 7 and 14 following DSS administration. (C–H) Micro CT analysis of control (open circles) and DSS-treated (closed circles) formalin-fixed femurs for trabecular BV/TV (C) representative 3-dimensional reconstruction of trabecular bone from mice with or without colitis at day 14 (D) trabecular thickness (E) trabecular number (G) and connective density (H) at day 7 or 14 following DSS treatment. Scale bar = 100 μm. (I–K) Fixed femurs were paraffin-embedded, sectioned, and stained for TRAP. (I) Representative image of TRAP-stained femurs from mice with or without colitis at day 7 (original magnification = 10×). (J) Number of osteoclasts per bone surface (N.Oc/BS). (K) Osteoclast surface per bone surface (Oc.S/BS). Error bars represent mean ± standard error of the mean. Data analyzed via 2-way ANOVA with Sidák multiple comparisons test. n = 5–18 mice per group. Post-hoc tests were only performed for significant (P < .05) ANOVAs. For (J) data analysis by 2-way ANOVA indicated that only DSS treatment (P = .02), but not time (P = .4) or interaction of these variables (P = .09) was significant. Post-hoc Sidák’s multiple comparisons test demonstrated an adjusted P = .02 at day 7 between control and DSS treated mice. For (K) by 2-way ANOVA, only DSS treatment (P = .04), but not time (P = .08), or interaction of these variables (P = .21) was significant. Post-hoc Sidák’s multiple comparisons test demonstrated an adjusted P = .06 at day 7 between control and DSS-treated mice. *P < .05; **P < .01; ****P < .0001.
Osteoclast Precursors Display Altered Surface Expression of Receptors Involved in Osteoclast Trafficking, Differentiation, and Function During DSS Colitis

Previous work has shown that the Cd11b−/−Ly6Chi OCP population is highly osteoclastogenic, has distinct surface cytokine receptor expression patterns compared with other myeloid cells, expresses receptors associated with osteoclast differentiation and function, and suppresses T cells. Therefore, we next determined if intestinal inflammation alters the surface expression of receptors important in osteoclast trafficking, differentiation, and function in OCPs from mice with and without DSS colitis. We focused on early time points after DSS administration, given that we...
observed an increase in osteoclast formation in vivo prior to trabecular bone loss (Figure 1, I–K). Because we observed significant increases in the abundance of MCP-1/CCL-2 in the femurs of mice treated with DSS prior to bone loss (Figure 4, C), we first examined the expression of the CCL-2 receptor, CCR2. We found that OCPs exhibited increased CCR2 expression \( (P < .0001) \) compared with controls (Figure 6, B). Given that CCL-2/CCR2 signaling governs monocyte egress to sites of inflammation, such as the inflamed intestine, we next examined the expression of the tethering factor, CXCR4, which promotes retention of Ly6C\(^{hi}\) monocytes within the bone marrow.\(^{48}\) OCPs from mice with DSS colitis also demonstrated increased CXCR4 expression \( (P < .001) \) compared with control mice (Figure 6, C). Next, we measured surface expression of receptors that govern osteoclast differentiation. We detected a significant decrease in RANK expression \( (P < .01) \) but not CSF1R \( (P = .053) \) (Figure 6, D–E). Because we observed significant increases in several MDL-1-associated cytokines (eg, G-CSF, TNF-\(\alpha\), IL-23, and MCP-1/CCL2) in the femurs of mice with colitis, we evaluated MDL-1 expression on OCPs during DSS treatment (Figure 3, D and Figure 4).\(^{22,24,26}\) OCP

Figure 3. Multiple murine models of intestinal inflammation lead to bone loss and alter the bone marrow cytokine milieu. Mice were subjected to ACT, *Citrobacter rodentium* infection, or STm infection prior to harvest of femurs for micro CT analysis. (A) *Rag1*\(^{-/-}\) mice were administered \( 5.0 \times 10^5 \) CD4\(^{+}\) CD25\(^{-}\)CD45RB\(^{hi}\) T cells (Tnaive) or PBS via intraperitoneal injection. (B) C57BL/6J mice were mock-infected with Luria broth (mock) or infected with \( 5.0 \times 10^6 \) CFUs *Citrobacter rodentium* (Citro) via oral gavage. (C) CBA/J mice were infected with \( 1.0 \times 10^9 \) CFUs of either WT STm strain IR715 (WT) or an invA spiB mutant via oral gavage. (D) Femurs from mice with or without intestinal inflammation were homogenized at the indicated time point and assessed for cytokine abundance via Luminex profiling. For DSS colitis, mice were administered 3% DSS for 7 days followed by 7 days of recovery with water. Heat map of cytokine abundance displayed as Log2 fold change of mice with colitis relative to respective non-colitis controls. Error bars represent mean ± standard error of the mean. Data analyzed via Student t test. \( n = 4–10 \) mice per group. *\( P < .05\); **\( P < .01\); ***\( P < .001\).
Figure 4. DSS colitis increases the abundance of MDL-1-associated cytokines. (A–I) Related to Figure 3, D. Mice were administered water (control, open circles) or 3% DSS (closed circles) for 7 days followed by 7 days of recovery with water. At the indicated time point, femurs from mice with or without intestinal inflammation were homogenized and assessed for cytokine abundance via Luminex profiling. Values were normalized to total protein as measured by a Pierce bicinchoninic acid (BCA) protein assay. Cytokines that were significantly elevated during colitis are shown. (A) G-CSF; (B) TNF-α; (C) MCP-1/CCL-2; (D) RANTES/CCL-5; (E) KC/CXCL1; (F) IL-12p40; (G) IL-12p70; (H) M-CSF; (I) VEGF. (J–L) A separate cohort of mice were administered water (control, open circles) or 3% DSS (closed circles) for 7 days followed by 7 days of recovery with water and assessed for Th17-related cytokine abundance via Luminex profiling and normalized to total protein as measured by a Pierce BCA protein assay. Cytokines that were significantly elevated during colitis are shown. (J) IL-23; (K) MIP-3α/CCL-20; and (L) TNF-α. Error bars represent mean ± standard error of the mean. Data analyzed via 2-way ANOVA with Sidák multiple comparisons test. Post-hoc tests were only performed for significant (P < .05) ANOVAs. n = 5–10 mice per group. *P < .05; **P < .01; ***P < .001; ****P < .0001.
MDL-1 expression and the percentage of MDL-1⁺ OCPs were significantly increased (P < .001 and P < .001, respectively) during DSS colitis (Figure 6, F–H). To determine if the enhanced OCP MDL-1 expression was dependent on mature B or T cells, we administered 3% DSS to Rag1⁻/⁻, which lack mature B and T lymphocytes. MDL-1 expression on OCPs and percentage of MDL-1⁺ OCPs was also significantly increased in Rag1⁻/⁻ mice given DSS (P < .0001 and P < .001) (Figure 6, I–K). To evaluate additional pro-osteoclastogenic co-receptors on OCPs during DSS colitis in WT mice, we measured surface expression of OSCAR, PIR-A/B, and TREM2. We noted similar increases in additional pro-osteoclastogenic co-receptors including OSCAR, PIR-A/B, and TREM2 (Figure 8, A–B). Increased OCP proliferation may represent one mechanism of increased osteoclast formation in vivo, as previous work has demonstrated that OCPs proliferate ex vivo in response to M-CSF.⁴⁷ To test the hypothesis that increased cellular proliferation may contribute to the increased osteoclast formation in vivo during intestinal inflammation, we evaluated intracellular Ki67 levels in OCPs from mice with or without DSS colitis. DSS treatment significantly increased Ki67 MFI as determined by analysis of variance [ANOVA] P < .05) but not percent positive Ki67 OCPs [ANOVA P = .09] during DSS colitis (Figure 8, C–D). When correcting for multiple comparisons, however, no significant difference in Ki67 MFI at days 1 or 3 was observed following DSS treatment in comparison to control mice. These results suggest that OCPs may proliferate early in DSS colitis. Collectively, these data highlight that OCPs derived from mice subjected to DSS-induced intestinal inflammation demonstrate changes in surface expression patterns of cytokine, chemokine, and osteoclast co-receptors associated with inflammation and enhanced osteoclastogenesis.

Anti-MDL-1 Antibody Treatment Blunts the Osteoclastogenic Potential of OCPs From Mice With Colitis and Ameliorates Colitis-associated Bone Loss

Given the increase in surface receptors associated with osteoclast differentiation and function, we next tested whether OCPs from mice with colitis are more prone to forming osteoclasts than those derived from healthy controls. To test this hypothesis, we sorted purified Cd11b⁻/⁻Ly6C⁰ OCPs, Cd11b⁺Ly6C⁺ monocytes, or Cd11b⁻Ly6C⁻ double-negative cells from the bone marrow of mice with or without DSS colitis and cultured these cells in the presence of exogenous M-CSF and RANKL. We observed that DSS treatment increased the ex vivo osteoclastogenic potential of purified monocytes, OCPs, and unsorted whole bone marrow (input) (Figure 9, A). OCPs displayed the highest osteoclastogenic potential (Figure 9, A). Because the surface expression of the pro-osteoclastogenic coreceptor MDL-1 was increased on DSS-derived OCPs, we assessed whether ex vivo anti-MDL-1 antibody treatment could blunt the enhanced osteoclast formation potential of these cells. In comparison to isotype control, treatment with anti-MDL-1 antibody abrogated the increased osteoclast formation of OCPs isolated from DSS-treated mice (P < .0001) (Figure 9, A).
Figure 6. OCPs isolated from mice with DSS colitis demonstrate altered expression of receptors involved in osteoclast differentiation and function. C57BL/6J (A–H) or Rag1−/− (I–K) mice were administered 3% DSS or water (control) for 7 days, after which bone marrow was processed for flow cytometry. (A) Gating strategy for Cd11b−/−Ly6C+ OCPs. (B–F) Mean fluorescence intensity (MFI) of surface markers related to osteoclast differentiation and function from C57BL/6J mice with (filled circles) or without (open circles) colitis including CCR2 (B) CXCR4 (C) colony stimulating factor 1 receptor (CSF1R) (D) receptor activator of nuclear factor kappa-B (RANK) (E) and MDL-1 (F). (G) Percent positive MDL-1 OCPs. (H) Representative histogram of MDL-1 distribution among OCPs. (I) OCP MDL-1 MFI, percent MDL-1+ OCPs (J) and representative histogram of MDL-1 staining (K) on OCPs derived from Rag1−/− mice with or without colitis. FMO, Fluorescence minus one control. Error bars represent mean ± standard error of the mean. Data analyzed via Student t test. n = 4–7 mice per group. **P < .05; ***P < .001; ****P < .0001.
Because anti-MDL-1 antibody could blunt ex vivo osteoclastogenesis of OCPs harvested from mice with colitis, we tested if this antibody could reduce bone loss in vivo in mice with experimental colitis. We first determined if anti-MDL-1 impacted baseline trabecular bone architecture in healthy control mice. No differences were observed in weight or trabecular bone parameters in mice receiving a single injection of anti-MDL-1 compared with IgG isotype.
We next evaluated whether in vivo anti-MDL-1 antibody treatment can reduce bone loss during DSS colitis. Because we observed an increase in OCP MDL-1 expression as early as day 3 following DSS administration (Figure 8, A–B), we administered monoclonal anti-MDL-1 antibody or IgG isotype control treatment to DSS-treated mice at day 3 following colitis induction (Figure 9, B). Anti-MDL-1 treated mice demonstrated no significant differences in histologic injury and demonstrated similar weight changes compared with isotype IgG control treated mice during colitis (Figure 9, C and E). Mice treated with anti-MDL-1 demonstrated a 27% increase in BV/TV compared with mice treated with isotype control (P < .05), which was associated with significantly increased trabecular thickness (P < .05) (Figure 9, F–K).

**Discussion**

The data from this study demonstrate that multiple murine models of IBD result in concomitant bone loss. Significant increases in the relative abundance of a highly osteoclastogenic pre-osteoclast population were observed across 4 models of intestinal inflammation. Furthermore, this study reveals that during DSS colitis, OCPs demonstrate altered cytokine-, chemokine-, and osteoclast-associated receptors, as compared with OCPs from control mice. The
changes to OCP surface receptor expression also occurred in Rag1−/− mice, suggesting that both the expansion and alteration of OCPs during DSS colitis are not solely dependent on interactions with inflammatory T cells, B cells, or their cytokines. OCPs from mice with colitis demonstrate increased osteoclast formation ex vivo when compared with OCPs derived from control mice, and this enhanced osteoclastogenesis is blunted by anti-MDL-1 antibody treatment. In vivo, a single dose of anti-MDL-1 antibody reduced bone loss during DSS colitis. Collectively, these data reveal that OCPs derived from mice with intestinal inflammation have enhanced osteoclastogenesis ex vivo, that this increased osteoclast formation is associated with increased MDL-1 surface expression on OCPs, and that in vivo anti-MDL-1 antibody treatment can ameliorate bone loss during DSS colitis.

We observed marked increases in cytokines and chemokines capable of inducing MDL-1 expression, most
notably G-CSF, in the femurs of mice subjected to models of colitis. This increase in MLD-1-associated cytokines corresponds with a robust increase in expression of MLD-1 on OCPs. Recent evidence has linked MLD-1 with IBD pathogenesis, as MLD-1\(^+\) TNF-α\(^+\) monocytes have been found to be significantly increased in the lamina propria of patients with IBD.\(^{49}\) Single-cell and bulk-RNA sequencing studies have additionally highlighted increases in MLD-1 (CLECSA) expression in inflamed intestinal tissue compared with control-matched non-inflamed tissue.\(^{50,51}\) Although human and murine correlates of bone marrow OCPs are poorly defined, bulk-RNA sequencing from human PBMCs has also revealed increases in CLECSA expression in patients with IBD compared with age and sex-matched controls.\(^{52}\) Furthermore, CLECSA expression has been identified by single-cell RNA-seq as a gene associated with an inflammatory monocyte cytokine module within the Risk Stratification and Identification of Immunogenetic and Microbial Markers of Rapid Disease Progression in Children with Crohn’s Disease (RISK) study.\(^{53}\)

In addition to mediating inflammatory responses, MLD-1 plays an important role in osteoclast formation and regulates osteoclastogenesis by forming a trimolecular complex with DAP-10 and DAP-12.\(^{54}\) Previous work has demonstrated that OCPs express MLD-1, but studies to date have not examined how MLD-1 or other co-receptors involved in osteoclast differentiation and function change during intestinal inflammation.\(^{47}\) MLD-1 has been reported to form a complex with the IL-23R in human monocytes, and IL-23 treatment of human monocytes leads to both osteoclast differentiation and increased MLD-1 expression.\(^{24}\) Although we did not evaluate OCPs from human bone marrow, these prior studies utilizing human monocytes suggest that MLD-1\(^+\) OCPs could potentiate IBD-associated bone loss in humans. Taken together, these observations suggest a mechanism whereby specific inflammatory cytokines associated with IBD pathogenesis impact the expression of osteoclast coreceptors on OCPs to drive osteoclastogenesis and bone loss.

In addition to increased MLD-1 expression, data from this study also demonstrate that OCPs exhibit increased expression of surface molecules important in osteoclast trafficking (CCR2 and CXCR4) during colitis. Elevated CCR2 may reflect that OCPs during colitis are primed to exit the bone marrow environment and traffic to the inflamed intestine. However, OCPs expressed higher levels of the tethering factor, CXCR4, indicating that these cells might instead be more tightly tethered to the bone marrow environment or contribute to replenishing monocyte pools.\(^{50}\) Although G-CSF, which was significantly elevated in the bone across all models of colitis, has been shown to reduce CXCR4 expression in Gr-1\(^+\) myeloid cells as a mechanism of mobilization, it conversely increases CXCR4 expression on HSCs.\(^{55,56}\) OCPs were originally reported to retain clonogenic potential as measured by colony formation.\(^{47}\) Therefore, G-CSF may induce CXCR4 expression in OCPs in a manner similar to that previously reported for HSCs. Future studies are needed to investigate how intestinal inflammation alters the tethering and mobilization properties of OCPs.

Although an expansion of OCPs represents one potential mechanism for increased osteoclastogenesis during DSS colitis, this study also demonstrates that colitis alters expression of surface receptors that directly govern osteoclast differentiation and function (RANK, CSF1R, MLD-1, OSCAR, TREM2, and PIR-A/B). OCPs from mice with DSS colitis demonstrated a subtle but statistically significant reduction in RANK expression. The functional consequences of reduced RANK are unclear, although DSS-derived OCPs maintained the ability to form robust osteoclasts ex vivo in response to treatment with respective ligands RANKL and M-CSF. TNF-α, which was increased in the femur homogenates across multiple murine models of colitis, has previously been shown to induce both OSCAR and PIR-A in addition to MLD-1.\(^{57,58}\) Accordingly, in addition to an increase in abundance of OCPs, enhanced sensitivity to pro-osteoclastogenic signals is likely a complementary mechanism that contributes to increased osteoclastogenesis and bone loss during DSS colitis.

Figure 9. (See previous page). Anti-MLD-1 antibody treatment blunts ex vivo osteoclastogenesis and protects against colitis-associated bone loss in vivo. (A) C57BL/6J mice were administered either 3% DSS or water (control) for 7 days. Bone marrow was isolated on day 7 following DSS treatment from the long bones of mice with or without colitis and processed for FACs. Unsorted whole bone marrow (input), CD11b\(^+\) Ly6C\(^+\) monocytes, CD11b\(^+\)Ly6G\(^+\) OCPs, or CD11b\(^+\)Ly6C\(^-\) (double negative) cells were seeded at either 5.0 \(\times\) 10\(^4\) (input) or 1.0 \(\times\) 10\(^4\) cells/mL (sorted), differentiated for 4 days with 5% v/v CMG14-12 supernatant and 35 ng/mL RANKL in the presence or absence of 10 \(\mu\)g/mL IgG isotype control or anti-MLD-1 antibody. After 4 days, cells were stained for TRAP, and multi-nucleated TRAP formation was manually enumerated. (B) Experimental design for in vivo anti-MLD-1 antibody treatment. C57BL/6J mice were administered 3% DSS or water (control) for 7 days, after which time mice were switched to water. At day 3 following DSS administration, mice received a single dose of 50 \(\mu\)g of either IgG isotype control or anti-MLD-1 antibody via intraperitoneal injection. Colons and femurs were harvested at day 14 following DSS administration. Created with BioRender.com. (C) Percent baseline weight of DSS-treated mice receiving either isotype control or anti-MLD-1 antibody. Arrow indicates time of IgG isotype or anti-MLD-1 antibody. (D) Micro CT analysis of formalin-fixed femurs from mice receiving either isotype or anti-MLD-1 antibody for trabecular BV/TV. (E) Histologic injury score of H&E sections from DSS-treated mice receiving either isotype control or anti-MLD-1 antibody. (F–K) Micro CT analysis of formalin-fixed femurs from DSS-treated mice receiving either isotype or anti-MLD-1 antibody for trabecular BV/TV (F) trabecular thickness (G) trabecular separation (H) trabecular number (I) connective density (J) and representative 3-dimensional reconstruction of trabecular bone from mice treated with either isotype or anti-MLD-1 antibody (K). Scale bar = 100 \(\mu\)m. Error bars represent mean \pm standard error of the mean. Data analyzed via 2-way ANOVA with Sidak multiple comparisons test for comparisons within each sorted population (A) or Student t test (D–J). Post-hoc tests were only performed for significant \((P < .05)\) ANOVAs. n = 3 independent cultures (A) 5 mice (controls, C–D), or 20 mice per group (DSS; C, E–J). * \(P < .05\); ** \(P < .01\); *** \(P < .001\); **** \(P < .0001\).
In this study, DSS colitis led to an increase in osteoclast formation in purified monocyte and OCP populations ex vivo. OCPs from DSS-treated mice displayed similar Ki67 levels as controls at day 7 following DSS administration. Therefore, cell cycling prior to exogenous M-CSF and RANKL administration likely does not explain the differences in osteoclast formation ex vivo. However, Ki67 MFI significantly increased during DSS administration, and this was most pronounced at early time points following DSS treatment. But these data did not reach statistical significance following correction for multiple comparisons, and therefore, additional experiments are necessary to test if increased cell cycling at days 1 and 3 following DSS colitis contributes to the expansion of OCPs.

There are some limitations of this work that will drive future studies. Because the goal of this study was to evaluate how intestinal inflammation alters osteoclasts and their precursors, we did not examine other important contributors to bone loss during IBD, such as nutrition and alterations to osteoblast biology. Therefore, these data do not exclude additional complementary mechanisms of IBD-associated bone loss that occur through nutritional deficits or altered osteoblast function, which have been highlighted in previous studies.31–33.59–62 We did observe bone loss in colitis models that were not associated with significant weight loss, which is consistent with previous reports and may point to mechanisms that are independent of nutritional status.33 Future studies should address the relative contributions of OCPs, nutritional status, and osteoblast function in contributing to bone loss during intestinal inflammation. Although several important osteoclast precursors were found to expand during colitis, this study did not evaluate alternative cell lineages (eg, dendritic cells) capable of osteoclastogenesis, and therefore cannot exclude their contribution to bone loss during intestinal inflammation. Anti-MDL-1 antibody treatment reduced osteoclast formation in OCPs isolated from DSS-treated mice ex vivo. Moreover, treating mice with a single dose of anti-MDL-1 antibody decreased bone loss in vivo during DSS colitis and did not significantly impact the severity of colitis. The ideal IBD therapeutic would not only target bone loss, but also treat the underlying intestinal inflammation to prevent inflammatory bone loss from occurring. However, targeting the MDL-1 axis to ameliorate bone loss during IBD may still be clinically efficacious in those patients who experience bone loss or non-healing fractures despite maximal intestinal-targeted therapy. Future studies should evaluate if MDL-1 blockade is effective in reducing IBD-associated bone loss when combined with standard of care therapy, such as anti-cytokine monoclonal antibodies. Future investigations should also test if anti-MDL-1 antibody treatment ameliorates bone loss in other models of intestinal inflammation. Furthermore, the generation of cell-specific knockouts of MDL-1 will also help to clarify cell-intrinsic versus cell-extrinsic roles of MDL-1 in colitis-associated bone loss. Previous work has demonstrated that blockade of MDL-1 prevents inflammatory-mediated bone loss in a murine model of inflammatory arthritis without impacting baseline bone formation, and we similarly did not observe an impact of anti-MDL-1 treatment on baseline bone parameters.23 Endogenous ligands for many osteoclast coreceptors, including MDL-1, remain poorly described. Deciphering the precise ligands that MDL-1 binds and understanding how monoclonal antibodies may disrupt these interactions remains an important area for future research. Ligands for DAP-12 associated receptors, such as MDL-1, are thought to be expressed on the same myeloid OCP populations.14 Additional work is also needed to evaluate MDL-1 expression on OCPs derived from humans with active vs quiescent IBD. Overall, this study demonstrates that intestinal inflammation significantly alters pre-osteoclast populations and implicates the osteoclast co-receptor, MDL-1, as a key mediator of bone loss during colitis.

Materials and Methods

Ethics Statement
All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center on the animal protocol M1600147-01. All experiments were conducted in accordance with National Institutes of Health guidelines, the Animal Welfare Act, and United States Federal Law, and performed with institutional biosafety committee approval. Mice were euthanized by CO2 asphyxiation with secondary confirmation by cervical dislocation and observation of heart rate and breathing. All authors had access to the study data and reviewed and approved the final manuscript.

Animal Use
Male and female 7- to 9-week-old C57BL/6J (Stock # 000664), CBA/J (Stock # 000656), and Rag1–/– (Stock # 002216) mice were purchased through The Jackson Laboratory and maintained in our colony for 1 week prior to experimentation. C57BL/6J, CBA/J, and Rag1–/– mice were bred homozygously. C57BL/6J and CBA/J mice were maintained in specific pathogen-free conditions. Rag1–/– mice were maintained in sterile conditions with autoclaved food and bedding prior to adoptive T cell transfer, after which they were switched to specific pathogen-free conditions.

Mouse Models of Colitis
For experiments involving DSS colitis, 3% DSS (TdB Labs) was administered ad libitum in the sterile drinking water of 7-week-old male or female WT or Rag1–/– mice for 1 week, after which time mice were switched back to sterile drinking water. Mice were euthanized at days 1, 3, 7, or 14 following the initiation of DSS treatment. Where indicated, mice were treated with 50 μg of either anti-MDL-1 (R&D, clone 226402) or IgG2a isotype control (R&D clone 54447) at day 3 following DSS-treatment or in control mice not treated with DSS. Mice were monitored for signs of clinical disease and weighed daily. For experiments involving adoptive T cell transfer, colitis was induced in male or female C57BL/6J Rag1–/– mice by adoptive transfer of sorted CD4+CD25 CD45RBhi cells. CD4+ T cells were isolated from the splenocytes of WT 8–12-week-old sex-matched C57BL/6 mice using negative selection magnetic enrichment (Stem
previously described. Brieﬂy, male C57BL/6J mice were infected by oral gavage with 5.0 × 109 colony-forming units (CFUs) of C. rodentium or mock-infected with Luria broth. Mice were weighed and monitored for disease progression. Colon tissue was homogenized, serially diluted, and plated on MacConkey Agar for enumeration of bacterial CFUs per gram of stool. For experiments involving STm, CBA/J female mice were infected via oral gavage with 1.0 × 109 CFUs of STm strain IR715 or an isogenic mutant lacking functional T3SS1 and T3SS2 (ΔinvA::tetRA ΔspiB::KSAC, herein referred to as invA spiB). Mice were weighed and monitored for disease progression. stool was collected throughout the course of infection and at the experimental endpoint, snap frozen, homogenized, serially diluted, and plated on MacConkey agar for enumeration of bacterial CFUs per gram of stool.

**Colitis Scoring**

Colons from mice with or without colitis were harvested at the indicated experimental time point, swiss-rolled, ﬁxed in 10% neutral buffered formalin for 24 hours, and subsequently processed for parafﬁn embedding, sectioning, and staining with hematoxylin and eosin (H&E). For STm infections, ceca were harvested at the indicated time point and processed as stated above for colons. H&E-stained colon sections were scored in a blinded manner by a gastrointestinal pathologist (M.R.P.) using previously published scoring criteria for DSS colitis, adoptive T cell transfer colitis, and C. rodentium induced colitis. H&E-stained cecal sections were scored in a blinded manner by a veterinary pathologist (M.X.B.) using previously published scoring criteria.

**Micro CT of Trabecular Bone**

Femurs were harvested at the indicated time point and ﬁxed with 10% neutral buffered formalin for 48 hours at 4 ºC and then placed in 70% ethanol. Trabecular bone was analyzed using a μCT40 (Scanco Medical, AG Bassersdorf, Switzerland) and Scanco software. Images were acquired at 55 kVp and 145 mA with an isotropic voxel size of 12 µm and an integration time of 250 ms with 1000 projections collected per 360° rotation. Images were reconstructed, filtered (sigma = 0.8; support = 1.0), and thresholded at 200 mg HA/ccm. Trabecular bone at the distal femur was manually contoured every 10 slices starting 30 slices proximal to the growth plate and advancing proximally for 100 slices such that trabeculae were included, and cortical bone was excluded in accordance with American Society for Bone and Mineral Research (ASBMR) guidelines. Sections between manual contours were automatically contoured.

**Bone Histology and Histomorphometry**

Following micro CT imaging, femurs were decalcified for 3 days in 20% EDTA (pH 7.4) at 4 ºC. Samples were then dehydrated, embedded in parafﬁn blocks, and sectioned longitudinally at 4 µm thickness through the medullary cavity with a Leica RM2255 microtome. Tissue sections were mounted onto Leica Superfrost glass slides and then stained with TRAP stain with hematoxylin counterstain. Bioquant software (Nashville, TN) was used to perform quantitative histomorphometry (osteoclast number, osteoclast surface, and bone perimeter) in accordance with ASBMR guidelines using 10× images generated from a Cytation 5 imaging system (Biotek).

**Flow Cytometry and Cell Sorting**

Bone marrow from murine femurs and tibias was ﬂushed with cold alpha-minimal essential media. Red blood cells were lysed for 5 minutes with ammonium chloride potassium lysing buffer, pelleted, resuspended in phosphate buffered saline (PBS), and passed through a 70-µm ﬁlter. Single-cell suspensions were washed with PBS, enumerated, and 1 million bone marrow cells per sample were pelleted in PBS prior to live/dead staining per the manufacturer’s protocol (Zombie Violet, Biologend). Cells were then washed with FACS buffer (PBS containing 3% fetal bovine serum and 0.1% sodium azide). Non-speciﬁc antibody staining was blocked with anti-CD16/32 (Biolegend, clone 93) for 15 minutes at room temperature. Single-cell bone marrow suspensions were stained with a cocktail of surface-staining antibodies to identify speciﬁc myeloid populations. Unless otherwise indicated, all antibodies were purchased from Biologend. For OCPs and monocytes, the following anti-mouse antibodies were used: Anti-CD45-AlexaFluor700 (clone 30-F11), anti-CD11b-BV605 (clone M1/70), anti-Ly6G-PE or anti-Ly6G-PE-Dazzle (clone 1A8), anti-Ly6C-FITC (clone HK1.4), anti-TER119-Pacific Blue (clone TER119), anti-B220-Pacific Blue (clone RA3-6B2), anti-CD3-Pacific Blue (clone 145-2C11). For LSK cells, the following antibodies were used: Anti-mouse lineage cocktail-FITC (clones 145-2C11, RB6-8C5, RA3-6B2, Ter-119, M1/70), anti-Sca-1-APC (clone D7), and anti-c-kit-PE (clone QA17A09). Additional surface markers were evaluated on monocytes and OCPs by staining with anti-CXCR4-BV-711 (clone L276F12), anti-MDL-1-APC (Miltenyi, clone REA582), anti-CX3CR1-APC (clone SA011F11), anti-CCR2-
APC/Fire-750 (clone SA203G11), anti-RANK-Pe (clone R12-31), and anti-CSF1R-PerCP/Cy5.5 (clone AFS98). Osteoclast co-receptors antibodies specific for OSCAR (Novus Biologicals, clone 5BB), PIR-A/B (clone 6C1), and TREM2 (R&D, clone 237920) were conjugated to APC using the APC conjugation kit – lightning link (Abcam) per the manufacturer’s instructions. Surface staining was accomplished by incubating single cell suspensions with a given antibody cocktail at 4 °C for 30 minutes. For intracellular staining, cells were fixed and permeabilized overnight at 4 °C using the Foxp3 / transcription factor staining buffer set (eBioscience) per manufacturer’s instructions followed by intracellular staining for Ki67-APC (clone 16A8). Cells were washed twice in FACS buffer and fixed in PBS with 2% paraformaldehyde and analyzed using a 3- or 5-Laser Fortessa analytical flow-cytometer. Single cells were gated using successive gates including side scatter-area by forward scatter-area (SSC-A × FSC-A), side scatter-height by area (SSC-H × SSC-A), and forward scatter-height by area (FSC-H × FSC-A). Gating for the indicated populations was done as described in Figure 5 and Figure 6, A. For cell sorting, single-cell bone marrow suspensions were obtained as described above, live-dead stained, and blocked as described above. Cells were stained with Anti-CD45-PE (clone RA3-6B2), and anti-CD3-PeCy7 (clone OTI3A3), and anti-CD45-APC (clone 123G8), anti-CD11b-PerCP/Cy5.5 (clone M1/70), anti-Ly6G-PE (clone 1A8), anti-Ly6C-FITC (clone H-200), anti-TER119-PeCy7 (clone M1/70), anti-Ly6C-PeCy7 (clone H-200), anti-Ly6C-APC (clone H-200), anti-Ly6C-AF700 (clone H-200), anti-CSF1R-PerCP/Cy5.5 (clone AFS98). Osteoclast progenitor (OCP) formation was evaluated using 5.0 × 10^5 unsorted whole bone marrow or 1.0 × 10^5 FACS purified populations were used immediately after sorting. 5.0 × 10^6 whole bone marrow or 1.0 × 10^5 FACs purified populations were seeded per well into 96-well plates supplemented with 5% v/v CMG14-12 supernatant (as a source of M-CSF) and 35 ng/mL RANKL. Cells were cultured for 4 days, at which point the cells were centrifuged twice at 4000 × g for 5 minutes at 4 °C to remove debris and then subjected to multiplexed cytokine detection using the Milliplex-MAP magnetic bead-based antibody detection kit (EMD Millipore, Billerica, MA) according to manufacturer’s instructions. Specifically, the 32-plex Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTMAg-70K-PX32) or the Mouse Th17 Magnetic Bead Panel (MT17MAG-47K) kits were used, and data were collected using the FLEXMAP 3D instrument. Measurements were corrected for total protein input as quantified by the Pierce BCA Protein Assay Kit per manufacturer’s instructions and reported as either log2 fold change in pg/mg of cytokine abundances in mice with colitis compared with controls or pg/mg normalized cytokine abundances.

**Statistical Analysis**

Statistical analyses were conducted using GraphPad Prism Software (Version 9). Statistical significance was assessed using the 2-tailed Student's t test, ordinary 1-way ANOVA with post-hoc Holm–Sidak multiple comparison’s test, or 2-way ANOVA with post-hoc Sidák multiple comparisons test as appropriate and as indicated in the figure legends. Differences were considered significant with a P value (P < .05). Post-hoc multiple comparisons test for significant differences within groups were only performed following a significant ANOVA P value (P < .05).

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Correspondence
Address correspondence to: Dr Jim Cassat, Division of Pediatric Infectious Diseases, Department of Pediatrics, Vanderbilt University Medical Center, 1035 Light Hall, 2215-B Garland Ave, Nashville, TN, 37232. e-mail: jim.cassat@vumc.org; tel: (615) 936-6494.

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CRediT Authorship Contributions
Christopher T. Peek (Conceptualization: Supporting; Data curation: Lead; Formal analysis: Equal; Investigation: Lead; Methodology: Lead; Writing – original draft: Lead; Writing – review & editing: Equal)
Caleb A. Ford (Investigation: Supporting; Writing – review & editing: Supporting)
Kara R. Eichelberger (Investigation: Supporting; Writing – review & editing: Supporting)
Justin Jacobse (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)
Teresa P. Torres (Investigation: Supporting; Writing – review & editing: Supporting)
Damian Maseda (Investigation: Supporting; Writing – review & editing: Supporting)
Yvonne L. Latour (Investigation: Supporting; Writing – review & editing: Supporting)
M. Blanca Piazzuelo (Writing – review & editing: Supporting; Blinded histopathologic analysis: Lead)
Joshua R. Johnson (Methodology: Supporting; Writing – review & editing: Supporting)
Mariana X. Byndloss (Conceptualization: Supporting; Funding acquisition: Supporting; Resources: Supporting; Supervision: Supporting; Writing – review & editing: Supporting; Blinded histopathologic analysis: Supporting)
Keith T. Wilson (Conceptualization: Supporting; Funding acquisition: Supporting; Resources: Supporting; Supervision: Supporting; Writing – review & editing: Supporting)
Jeffrey C. Rathmell (Conceptualization: Supporting; Funding acquisition: Supporting; Resources: Supporting; Supervision: Supporting; Writing – review & editing: Supporting; Blinded histopathologic analysis: Supporting)
Jeremy A. Goettel (Conceptualization: Supporting; Data curation: Equal; Investigation: Lead; Methodology: Lead; Writing – original draft: Lead; Writing – review & editing: Equal)

Conflicts of interest
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