INFLAMMATORY BOWEL DISEASE AND BONE: MECHANISMS AND INTERVENTIONS FOR INFLAMMATION-INDUCED BONE ALTERATIONS

A Dissertation

by

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ABSTRACT

Inflammatory bowel disease (IBD) effects approximately 1.6 million people in the United States with the incidence and prevalence increasing worldwide. All current treatments for IBD aim to simply mitigate the disease symptoms and come with negative consequences. A common comorbidity of IBD is inflammation-induced bone loss, which is characterized by increased bone resorption and decreased bone formation. Osteocytes, cells embedded in the bone matrix, are considered the primary regulatory cell type in bone; however, the role of osteocytes in inflammation-induced alterations in bone is unknown. The goals of the current project are to examine the role of osteocyte signaling proteins in inflammation-induced changes in bone turnover during chronic IBD and secondly, to explore lifestyle changes and therapeutic targets for IBD-induced alterations in bone.

Male Sprague-Dawley rats (2 months old) were given gut inflammation via rectal instillations of 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 30% ethanol while vehicle-treated rats received only 30% ethanol for four weeks. Osteoclast surfaces of cancellous bone were increased after TNBS while bone formation rate was decreased. These changes in bone turnover were coincident with higher osteocytes positive for pro-inflammatory markers, osteoclastogenesis regulators, and bone formation inhibitors. In a second experiment, TNBS and vehicle-treated rats were fed a moderately elevated soy protein diet during the experimental period. TNBS-treated animals fed the moderately elevated soy protein diet had reductions in osteoclast surfaces and increased bone formation rates corresponding with declines in osteocytes positive for pro-inflammatory factors. Finally, a third group of TNBS and vehicle-treated rats received exogenous administration of irisin, a protein released during exercise. TNBS-treated rats receiving irisin had significantly higher bone formation rates and lower osteoclast surfaces

than those receiving TNBS alone. Additionally, irisin-treated rats had lower osteocytes positive for pro-inflammatory factors.

These results indicate that osteocytes respond to inflammatory signals and may orchestrate changes in bone turnover. Secondly, a moderately elevated soy protein diet reduced the inflammatory alterations in bone during chronic IBD. Additionally, designing methods to increase endogenous irisin, possibly through exercise, could potentially reduce inflammatory changes in bone during IBD. Finally, exogenous irisin administration is a potential novel therapeutic target for inflammation-induced bone loss.

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1. INTRODUCTION AND LITERATURE REVIEW

Bone is a dynamic tissue that adapts to its environment. In systemic physiology, the skeletal system plays many roles including protection, structure for locomotor ability, mineral homeostasis, and hematopoiesis. Bone tissue is sensitive to changes and disturbances in mechanical loads, energy availability and nutritional status, hormonal changes, and immune system alterations. Bone is continuously undergoing formation and resorption and, under normal physiological conditions, these processes are tightly regulated to ensure homeostasis and maintenance of bone mass. However, pathological disturbances can disrupt this tight balance of formation and resorption leading to bone loss and increased risk of fracture. Bone tissue adaptations are primarily carried out by three bone cells: osteoblasts, osteoclasts, and osteocytes.

1.1 Cells of Bone

Osteoclasts make up less than 1% of total bone cells and are responsible for degrading the mineralized matrix of bone leading to bone resorption. Derived from the monocyte-macrophage lineage, osteoclasts are large, highly motile, multi-nucleated cells with a unique sealing zone which attaches to the bone surface. Within the sealing zone, a ruffled border of the cell releases proteolytic enzymes and hydrogen ions that degrade both the mineral and organic matrix of bone (1). Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) are critical factors for osteoclastogenesis. RANKL binds to its receptor, RANK, and triggers osteoclastogenesis (2, 3). Another molecule, osteoprotegerin (OPG), has no signaling capabilities, but is capable of binding to RANKL. When OPG binds to RANKL, it prevents RANKL from interacting with RANK, decreasing osteoclast differentiation

(4; Figure 1.1). Therefore, the ratio of RANKL to OPG plays a critical role in osteoclast formation and activation.

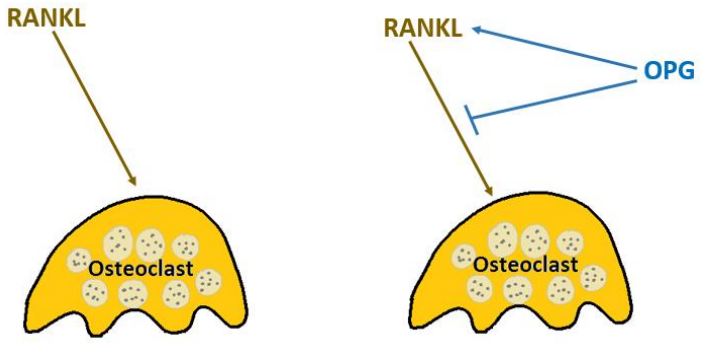

Figure 1.1: RANKL and OPG in osteoclastogenesis. RANKL stimulates osteoclast formation when binding to RANK while OPG binds to RANKL preventing its interaction with RANK and preventing osteoclast formation.

Making up less than 5% of total bone cells, osteoblasts work in teams to deposit a protein matrix on bone surfaces which is then mineralized in a process known as bone formation. Osteoblasts are derived from mesenchymal progenitors that also can develop into chondrocytes, muscle cells, and adipocytes (2). Mature osteoblasts are non-proliferating cuboidal cells that secrete type 1 collagen and other proteins to form an organic matrix known as osteoid. This organic matrix can then be mineralized to form bone. Bone morphogenetic protein (BMP), transforming growth factor-β (TGFβ), and Wnt signaling factors are all involved in triggering osteoblastogenesis (5). Additionally, the transcription factors Runx2 and Sp7/osterix are critical for developing osteoblasts (2). The majority of osteoblasts likely undergo apoptosis after completing their tasks in bone formation; however, some osteoblasts are encased in the mineralized matrix of bone becoming osteocytes.

Osteocytes are the longest living and most abundant bone cells making up 90-95% of the cells in bone tissue (6). Although osteocytes form when osteoblasts become buried in the bone matrix, osteocytes are a unique cell type with distinct features. Osteocytes are situated in lacuna within the mineralized matrix of bone and form long dendritic processes that extend out from their cell bodies into spaces known as canaliculi. Through these dendritic processes osteocytes form a network of communication with other osteocytes, cells on bone surfaces, and the marrow (6). Due to these vast communication networks, osteocytes are perfectly situated to sense the systemic environment as well as local signals within the bone and orchestrate specific adaptations. It is now understood that these cells, formerly considered simply place-holders in bone, are the master regulators of bone tissue.

Osteocytes can orchestrate the actions of osteoblasts and osteoclasts through several mechanisms. Osteocyte distress signals and apoptosis are potent signals to recruit osteoclasts to resorb bone in that region (7, 8). Certain signaling pathways like the Wnt/β-catenin pathway appear to be critical for maintaining osteocyte viability and, therefore, overall bone health (7, 9). Secondarily, osteocytes express and release proteins that signal to osteoblasts, osteoclasts, and other cells to orchestrate changes in response to the environment. Osteocytes express multiple factors important for the maintenance of mineral homeostasis including SOST, Phex, dentin matrix acidic phosphoprotein-1 (DMP1), and fibroblast growth factor 23 (FGF23) (7). Sclerostin, the protein encoded by the SOST gene, is one of the most well-studied osteocyte proteins. As an antagonist of the Wnt/β-catenin system, increased sclerostin expression leads to a suppression of osteoblasts and, therefore, is a negative regulator of bone formation (10, 11). Mice overexpressing SOST have low bone mass (11) and transgenic mice lacking sclerostin have increased bone formation rate and bone strength (12). Osteocytes also express RANKL and

OPG, the critical regulators of osteoclastogenesis (2). It is now appreciated that osteocyte RANKL is the primary source of RANKL needed for osteoclast formation for bone remodeling (13, 14).

One of the most well examined actions of osteocytes is their mechanosensory capabilities. It has long been known that bone adapts to the mechanical strains placed on it; however, only in the past couple decades has the important role of the osteocyte been explored. Osteocytes sense mechanical strains through tissue strain, fluid flow shear stress through the lacuna-canalicular network, and movement on cell bodies and dendrites (9, 15). These mechanosensory signals on osteocytes can trigger them to release various proteins that impact bone turnover. For example, a lack of mechanical loading results in increased sclerostin while increased loading decreases osteocyte sclerostin (16, 17). RANKL and OPG are also mechanosensitive osteocyte proteins (18); for example, osteocyte RANKL release is increased due to lack of mechanical loading. Furthermore, mice lacking osteocyte RANKL are protected from disuse-induced bone loss (14). Additionally, osteocytes release insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6) in response to increased mechanical loading, leading to signaling to osteoblasts to increase bone formation (17, 19-21; Figure 1.2). The role of osteocytes in the mechanosensory capabilities of bone highlight the important role these cells play in bone adaptations to the environment.

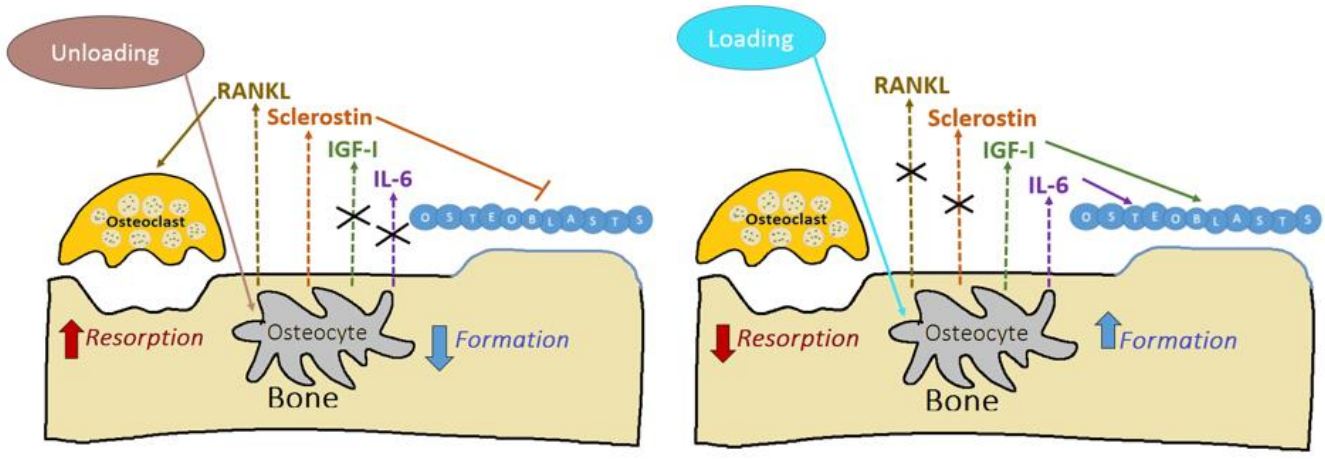

Figure 1.2: Response of osteocytes to mechanical unloading and loading. Mechanical unloading (left) and mechanical loading (right) impact osteocyte proteins controlling bone formation and bone resorption.

1.2 Inflammation and Bone

Another environmental stimulus that impacts skeletal tissue is inflammation. Inflammation is a complex response of the immune system to an injury or threat, where components of the immune system try to minimize and repair damage caused by the threat/injury. The innate arm of the immune system responds immediately to threats and damage in a non-specific manner while the adaptive immune system takes time to respond, but has specific and long-lasting memory to unique pathogens. Both arms of the immune system, once activated, release soluble factors known as cytokines. Many cytokines are also released by parenchymal cell types to aid in the immune response. Cytokines regulate the intensity, direction, and duration of the immune response by stimulating or inhibiting various cell types, regulating the secretion of other cytokines or antibodies, and sometimes can program cell death in the target cell (22). Cytokines are, therefore, capable of inducing communication within a vast network of cells. In some cases, the immune system remains active due to loss of ability to recognize "self"

vs. “non-self” resulting in autoimmunity which leads to chronic inflammation and a constant barrage of cytokines. Cytokines are sometimes classified by the type of T helper lymphocyte they are largely produced by – Th1, Th2, or Th17. Th1 cells produce many cytokines including interferon-γ (IFN-γ) and the tumor necrosis factor (TNF) cytokines while Th2 cells produce factors such as interleukin-4 (IL-4) and interleukin-10 (IL-10) among others (23). Th17 are the most recently identified class of T helper lymphocytes which primarily release interleukin-17 (24). While these classifications have merit, the immune system is vastly complex and no cytokine perfectly fits into a single grouping. Nonetheless, organ-specific autoimmune diseases like Crohn’s disease are often Th1-driven while allergen-specific conditions are often Th2-driven (23).

The interaction between the immune system and skeletal tissue has recently garnered interest, leading to the development of a field of study known as osteoimmunology (25-28). This cross-talk between the immune system and bone has become more evident with the accumulating knowledge of the shared signaling pathways between immune cells and osteoclasts, which are essentially specialized macrophages which develop from the same lineage as immune cells. Additionally, immune cells develop in the marrow of bone in the same environment as osteoclasts and osteoblasts. The first molecule to establish the concept of crosstalk between these two systems was RANKL (25, 27). This molecule is not only a key factor in osteoclastogenesis, but also plays critical roles in immune function. Bone biologists and immunologists unexpectedly found that the long sought after osteoclast differentiation factor was the same factor expressed by T cells to stimulate dendritic cells (27). RANKL is expressed by many immune cells including T cells and B cells, while its receptor RANK is expressed on macrophages and monocytes (29). Some candidate roles for RANKL in the immune system

include stimulating antigen presentation to T cells, modulation of T regulatory cells, development of B cells, and lymph node development (29). Mice lacking RANKL have both severe osteopetrosis due to a lack of osteoclasts as well as a deficiency of B cells and a lack of peripheral lymph nodes (30). Importantly, RANKL, RANK, and OPG are all members of the tumor necrosis factor (TNF) and TNF receptor superfamilies with broad expression throughout the body and association with immune cells (31). Therefore, these molecules are not simply osteoclastogenesis factors and must be viewed in their broader context of immune and systemic physiology.

Beyond RANKL/RANK/OPG, multiple other immune factors influence bone homeostasis. In particular, cytokines can interact with bone cells leading to increased bone resorption that, over time, leads to inflammatory bone loss (32). Multiple conditions with chronic inflammation are associated with low bone mass including rheumatoid arthritis, where there is both local bone erosion and systemic bone loss (33, 34), systemic lupus erythematosus (35), psoriasis and psoriatic arthritis (36), ankylosing spondylitis (37), and celiac disease (38). A key driver of inflammation is tumor necrosis factor-α (TNF-α). TNF-α is a strong stimulator of osteoclastogenesis even with extremely low levels of RANKL (39, 40). In a cell culture model, Lam *et al* demonstrated that less than 1% of the RANKL needed to induce osteoclastogenesis was necessary in the presence of TNF-α to induce a robust increase in osteoclast development (40). TNF-α also mediates and synergizes with RANKL to increase osteoclastogenesis (41, 42). TNF-α also increases the production of OPG (43). Another cytokine, interleukin-6 (IL-6), has equivocal roles in bone physiology, but appears to not stimulate osteoclasts alone unless other cytokines are present normal physiological conditions; however, with TNF-α, IL-6 synergizes to stimulate osteoclasts and increase production of RANKL and OPG (42; Figure 1.3). Cytokines

classified as Th2, often considered "anti-inflammatory" in nature, remain more poorly understood in the context of bone physiology. Both interleukin-4 (IL-4) and interleukin-10 (IL-10) have been shown to inhibit osteoclasts and reduce RANKL in cell culture models (44-47). *In vivo* models reflect a more complex picture with mice lacking IL-10 having low bone mass and increased fragility (48). This observation suggests that IL-10 and also likely IL-4 have more complex roles in skeletal physiology than simply inhibiting osteoclasts.

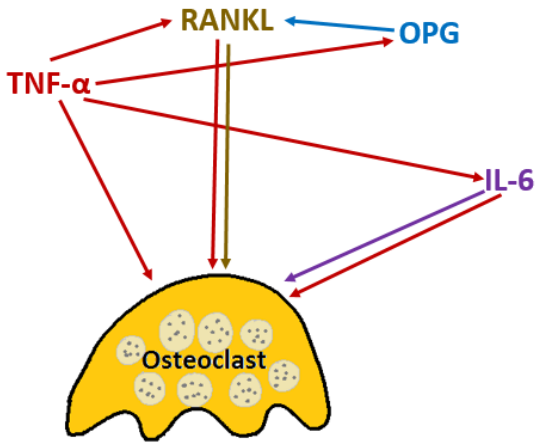

Figure 1.3: Proposed interactions of TNF-α, RANKL, OPG, and IL-6 on osteoclast formation. TNF-α strongly stimulates osteoclastogenesis as well as stimulating and synergizing with RANKL and IL-6 to enhance osteoclast formation. TNF-α also stimulates OPG.

Pro-inflammatory factors also interact with osteoblast development and function. TNF-α inhibits osteoblast genes and differentiation factors and inhibits bone collagen synthesis (41, 49, 50). Runx2, a key factor in osteoblast differentiation, is degraded by TNF-α; mice injected with TNF-α have decreased Runx2 protein levels (51). Additionally, TNF-α inhibits the anabolic signals of IGF-I in osteoblasts (41, 52). Osteoblast apoptosis is induced in the presence of TNF-α (41, 53). Finally, sclerostin, the inhibitor of osteoblast formation, is transcriptionally

activated by TNF-α (54; Figure 1.4). The role of IL-6 in osteoblasts in the context of inflammation is unknown; however, IL-6 appears to be able to signal to osteoblasts in the absence of TNF-α in conditions of increased mechanical loads (17, 19). The role of IL-10 and IL-4 on osteoblast differentiation and function is uncertain. Mice lacking IL-10 have low bone formation rate (48), indicating some potential role of IL-10 with osteoblasts.

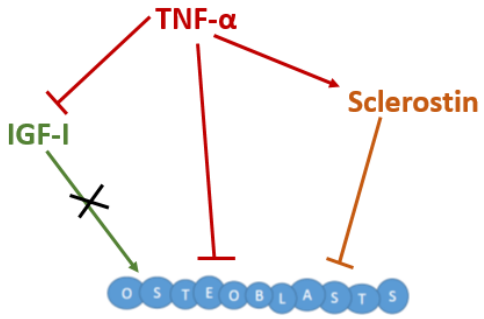

Figure 1.4: Influence of TNF-α on osteoblasts. TNF-α inhibits the anabolic signals of IGF-I on osteoblasts. TNF-α also directly inhibits osteoblasts while stimulating sclerostin which also inhibits osteoblasts.

There is a great lack of literature on how inflammation impacts osteocytes, even though osteocytes are considered to be the master regulators of bone cell activity. What is known is that TNF-α can trigger osteocyte apoptosis (55) and that TNF-α is a transcriptional activator of the osteocyte protein, sclerostin (Baek; Figure 1.5). Due to their central role in orchestrating changes in bone turnover during increased or decreased mechanical loads and the fact that many of these same factors are associated with inflammatory processes (Table 1.1), it seems highly probable that osteocytes also play a central role in orchestrating adaptations to inflammatory signals.

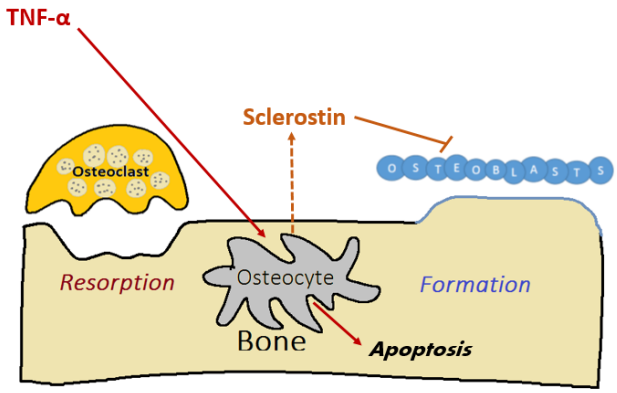

Figure 1.5: TNF-α interactions with osteocytes. TNF-α induces osteocyte apoptosis and increases sclerostin expression.

Table 1.1: Factors associated with inflammatory bone loss, their interactions with bone cells, and expression in osteocytes.

Factor	**Role in Bone**	**Expression in Osteocytes**
Tumor necrosis factor-α (TNF-α)	Stimulates osteoclast formation, suppresses osteoblasts, triggers osteoblast and osteocyte apoptosis	Unknown
Interleukin-6 (IL-6)	Equivocal role in bone, with TNF-α synergizes osteoclast formation, may stimulate osteoblasts alone	Yes; known to be mechanosensitive
Interleukin-10 (IL-10)	Inhibits osteoclastogenesis	Unknown
Interleukin-4 (IL-4)	Inhibits osteoclastogenesis, inhibits RANKL	Unknown
Sclerostin	Inhibits bone formation, transcriptionally activated by TNF-α	Yes; known to be mechanosensitive
Receptor activator of nuclear factor KB ligand (RANKL)	Final step in osteoclastogenesis, activated by TNF-α, synergizes with TNF-α to increase osteoclast formation	Yes; known to be mechanosensitive
Osteoprotegerin (OPG)	Binds to RANKL preventing osteoclastogenesis, stimulated by TNF-α	Yes; known to be mechanosensitive
Insulin-like growth factor-I (IGF-I)	Stimulates osteoblasts and increases bone formation rate, inhibited by TNF-α	Yes; known to be mechanosensitive

1.3 Inflammatory Bowel Disease and Bone

One such chronic inflammatory condition with skeletal morbidities is inflammatory bowel disease (IBD). The primary IBD conditions are ulcerative colitis and Crohn's disease, which are caused by prolonged inflammation along all or parts of the digestive tract. Approximately 1.6 million people in the United States have IBD and the incidence and prevalence is increasing globally (56-58). Particularly of concern is the increasing incidence of pediatric IBD, especially Crohn's disease (59). Pediatric IBD is characterized by more extensive disease and lifelong complications (56). While the exact etiology of IBD remains largely unknown, it is considered an autoimmune condition in which the immune system mounts an attack against tissues within the digestive tract leading to chronic inflammation; however, genetic and environmental factors are also likely involved in the development of the pathology (60-64). IBD pathogenesis is believed to be largely driven by Th_1 cytokines like TNF-α (61, 62, 65, 66).

Beyond the inflammation, significant damage, and complications that can arise within the gastrointestinal tract due to IBD, the inflammatory insult initiated in the gut can lead to multiple extra-intestinal complications that can lead to additional morbidity (67). One such complication is bone loss which is a leading comorbidity of IBD (68, 69). Osteopenia (T score between -1 and -2.5 below healthy age-matched controls) and osteoporosis (T score below -2.5) prevalence are common in patients with IBD, with reported incidence of osteoporosis in this population ranging from 12-70% (68, 70, 71). Of a cohort of forty-nine patients with IBD (average age = 37), 42% met the criteria for osteopenia in the femoral neck while 41% met the criteria for osteoporosis. In this same cohort, 34% had osteopenia in the lumbar spine and 42% had osteoporosis in this bone site (72). Other studies have shown similar loss of bone mineral density at the lumbar spine and

femoral neck (73). Osteopenia is also common in children with IBD, a population rarely plagued with low bone mass (74, 75). At time of diagnosis, many patients with IBD already have lower bone mineral density than healthy controls (76, 77) and decreased trabecular BMD and cortical section modulus (75). Children just 35 days after diagnosis with Crohn's disease had low total body bone mineral content for age and sex as well as reduced trabecular volumetric BMD at the tibia metaphysis and thin cortices at the tibia diaphysis (78). This is particularly concerning since approximately 35% of total bone mineral accrual occurs during the four years of adolescence surrounding the age of peak linear growth velocity (79). Thus, during a time when most adolescents are rapidly gaining bone mineral, those with IBD are losing bone mass. This bone loss in patients with IBD can be rapid. In a cohort of 54 adults with IBD, the mean loss of vertebral BMD was 3% over the course of a year, but eleven of the 54 patients had bone loss 3-fold greater than the mean, indicating a wide range of rates of bone loss (80). Of the conditions within IBD, Crohn's disease is often associated with more severe bone loss compared to ulcerative colitis, particularly in cancellous bone, the three-dimensional lattice-like structure in the vertebral bone and ends of long bones (81).

Concurrent with low bone mass and increased rates of bone loss, patients with IBD have increased serum or urinary measures of bone resorption and decreased circulating measures of bone formation (69, 82-85). Given the pro-inflammatory status in IBD patients, this suppression in formation and increase in resorption in patients with IBD indicates a systemic effect of inflammation on bone cell activity that promotes net bone loss. Additionally, serum RANKL and OPG are dysregulated in IBD patients, with increases particularly in circulating OPG levels (69, 86-89). Of cytokines, serum IL-6 is elevated in IBD patients (72, 90-92). Other cytokines, like TNF-α, are often not different in the serum (92). One study treated fetal rat parietal bones with

serum from newly diagnosed children with IBD and discovered uneven mineralized bone matrix, altered osteoblast morphology, and disorganized osteoblasts; however, treatment with an IL-6 neutralizing antibody prevented these changes (92). Therefore, pro-inflammatory cytokines like IL-6 are believed to be drivers of the altered bone turnover seen in IBD.

With decreased formation, increased resorption, and low bone mass, patients with IBD are at an increased risk of fractures. Indeed, IBD patients at any age have 40% greater fracture incidence than the general population (93). In comparison, the fracture risk over an entire lifetime for white postmenopausal women, the population at the greatest risk for osteoporosis, is 30-40% (94, 95). Vertebral fractures are particularly common in IBD even in patients with normal BMD (96), with prevalence strikingly high at approximately 22% even in those aged less than 30 years (97). Incidence of hip and vertebral fractures are higher in patients with Crohn's disease versus ulcerative colitis (98), reflecting the more significant bone loss that is often seen in Crohn's disease patients. Disease severity determined by the number of disease symptoms predicts fracture incidence in IBD patients (98).

It is important to note bone loss due to IBD is not caused by corticosteroid use, a frequently used therapeutic agent in IBD. First, as previously mentioned, newly diagnosed patients who have yet to receive any treatments have lower bone mass than healthy controls (75-77). Another study demonstrated that low BMD in IBD patients did not correlate with current or previous corticosteroid use (82). Likewise, a significant portion of corticosteroid naïve Crohn's disease patients have osteopenia (70). Vertebral fractures occur at the same rates in Crohn's disease patients whether or not they use corticosteroids (96). In pediatric patients, bone mass was inversely correlated with disease activity indices (90) and fracture incidence is predicted by

disease severity as well (98), indicating it is the disease itself that leads to bone loss and not complications due to corticosteroid treatment.

1.4 IBD and Bone in Animal Models

Multiple animal models can be utilized to examine the mechanisms of IBD pathology and to experiment with novel therapeutics. There are many transgenic mouse models that develop symptoms similar to human colitis or Crohn's disease (99-101). For example, interleukin-10 deficient mice have a primarily Th_1-driven response with inflammation in both the small intestine and colon (99). In another model, $TNF^{\Delta ARE}$ transgenic mice, elevated TNF-α due to depressed mRNA degradation triggers the development of inflammation in the proximal colon and ileum similar to human Crohn's disease (99, 100). There are two predominantly used chemically-induced models of gut inflammation that can be utilized in both mice and rats. A haptenizing agent named 2,4,6-trinitrobenzenesulfonic acid (TNBS) can be dissolved in ethanol and instilled rectally to induce gut inflammation (101). The ethanol breaks the mucosal barrier in the colon while the TNBS makes the colonic cells and potentially the gut microbiota immunogenic to the host, triggering an immune reaction (99, 101). Cytokine profiles with TNBS-induced IBD are similar to human Crohn's disease (99). Prepubertal rats treated with TNBS had elevated serum IL-6 levels similar to those seen in children with Crohn's disease (102). The second chemically induced model utilizes dextran sodium sulfate (DSS) dissolved in drinking water. It is believed DSS is directly toxic to the epithelial cells of the colon, thereby initiating an inflammatory response (99, 101). DSS typically induces inflammation along the colon and, therefore, may be more analogous to human colitis (100). While murine TNBS-induced IBD is largely Th1 driven, DSS-induced IBD in mice shifts from a Th1 acute phase to Th2 as the disease becomes chronic (103).

Few studies have addressed bone outcomes in animal models of IBD. One study examined two weeks of DSS-induced gut inflammation in mice, demonstrating that the DSS-induced IBD lead to low bone mass, depressed bone formation, and increased osteoclast number (104). Two other studies found suppressed osteoblastic activity and bone formation rate due to chemically-induced IBD that was reversible with recovery from IBD (105, 106). In general, there is a lack of studies examining chronic IBD in animal models, as most studies focus on much more acute time frames (5-14 days). Since IBD is a chronic disease, studies addressing more long-term outcomes are needed to understand the pathophysiology of prolonged gut inflammation as well as the impact on bone. . Additionally, there are no published data addressing the role of osteocyte proteins in mediating the alterations in bone turnover that result in IBD-induced bone loss.

1.5 Current Treatments for IBD

IBD is a chronic condition that currently has no cure. All current treatments aim to simply mitigate disease symptoms and improve the quality of life of patients. Corticosteroids can be used to decrease inflammation; however, the negative effect of corticosteroids on bone health is well documented, with long-term use associated with development of osteoporosis (107, 108). Previously, immunosuppressant agents like azathioprine were a common therapy for IBD; however, azathioprine can cause leucopenia and bone marrow suppression or toxicity leading to potentially lethal complications (109). A great advance in treating chronic inflammatory conditions like rheumatoid arthritis and Crohn's disease came with the development of anti-cytokine therapies, specifically anti-TNF (110). Anti-TNF treatments (infliximab, etanercept, adalimumab) often lead to reductions in other cytokines and chemokines including IL-6 (110). With regards to bone, anti-TNF therapy has been shown to improve BMD (111) and systemic

measures of bone formation (112) in patients with IBD. However, there are multiple unknown factors with anti-TNF treatment, including its impact on bone parameters and fracture risk with long-term use (113). Additionally, whether the cessation of anti-TNF treatment is negative to bone health is uncertain (113). Other complications of anti-TNF treatment make its use, although valuable in many autoimmune inflammatory conditions, not ideal. There is nearly a doubled risk of developing a serious infection while on anti-TNF therapy (114, 115) and a dose-dependent increased risk of developing malignancies (115). Additionally, development of autoantibodies can occur with anti-TNF pharmaceuticals, which can interfere with the pharmacokinetics of the drug and, in some rare cases, lead to the development of other autoimmune conditions (116, 117). Other anti-cytokine treatments, like anti-IL-6, have been explored for use in IBD (118), but are not currently available to patients.

Treatments specific to bone typically fall into two categories – anti-resorptive therapies and anabolic therapies. Bisphosphonates are one class of anti-resorptive agents that inhibit bone resorption by triggering osteoclast apoptosis. Risedronate, one type of bisphosphonate, improved bone mass at the hip and spine in IBD patients with low bone mass (119). Bone loss due to another inflammatory condition, inflammatory arthritis in human TNF transgene mice, was retarded due to treatment with zoledronate, another bisphosphonate, but it had no effect on inflammation (120). A common side effect of some bisphosphonate treatments is gastrointestinal complications, reported in close to 50% of patients, often resulting in low adherence to treatment (121). Gastrointestinal complications due to bisphosphonates in IBD may make adherence even more difficult than for other populations of patients. Another anti-resorptive drug, denosumab, works by binding RANKL, therefore preventing osteoclast formation. In patients with rheumatoid arthritis who experience inflammatory bone loss, denosumab combined with the

immunosuppressant, methotrexate, helped maintain joint structure and improved bone turnover markers, but had no effect on disease activity (122). Intermittent parathyroid hormone (teriparatide) treatment is the only approved anabolic agent for bone at this time. There are no trials evaluating its effectiveness in preventing bone loss in IBD. Another anabolic treatment currently in clinical trials, an antibody against sclerostin, was examined in an animal model of IBD and was found to improve bone mass, but had no impact on histological changes in the colon (123). In general, the few studies that have examined bone specific therapies in inflammatory disorders show potentially improved bone mass, but no changes in the underlying pathology or inflammatory status.

There is a great need for holistic treatments for IBD that mitigate the gut pathology as well as the bone comorbidity. Current anti-inflammatory treatments like corticosteroids and anti-TNF therapy mitigate the inflammatory status, but lead to significant negative side effects and consequences like increased risk of serious infections. Additionally, these treatments are either already known to not be safe in the long-term (i.e. corticosteroids) or have unknown implications with long-term use. To date, bone-specific therapies appear to only impact bone itself and not the actual inflammatory disease causing the bone loss, which increases the likelihood of needing multiple concurrent pharmacological therapies and thereby increasing the risk of negative side effects and drug interactions. Therefore, novel therapeutics and lifestyle/dietary interventions for IBD that influence the entire system, gut and bone and all other extraintestinal organs influenced by inflammation, would be of great value in mitigating disease symptoms, preventing comorbidities, and improving the quality of life of patients.

1.6 Specific Aims of Current Studies

Inflammation-induced bone loss from IBD causes increased bone resorption and suppressed bone formation. While the systemic actions of multiple cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are more well understood in inflammatory states, how these factors are altered specifically in bone are unknown. To date, no study has comprehensively addressed the osteocyte-specific response in the pathogenesis of IBD-induced inflammation and bone loss. Understanding the role of osteocytes and their regulatory proteins will provide greater insight into the mechanisms behind IBD-induced bone loss and subsequent targets for elucidating countermeasure efficacy. Secondly, as previously discussed, there is a great need for holistic treatments for IBD to prevent gut and bone damage and improve quality of life. Non-pharmacological measures to treat bone loss particularly with reference to inflammatory mechanisms have not been fully explored. We propose examining both a dietary intervention and exogenous treatment with irisin, a factor released during exercise, in an animal model of chronic IBD. No published investigations have addressed these proposed interventions in chronic IBD or their influence on bone outcomes. The objectives are as follows:

Specific Aim 1 (SA1): *Determine the impact of inflammation in a chronic IBD rodent model on bone turnover, osteocyte inflammatory cytokines, and osteocyte regulators of bone turnover*. I hypothesize chronic IBD will result in increased osteoclast surfaces, decreased bone formation rate, increased osteocyte TNF-α and IL-6, decreased osteocyte IGF-I, alterations in osteocyte RANKL/OPG, and increased osteocyte sclerostin. The changes in osteocyte proteins will correlate with changes in bone formation rate and osteoclast surface.

Specific Aim 2 (SA2)*: Evaluate the impact of a 35% soy protein isocaloric diet during chronic IBD on bone turnover, osteocyte inflammatory cytokines, and osteocyte regulators of*

bone turnover. I hypothesize a moderately elevated soy protein diet will decrease pro-inflammatory cytokines and favorably alter osteocyte regulators of bone turnover. These changes will correspond with increases in bone formation rate and reduced osteoclast surfaces.

Specific Aim 3 (SA3)*: Evaluate the impact of exogenous treatment with recombinant irisin during chronic IBD on bone turnover, osteocyte inflammatory cytokines, and osteocyte regulators of bone turnover.* I hypothesize treatment with irisin will decrease osteoclast surfaces and exert an anabolic effect on bone leading to increased bone formation rate. Additionally, exogenous irisin treatment during chronic IBD will decrease pro-inflammatory cytokines and favorably alter osteocyte regulators of bone turnover.

The work proposed in these specific aims will allude to the involvement of osteocytes in inflammation-induced bone loss as well as characterize potential holistic treatments for IBD. Applications of this project can aid in dietary recommendations, the potential development of exercise therapies, and the development of new pharmacologic treatments for IBD. In addition, these results could potentially be applied to other chronic systemic inflammatory conditions like autoimmune conditions and spinal cord injury.

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2. INFLAMMATORY BOWEL DISEASE IN A RODENT MODEL ALTERS OSTEOCYTE PROTEIN LEVELS CONTROLLING BONE TURNOVER*

Inflammatory bowel disease (IBD) is a group of diseases, including Crohn's disease and ulcerative colitis, that affects approximately 1.6 million people in the United States and 2.2 million in Europe (1) with its incidence and prevalence increasing worldwide (2, 3). Inflammation occurring along all or multiple sections of the gastrointestinal (GI) tract or in specific sections (i.e. colon in ulcerative colitis) leads to chronic systemic inflammation (4). One common comorbidity seen in IBD patients is bone loss (4-8). Low bone mass affects 40-50% of patients with IBD with 10-20% having clinical osteoporosis (5, 6, 9). Not surprisingly, there is a 40% greater fracture incidence in patients with IBD (10). Therapeutic use of corticosteroids by these patients can further exacerbate bone loss, but significant bone loss in IBD occurs even in those not on corticosteroid therapy (11, 12). Rapid loss of vertebral bone occurs in IBD and vertebral fractures are estimated to develop in up to 1 in every 5 adults (13-15). Similarly, bone loss observed at the clinically relevant fracture site of the femoral neck mirrors bone loss seen in vertebral bone (7, 16). Although IBD is most prevalent in young adults and adolescents, the incidence of diagnosis in children is increasing (17). This is a critical stage in bone development and children diagnosed with Crohn's disease experience losses in cancellous volumetric bone mineral density at the tibia (18) as well as decreased total body and femoral neck bone mineral content (19).

It is generally believed systemic inflammation due to IBD leads to bone loss by stimulating osteoclast activity (increasing bone resorption) and inhibiting osteoblast activity (decreasing bone formation) (20). Systemic increases in circulating pro-inflammatory cytokines coincide with IBD (4, 5, 21) particularly during symptom flare-ups. Tumor necrosis factor-α (TNF-α), a potent pro-inflammatory cytokine, plays a crucial role in inflammatory bone loss by stimulating osteoclastogenesis and inhibiting osteoblast function (22, 23). TNF-α also indirectly lowers bone formation by suppressing the anabolic growth factor, insulin-like growth factor-I (IGF-I) (22). Serum IGF-I concentrations are 21% lower in IBD patients compared to healthy controls (24). Additionally, interleukin-6 (IL-6) concentrations are significantly elevated, up to 34-fold higher, in serum of both children and adults with IBD (9, 21, 24). Although the exact role of IL-6 in bone turnover is equivocal, in the context of IBD *in vitro* studies have implicated it as a prominent factor leading to bone loss (21). Although interleukin-10 (IL-10) was originally described as an anti-inflammatory cytokine, recent studies have shown that its immunomodulatory effects are complex, as well as tissue- and context-dependent (25). Aside from its role in gut immunomodulation, IL-10 has been shown to increase in serum concentration of patients with IBD (26, 27). Since IL-10 inhibits osteoclastogenesis (28, 29), it is hypothesized that elevated serum IL-10 may be a compensatory mechanism with respect to bone to prevent excessive bone resorption. Another proposed mechanism for the increase in bone resorption in IBD is alterations in receptor activator of nuclear factor κ-B ligand (RANKL) and osteoprotegerin (OPG); both are the final regulatory step in osteoclastogenesis. Interestingly, OPG concentrations, rather than RANKL concentrations, are elevated in serum of patients with IBD and are inversely correlated with low BMD (4, 6, 30-32).

Research over the past decade has revealed how osteocytes, bone cells embedded in the bone matrix, are critical regulators of bone metabolism releasing factors influencing the development and actions of both osteoblasts and osteoclasts (33). While the role of osteocytes in bone homeostasis is increasingly appreciated, the impact of systemic inflammation on osteocytes is poorly understood. Sclerostin, an osteocyte protein which inhibits the Wnt/β-catenin signaling pathway in osteoblasts, is upregulated by TNF-α (34) suggesting a potentially significant link between inflammation and osteocyte function. Besides sclerostin, many of the systemic circulating factors implicated as players in IBD-induced bone loss are proteins expressed by osteocytes including RANKL, OPG, IL-6, and IGF-I (35-39). Previously, these osteocyte proteins have been primarily studied in the context of loading and unloading and mechanical strain (35, 37, 39). How these osteocyte proteins are altered in response to inflammation at distant sites (i.e. gastrointestinal systems) and how these osteocyte protein alterations influence osteoblast and osteoclast activity in bone is unknown.

Goals of this current project were to examine the impact of these factors, all of which are altered in IBD patients' serum in clinical trials, on tissue-specific bone outcomes in an established rodent model of IBD, specifically focusing on the osteocyte response. To our knowledge, this is the first investigation to examine the role osteocytes might play in mediating the loss of bone in the context of IBD. We hypothesized that 4 weeks of IBD in young male rats would result in bone loss, increased bone resorption, and decreased bone formation. In addition, we hypothesized the prevalence of osteocytes positive for TNF-α, IL-6, IL-10, RANKL, OPG, and sclerostin would be higher in IBD, while prevalence of osteocytes positive for IGF-I would be lower compared to age-matched vehicle treated controls.

2.1 Materials and Methods

Animals: Sixteen two-month-old male Sprague Dawley rats (weighing 250-274 grams) were purchased from Charles River Laboratories (Houston, TX), pair housed in a room with 12 hour light-dark cycles, and allowed *ad libitum* access to LabDiet 5053 rodent chow (LabDiet; St Louis, MO) and water. After one week of acclimation, rats were randomly divided into TNBS and vehicle control (n=8 per group). Tissues were collected from these animals for this study, but the parent protocol using different tissues set the group size; however, statistical power analyses for our outcomes showed n=4 as sufficient power for bone formation rate analyses and n=6 for all other histomorphometric and histological analyses. Gut inflammation was induced by 250-274 uL (1 uL/gram body weight) rectal instillations of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma Aldrich, St Louis, MO) in 30% ethanol:DiH_2O solutions (40-42). The 30% ethanol was used to disrupt the mucosal barrier while the haptenizing agent, TNBS, stimulated an immune response leading to gut inflammation. At days 1, 7, 14, 21, and 26 days non-fasted rats were anesthetized via inhaled isoflurane with a precision vaporizer and given enemas with either vehicle (30% ethanol:DiH_2O) or TNBS (30mg/kg trinitrobenzene sulfonic acid dissolved in 1mL 30% ethanol:DiH_2O) using an 18-gauge catheter with a blunted end inserted 7.5 cm into the rectum. The anus was held closed for 5 minutes after instillation to control the contact of the enema solution with the colon, after which animals were allowed to recover from anesthesia. Animal handling could not be completely blindly due to the nature of the study, but all collected tissues were analyzed blindly by the investigators. Fluorochrome calcein labels (Sigma Aldrich, St Louis, MO) were injected intraperitoneally 9 and 2 days prior to termination to label mineralized surfaces on bone. At day 28 animals were anesthetized via inhaled isoflurane, euthanized via thoracotomy, and tissues collected. All animal procedures

were approved by Scott & White Healthcare and Texas A&M Health Science Center Institutional Animal Care and Use Committees and conform to the NIH Guide for the Care and Use of Laboratory Animals.

Colon histopathology: Upon removing rat large intestinal loops, sections were cut from the colon (3 cm distal to the caecum), flushed of fecal matter, washed in DPBS, and fixed in 4% paraformaldehyde for 2 hours at room temperature; one more brief wash in PBS occurred before tissue was placed in 70% ethanol to dehydrate overnight at 4°C. Sections were then further dehydrated via a Thermo-Scientific STP 120 Spin Tissue Processor, paraffinized via a Thermo Shandon Histocenter 3 Embedding tool, sectioned (6 μm) via microtome, and adhered to positively charged glass slides for staining via Hematoxylin and Eosin (H&E) staining solutions. Colon damage scoring was performed on a 0-4 scale (0 being normal, and 4 being severe damage or alteration) for epithelial cell loss, crypt loss, edema, and cellularity.

Peripheral Quantitative Computed Tomography: Right tibia were saved in phosphate buffered saline in -35° C. Once thawed, *ex vivo* pQCT scans of the proximal tibia metaphysis (mixed cortical and cancellous bone site) and mid-shaft tibia (purely cortical bone site) were completed on a Stratec XCT Research-M device (Norland Corp., Fort Atkinson, WI). Metaphyseal volumetric BMD was measured at the proximal tibia from 4 slices located at least 1 mm distal of the growth plate. Three contiguous slices were averaged to provide one value for each variable at the proximal tibia metaphysis. One mid-shaft tibia slice was taken at approximately 50% of the total bone length. Scans were completed at 2.5 mm/sec scan speed, 100 μm voxel resolution, and 0.5 mm slice thickness. Measures obtained from the *ex vivo* pQCT scans include cortical and cancellous volumetric bone mineral density (vBMD).

Dynamic and static cancellous histomorphometry: For cancellous histomorphometry measures, undemineralized proximal tibia and 4th lumbar vertebra were fixed in 4% phosphate-buffered formalin for 24 hours and then subjected to serial dehydration and embedded in methyl methacrylate (Aldrich M5, 590-9, St. Louis, MO, USA). Serial frontal sections were cut 8 μm-thick and left unstained for fluorochrome calcein label measurements. The histomorphometric analyses were performed using OsteoMeasure Analysis System, version 3.3 (OsteoMetrics, Inc., Atlanta, GA, USA). A defined region of interest was established approximately 500 μm from the growth plate and within the endocortical edges encompassing approximately 8 mm^2 at 20x magnification. Total bone surface (BS), single-labeled surface (sLS/BS), double-labeled surface (dLS/BS), mineralized surface (MS/BS), and interlabel distances were measured at 20x magnification. Mineral apposition rate (MAR) was calculated from the interlabel distance and time of labels. Bone formation rate (BFR/BS) was determined by multiplying MS/BS by MAR. Additionally, 4 μm-thick sections were treated with von Kossa stain and tetrachrome counterstain to measure cancellous microarchitecture and osteoid (OS/BS) and osteoclast (Oc.S/BS) surfaces as a percent of total cancellous surface measured at 40x magnification. All nomenclature for cancellous histomorphometry follows standard usage (45).

Dynamic cortical histomorphometry: Undemineralized left distal tibia were fixed in 4% phosphate-buffered formalin for 24 hours and then serially dehydrated and embedded in methyl methacrylate. Cross sections of the bone closest to the mid-shaft were made on an IsoMet Low Speed Saw (Buehler, Lake Bluff, IL) approximately 100 μm thick. Cross sections were analyzed at 20x magnification using OsteoMeasure Analysis System, version 3.3 (OsteoMetrics, Inc., Atlanta, GA, USA) for MS/BS and MAR; bone formation rate was calculated as stipulated above.

Immunohistochemistry: Left distal femora were fixed in 4% phosphate-buffered formalin for 24 hours at 4°C and then decalcified in a sodium citrate/formic acid solution for approximately 14 days then stored in 70% ethanol. Sections were then further dehydrated in Thermo-Scientific STP 120 Spin Tissue Processor, paraffinized via a Thermo Shandon Histocenter 3 Embedding tool, sectioned to approximately 8 µm thickness, mounted on positively charged slides, and immunostained using an avidin-biotin method. Briefly, samples were rehydrated, peroxidase inactivated (3% H2O2/Methanol), permeabilized (0.5% Triton-X 100 PBS), blocked with species-appropriate serum for 30 minutes at room temperature (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) and incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-rat TNF-α, (1:100, LifeSpan BioSciences, Inc, Seattle, WA), polyclonal rabbit anti-IL-6 (1:300, Abcam, Inc, Cambridge, MA), goat polycolonal anti-IL-10 (1:100, Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-IGF-I (1:200, Abcam, Inc, Cambridge, MA), goat polyclonal anti-mouse RANKL (1:100, Santa Cruz Biotechnology, Dallas, TX), goat polyclonal anti-mouse OPG (1:100, Santa Cruz Biotechnology, Dallas, TX), polyclonal goal anti-mouse sclerostin (1:60, R&D Systems, Minneapolis, MN). On the subsequent day, sections were incubated at room temperature for 45 minutes with the appropriate species' biotinylated anti-IgG secondary antibody according to manufacturer specifications. Peroxidase development was performed with an enzyme substrate kit (DAB, Vector Laboratories). Counterstaining was conducted with 0.2% methyl green counterstain (Vector Laboratories) for 90 seconds; sections were subsequently dehydrated into organic phase and mounted with xylene-based mounting media (Polysciences, Warrington, PA). Negative controls for all antibodies were completed by omitting the primary antibody. Sections were analyzed by quantifying the proportion of all osteocytes staining positively for the protein in

three distinct bone compartments – cancellous bone (~500 microns from the growth plate, an area of approximately 4 mm^2), metaphyseal cortical bone (~500 microns from growth plate, an area of approximately 2 mm^2), and the cortical shaft (~100 microns distal to midshaft of bone, an area of approximately 2 mm^2; Figure 1). Negative controls (still containing methyl green stain) were used to quantify osteocyte density in the same bone compartments as listed above.

Statistical analyses: A t-test was completed between TNBS treated and Vehicle for each variable. Significance was determined at $p<0.05$. Effect size (partial eta-squared) was determined for values of $p<0.05$. All data is represented as mean ± standard deviation. Regression analysis was completed testing cancellous %$RANKL^+$ osteocytes to cancellous osteoclast surface, %TNF-α^+ osteocytes to %$sclerostin^+$ osteocytes, and %$sclerostin^+$ cancellous osteocytes to osteoid surface, %$sclerostin^+$ cancellous osteocytes to cancellous osteoid surface and cancellous bone formation rate, and %$sclerostin^+$ cortical shaft osteocytes to cortical bone formation rate. Statistics were completed using JMP Statistical Software (Cary, NC).

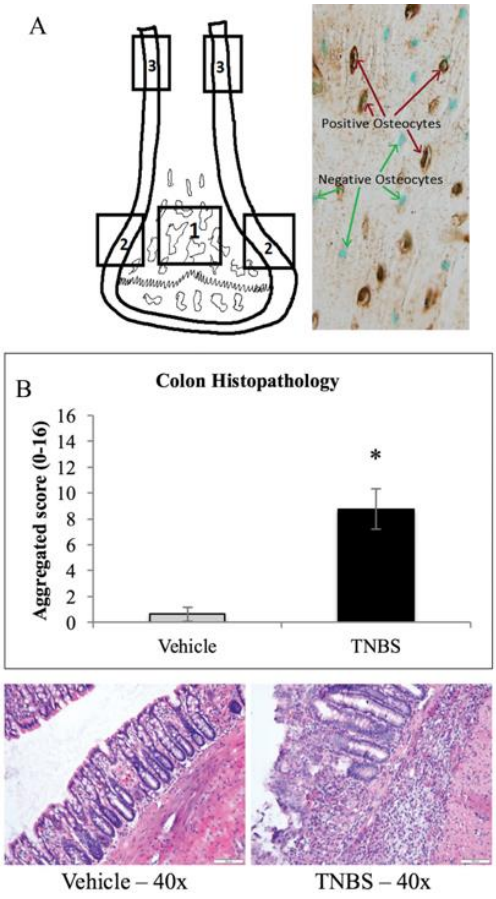

Figure 2.1: Bone regions and colon histopathology (A) Three regions of the distal femur analyzed via immunohistochemistry for percent-positive osteocytes and a representative image of regions of interest for analysis of immunohistochemistry of osteocytes. (B) Colon histopathology and representative images of colon from vehicle and TNBS. Histopathology score based on edema, crypt cell loss, cellularity, and intestinal epithelial cell damage was higher in TNBS versus vehicle ($p = 0.049$). *Indicates difference from vehicle.

2.2 Results

Animals: After 28 days of either TNBS or Vehicle treatment, there were no differences in bodyweight between the groups (average weight = 345±19 grams, p=0.248). There were no animal losses throughout the experiment and all animals maintained normal eating and grooming behavior. No modifications were made to the experimental protocol.

Histopathology of the colon is elevated in TNBS treated animals: In TNBS treated animal colons, there were notable perforations in the mucosal layer as well as increased cellular

investiture in the lamina propria. A large and significant increase in the histopathology score (the sum of individual scored determinants - intestinal epithelial damage, crypt loss, cellularity, and edema) occurred in TNBS versus Vehicle treated animals ($p<0.001$; Figure 2.1).

TNBS resulted in slight declines in volumetric bone mineral density: Total vBMD at the proximal tibia metaphysis was 7% lower in TNBS compared to Vehicle treated animals, trending towards significance ($p=0.058$; Veh average = 414±29 mg/cm^3, TNBS average = 385±27 mg/cm^3). There were no differences in vBMD at the midshaft tibia ($p=0.328$; data not shown).

Deficits in %BV/TV in proximal tibia metaphysis and 4th lumbar vertebrae (L4) are mediated by increased %Oc.S/BS and large declines in indices of bone formation. Cancellous bone volume (BV/TV) was lower in TNBS compared to Vehicle treated animals in both the proximal tibia metaphysis and the 4th lumbar vertebrae ($p=0.027$, effect size=0.347 and $p=0.026$, effect size=0.35, respectively; Figure 2.2A). Except for a trend for a 21% increase in 4th lumbar vertebral trabecular separation in TNBS treated animals ($p=0.058$, proximal tibia $p=0.121$; Figure 2.2E), there were no other significant differences in trabecular thickness (proximal tibia $p=0.180$, L4 $p=0.277$; Figure 2.2D) or trabecular number (proximal tibia $p=0.327$, L4 $p=0.282$; Figure 2.2E) between the two groups. Cancellous osteoclast surface (Oc.S/BS) was higher in both the proximal tibia and 4th lumbar vertebrae in the TNBS-treated group ($p=0.018$, effect size=0.385 and $p=0.004$, effect size=0.511, respectively; Figure 2.2B). Osteoid surface (OS/BS) was lower in both the proximal tibia and 4th lumbar vertebrae in the TNBS treatment ($p=0.001$, effect size=0.630 and $p=0.02$, effect size=0.375, respectively; Figure 2.2C). In the proximal tibia metaphysis and the 4th lumbar vertebrae, mineralized surface (MS/BS) was lower in TNBS compared to Vehicle treatment ($p<0.001$, effect size=0.912 and $p=0.006$, effect size=0.809,

respectively; Figure 2.2G). In both bone compartments, mineral apposition rate (MAR) was also lower in TNBS (p=0.009, effect size=0.710 and p=0.018, effect size=0.709, respectively; Figure 2.2H). Bone formation rate (BFR/BS) was lower in both cancellous bone sites in TNBS (p=0.002, effect size=0.828 and p=0.001, effect size=0.925, respectively; Figure 2.2I).

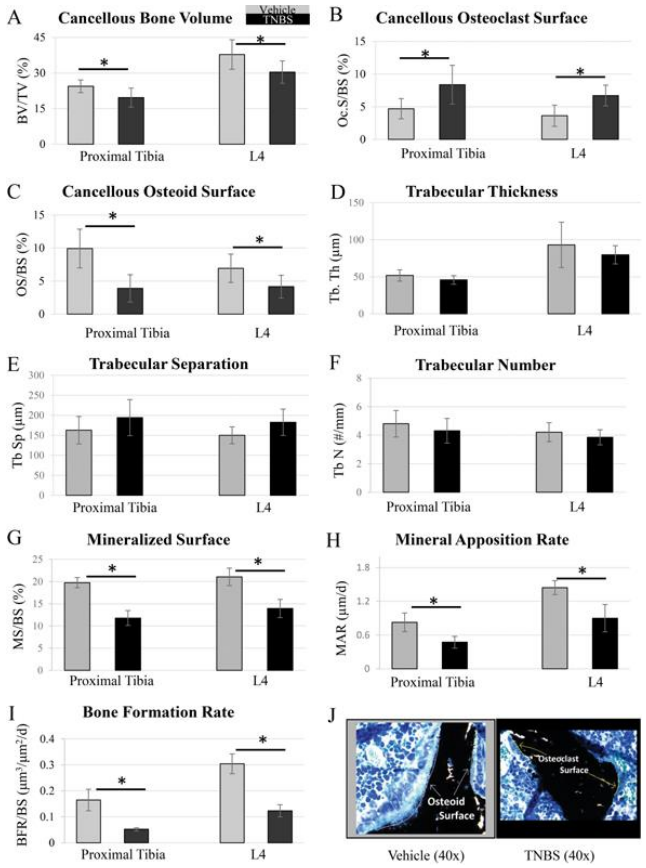

Figure 2.2: Cancellous histomorphometry at the proximal tibia metaphysis and fourth lumbar vertebra (L4). (A) Cancellous bone volume was lower in TNBS in both the proximal tibia ($p = 0.027$) and L_4 ($p = 0.026$). (B) Cancellous osteoclast surface was higher in TNBS at the proximal tibia ($p = 0.018$) and L_4 ($p = 0.004$).
Figure 2.2, continued. (C) Cancellous osteoid surface was lower in TNBS at the proximal tibia ($p = 0.001$) and L_4 ($p = 0.02$). (D) No statistical differences in trabecular thickness.
(E) No statistical differences in trabecular separation. (F) No statistical differences in trabecular number. (G) Mineralized surface was lower in TNBS at the proximal tibia ($p < 0.001$) and L_4 ($p = 0.006$). (H) Mineral apposition rate was lower in TNBS at the proximal tibia ($p = 0.009$) and L_4 ($p = 0.018$). (I) Cancellous bone formation rate was lower in TNBS at the proximal tibia ($p = 0.002$) and L_4 ($p = 0.001$). (J) Representative images of static histomorphometry of the proximal tibia at ×400 magnification of a vehicle animal (right) and TNBS animal (left).
*Indicates difference between TNBS and vehicle.

Midshaft tibia bone formation rate was lower in TNBS treated animals. Mineralized surface was lower in the TNBS treated group (p=0.009, effect size=0.709; Figure 2.3C), but there were no differences between groups in mineral apposition rate (p=0.710; Figure 2.3D). Bone formation rate was approximately 35% lower in the TNBS treatment group (p=0.025, effect size=0.595; Figure 2.2A).

Osteocyte density was lower in TNBS animals. The total numbers of osteocytes quantified in the cancellous compartment of bone were lower in TNBS animals compared to Vehicle (p=0.005, effect size=0.527; Figure 2.4A). Osteocyte density was also lower in TNBS treated animals in the metaphyseal cortical bone (p=0.003, effect size=0.612; Figure 2.4B) and the cortical shaft (p=0.036, effect size=0.340; Figure 2.4B).

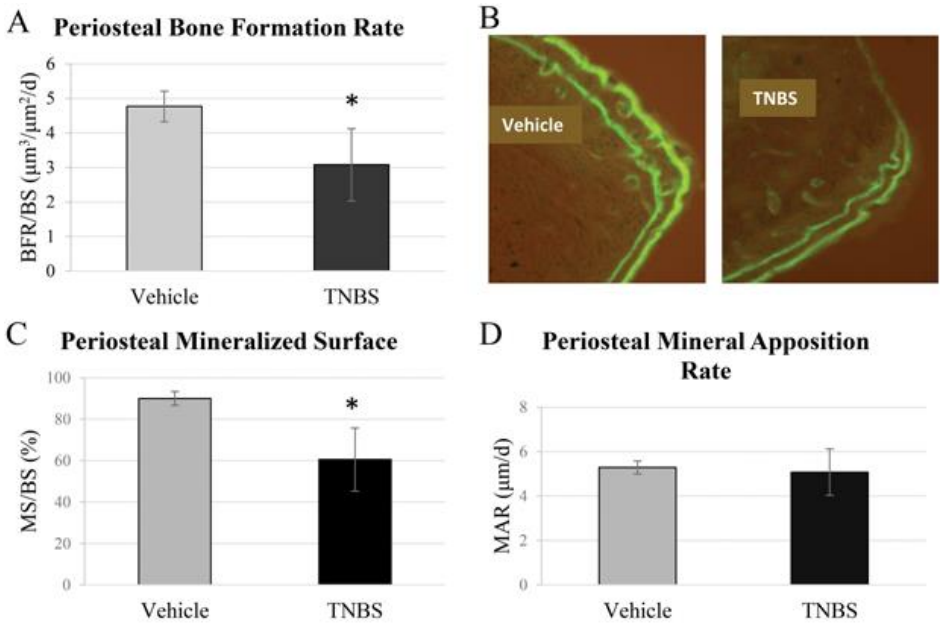

Figure 2.3: Cortical bone formation rate at the midshaft tibia. (A) Cortical bone formation rate was lower in TNBS compared to vehicle ($p = 0.025$). (B) Representative images of periosteal BFR at the midshaft tibia in a vehicle animal and TNBS animal at ×10 magnification. (C) Periosteal mineralized surface was lower in TNBS compared to vehicle ($p = 0.009$). (D) No statistical difference in mineral apposition rate at the midshaft tibia. *Indicates difference from vehicle.

Osteocyte prevalence of inflammatory markers were altered in TNBS animals: TNF-α: %TNF-α^+ osteocytes were higher in TNBS-treated rats in both the cancellous bone and metaphyseal cortical bone (p=0.01; effect size=0.499 and p=0.046; effect size=0.341 respectively; Figure 2.4C). There were trending increases in the cortical shaft in TNBS-treated rats (p=0.062; Figure 2.4C). IL-6: %IL-6^+ osteocytes were higher in TNBS-treated rats in the cancellous bone (p=0.005; effect size=0.548, and p=0.029; effect size=0.392, respectively; Figure 2.4D) and the metaphyseal cortical bone. There were no differences at the cortical shaft (p=0.134). IL-10: %IL-10^+ osteocytes were not different between TNBS-treated rats and Vehicle in any bone compartment (cancellous p=0.620, metaphyseal p=0.808, shaft p=0.344; Figure 2.4E). IGF-I: %IGF-I^+ osteocytes were higher in TNBS-treated rats in the cancellous bone and the metaphyseal cortical bone (p=0.003; effect size=0.591 and p=0.013; effect size=0.471, respectively; Figure 2.4F). There were no differences at the cortical shaft (p=0.592). RANKL: %RANKL$^+$ osteocytes were higher in the cancellous bone and the cortical shaft in TNBS-treated rats (p=0.011; effect size=0.455 and p=0.049; effect size=0.307, respectively; Figure 2.5A). There were no differences at the metaphyseal cortical bone between the groups (p=0.126). OPG: %OPG$^+$ osteocytes were higher in the cancellous bone and the metaphyseal cortical bone in TNBS-treated rats (p=0.012; effect size=0.418 and p=0.035; effect size=0.320, respectively; Figure 2.5B). There were no differences at the cortical shaft (p=0.644). Sclerostin: %Sclerostin$^+$ osteocytes were higher in the cancellous bone (p<0.0001; effect size=0.782), the metaphyseal cortical bone (p=0.005; effect size=0.514), and the cortical shaft in TNBS treated animals (p=0.03; effect size=0.352; Figure 2.5E).

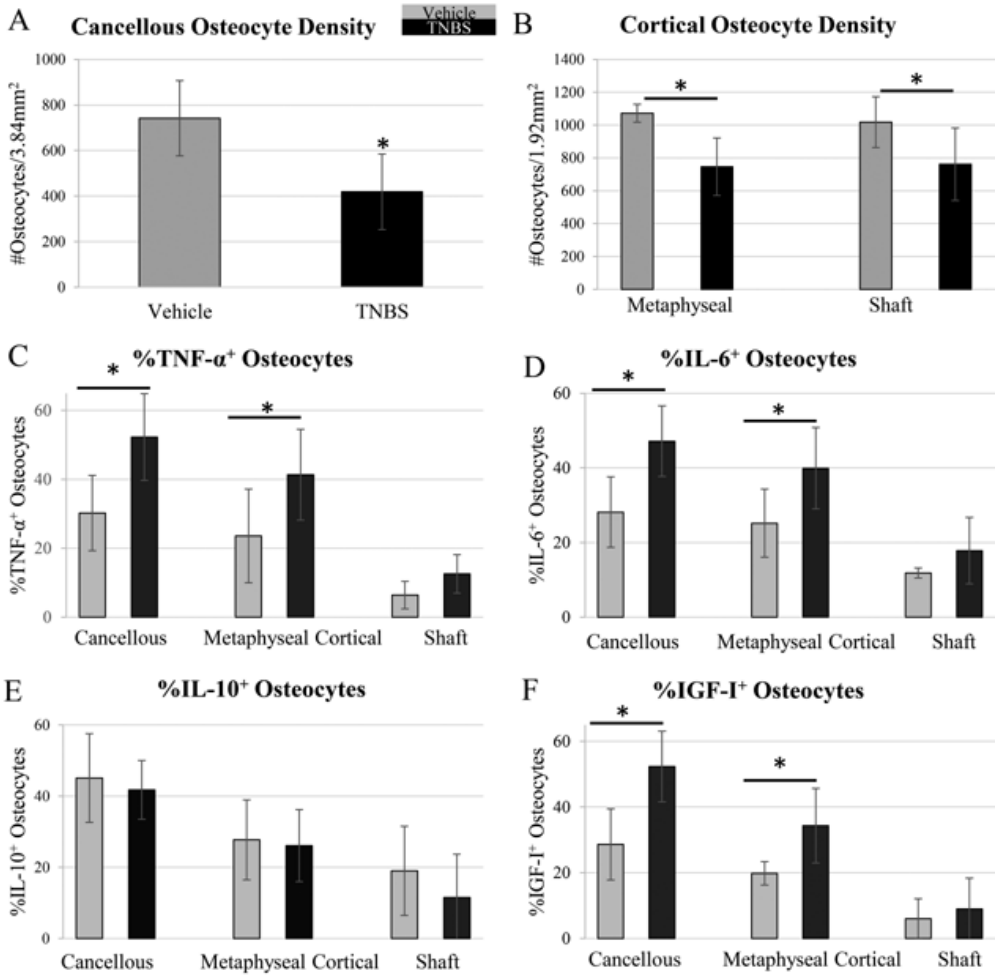

Figure 2.4: Immunohistochemical staining of osteocytes for density and percent positive TNF-α, IL-6, IL-10, and IGF-I. (A) Cancellous osteocyte density was lower in TNBS ($p = 0.005$). (B) Osteocyte density was lower in TNBS in both the metaphyseal cortical bone ($p = 0.003$) and the cortical shaft ($p = 0.036$). (C) %TNF-α^+ osteocytes were higher in cancellous ($p = 0.01$) and metaphyseal cortical bone ($p = 0.046$) in TNBS compared to vehicle. (D) %IL-6^+ osteocytes were elevated in TNBS in the cancellous ($p = 0.005$) and metaphyseal cortical ($p = 0.029$) bone compartments. (E) %IL-10^+osteocytes were not different between TNBS and vehicle. (F) %IGF-I^+ osteocytes were higher in TNBS in both the cancellous ($p = 0.003$) and metaphyseal cortical bone ($p = 0.013$). *Indicates difference between TNBS and vehicle within specific bone compartment.

The increase in %OPG$^+$ osteocytes and %RANKL$^+$ osteocytes statistically predicted the increase in osteoclast surface. %RANKL$^+$ cancellous osteocytes alone statistically predicted the increase cancellous osteoclast surface (R^2=0.565, adjusted R^2=511, p=0.012; Figure 2.5C). Additionally, the ratio of %OPG$^+$ cancellous osteocytes to %RANKL$^+$ cancellous osteocytes

statistically predicted cancellous osteoclast surface (R^2=0.405, adjusted R^2=0.339, p=0.035; Figure 2.5D).

The increase in %TNF-α^+ osteocytes statistically predicted the increased in %sclerostin$^+$ osteocytes. %TNF-α^+ cancellous osteocytes explained approximately 60% of the variability in %sclerostin$^+$ cancellous osteocytes (R^2=0.588, adjusted R^2=0.546, p=0.004; Figure 2.5F).

The increase in %sclerostin$^+$ osteocytes statistically predicted declines in bone formation. %Sclerostin$^+$ cancellous osteocytes statistically predicted the decline in cancellous osteoid surface (R^2=0.581, adjusted R^2=0.539, p=0.004; Figure 2.5G) and cancellous bone formation rate (R^2=0.674, adjusted R^2=0.609, p=0.024). %Sclerostin$^+$ cortical shaft osteocytes explained approximately 90% of the variability in cortical bone formation rate (R^2=0.908, adjusted R^2=0.890, p=0.001; Figure 2.5H).

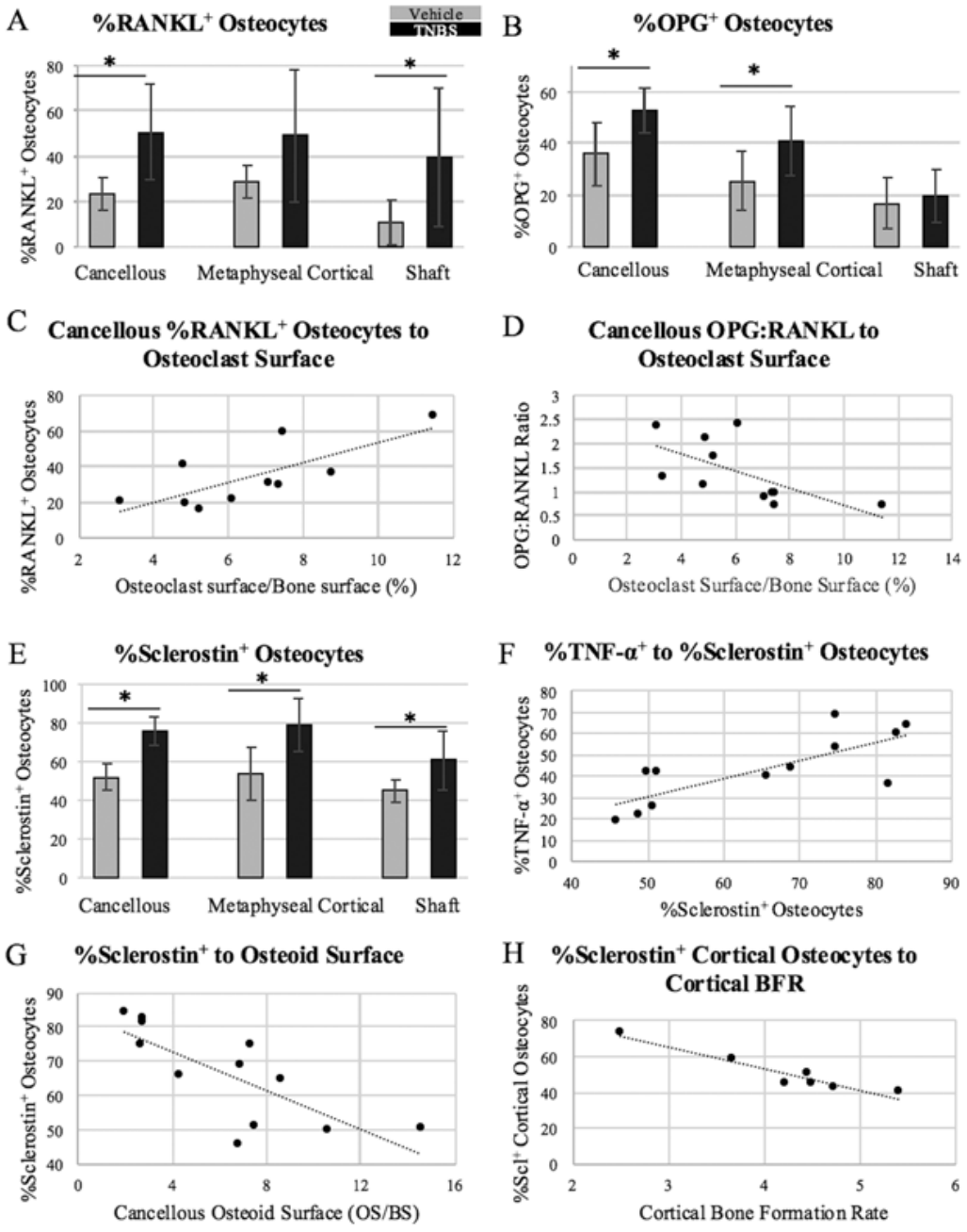

Figure 2.5: Immunohistochemical staining of osteocytes for RANKL, OPG, and sclerostin with regression analyses. (A) %RANKL$^+$osteocytes were higher in TNBS in the cancellous ($p = 0.011$) and cortical shaft ($p = 0.049$) bone compartments. (B) %OPG$^+$ osteocytes were elevated in the cancellous ($p = 0.012$) and metaphyseal cortical bone in TNBS ($p = 0.035$). (C) Cancellous %RANKL$^+$ osteocytes predicted the increase in cancellous osteoclast surface ($R^2 = 0.565$, $p = 0.012$). (D) The ratio of %positive-osteocytes of OPG:RANKL predicted the increase in cancellous osteoclast surface ($R^2 = 0.405$, $p = 0.035$). (E) %Sclerostin$^+$ osteocytes were higher in TNBS in the cancellous ($p < 0.0001$), metaphyseal cortical ($p = 0.005$), and shaft ($p = 0.03$). (F) %TNF-α$^+$ cancellous osteocytes predicted the increase in %sclerostin$^+$ cancellous osteocytes ($R^2 = 0.588$, $p = 0.004$). (G) %Sclerostin$^+$ cancellous osteocytes predicted the decline in cancellous osteoid surface ($R^2 = 0.674$, $p = 0.024$). (H) %Sclerostin$^+$ cortical osteocytes predicted the decline in cortical bone formation rate ($R^2 = 0.908$, $p = 0.001$). *Indicates difference between TNBS and vehicle within specific bone compartment.

2.3 Discussion

The findings of this study, first, provide confirmation in a rodent model of IBD that the magnitude of bone loss, increased bone resorption, and decreased bone formation are all associated with chronic inflammation initiated by the gastrointestinal pathology. Secondly, this study provides novel evidence of the role of osteocytes and osteocyte proteins in the systemic inflammation associated with IBD.

Several other previously published studies have documented changes in bone mass and bone turnover due to IBD in rodent models similar to those seen in clinical patients (44, 45). This study corroborates these findings demonstrating loss of cancellous bone volume, increased bone resorption, and decreased bone formation in young male rats with an experimental model of IBD (Figure 2). These skeletal changes are concurrent with higher intestinal epithelial damage, crypt loss, increased cellularity, and edema in the colon of the IBD animals (Figure 2.1). Therefore, the primary inflammation and damage initiated in the gut appears capable of having a negative impact on multiple distant bone sites. The patterns of loss of cancellous bone volume and altered bone turnover were similar in the 4^{th} lumbar vertebrae and the proximal tibia metaphysis. In patients with IBD, it is cancellous-rich bone sites like vertebral bone and the femoral neck that exhibit the most significant declines in bone mineral density and the greatest increase in fracture incidence (6, 13-16). In our study, the changes in the proximal tibia cancellous resorbing and forming surfaces were significantly altered (TNBS osteoclast surface was 76% higher than vehicle and osteoid surface was 60% lower than Vehicle; see Figure 2.2), while there were only tendencies towards lower volumetric bone mineral density (7% lower) in TNBS treated animals.. Therefore, the bone loss measured in clinical studies via dual x-ray absorptiometry or computed tomography may under-estimate or not have adequate sensitivity to quantify the changes in bone

cell activity occurring in local bone sites. These patterns imply that inflammatory signals from the primary site of damage/inflammation in the gut are somehow transmitted to multiple distant bone sites. Additionally, in our study the rats only experienced four weeks of IBD and it is likely that greater loss of BMD would occur after an even longer period of IBD. Since IBD currently has no cure, should the alterations in bone turnover favoring bone resorption found in this animal model be maintained over longer periods of time, devastating losses of bone density and resistance to fracture could occur.

Pro-inflammatory cytokines, like TNF-α, are potent stimulators of osteoclastogenesis and osteoclast activity while also suppressing bone formation (22, 23). In a mouse model of IBD, elevated TNF-α gene expression was seen in the colon along with an elevated inflammation score (46). In previous work using the same experimental model of IBD, our group has shown rapid, large increases in TNF-α RNA expression in the mesenteric lymphatics (40). In this study, the %TNF-α-positive osteocytes in the cancellous and metaphyseal cortical bone were approximately 75% higher in TNBS rats than in vehicle-treated animals (Figure 4). Likewise, IL-6, another pro-inflammatory cytokine, was approximately 60% higher in the osteocytes of TNBS treated animals in both metaphyseal bone compartments (Figure 2.4). These changes were coincident with increased osteoclast surface, decreased osteoid surface, and decreased bone formation rate in cancellous bone. In human IBD patients, serum IL-6 concentrations are significantly elevated in both children and adults (21, 24). Adding the serum from children with Crohn's disease to fetal rat parietal bone cultures resulted in demineralization, while neutralization of IL-6 prevented changes in osteoblasts and morphology (21). Therefore, it appears that serum IL-6 plays a significant role in the altered bone turnover in IBD. Since IL-6 is also a product of osteocytes (35, 36), it is possible that the local inflammatory state in bone is

dependent on direct osteocyte contributions to IL-6 and not merely responding to systemic signals. In our study, the osteocyte prevalence of IL-10 was not different in osteocytes in TNBS-treated versus Vehicle-treated animals (Figure 2.4). While the role of IL-10 in IBD in humans remains poorly understood and equivocal in the pathology of IBD, the osteocyte IL-10 we observed was not elevated like the increased serum IL-10 concentrations in patients with IBD (26, 27). Clinical studies have been inconsistent regarding serum IL-10 levels, with some patients showing elevated IL-10 (presumably as a compensatory mechanism to gut inflammation), but other studies have shown no elevation in IBD patients compared to healthy populations (47). It is known IL-10 -/- mice develop systemic osteopenia, and that the degree of osteopenia is more severe in IL10 -/- mice with spontaneous colitis than without, supporting a critical role of inflammation beyond IL-10 mediated mechanisms (48). Furthermore IL-10's role in influencing bone turnover or resorption is equivocal, with the interaction of IL-10 and its impact on osteocyte biology unknown (48). Therefore in the local bone tissue, there does not appear to be a compensatory, anti-inflammatory response via IL-10 mechanisms but this does not exclude other members of the IL-10 family such as the IL-20 subfamily, which will be the subject of future studies (47, 48). Taken together, these data suggest an established pro-inflammatory state in bone, specifically in osteocytes, after four weeks of IBD in this rodent model.

Alterations in RANKL and OPG have also been implicated as playing roles in elevated bone resorption in IBD due to altered serum concentrations in patients, with high OPG:RANKL ratios favoring osteoclast formation (4, 6, 32-34). Studies of colonic explant cultures have identified inflamed colon tissue as source of OPG (34); however, RANKL and OPG are also produced by osteocytes and osteoblasts, hence one of our goals was to examine osteocyte

RANKL and OPG in these IBD animals. Percent-positive RANKL and OPG osteocytes were indeed higher in TNBS compared to Vehicle-treated rats (Figure 2.5). Using a regression model, %RANKL$^+$ osteocytes explained approximately 57% of the variability in the increase in cancellous osteoclast surface (R^2=0.565) and the ratio of OPG to RANKL positive osteocytes explained approximately 40% of the variability in the increase in osteoclast surface (R^2=0.405). The direction of these changes in osteocyte prevalence of RANKL and OPG is similar to that seen in circulating serum concentrations in human IBD patients. The increased RANKL is likely due to increased inflammation, particularly from TNF-α, as TNF-α cooperates with RANKL in osteoclast formation (49-50). It has been hypothesized that the increase in OPG is either a compensatory response to increased bone resorption or that it is simply a response to the inflammatory state (33). In addition, cell culture studies have found OPG mRNA in osteoblasts is increased by TNF-α (51) indicating it, too, may be directly influenced by an increased pro-inflammatory state.

Inflammatory bone loss is not only characterized by an increase in bone resorption, but also a decrease in bone formation (20). Both TNF-α and IL-6 have been implicated in this decline in bone formation via suppression of osteoblasts (21, 22). In this study, we investigated two other osteocyte proteins that directly regulate osteoblast function and bone formation. IGF-I is a potent stimulator of bone formation (52) and TNF-α suppresses its actions in osteoblasts (22). Osteocyte-derived IGF-I plays a role in the adaptation of bone to mechanical loading (40, 41), but how it is altered with systemic inflammation has not been explored. We hypothesized that osteocyte IGF-I would decrease in IBD rats, based on documented declines observed in serum IGF-I concentrations in IBD patients (24, 53). Contrary to our hypothesis, we observed an increase in osteocyte-specific IGF-I in IBD animals (Figure 2.4). This increase in %IGF-I$^+$

osteocytes could represent a local mechanism compensating for declines in systemic IGF-I by local secretions of this potent anabolic factor; however, the elevated IGF-I levels in osteocytes did not rescue the observed decline in bone formation in these IBD rats. Future studies will address whether circulating concentrations of IGF-I are lower in this rodent model of IBD similar to what is seen in human patients.

Another osteocyte protein that regulates bone formation is sclerostin, which inhibits the Wnt/β-catenin signaling pathway, thereby suppressing osteoblast activity (54). TNF-α is a transcriptional activator of sclerostin; in a recently published study, diet-induced obesity in mice resulted in elevated TNF-α and sclerostin in bone (36). In our study, we found elevated osteocyte sclerostin prevalence in all bone compartments of the distal femur (Figure 2.5). Nearly 60% of the increase in sclerostin was statistically predicted by increases in osteocyte TNF-α via regression analysis. In turn, %sclerostin^{+} osteocytes closely predicted the declines in cancellous osteoid surface and cancellous and cortical bone formation rate. Therefore, we demonstrate that an increased pro-inflammatory state (i.e. increased osteocyte TNF-α) was strongly associated with elevated osteocyte sclerostin and decreased bone formation activity.

Interestingly, in this study the largest significant changes in %positive osteocyte proteins were in the metaphyseal bone – primarily the cancellous compartment followed by the metaphyseal cortical bone. Except for sclerostin and RANKL, the other proteins that did change in the metaphyseal bone were not significantly altered in the cortical shaft of the bone. This corresponds with the clinical evidence of increased bone loss and fracture risk in cancellous-rich bone sites like vertebral bone. Additionally, we found lower osteocyte density in all compartments of the bone in IBD animals (Figure 2.4). This effect was not localized solely to the cancellous bone compartment but to both cancellous and cortical compartments. IBD animals

had fewer osteocytes in all compartments, but of those osteocytes still present (particularly in the cancellous bone), there was a higher prevalence of osteocytes positive for all proteins measured in this study except IL-10. The lower number of osteocytes in IBD animals may suggest increased apoptosis, which warrants further study.

In conclusion, we found that four weeks of inflammatory bowel disease in young male rats resulted in increased bone resorption, decreased bone formation, and loss of cancellous bone volume. Distant inflammatory changes initiated in the gut in this animal model of IBD resulted in increased prevalence in the distal femur osteocytes positive for TNF-α, IL-6, RANKL, OPG, IGF-I, and sclerostin. The altered prevalence of these factors was seen as a local increase in the bone inflammatory state coincident with increased bone resorption and decreased bone formation in all bone compartments tested. Osteocytes, often considered the primary cell type regulating bone formation/resorption, were impacted by the gut inflammation caused by IBD. The pathways by which the signals initiated in the gut alter the systemic immune response and produce the serum signals that in turn affect bone at multiple distant sites are not yet understood. However, these data suggest that bone cells are not simply passively responding to the circulating inflammatory signals from the gut, but that the osteocyte response plays a key regulatory role in the development of IBD-induced bone loss.

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3. A MODERATELY ELEVATED SOY PROTEIN DIET MITIGATES INFLAMMATORY CHANGES IN CANCELLOUS BONE TURNOVER DURING CHROINC TNBS-INDUCED INFLAMMATORY BOWEL DISEASE

The influence of diet on the course of inflammatory bowel disease is poorly understood. Food antigens are the second most common antigen present in the intestinal lumen second only to bacterial antigens; therefore, nutritional factors could play important roles in the development of gut inflammation (1). However, this area of research remains inconclusive. Some evidence links high sugar intake, high animal protein and cholesterol intake with the development of IBD (2-5) and high consumption of vegetables with lower risk of developing IBD (5). The results of the few clinical trials for nutritional interventions during active IBD, like low sugar diets, have been inconsequential (1, 2). Despite the lack of evidence for nutritional influence on IBD, determining a specific diet or nutrient that could produce anti-inflammatory benefits to protect gut and bone health would improve clinical care and quality of life in patients.

While there are some links between high animal protein consumption and the development of gut inflammation, there is some preliminary evidence demonstrating that plant-based soy protein may actually exert anti-inflammatory effects. In postmenopausal women, soy protein intake decreases circulating C reactive protein levels, a non-specific measure of inflammation, as well as the pro-inflammatory marker, interleukin-18 (6). Soy protein consumption decreases macrophage infiltration in an animal model of polycystic kidney disease (7). Additionally, the severity of atherosclerotic lesions is lessened with a soy protein diet in apolipoprotein E-deficient mice potentially through inhibiting monocyte chemoattractant protein-1, part of the inflammatory response (8). Two animal models of IBD have examined the use of soy protein. In dextran sodium sulfate-induced colitis in piglets, soy-derived di- and tri-peptides

attenuate colonic expression of pro-inflammatory cytokines and inflammatory gut changes (9). In mice with dextran sodium sulfate colitis, consumption of soy protein, but not casein or probiotic supplementation, decreases gut inflammatory scores and colonic gene expression of *TNFα* (10). These data indicate that soy protein may exert unique anti-inflammatory effects in the gut and beneficially impact the course of inflammatory conditions like IBD. These studies all only examine the impact of soy on acute gut inflammation and do not address distant tissues affected by IBD like bone.

With regards to bone health, protein consumption has a controversial history due to concerns of excess protein creating a negative calcium balance in the body; however, a controlled study with stable isotope tracing in healthy young females demonstrated no negative alterations in bone turnover with high protein diets (11). Additionally, large epidemiological studies demonstrate the opposite of the concern with low protein intake associated with increased bone loss in the elderly (12). A meta-analysis also demonstrates slight improvements in lumbar spine BMD with protein supplementation (13). Importantly, there is also no evidence of a detrimental effect of protein consumption on fracture incidence (13). In overweight participants in a weight loss trial, protein supplementation mitigates the decline in bone mass seen due to energy restriction (14). Whether a moderately elevated protein diet would attenuate bone loss in chronic inflammation is unknown. A diet high in soy isoflavones, but without elevated protein consumption, mitigates lumbar spine bone loss in perimenopausal women (15). In young male rats with a high protein diet, soy protein increases cancellous and cortical volumetric bone mineral density (16). Whether soy protein can mitigate inflammatory bone loss has not been examined.

The purpose of this current project is to examine the impact of a moderately elevated soy protein diet during chronic gut inflammation in an animal model of IBD. We hypothesized that a moderately elevated soy protein diet would improve gut pathology and mitigate the inflammatory status of bone compared to a standard rodent control diet with protein from casein, a primary protein in dairy milk. Specifically, we hypothesized our dietary intervention would mitigate gut pathology and decrease osteocyte TNF-α, IL-6, RANKL, OPG, and sclerostin. These changes would correlate with an increase in bone formation rate and a decrease in osteoclast surface, an indicator of resorptive activity. Additionally, we hypothesized that osteocyte IGF-I would increase with the dietary protein intervention concurrent with increased bone formation rate.

3.1 Materials and Methods

Animals: Thirty-two male Sprague-Dawley rats (1.5 months old) were ordered from Envigo (Houston, Texas) and singly housed in an institutionally approved animal facility with 12 hour light dark cycles. Animals were allowed approximately four days to acclimate to the facility before being switched from standard rodent chow (Teklad 2018, Envigo) to the purified AIN93G chow (Research Diets, Inc., New Brunswick, NJ). Animals were allowed free access to food and water. Following the one week acclimation to the diet, animals were randomly divided into four different groups (n=8/group): Vehicle (Veh), Vehicle with soy protein (Veh+Pro), IBD induced via 2,4,6-trinitrobenzenesulfonic acid (TNBS), and IBD with soy protein (TNBS+Pro). Gut inflammation was induced by rectal instillations of 1 uL/gram body weight, of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 30 mg/kg, Sigma Aldrich, St Louis, MO) in 30% ethanol:DiH_2O solutions, as previously described (17, 18). The 30% ethanol was used to disrupt the mucosal barrier while the haptenizing agent, TNBS, stimulated an immune response leading

to gut inflammation. On days 1, 7, 14, 21, and 26 non-fasted rats were anesthetized via inhaled isoflurane with a precision vaporizer and given enemas with either vehicle or TNBS using an 18-gauge catheter with a blunted end inserted 7.5 cm into the rectum. The anus was held closed for 5 minutes after instillation to control the contact of the enema solution with the colon, after which animals were allowed to recover from anesthesia. Rectal instillations began at 2 months of age and continued for up to four weeks. Fluorochrome calcein labels (Sigma Aldrich, St Louis, MO) were injected intraperitoneally eight and three days prior to termination to label mineralized surfaces on bone. Animal health was monitored daily and food intake and bodyweight were measured several times per week for the entire experimental period. After four weeks of TNBS or Veh treatment, rats were anesthetized via inhaled vaporized isoflurane, euthanized via thoracotomy, and tissues were collected. All animal procedures were approved by the Texas A&M Institutional Animal Use and Care Committee and conform to the NIH Guide for the Care and Use of Laboratory Animals.

Diets: Beginning on the first day of rectal instillations, all Pro animals were switched from the AIN93G diet formulated with 20% of the kilocalories coming from casein to a specialized diet based on the AIN93G with 35% of the kilocalories coming from soy protein (Research Diets, Inc). Diet specifications are detailed in Table 3.1 and Table 3.2. Rats were kept on the Pro diet continuously for the remainder of the study. Food intake was monitored throughout the entire study.

Table 3.1: Percent kilocalories of macronutrients in the control and moderately elevated soy diet.

	Control Diet %kcal	**Soy Protein Diet %kcal**
Protein	20	35
Carbohydrate	64	49
Fat	16	16
Total	*100*	*100*

Table 3.2: Control and moderately elevated soy protein diet composition. Information provided by Research Diets, Inc.

Ingredient	**Control Diet**	**Soy Protein Diet**
Casein	200 gm	0
Soy protein	0	345 gm
DL-methionine	0	5 gm
L-cystine	3 gm	0
Corn starch	397 gm	250 gm
Maltodextrin 10	132 gm	132 gm
Sucrose	100 gm	100 gm
Cellulose	50 gm	50 gm
Soybean oil	70 gm	70 gm
t-Butylhydroquinone	0.014 gm	0.014 gm
Mineral mix	35 gm	35 gm
Vitamin mix	10 gm	10 gm
Choline Bitartrate	2.5 gm	2.5 gm
Total	*1000 gm*	*1000 gm*

Tissue processing and histological analysis: Whole length colons were removed, processed, and were scored from H&E stained sections based on epithelial structure, crypt structure, cellularity, and edema. Scores were adjusted to account for area of tissue affected. All scores were conducted blindly.

Dynamic and Static Histomorphometry: Undemineralized right proximal tibia and fourth lumbar vertebrae (L4) were fixed in 4% phosphate buffered formalin and then subjected to serial dehydration and embedded in methyl methacrylate (J.T. Baker, VWR, Radnor, PA). Serial frontal sections at 8 µm thickness were left unstained for analysis of fluorochrome labels for

dynamic histomorphometry. The histomorphometric analyses were performed using OsteoMeasure Analysis System, version 3.3 (OsteoMetrics, Inc., Atlanta, GA, USA). For the proximal tibia, a defined region of interest was established approximately 500 μm from the growth plate and within the endocortical edges encompassing approximately 8 mm^2 at 20x magnification. Total bone surface (BS), single-labeled surface (sLS/BS), double-labeled surface (dLS/BS), mineralized surface (MS/BS), and inter-label distances were measured at 20x magnification. Mineral apposition rate (MAR) was calculated from the time lapsed between label deliveries. Bone formation rate (BFR/BS) was determined by multiplying MS/BS by MAR. Additionally, 4 μm-thick sections were treated with von Kossa stain and tetrachrome counterstain to measure cancellous microarchitecture and osteoid (OS/BS) and osteoclast (Oc.S/BS) surfaces as a percent of total cancellous surface measured at 40x magnification. All nomenclature for cancellous histomorphometry follows standard usage (19).

Immunohistochemistry of Osteocyte Proteins: Left distal femora were fixed in 4% phosphate-buffered formalin for 24 hours at 4°C and then decalcified in a sodium citrate/formic acid solution for approximately 14 days then stored in 70% ethanol. Sections were then further dehydrated in Thermo-Scientific STP 120 Spin Tissue Processor, paraffinized via a Thermo Shandon Histocenter 3 Embedding tool, sectioned to approximately 8 μm thickness, mounted on positively charged slides, and immunostained using an avidin-biotin method. Briefly, samples were rehydrated, peroxidase inactivated (3% H2O2/Methanol), permeabilized (0.5% Triton-X 100 PBS), blocked with species-appropriate serum for 30 minutes at room temperature (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) and incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-rat TNF-α, (LifeSpan BioSciences, Inc, Seattle, WA), polyclonal rabbit anti-IL-6 (Abcam, Cambridge, MA), polyclonal rabbit anti-IL-10

(Abcam), polycloncal rabbit anti-IL-4 (Abcam), polyclonal rabbit anti-annexin V (Abcam), polyclonal goat anti-mouse sclerostin (R&D Systems, Minneapolis, MN), polyclonal rabbit anti-RANKL (Abcam), and polyclonal rabbit anti-OPG (Biorbyt, San Francisco, CA). On the subsequent day, sections were incubated at room temperature for 45 minutes with the appropriate species' biotinylated anti-IgG secondary antibody according to manufacturer specifications. Peroxidase development was performed with an enzyme substrate kit (DAB, Vector Laboratories). Counterstaining was conducted with 0.2% methyl green counterstain (Vector Laboratories) for 90 seconds; sections were subsequently dehydrated into organic phase and mounted with xylene-based mounting media (Polysciences, Warrington, PA). Negative controls for all antibodies were completed by omitting the primary antibody. Sections were analyzed by quantifying the proportion of all osteocytes staining positively for the protein in the cancellous bone (~500 microns from the growth plate, an area of approximately 4 mm^2). Previous data demonstrated that the cancellous bone region was the bone compartment with osteocytes most responsive to inflammation due to IBD.

Statistical analyses: All data were tested for homogeneity and analyzed using a 2x2 factorial ANOVA to determine main effects of diet and TNBS. If a diet-by-TNBS interaction was present ($p<0.05$), all-groups analysis was completed. If the main effects were significant ($p<0.05$), a Duncan post-hoc test was used to determine differences between groups. Statistical analyses were completed on SPSS (IBM; Armonk, NY). All data are represented as mean ± standard deviation.

3.2 Results

All animals maintained bodyweight and consistent food intake across the course of the study regardless of treatment or diet. There were no statistical differences in bodyweight

between any groups at any time point in the study. Additionally, food intake was not different due to treatment or diet (Table 3.3).

Table 3.3: Average daily food intake and final bodyweights. No groups were statistically different from each other at any time point.

Measure	**Veh**	**Veh+Pro**	**TNBS**	**TNBS+Pro**
Average daily food intake	17.16±1.2	16.3±2.1	17.3±1.6	16.4±1.3
Final bodyweight	355±17	351±17	348±30	333±14

The moderately elevated soy protein diet mitigated gut damage and inflammation during chronic TNBS. There were significant main effects on colon pathology scores for treatment, diet, and a significant treatment-by-diet interaction ($p<0.0001$ for all). This aggregated score for TNBS-treated rats was higher than those for all other groups, with TNBS+Pro values no different from both Veh groups (Figure 3.1).

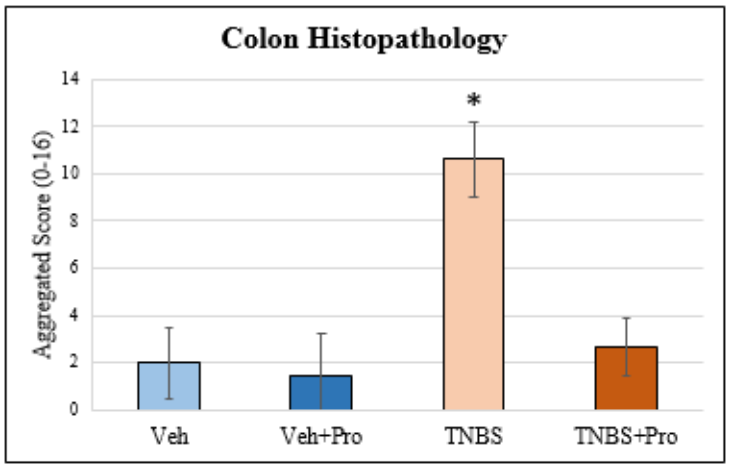

Figure 3.1: Colon histopathology. TNBS was higher than all other groups ($p < 0.0001$).
*Indicates difference from all other groups.

The soy protein diet mitigated the increase in osteoclast surface seen in TNBS. At both the proximal tibia and L4, there was a main effect of TNBS ($p=0.001$ for both) and a main effect of diet ($p=0.026$, $p=0.039$, respectively). There was a significant interaction effect only at the proximal tibia ($p=0.04$). For both proximal tibia and L4, the TNBS group had the highest osteoclast surface of all groups. TNBS+Pro animals were no different than both vehicle-treated rats at both sites (Figure 3.2A).

The soy protein diet increased bone formation rate in both TNBS and Veh treated animals. For bone formation rate, there were significant main effects of treatment and diet at both the proximal tibia and L4 ($p<0.0001$ for all) with a significant interaction effect between treatment and diet at the proximal tibia ($p<0.0001$). At the proximal tibia, the TNBS group BFR values were lower than all other groups while the protein diet increased BFR. At L4, TNBS animals had the lowest BFR with TNBS+Pro animals no different from vehicle-treated (Figure 3.3A). At the proximal tibia there were significant main effects for both treatment and diet ($p<0.0001$ for both) in MS/BS and main effects for treatment and diet ($p=0.003$, $p=0.004$) for MAR. There were non-significant interaction effects for both MS/BS ($p=0.067$) and MAR ($p=0.077$) at the proximal tibia. At the proximal tibia, both TNBS groups had lower MS/BS than both Vehicle groups (Figure 3.3B). For MAR, the TNBS group was lower than all other groups. At L4, there were main effects for both treatment and diet ($p<0.0001$ for both) for MS/BS, but only a main effect for diet on MAR ($p=0.009$). MS/BS was lowest in the TNBS group with the TNBS+Pro group no different than vehicle-treated. For MAR, the TNBS group was lower than both soy protein groups with the vehicle group no different from any other group (Figure 3.3C). In osteoid surface measured via static histomorphometry, similar changes were seen as in MS/BS

with both TNBS groups lower than Veh groups in the proximal tibia and TNBS lower than all other groups in L4 (Figure 3.2B).

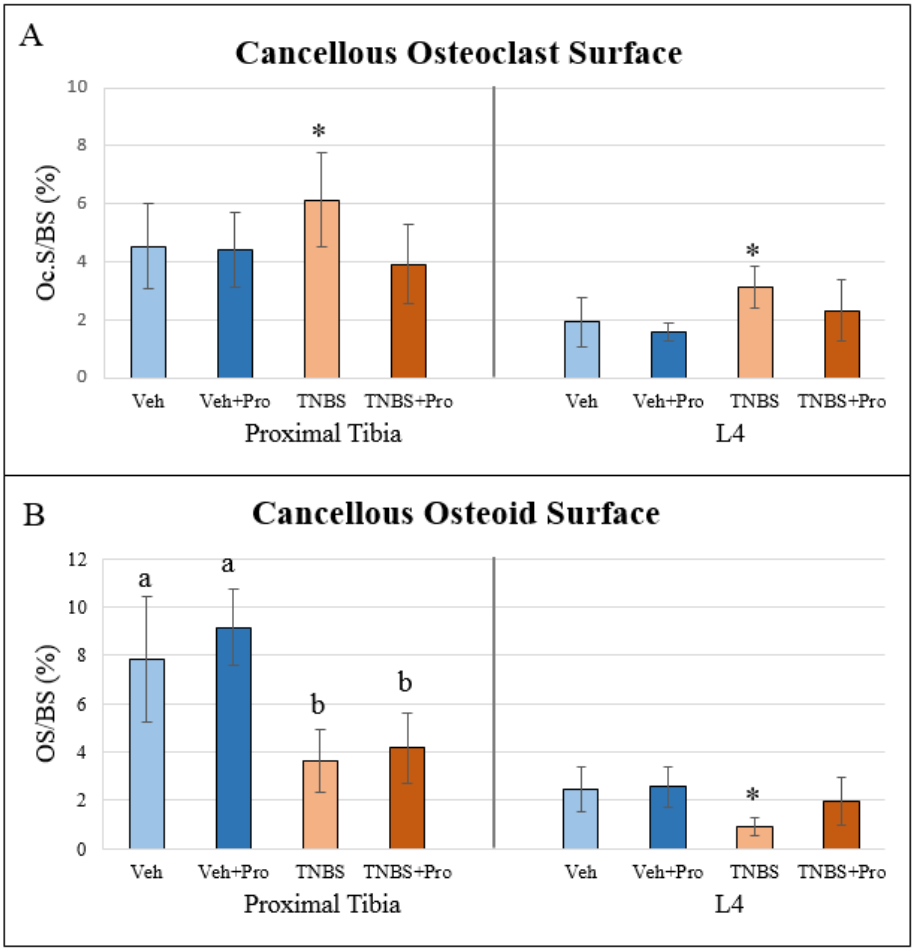

Figure 3.2: Histomorphometric analysis of the proximal tibia and 4th lumbar vertebrae. (A) Osteoclast surfaces were higher in TNBS in both bone sites. TNBS+Pro was not different than vehicle groups. (B) Osteoid surface was lower in both TNBS groups at the proximal tibia, but only lower in TNBS in L4. *Indicates difference from all other groups. Bars not sharing the same letter are statistically different.

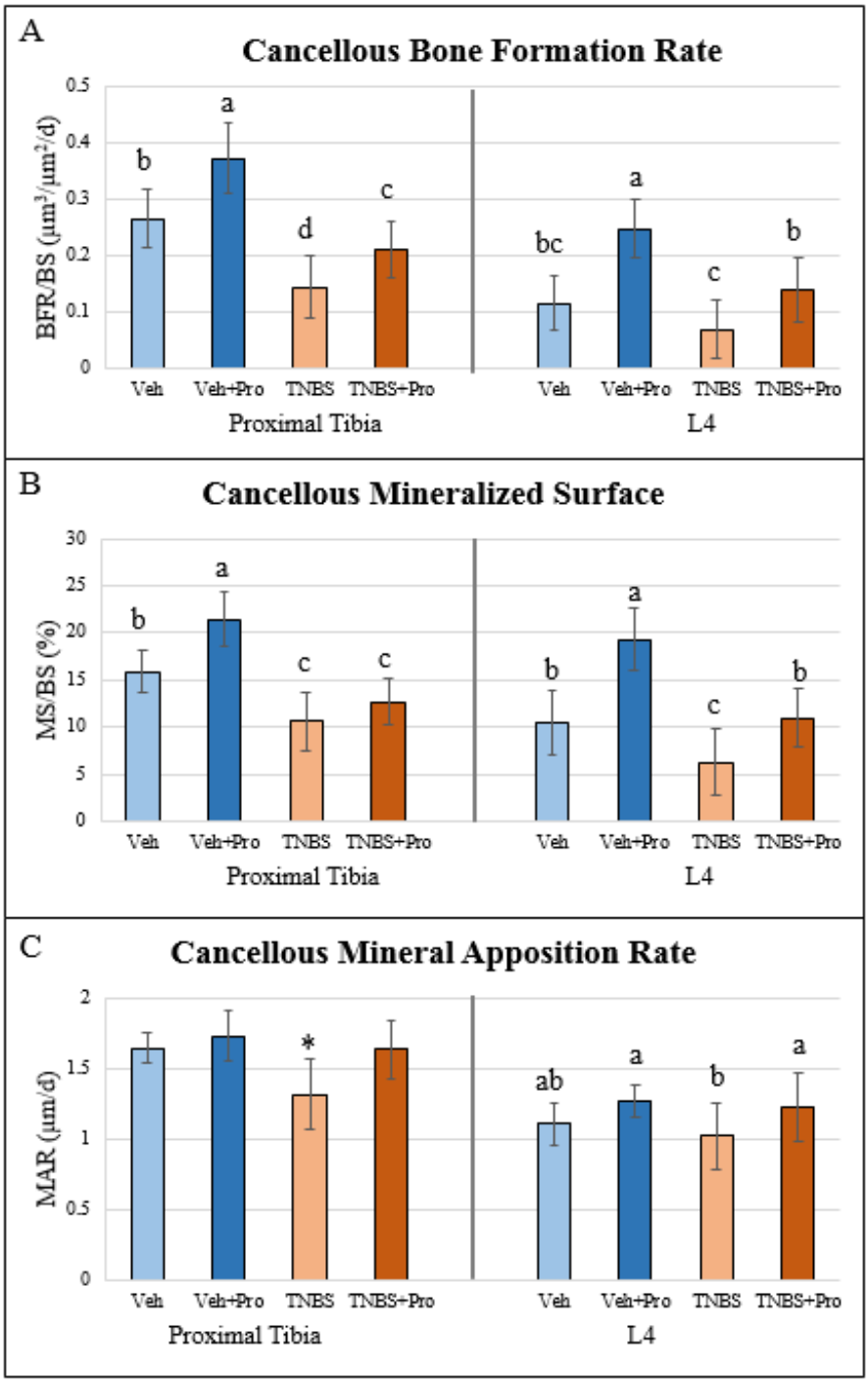

Figure 3.3: Dynamic histomorphometry of the proximal tibia and 4th lumbar vertebrae. (A) Cancellous BFR at the proximal tibia was lowest in TNBS and elevated due to Pro. At L4, TNBS+Pro increased BFR to Veh levels. (B) Pro had no effect on MS/BS at the proximal tibia, but increased MS/BS at L4. (C) Mineral apposition rate was lower in TNBS at the proximal tibia, but no different from Veh in TNBS+Pro. At L4, MAR was increased due to Pro. *Indicates difference from all other groups. Bars not sharing the same letter are statistically different.

Pro-inflammatory osteocyte proteins were reduced due to soy protein diet in TNBS animals. TNF-α: There were significant main effects of both TNBS and diet ($p<0.0001$) on %TNF-α+ osteocytes as well as a significant interaction effect ($p=0.028$). The TNBS group was higher than all other groups with the TNBS+Pro group no different than the Veh group (Figure 3.4A). IL-6: For %IL-6+ osteocytes there was a main effect of treatment ($p=0.001$) and diet ($p=0.01$), but no interaction effect ($p=0.882$). The TNBS group had the highest IL-6+ osteocytes with TNBS+Pro no different from either the TNBS alone group or the Veh+Pro group (Figure 3.4B). IL-4: There was a main effect of TNBS treatment ($p<0.0001$), a main effect of diet ($p=0.025$), and an interaction effect ($p=0.033$) for IL-4+ osteocytes. The TNBS group had lower osteocyte IL-4 than all other groups (Figure 3.4C). IL-10: There was only a main effect of diet on IL-10+ osteocytes ($p=0.002$) and no effect of treatment ($p=0.934$) or an interaction ($p=0.131$). The TNBS group had the highest IL-10 with the Veh group no different from either TNBS or Veh+Pro groups. The TNBS+Pro group had the lowest IL-10+ osteocytes (Figure 3.4D). RANKL: There was a significant main effect of treatment ($p<0.0001$) and a significant interaction effect ($p=0.004$), but no effect of diet on RANKL+ osteocytes ($p=0.120$). The TNBS group had the highest levels of RANKL, significantly higher than all other groups (Figure 3.5A). OPG: Main effects for treatment and treatment-by-diet were present in OPG+ osteocytes ($p<0.0001$, $p=0.004$, respectively), but no effect of diet ($p=0.061$). The TNBS group had the highest OPG followed by TNBS+Pro. The Veh+Pro was no different from either the TNBS+Pro or Veh groups (Figure 3.5B). IGF-I: For IGF-I+ osteocytes, there was only a significant interaction effect ($p=0.01$). There were no effects of treatment ($p=0.463$) or diet ($p=0.528$). The TNBS group had the highest osteocyte IGF-I with TNBS+Pro no different than Veh (Figure 3.5C). Sclerostin: While there was a non-significant interaction effect in sclerostin+ osteocytes

(p=0.077), there were main effects of both treatment (p=0.038) and diet (p=0.018). The TNBS group had the highest osteocyte sclerostin of all groups (Figure 3.5D). Annexin V: There was a main effect of annexin V-positive osteocytes for treatment (p=0.048) and diet (p=0.001) and a significant interaction effect (p=0.011). The TNBS group had the highest Annexin V positive osteocytes of all groups. TNBS+Pro was no different from either vehicle-treated group (Figure 3.5E).

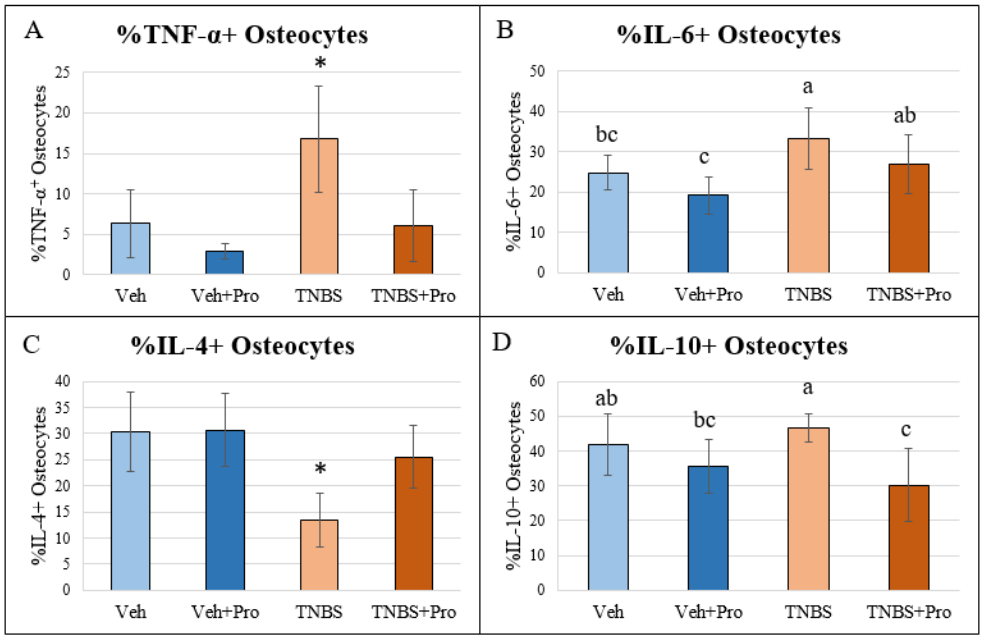

Figure 3.4: Immunohistochemistry of the distal femur cancellous osteocytes for inflammation-related factors. (A) TNF-α-positive osteocytes were highest in TNBS. TNBS+Pro was no different than Veh. (B) IL-6-positive osteocytes were highest in TNBS and lowered due to Pro, but TNBS+Pro was not different from either TNBS or Veh. (C) IL-4-positive osteocytes were lower in TNBS than all other groups. TNBS+Pro was not different from Veh. (D) IL-10-positive osteocytes were no different between TNBS and Veh, but lower due to Pro. *Indicates difference from all other groups. Bars not sharing the same letter are statistically different.

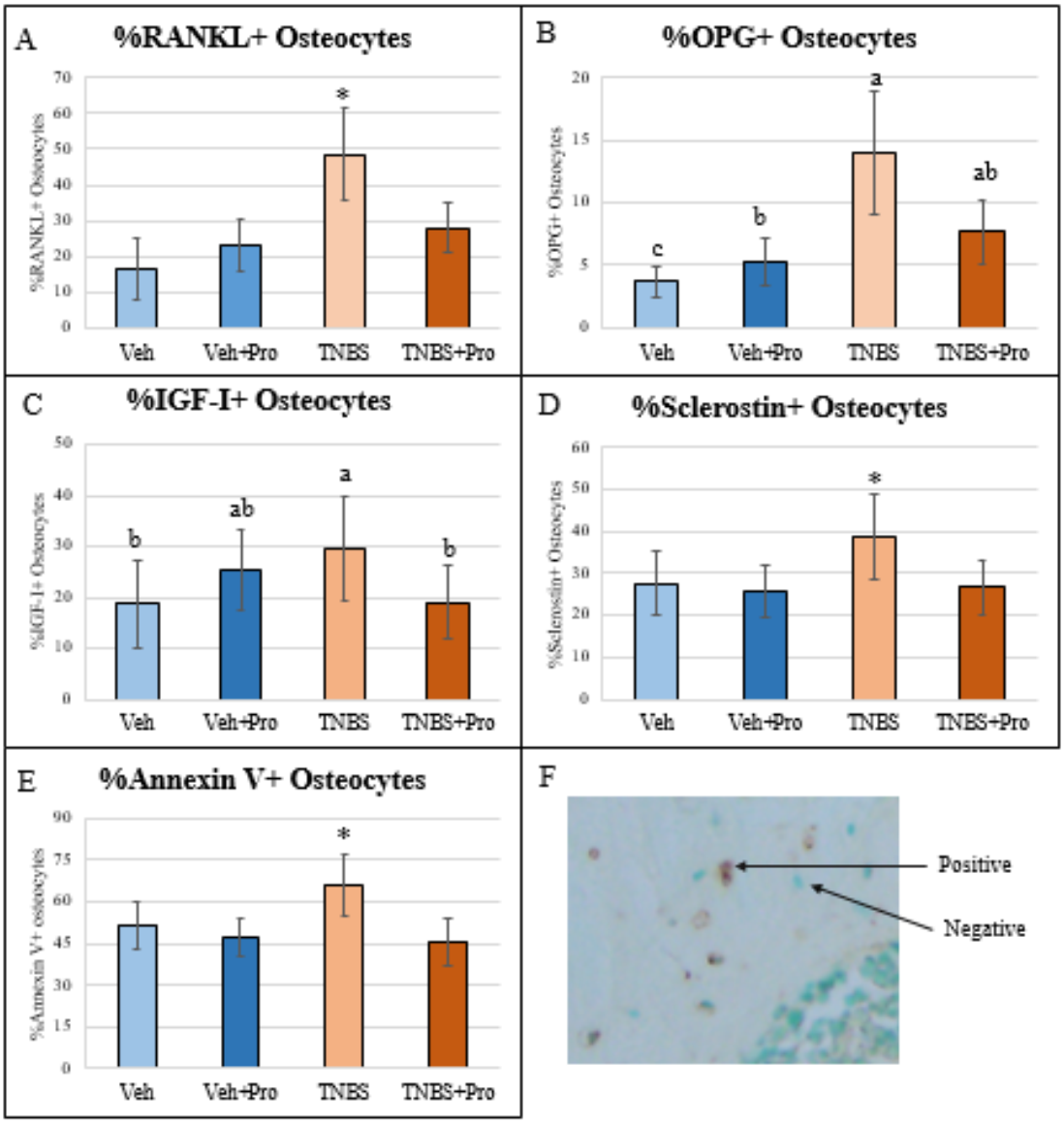

Figure 3.5: Immunohistochemistry of the distal femur cancellous osteocytes. (A) RANKL-positive osteocytes were highest in TNBS. TNBS+Pro was no different than Veh. (B) OPG-positive osteocytes were highest in TNBS. TNBS+Pro was not statistically different from TNBS or Veh. (C) IGF-I-positive osteocytes were elevated due to TNBS. TNBS+Pro was no different from Veh. (D) Sclerostin-positive osteocytes were higher in TNBS and TNBS+Pro was no different from Veh. (E) Annexin V-positive osteocytes were higher in TNBS, but no different from Veh in TNBS+Pro. (F) Representative image of immunohistochemistry of cancellous osteocytes.*Indicates difference from all other groups. Bars not sharing the same letter are statistically different.

3.3 Discussion

The primary finding of this study is that a moderately elevated soy protein diet successfully mitigated gut inflammation and beneficially altered bone turnover in an animal model of chronic inflammatory bowel disease. Secondly, this study demonstrates that osteocyte proteins are altered with increased dietary intake of soy protein coincident with positive changes in bone turnover. These results suggest a potential regulatory role of osteocytes in inflammatory bone loss whereby osteocytes release proteins to signal to osteoblasts and osteoclasts.

The beneficial effects of soy protein on gut inflammation have been demonstrated previously in animal models. In piglets with induced IBD via dextran sodium sulfate (DSS), soy protein isolate supplementation, while it did not fully alleviate the symptoms associated with gut inflammation, macroscopic analysis of the colons reveal the soy protein isolate treatment reduced colon inflammation and gut permeability (9). Similarly, DSS mice on a diet with 20% caloric intake from soy protein had a decrease in colon shortening and a reduction in colon inflammation, while diets consisting of 20% protein from casein and whey had no beneficial effect on gut inflammation (10). Both these studies examined acute outcomes with only 4-5 days of DSS administration to induce gut inflammation. In our study, we examined chronic gut inflammation after 28 days of TNBS-induced IBD. Our rats on a diet with 35% caloric content from soy protein had colon histopathology scores no different from vehicle-treated rats indicating successful mitigation of gut inflammation due to the moderately elevated soy protein diet. Our data along with the previous studies utilizing soy protein highlight the beneficial impact of soy protein on gut parameters during acute and chronic gut inflammation.

In the current study, we found similar increases in osteoclast surface and decreases in osteoid surface and bone formation rate due to chronic IBD in our young male rats to those previously reported (17). The moderately elevated soy protein diet in TNBS rats reduced osteoclast surface at both the proximal tibia and L4 by 26-36% compared to that value observed in TNBS rats on normal rodent chow resulting in osteoclast surface values no different from those of vehicle-treated rats. In osteoid surface, there was no beneficial effect of soy protein during TNBS in the proximal tibia, but in the lumbar vertebrae the diet increased osteoid surface by ~110% compared to TNBS alone; however, this was still ~20% lower than in vehicle-treated. Dynamic histomorphometry at both sites revealed similar patterns in mineralized surface, with no differences between TNBS and TNBS+Pro at the proximal tibia, but elevated mineralized surface in TNBS+Pro compared to TNBS at L4. Despite minimal changes in mineralized surface (MS/BS) and osteoid surface at the proximal tibia due to the dietary intervention in TNBS animals, bone formation rate was approximately 50% higher in both soy protein groups versus the control diet comparators. This increase in BFR at the proximal tibia was due largely to increased mineral apposition rate (MAR). In L4, both MS/BS and MAR were elevated due to the soy protein intervention. This indicates a site-specific effect of soy protein on osteoblasts with potentially greater recruitment of osteoblast teams at the L4 cancellous bone, but greater activity of osteoblast teams in both long bone and vertebral cancellous compartments. Regardless of the exact mechanisms, the moderately elevated soy protein diet did mitigate the increased osteoclasts due to TNBS and increased bone formation rate even during chronic gut inflammation.

The inflammatory insult in the gut during chronic IBD leads to an increase in Th1 cytokines such as TNF-α and IL-6. Previously, we discovered these pro-inflammatory cytokines are elevated in osteocytes in bone during chronic IBD correlating with increased osteoclast

surface and decreased BFR (17). We hypothesized that mitigation of the inflammation in the gut would result in mitigation of the bone inflammatory changes as well. The two previously published studies examining soy protein's impact on acute gut inflammation in animal models demonstrate that soy protein attenuates colonic expression of TNF-α, corresponding with decreased inflammatory scores (9, 10). Therefore, these studies concluded that soy protein can act as an anti-inflammatory agent; however, neither of these studies examined bone outcomes. In our study, we found nearly a three-fold decrease in TNF-α-positive osteocytes in TNBS+Pro animals compared to those animals TNBS alone resulting in no osteocyte TNF-α difference between TNBS+Pro rats and vehicle-treated rats. Osteocyte IL-6 was 18% lower in TNBS+Pro rats compared to the TNBS group alone. These reductions in osteocytes positive for pro-inflammatory cytokines correspond with the reduced osteoclast surface in IBD animals fed the moderately elevated soy protein diet. Also, since TNF-α suppress osteoblast formation and activity (20), the decrease in TNF-α in bone likely supports the increase in BFR.

Th2 cytokines, like interleukin-10, are often considered "anti-inflammatory" and have a more equivocal role in IBD pathology, as discussed in our previous work (17) in which we found no alterations in IL-10-positive osteocytes in IBD rats after four weeks of treatment. We confirmed that finding in this current study but did, however, find a significant effect of our soy protein diet on decreasing IL-10-positive osteocytes in rats. In cell culture models, IL-10 inhibits osteoclasts (21, 22), but the functional role of osteocyte IL-10 is unknown. What the decreased osteocyte IL-10 with the soy protein intervention indicates is uncertain since osteoclast surface is also depressed with moderately elevated soy protein. Another Th2 cytokine, interleukin-4 (IL-4), we discovered to be decreased in TNBS-treated animals, but restored back to vehicle levels with the soy protein diet. IL-4, similar to IL-10, has implicated actions in inhibiting RANKL and

preventing osteoclastogenesis (23, 24). The role of osteocyte IL-4 is also unknown at this time. In summary, our data indicate that the moderately elevated soy protein diet alters Th2 cytokines as well as Th1, but further work must be done to define the functional roles these Th2 cytokines play in osteocytes, osteoclasts, and osteoblasts in the context of inflammation.

RANKL and OPG, regulators of osteoclast formation, are altered in the serum of IBD patients (25) and in osteocytes of IBD rats (17). We found osteocyte-specific RANKL and OPG to be 3- to 4-fold higher, respectively, in the TNBS group as compared to those in vehicle-treated rats. The soy protein intervention reduced the percentage of RANKL-positive osteocytes by 43% and OPG-positive osteocytes by 41% in TNBS animals. These changes corresponded with decreased osteoclast surface at both the proximal tibia and L4 in the TNBS+Pro group. Since RANKL and OPG are regulated in part by TNF-α (26, 27), the decrease in TNF-α seen with the soy protein diet likely contributed to the decrease in both RANKL and OPG. Additionally, another osteocyte protein, sclerostin, is upregulated by TNF-α (28) and elevated in IBD (17). The soy protein intervention reduced osteocyte sclerostin by 31% as compared to TNBS rats on normal diet and to the same level as observed in vehicle-treated rats. This decline in sclerostin, a negative regulator of osteoblasts, corresponded with increased bone formation rate. Finally, TNF-α can also induce apoptosis of osteocytes (29) which, in turn, can lead to increased recruitment of osteoclasts to that area to resorb bone (30). We found annexin V, a marker of apoptosis, to be more prevalent in osteocytes due to IBD, but our soy protein treatment prevented the increased in osteocytes positive for annexin V. Therefore, our data indicates our soy protein diet decreased osteocyte apoptosis, which also may contribute to the reduced recruitment of osteoclast surface in the IBD rats with the moderately elevated soy protein diet.

Our original hypothesis was that the soy protein would act as an anti-inflammatory agent and the moderate elevation in protein content in the diet would act to increase bone formation potentially due to an increase in insulin-like growth factor-I (IGF-I). Our original hypothesis was supported in that the moderately elevated soy protein intervention during IBD reduced pro-inflammatory markers and increased bone formation rate; however, we cannot conclude that osteocyte IGF-I played a role in this effect. Serum IGF-I is decreased in patients with IBD (31), but we previously demonstrated that osteocyte IGF-I was, in fact, elevated in TNBS rats along with elevations in pro-inflammatory markers (17). Osteocyte IGF-I has mostly been examined in response to increased or decreased mechanical loading on bone (32, 33, 34) and the role of osteocyte IGF-I in inflammatory states is unknown. In this current study, we found similar elevations in IGF-I-positive osteocytes due to TNBS, but a 35% decrease in osteocytes positive for IGF-I in TNBS animals fed the soy protein diet, returning to levels similar to vehicle controls. The moderately elevated soy protein diet did increase bone formation rate, but our data provide no support for osteocyte IGF-I as a mechanism for these observed changes.

We did not find differences in bone mass or cancellous microarchitecture due to IBD or to the dietary intervention. The inflammatory insult with TNBS instillation in our experiment did not cause serious side effects like rapid weight loss or altered eating patterns as many other animal studies using induced IBD find, but the resulting changes in gut and bone are consistent with a pro-inflammatory state that, if it continued over a longer period, would undoubtedly result in bone loss. While we could not detect changes in bone mass over the four weeks of this experiment, the elevated resorptive and decreased formation activity indicate what would likely become progressive bone loss in IBD and a mitigation of bone loss or preservation of bone mass with the soy protein intervention. Additionally, since our animals had no changes in body weight

or food intake, we hypothesize the changes in bone and the colon observed in TNBS rats that were mitigated by the soy protein diet are due to inflammation and not malnutrition or poor nutrient absorption. Since IBD is a chronic condition that often develops in childhood or young adulthood, even a mild inflammation like that of our rats would result in bone loss over time and increased fracture risk.

In conclusion, this study demonstrated that a moderately elevated soy protein diet during chronic IBD in young male rats mitigated the inflammation-induced increases in osteoclast surfaces and decreases in bone formation rate. Additionally, osteocyte proteins reflected a shift away from a pro-inflammatory state resulting in reductions in bone specific proteins responsible for controlling osteoclast formation and inhibiting osteoblasts. Therefore, our data show potential for a dietary intervention that could be used in conjunction with other therapies to beneficially alter the inflammatory insult during this chronic disease.

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4. EXOGENOUS TREATMENT WITH IRISIN PREVENTS INFLAMMATORY BONE CHANGES DURING CHRONIC TNBS-INDUCED INFLAMMATORY BOWEL DISEASE

Exercise has long been lauded as anti-inflammatory; however, the exact mechanism by which exercise exerts immunological change or the specifics of those immunological changes remains largely unknown. Increasing levels of physical activity are associated with lower C-reactive protein (1); however, data from large controlled studies examining inflammatory factors influenced by exercise are lacking (2). Despite the lack of empirical evidence demonstrating the anti-inflammatory benefits of exercise, exercise has been recommended in multiple inflammatory conditions including inflammatory bowel disease (3). A couple small intervention studies found moderate walking exercise improved IBD symptoms (4, 5). Another study found Crohn's disease patients in remission engaging in higher levels of exercise activity were less likely to develop active disease (6). Correlational evidence also shows relationships between physical activity and the development of IBD, with sedentary occupations correlated with higher incidence of IBD (7) and participation in a lower number of childhood sports also associated with increased risk of developing IBD (8). The relative risk of Crohn's disease is inversely related to regular physical activity (9). Combined, these studies suggest there may be a preventive effect of regular exercise in the development of IBD and a potential mitigation of inflammation due to exercise during IBD. An understanding of what factors are released during exercise and their exact roles in immune function and inflammatory processes could lead to novel therapeutic targets for treating IBD as well as specified exercise training programs targeted to mitigating disease symptoms.

Irisin, a protein released from exercising muscle due to an increase in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), has recently emerged as a potential modulator of exercise-related physiological adaptations (10). Originally proposed to be a myokine, evidence indicates irisin could be released from other tissues including adipose and cardiac muscle (11, 12). Irisin was originally defined as a mediator of increased metabolism due its induction of uncoupling protein-1 (UCP-1) in white adipose leading to the browning of white adipose (10). Following acute exercise, irisin is elevated in serum in humans, rats, and mice (10, 11, 13). Young male rats exposed to 10 minutes of stressful exercise via water floating had approximately 75% increases in serum irisin compared to control non-exercised rats (11). Three weeks of free wheel running in mice followed by 12 hours of rest revealed approximately 66% higher irisin present in the plasma of exercised mice (10). Serum irisin was approximately 60% higher after acute submaximal treadmill running in mice compared to control levels (13). Following 10 weeks of cycle training (4-5 sessions/week for 20-35 minutes/session at approximately 65% of maximal oxygen consumption) in male humans, plasma irisin increased by approximately 75% compared to baseline (10). It remains uncertain whether there are chronic adaptations of irisin to exercise training or if irisin only transiently increases after a bout of exercise. One study found serum irisin to increase after heavy resistance exercise or aerobic training, but returned to baseline several hours after exercise (14).

Interestingly, aside from irisin's originally proposed actions in metabolism, several studies have shown decreased serum irisin in conditions with inflammatory components. In chronic kidney disease where circulating pro-inflammatory markers predict disease stages (15), serum irisin levels decrease with increasing severity of disease (16). Likewise, type 2 diabetes, which is characterized by chronic systemic low grade inflammation (17), is associated with low

serum irisin (18). Finally, in an animal model of chronic heart failure, there is a negative correlation between circulating irisin and circulating TNF-α (19). Additionally, recent studies have demonstrated exogenous irisin treatment improved inflammatory outcomes in rodent models of atherosclerosis (20), ischemic stroke (21), and LPS-induced lung injury (22). Irisin has also recently emerged as a bone anabolic factor stimulating osteoblast proliferation and increased bone formation rate (23, 24, 25). The role of irisin in preventing inflammation-induced bone alterations has not yet been explored nor has the use of irisin as a therapeutic agent for chronic IBD.

The goal of this current project is to examine exogenous irisin treatment during chronic IBD in a rodent model. We hypothesized intraperitoneal injections of irisin after establishment of TNBS-induced gut inflammation would mitigate the pro-inflammatory response in bone. Specifically, we hypothesized that exogenous irisin treatments would increase bone formation rate and decrease osteoclast surfaces. These changes in bone turnover would correspond with decreased osteocyte TNF-α, IL-6, RANKL, OPG, and sclerostin. Additionally, we hypothesized irisin treatments during TNBS would restore osteocyte IGF-I levels to that of vehicle-treated rats.

4.1 Materials and Methods

Animals: Thirty-two male Sprague-Dawley rats (1.5 months old) were ordered from Envigo (Houston, Texas) and singly housed in an institutionally approved animal facility with 12 hour light:dark cycles. Animals were allowed approximately four days to acclimate to the facility before being switched from standard rodent chow (Teklad 2018, Envigo) to the purified AIN93G chow (Research Diets, Inc., New Brunswick, NJ). Animals were allowed free access to food and water. Following the one-week acclimation to the diet, animals were randomly divided into four different groups (n=8/group): Vehicle (Veh), Vehicle with irisin (Veh+Ir), IBD induced

via 2,4,6-trinitrobenzenesulfonic acid (TNBS), and IBD with irisin (TNBS+Ir). Rectal instillations of either 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 30% ethanol or 30% ethanol (for Veh) began at 2 months of age and continued for four weeks. Instillations occurred once per week with the fifth and final instillation occurring 3-4 days prior to termination. Intraperitoneal irisin injections were delivered, 2x per week beginning the second week of instillations, for a total of six injections. Fluorochrome calcein labels (Sigma Aldrich, St Louis, MO) were injected intraperitoneally 8 and 3 days prior to termination to label mineralized surfaces on bone. Animal health was monitored daily and food intake and bodyweight were measured several times per week for the entire experimental period. After four weeks of TNBS or Veh treatment, rats were anesthetized via inhaled vaporized isoflurane, euthanized via thoracotomy, and tissues were collected. All animal procedures were approved by the Texas A&M Institutional Animal Use and Care Committee and confirm to the NIH Guide for the Care and Use of Laboratory Animals.

Rectal Instillations: Gut inflammation was induced by rectal instillations of 1 uL/gram body weight rectal of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma Aldrich, St Louis, MO) in 30% ethanol:DiH_2O solutions as previously described (26). Instillations were given at days 1, 7, 14, 21, and 26 of the experimental period to non-fasted rats anesthetized via inhaled isoflurane with a precision vaporizer.

Irisin Injections: Recombinant irisin (Adipogen Life Sciences, San Diego, CA) was dissolved in sterile phosphate buffered saline. Doses of 18 ng/mL dissolved in sterile phosphate-buffered saline (2 µL) were injected intraperitoneally into all irisin treated rats. Previous work of ours identified normal circulating levels of irisin in serum in rats of the same age and strain from the same vendor to be 6 ng/mL. Therefore, our goal was to provide 4x that of normal circulating

levels. Irisin-treated rats received two doses per week (3.5 days apart) for 3 weeks. The first irisin dose coincided with the second rectal instillation.

Dynamic and Static Histomorphometry: Undemineralized right proximal tibia and fourth lumbar vertebrae (L4) were fixed in 4% phosphate buffered formalin and then subjected to serial dehydration and embedded in methyl methacrylate (J.T. Baker, VWR, Radnor, PA). Serial frontal sections at 8 µm thickness were left unstained for analysis of fluorochrome labels for dynamic bone formation rate (BFR). All analyses were completed on OsteoMeasure Analysis System, version 3.3 (OsteoMetrics, Inc., Atlanta, GA). For the proximal tibia, a defined region of interest was established approximately 500 µm from the growth plate and within the endocortical edges encompassing approximately 8 mm^2 at 20x magnification. For L4, a region was defined 500 µm above the end of the bone within endocortical edges at approximately 3 mm^2 at 20x magnification. For 8 µm sections, total bone surface (BS), single-labeled surface (sLS/BS), double-labeled surface (dLS/BS), mineralized surface (MS/BS), and inter-label distances were measured at 20x magnification (Figure 4.1). Mineral apposition rate (MAR) was calculated from the inter-label distance and time of labels. Bone formation rate (BFR/BS) was determined by multiplying MS/BS by MAR. Further frontal sections of the proximal tibia and L4 were sectioned at 4 µm thickness treated with a Von Kossa stain with a tetrachrome counterstain. These sections were measured at 40x magnification for identification of osteoclast surface (OcS/BS) and osteoid surface (OS/BS). All analyses were completed by the same individual to ensure consistency in measures. All nomenclature for cancellous histomorphometry follows standard usage (27).

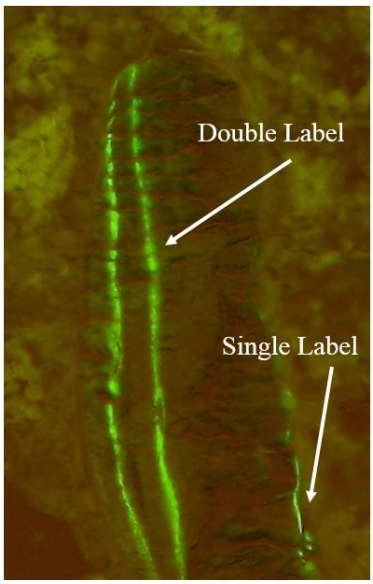

Figure 4.1: Identification of single vs. double fluorochrome labels on cancellous bone.

Immunohistochemistry of Osteocyte Proteins: Left distal femurs were fixed in 4% phosphate buffered formalin and then stored in 70% ethanol. Tissues then underwent decalcification in a formic acid/sodium citrate solution for approximately 18 days. Sections were then further dehydrated in Thermo-Scientific STP 120 Spin Tissue Processor, paraffinized via a Thermo Shandon Histocenter 3 Embedding tool. Frontal sections at 8 µm thickness were mounted on positively charged slides and immunostained using an avidin-biotin method as previously described (26). Incubation occurred with the following primary antibodies: polyclonal rabbit anti-rat TNF-α, (LifeSpan BioSciences, Inc, Seattle, WA), polyclonal rabbit anti-IL-6 (Abcam, Cambridge, MA), polyclonal rabbit anti-IL-10 (Abcam, Cambridge, MA), polycloncal rabbit anti-IL-4 (Abcam, Cambridge, MA), polyclonal rabbit anti-annexin V (Abcam, Cambridge, MA), polyclonal goat anti-mouse sclerostin (R&D Systems, Minneapolis, MN), polyclonal rabbit anti-annexin V (Abcam, Cambridge, MA). All sections were counterstained with methyl green. Sections were analyzed as the percentage of osteocytes stained positively for

the protein with a 4 mm^2 region in the distal femur cancellous bone compartment approximately 500 μm away from the growth plate and excluding endocortical surfaces. All analyses were completed by the same individual. Previous data demonstrated the cancellous bone region is the bone site that responds most vigorously to inflammation due to IBD (26).

Statistical analyses: All data were tested for homogeneity and analyzed using a 2x2 factorial ANOVA to determine main effects of irisin and TNBS. If an irisin-by-TNBS interaction was present ($p<0.05$), all-groups analysis was completed. If the main effects were significant ($p<0.05$), a Duncan post-hoc test was used to determine differences between groups. Statistical analyses were completed on SPSS (IBM; Armonk, NY). All data are represented as mean ± standard deviation.

4.2 Results

All animals maintained bodyweight and consistent food intake across the course of the study regardless of TNBS or irisin treatment. There were no statistical differences in bodyweight between any groups at any time point in the study. Additionally, food intake was not different due to any treatment.

Exogenous irisin treatment mitigated gut damage and inflammation during chronic TNBS. There were significant main effects for TNBS treatment, irisin, and a significant TNBS-by-irisin interaction ($p<0.0001$ for all) on histopathological scores. The TNBS group was higher than all other groups, with both Veh groups no different from the TNBS+Ir group (Figure 4.2).

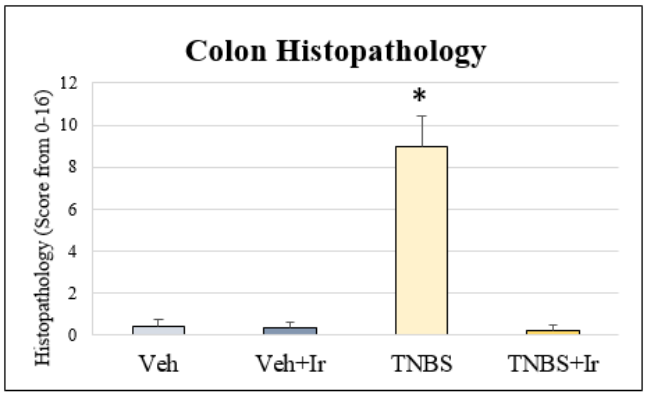

Figure 4.2: Colon histopathology. TNBS had the highest aggregated score with TNBS+Ir no different from either vehicle-treated group. *Indicates different from all other groups.

Irisin decreased osteoclast surface at both the proximal tibia and L4 regardless of gut inflammation. At the proximal tibia, there was a main effect of irisin ($p<0.0001$) and an interaction effect ($p=0.045$), but no main effect of TNBS treatment ($p=0.278$). At this bone site, TNBS animals had the highest osteoclast surface followed by vehicle-treated rats. TNBS+Ir rats had the lowest osteoclast surface with Veh+Ir animals no different from those in Veh or TNBS+Ir groups. At L4, there was also a significant main effect of irisin ($p<0.0001$) and an interaction effect ($p=0.031$), but no main effect of TNBS treatment ($p=0.141$). At L4, TNBS rats had the highest osteoclast surface followed by Veh animals, with both irisin groups having the lowest osteoclast surface (Figure 4.3A).

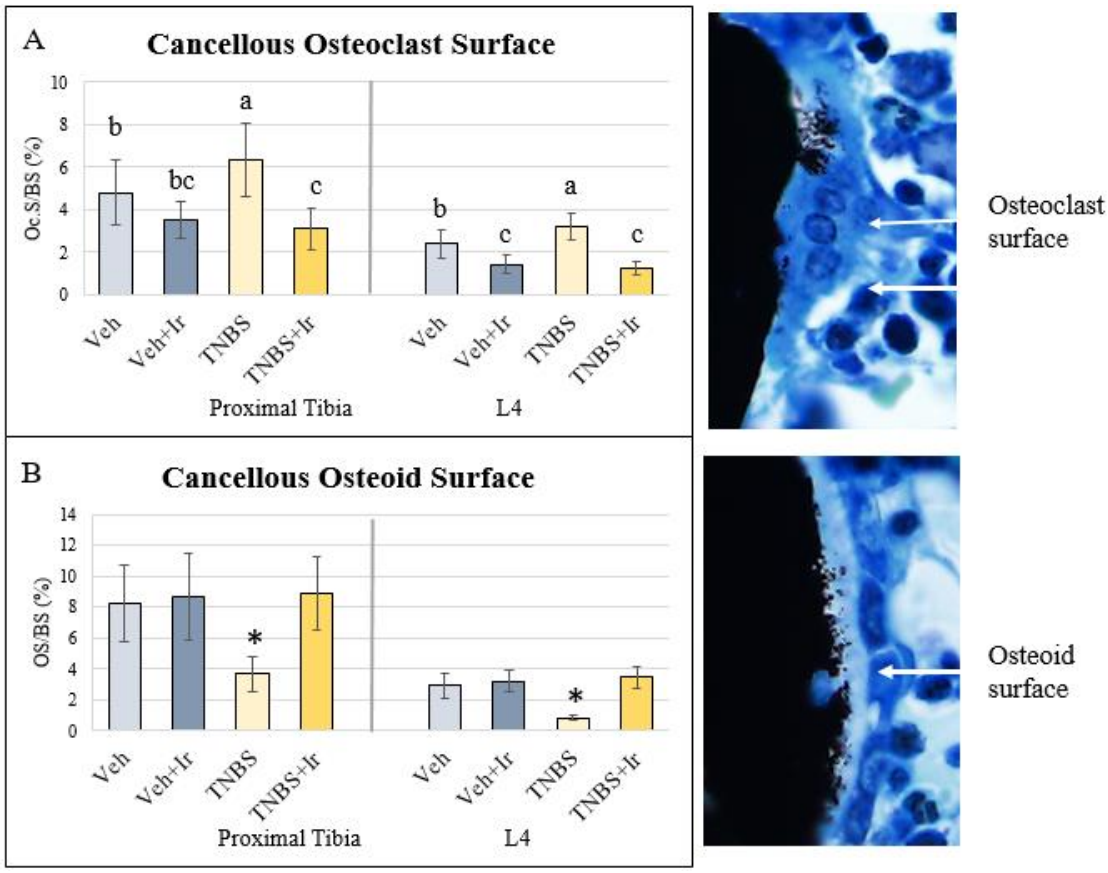

Figure 4.3: Osteoclast and osteoid surfaces on cancellous bone of the proximal tibia and 4th lumbar vertebrae. (A) Osteoclast surface was highest in TNBS in the proximal and lowest in both Ir groups. In L4, osteoclast surface was highest in TNBS and both Ir groups were lower than Veh. (B) Osteoid surface was lower in TNBS in both the proximal tibia and L4. TNBS+Ir was no different than Veh. *Indicates different from all other groups. Bars not sharing the same letter are statistically different.

Exogenous irisin caused a robust increase in bone formation rate in all animals regardless of gut inflammation. At the proximal tibia, there were significant main effects of TNBS treatment and irisin ($p<0.0001$ for both), but no interaction effect ($p=0.738$). BFR was lowest in TNBS with both irisin-treated groups having BFR higher than Veh rats (Figure 4.4A). For mineralizing surface, there were significant effects of TNBS treatment ($p=0.006$) and irisin ($p<0.0001$) and an interaction effect ($p=0.006$). Both irisin groups had the highest MS/BS with TNBS having the lowest (Figure 4.4B). For mineral apposition rate, there were main effects for

TNBS treatment ($p=0.001$) and irisin ($p<0.0001$), but no interaction ($p=0.927$). MAR was lowest in the TNBS group with the TNBS+Ir having MAR higher than Veh rats (Figure 4.4C). For BFR at L4, there were main effects of TNBS treatment ($p=0.01$) and irisin ($p<0.0001$), but no interaction effect ($p=0.912$). BFR was higher in both irisin groups than Veh and TNBS (Figure 4.4A). For mineralizing surface at L4, there were main effects of TNBS treatment ($p=0.008$) and irisin ($p<0.0001$), but no interaction ($p=0.175$). MS/BS was lowest in the TNBS group with the TNBS+Ir group not statistically different from Veh rats (Figure 4.4B). For MAR at L4, there were also main effects of TNBS treatment ($p=0.029$) and irisin ($p<0.0001$) and no interaction effect ($p=0.912$). MAR was greater in both irisin-treated groups compared to Veh and TNBS (Figure 4.4C). Osteoid surface showed reversal of depressed osteoid due to gut inflammation in the irisin-treated groups. At the proximal tibia, there were main effects of TNBS treatment ($p=0.018$), irisin ($p=0.003$), and a significant interaction effect ($p=0.011$). TNBS had the lowest osteoid surface of all groups. At L4, there were significant effects of TNBS treatment ($p=0.002$), irisin ($p<0.0001$), and an interaction effect ($p<0.0001$). At L4, similar to the proximal tibia, TNBS had the lowest osteoid surface of all groups (Figure 4.3B).

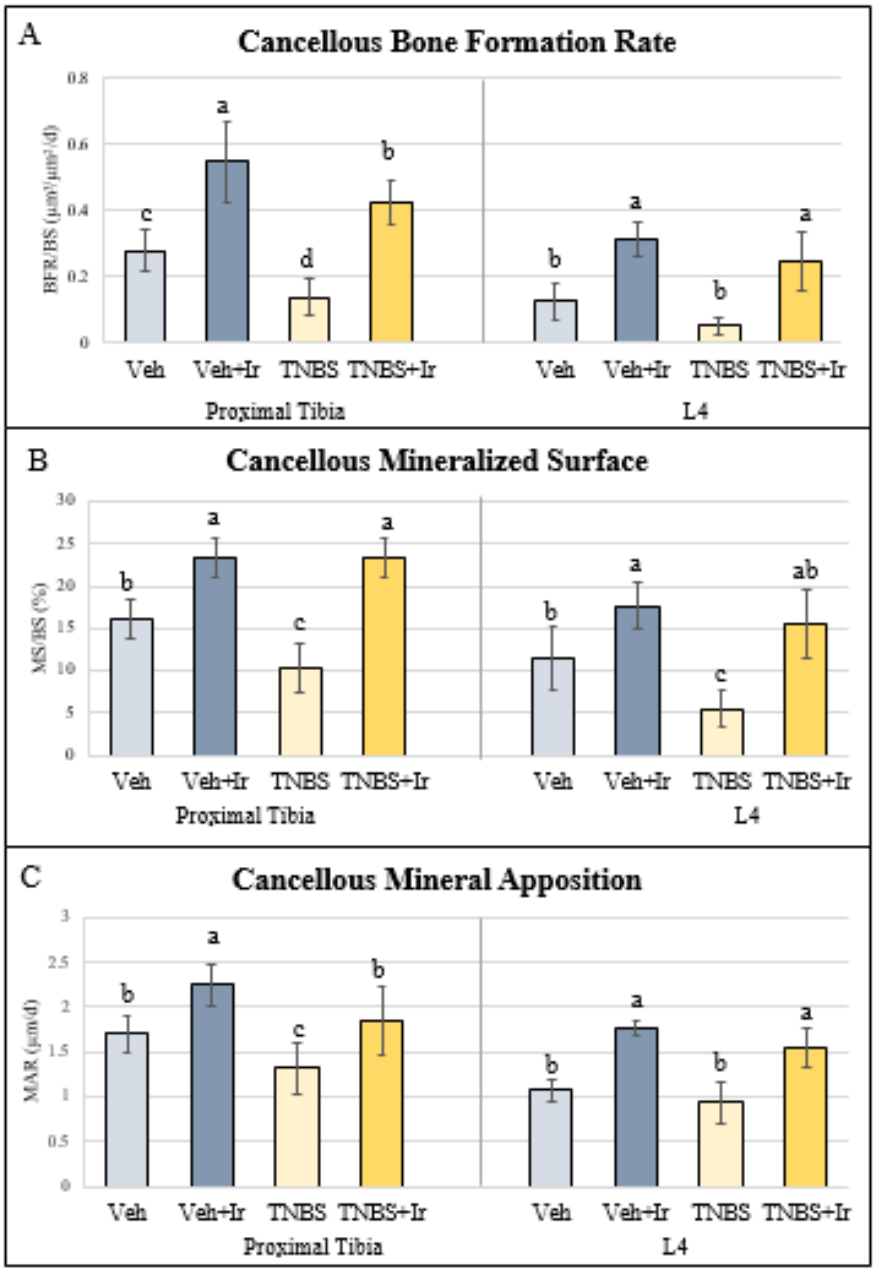

Figure 4.4: Cancellous bone formation rate at the proximal tibia and L4. (A) Bone formation rate was highest in Veh+Ir, followed by TNBS+Ir. TNBS alone had the lowest BFR. (B) Mineralized surface was highest in both Ir treated groups in both sites followed by Veh with TNBS having the lowest BFR. (C) MAR at the proximal tibia was highest in Veh+Ir with TNBS+Ir no different than Veh and TNBS having the lowest MAR. At L4, both Ir groups had the highest MAR. Bars not sharing the same letter are statistically different.

Exogenous irisin treatment reduced pro-inflammatory cytokines in osteocytes and altered regulators of bone turnover. TNF-α: There were significant main effects of both TNBS treatment ($p=0.001$) and irisin ($p<0.0001$) on %TNF-α+ osteocytes as well as a significant interaction effect ($p=0.001$). The TNBS group had the highest TNF-α followed by Veh with both irisin groups having the lowest %TNF-α+ osteocytes (Figure 4.5A). IL-6: For %IL-6+ osteocytes there was a main effect for both TNBS treatment and irisin ($p<0.0001$ for both), but no interaction effect ($p=0.123$). TNBS rats had the highest IL-6+ osteocytes followed by Veh. Veh+Ir had the lowest IL-6 with TNBS+Ir no different from Veh or Veh+Ir (Figure 4.5B). IL-4: There was no main effect of treatment on IL-4+ osteocytes ($p=0.074$), but a significant effect of irisin treatment ($p<0.0001$) and an interaction effect ($p=0.023$). Vehicle rats had the highest IL-4+ osteocytes followed by TNBS rats with both irisin-treated groups having the lowest IL-4+ osteocytes (Figure 4.5C). IL-10: There was only a main effect of irisin on IL-10+ osteocytes ($p<0.0001$). There was no effect of TNBS treatment ($p=0.914$) or an interaction ($p=0.425$). IL-10+ osteocytes were lower in both irisin-treated groups (Figure 4.5D). RANKL: There were significant main effects of TNBS treatment and irisin ($p<0.0001$ for) and a significant interaction effect ($p=0.002$) for RANKL-positive osteocytes. The TNBS group had higher RANKL-positive osteocytes than all other groups (Figure 4.6A). OPG: Main effects for TNBS treatment, irisin, and treatment-by-diet were present in OPG+ osteocytes ($p<0.0001$ for all). TNBS rats had the highest OPG-positive osteocytes of all groups (Figure 4.6B). IGF-I: There was a significant main effect of only irisin treatment on IGF-I-positive osteocytes ($p<0.0001$) with non-significant effects of TNBS treatment ($p=0.094$) and TNBS-by-irisin interaction ($p=0.056$). The TNBS group had the highest osteocyte IGF-I followed by Veh rats with both irisin-treated groups having the lowest %IGF-I+ osteocytes (Figure 4.6C). Sclerostin:

There was a main effect of both TNBS treatment (p=0.001) and irisin (p<0.0001) as well as an interaction effect (p=0.042). TNBS rats had the highest levels of sclerostin-positive osteocytes followed by Veh rats. The Veh+Ir group had the lowest %sclerostin+ osteocytes with TNBS+Ir rats no different from either Veh or Veh+Ir (Figure 4.6D). Annexin V: For annexin V-positive osteocytes, a marker of cell undergoing apoptosis, there were main effects of TNBS (p=0.008), irisin treatment (p<0.0001), and an interaction (p=0.003). TNBS rats had higher annexin V-positive osteocytes than all other groups (Figure 4.6E).

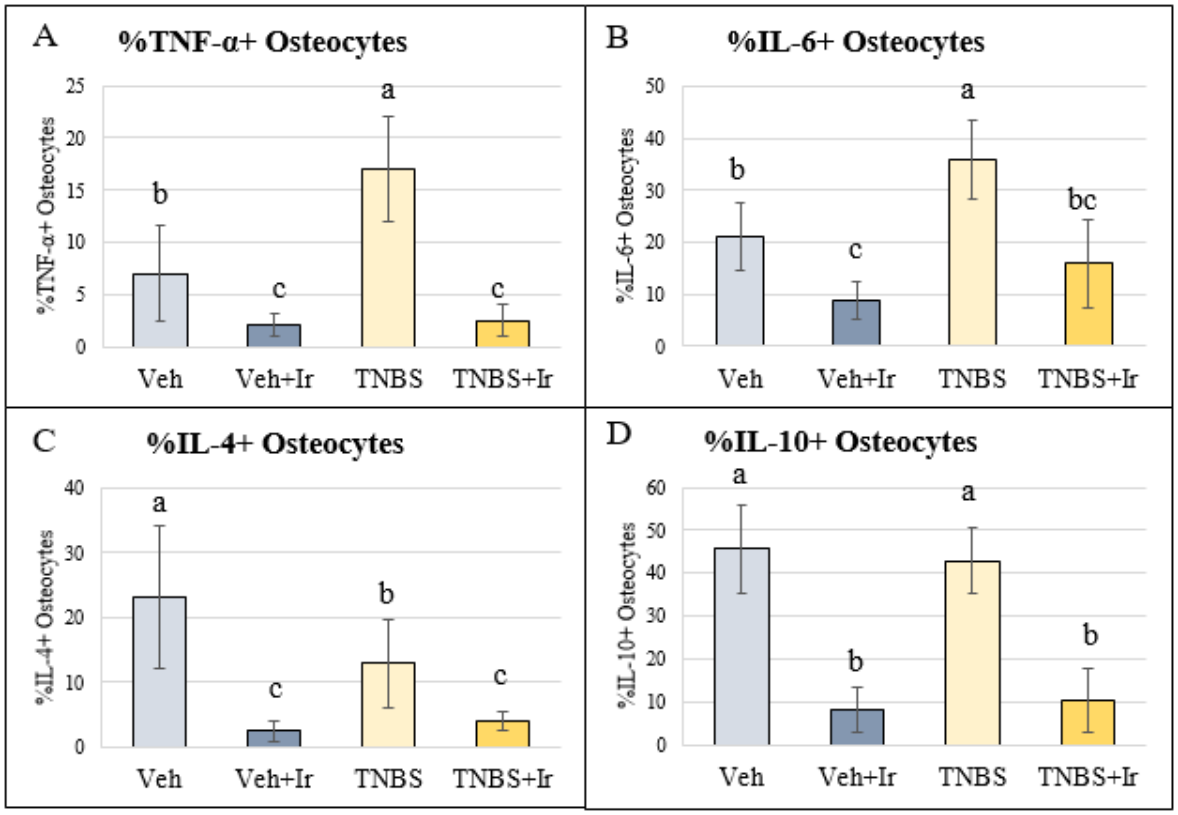

Figure 4.5: Immunohistochemistry for inflammatory proteins in osteocytes in the cancellous bone of the distal femur. (A) TNF-α-positive osteocytes were highest in TNBS with both Ir treated groups lower than Veh. (B) IL-6-positive osteocytes were highest in TNBS with TNBS+Ir no different from Veh or Veh+Ir. (C) IL-4-positive osteocytes were lower in TNBS vs.Veh, but both Ir groups were lower than TNBS. (D) Both Ir groups had lower IL-10-positive osteocytes than Veh and TNBS. Bars not sharing the same letter are statistically different.

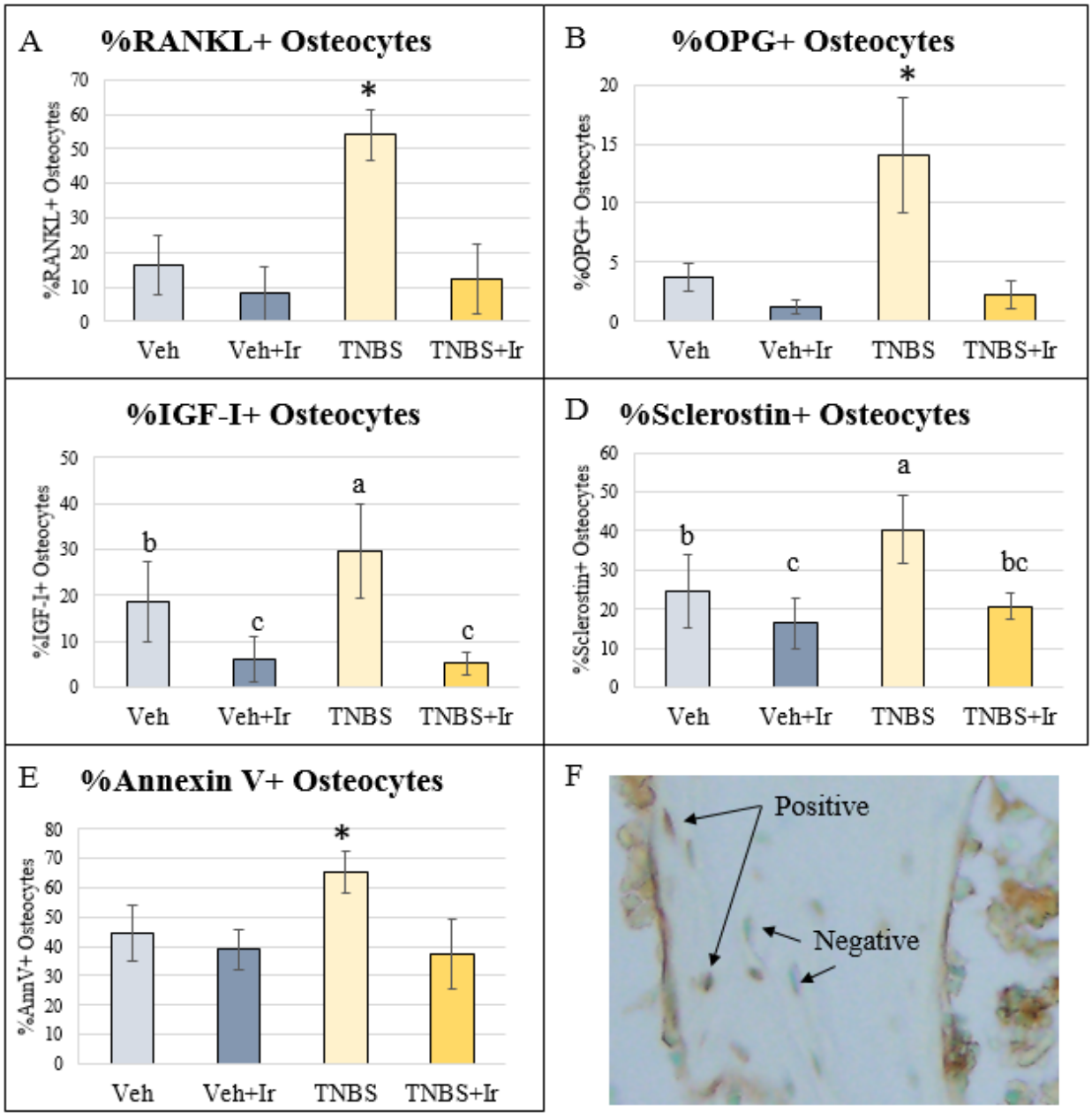

Figure 4.6: Immunohistochemistry for osteocyte proteins in the distal femur cancellous bone. (A) RANKL-positive osteocytes were elevated in TNBS, but TNBS+Ir was no different from Veh. (B) OPG-positive osteocytes were higher in TNBS than any other group. (C) IGF-I-positive osteocytes were highest in TNBS with both Ir treated groups lower than Veh. (D) Sclerostin-positive osteocytes were highest in TNBS with TNBS+Ir no different from Veh or Veh+Ir. (E) Annexin V-positive osteocytes were highest in TNBS with TNBS+Ir no different than either Veh group. (F) Representative image of immunohistochemistry of osteocytes in cancellous bone. *Indicates different from all other groups. Bars not sharing the same letter are statistically different.

4.3 Discussion

The primary findings of this study are that exogenous treatment with the exercise-related myokine, irisin, ameliorated gut inflammation in chronic IBD, reversed inflammation-induced changes in bone turnover, and altered osteocyte proteins. We found that exogenous irisin treatments reduced bone-specific, pro-inflammatory markers and significantly stimulated bone formation rate.

Our analyses of the colons of TNBS rats demonstrated that irisin treatment completely mitigated the edema, crypt cell loss, and increased cellularity seen in the TNBS-treated animals. The IBD rats with irisin treatment had colon structure and cellularity no different from all vehicle- treated rats. The role of irisin on gut physiology, healthy or diseased, has not been explored, but our data indicate that irisin acts to reduce the inflammatory insult of TNBS in the gut.

Irisin has recently been introduced as a bone anabolic factor. Cell culture studies have revealed increased osteoblast proliferation and differentiation with irisin treatment (24, 25). Additionally, injections of recombinant irisin into healthy mice resulted in increased periosteal bone formation rate (23) and increased trabecular and cortical osteoblast numbers (25). Our rats had robust increases in cancellous bone formation rate with approximately 1- to 2-fold higher BFR at the proximal tibia and 1.5- to 3.6-fold higher BFR at L4, regardless of TNBS treatment. These increases in BFR were due to increases in both mineralized surface and mineral apposition rate indicating both an increase in recruitment of osteoblasts and increased activity of those osteoblast teams. Interestingly, there were TNBS-by-irisin interaction effects only in mineralized surface, with mineralized surface no different between Veh+Ir and TNBS+Ir groups, but there were no interactions in MAR or BFR where the TNBS+Ir group was still lower than Veh+Ir rats.

This indicates that exogenous irisin treatment may prevent the suppression of osteoblast recruitment (MS/BS) during inflammation, but may not completely alleviate the suppression on the activity (MAR) of those osteoblasts. However, it must be noted that BFR was still higher in IBD rats treated with irisin compared to vehicle controls (~50% higher at the proximal tibia and ~95% higher at L4). Therefore, our data clearly demonstrate the anabolic actions of irisin on bone.

With regards to bone resorption, *in vitro* studies have found irisin decrease RANKL-induced osteoclastogenesis (25) and treatment with exogenous irisin decreased bone resorption (23). In our study, we found declines in osteoclast surface due to exogenous irisin treatment (51% lower in TNBS+Ir vs TNBS at the proximal tibia and 61% lower at L4). Irisin treatment completely prevented the increase in osteoclast surface seen due to IBD. Additionally, osteoclast surfaces were lower in both irisin-treated groups than in vehicle-treated rats (26-41% lower). Therefore, we demonstrated for the first time that irisin treatment can prevent inflammation-induced increases in osteoclast-resorbing surfaces.

Our key hypothesis was that irisin treatment would prevent inflammatory bone changes by decreasing pro-inflammatory cytokines. A few other recent studies show that exogenous irisin treatment can reduce pro-inflammatory cytokines in various inflammatory conditions. In a model of ischemic stroke, exogenous irisin administration reduces the magnitude of the re-perfusion injury and expression of TNF-α and IL-6 mRNA (21). Likewise, in LPS-induced lung injury, treatment with irisin is protective and reduces the production of multiple pro-inflammatory cytokines including TNF-α and IL-6 (20). Finally, irisin reduces vascular inflammation in atherosclerotic mice (22). In our study, exogenous irisin treatment reduced TNF-α-positive osteocytes by 85% and IL-6-positive osteocytes by 55% in TNBS animals. These

osteocyte-specific data match previous work in other animal models demonstrating the effect irisin has in reducing pro-inflammatory cytokines. Importantly, these changes in osteocyte pro-inflammatory cytokines correspond with decreased osteoclast surfaces and increased bone formation rate. Additionally, we examined two Th2 cytokines, IL-10 and IL-4, and found both were significantly reduced due to irisin treatment, indicating a potential for irisin modulating both Th1- and Th2-related cytokines. As previously mentioned, the role of Th2 cytokines in bone cells is largely unknown and we cannot speculate what these alterations due to irisin mean for bone homeostasis. Our data indicate a clear immunological modulation via irisin resulting in reductions in pro-inflammatory cytokines.

With regard to bone-specific factors, in vitro experiments reveal decreased RANKL-induced osteoclastogenesis due to irisin treatment (25). Furthermore, recombinant irisin administration in mice leads to decreased SOST expression, the gene for sclerostin (23). We, however, are the first to examine specific osteocyte protein response to exogenous irisin treatment. RANKL-positive osteocytes were 4.5-fold lower in TNBS+Ir compared to the TNBS alone animals. Likewise, OPG, the decoy receptor for RANKL, was approximately 6-fold lower in TNBS animals treated with irisin. Since both RANKL and OPG are regulated in part by TNF-α and are the key regulatory step in osteoclastogenesis, the reduction in TNF-α and the reduction in osteoclast surfaces is consistent with our data demonstrating reduced RANKL and OPG. TNF-α also regulates sclerostin, leading to our hypothesis that sclerostin would be reduced due to irisin treatment. Our data confirm our hypothesis, with the percentage of sclerostin-positive osteocytes nearly 2-fold lower in irisin-treated TNBS animals than TNBS alone rats. Our sclerostin data correspond with the robust increase in bone formation rate and mineralizing surface, since sclerostin is a negative regulator of osteoblastogenesis. Additionally, we found

irisin treatment prevented the increase in annexin V in osteocytes indicating normalized apoptosis rates which is also likely due to the decrease in TNF-α and matches our data showing lower osteoclast levels. Due to our previous data showing the confounding data with osteocyte IGF-I, we hypothesized that irisin treatment would return osteocyte IGF-I to vehicle levels, but our data revealed IGF-I-positive osteocytes were lower in both irisin treated groups than in vehicle rats. Since irisin is clearly an anabolic factor inducing an increase in bone formation rate and osteocyte IGF-I is known to induce osteoblasts in conditions of increased loading (28, 29, 30), the current data indicate that we do not yet understand the complexities of osteocyte IGF-I, its role in inflammatory-related conditions, nor its potential interaction with irisin. Nonetheless, our data indicate that irisin treatment alters osteocyte-specific proteins and, in the case of inflammatory bone changes, beneficially alters proteins to favor bone formation and decreased bone resorption.

Similar to our previous work with soy protein interventions, our animals in this study did not show any differences in body weight, food intake, bone mineral density, or cancellous microarchitecture. As previously discussed, our model create a modest inflammatory insult; however, over the long term the changes in bone turnover we see in our TNBS animals would lead to bone loss. Additionally, our irisin treatment did not show changes in bone mass. Due to the slow nature of bone adaptations, the changes in bone turnover markers with irisin treatment indicate that with time there would likely be increased bone mass.

Our data indicate that exogenous irisin treatment acts to decrease pro-inflammatory cytokines and, consequently, reverse inflammatory-induced changes in bone turnover. Since irisin is increased during exercise, targeted exercise therapies to increase irisin could potentially be created to treat inflammatory conditions like IBD; however, the question is if irisin is a major

factor driving immunological changes during exercise. To examine this, Li *et al* injected exercising mice with a neutralizing antibody for irisin prior to ischemic injury and found those mice had more profound neuronal injury than exercising mice injected with control IgG (21). Therefore, it is reasonable to hypothesize that irisin is, in fact, a major driver of the so-called "anti-inflammatory" effects of exercise. It is important to note that most studies examining exercise-induced changes demonstrate only a modest (60-75%) magnitude of acute exercise-induced increase in serum irisin with the training protocols utilized (10, 11, 13). In our study, rats were injected twice weekly with a dose 4-times that of circulating levels, potentially resulting in an increase greater than with exercise training protocols. Therefore, studies to determine the amount and type of exercise needed to naturally induce changes in serum irisin to achieve similar changes in inflammatory markers are needed. Also, it must be noted that exercise is a complex physiological process and there may be a host of other factors released during exercise that either enhance or depress the influence of irisin. Our data indicate that exogenous irisin, reverses chronic inflammatory bone changes, highlighting the potential therapeutic effect of both exercise-induced and exogenous irisin.

In conclusion, this study demonstrates for the first time that exogenous irisin treatment during chronic inflammatory bowel disease preserved normal gut morphology, induced an increase in bone formation rate, and suppressed osteoclast surfaces. Additionally, irisin treatment impacted the inflammatory response of bone by reducing osteocyte pro-inflammatory markers as well as bone-specific regulators of bone turnover. Overall, irisin treatment reversed the inflammatory-induced changes in bone seen with IBD alone. Therefore, our data indicate that irisin may be a key regulatory mediator of the proposed anti-inflammatory effects of exercise, suggesting that targeted exercise regimens designed to maximize increases of circulating irisin

may prove to be beneficial for individuals with chronic systemic inflammatory conditions like IBD. Secondly, our data indicates that irisin administration, alone, could be a novel therapy for chronic inflammatory conditions and a potential treatment for inflammation-induced bone loss.

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5. CONCLUSIONS

Together these studies demonstrate that 1) chronic inflammatory bowel disease leads to inflammatory-induced changes in bone turnover and a pro-inflammatory response of osteocytes, 2) a moderately elevated soy protein diet can mitigate the pro-inflammatory state of osteocytes as well as inflammation-induced changes in bone turnover, and 3) irisin, an adipomyokine released during exercise, alleviates the inflammatory state of bone during chronic IBD and altered osteocyte protein response.

To date, the role of osteocytes in inflammation-induced bone loss has been neglected. While inflammation is known to lead to an increase in bone resorption and a decrease in bone formation, whether or not osteocytes play a role in regulating these changes has not been addressed. In our model of chronic inflammatory bowel disease, there was an increase in osteocytes positive for multiple pro-inflammatory cytokines (TNF-α and IL-6) as well as many factors activated by TNF-α (RANKL, OPG, sclerostin; Figure 5.1). These changes in osteocyte proteins strongly predicted the increases in osteoclast surfaces and declines in bone formation rate in IBD rats. These data indicate that osteocytes respond to systemic signals of inflammation. Similar to conditions of mechanical loading or unloading where osteocytes are known to play a key regulatory role, osteocytes may also be orchestrating the changes observed in bone turnover due to systemic inflammation.

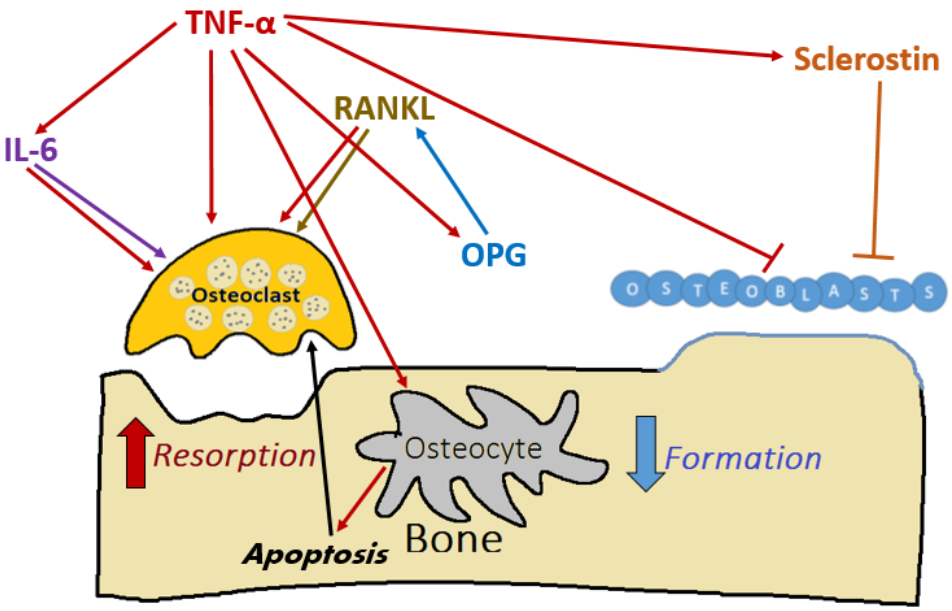

Figure 5.1: Influence of pro-inflammatory factors on bone cells during chronic IBD. Together these alterations lead to an increase in bone resorption and a decrease in bone formation. Our data indicate that all these factors are either expressed or bind to osteocytes.

With current treatments for IBD all aiming to simply mitigate the disease symptoms often with negative side effects, there is a need for lifestyle interventions that can mitigate inflammation, thus lessening the need for medications or enhancing the influence of concurrent treatments. In our second study, we examined the use of a moderately elevated soy protein diet during chronic IBD and discovered it successfully mitigated the inflammatory-induced changes in gut structure and the pro-inflammatory status of osteocytes, as well as decreased osteoclast surfaces and increased bone formation rate. In most all of our measures, the IBD animals with the soy protein intervention had values no different from their vehicle-treated controls. Additionally, the beneficial changes in bone turnover were all concurrent with alterations in osteocyte proteins, again alluding to a potential key regulatory role of osteocytes in inflammatory

conditions. The mechanisms by which the effects of the soy protein diet influenced the GI and bone inflammation remain to be determined (Figure 5.2).

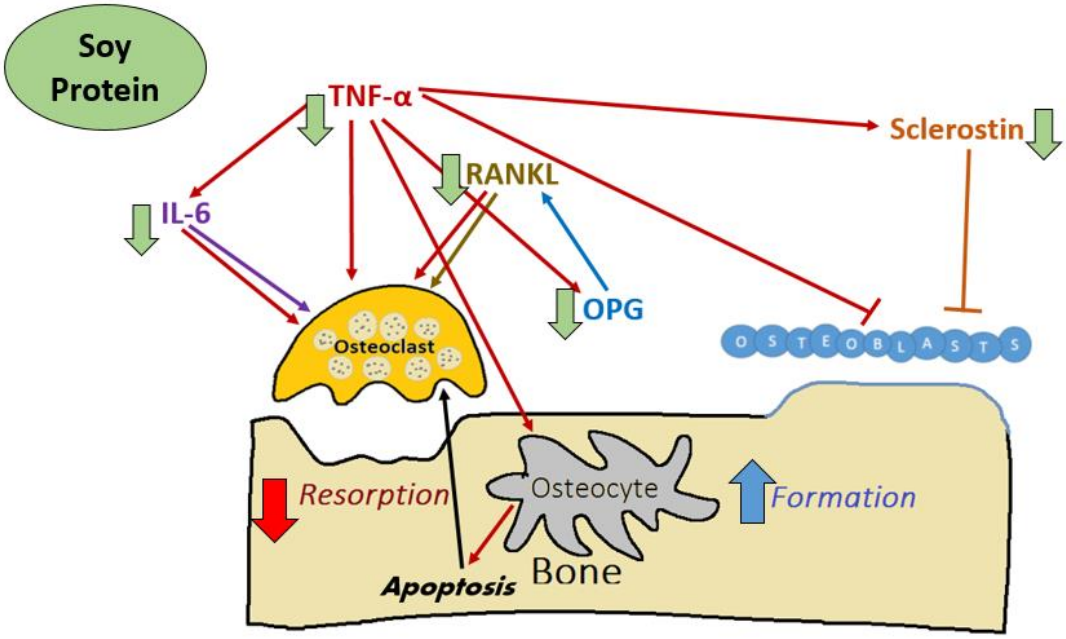

Figure 5.2: Intervention of soy protein during chronic IBD. Moderately elevated soy protein during chronic IBD resulted in decreases in key pro-inflammatory factors, osteoclastogenesis factors, and osteoblast inhibitors. The soy protein intervention mitigated osteoclast surfaces and increased bone formation.

Although exercise has long been lauded as anti-inflammatory, there is minimal evidence of what factors may actually be inducing such a change or what type of immunological change is occurring due to exercise. In our final study, we explored the use of exogenous treatment with irisin, a factor released during exercise, during chronic IBD. We discovered that exogenous irisin mitigated the inflammatory insult in both the gut and the bone. Irisin treatment lead to an increase in bone formation rate and a concurrent decrease in osteoclast surfaces beyond those of vehicle-treated rats. These changes in bone turnover were concurrent with

declines in pro-inflammatory factors, osteoclastogenesis regulators, and osteoblast inhibitors in osteocytes (Figure 5.3).

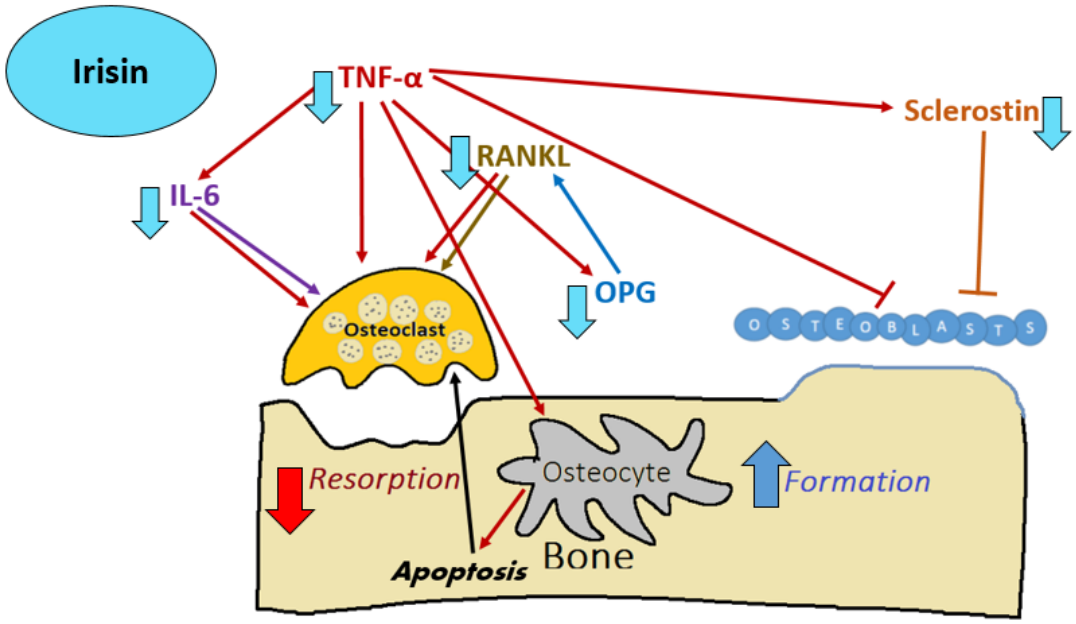

Figure 5.3: Actions of exogenous irisin administration during chronic IBD. Exogenous irisin administration resulted in declines in TNF-α, IL-6, RANKL, OPG, and sclerostin. These changes were concurrent with increased bone formation rate and decreased osteoclast surfaces.

In conclusion, our data demonstrate that osteocytes may be regulators of inflammation-induced bone loss and, therefore, treatments that alter osteocyte proteins may be key therapeutic targets for diseases impacting bone. Additionally, we found that both a moderately elevated soy protein diet and exogenous treatment with irisin successfully mitigated the pro-inflammatory status of bone and attenuated or reversed inflammation-induced changes in bone turnover. Therefore, a soy protein diet may be useful as a simple and cost effective lifestyle addition for patients with IBD. Regular exercise therapy may also be able to aid in protection against the inflammatory insult of chronic IBD and weight-bearing exercise would have the added benefit on bone of increasing mechanical loads. Finally, exogenous irisin may also be a

novel anti-inflammatory therapeutic for IBD and other chronic systemic inflammatory conditions.