

ALTERED BONE RESORPTION IN TYPE 1 AND TYPE 2 DIABETES

By

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To my mother, father, and brother for being a constant support to me during this
challenging endeavor

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LIST OF ABBREVIATIONS

ACPA	Antibodies to citrullinated protein antigens
ADAM	A disintegrin and metalloproteinase domain-containing protein
AGE	Advanced glycated end-product
ALP	Alkaline phosphatase
α -MEM	Minimum essential medium, alpha modification
AP-1	Activator protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
α v β 3	Alpha v beta 3 integrin
B-cells	Beta cells
BM-OC	Bone marrow-derived osteoclast
BMP	Bone morphogenic protein
Breg	Regulatory B cell
C57BL/6	C57 black 6 mouse strain
CA	Carbonic anhydrase
CaCl ₂	Calcium chloride
Cbfa1	Core-binding factor alpha 1
CD25	Interleukin 2 receptor
c-fms	Receptor for macrophage colony stimulating factor
CFU-GM	Colony forming unit granulocyte macrophage
ClC-7	Chloride channel 7
CRP	C-reactive protein
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTR	Calcitonin receptor

CTx	Carboxy-terminal collagen crosslink
db/db	Diabetic mutation
DC	Dendritic cell
DC-STAMP	Dendritic cell-specific transmembrane protein
DPD	Deoxypyridinoline crosslink
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
Flt3	Fms-like tyrosine kinase receptor-3
GAD65	65kDa isoform of glutamic acid decarboxylase
G-CSF	Granulocyte colony stimulating factor
GLUT2	Glucose transporter 2
GM-CSF	Granulocyte macrophage colony stimulating factor
HbA1c	Glycated hemoglobin A1c
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
hOC	Human osteoclast
HRP	Horse radish peroxidase
IA-2A	Insulinoma-associated protein 2 antibody
ICA	Islet cell antibody
IDDM	Insulin-dependent diabetes susceptibility loci
IFN- γ	Interferon gamma
IGF-1/2	Insulin-like growth factors 1 and 2
IgM	Immunoglobulin M
I κ B	Inhibitor of kappa B

IL-2	Interleukin 2
IP-10	Interferon gamma-induced protein 10
IRB	Institutional review board
KCl	Potassium chloride
Lepr	Leptin receptor
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
mBSA	Methylated bovine serum albumin
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein 1 alpha
Mitf	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
mOC	Mouse osteoclast
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NFATc1	Nuclear factor of activated T cells, cytoplasmic 1
NF- κ B	Nuclear factor kappa B
NK	Natural killer cell
NO	Nitric oxide
NOD	Non-obese diabetic mouse strain
NOR	Non-obese resistant mouse strain
NSAID	Non-steroidal anti-inflammatory drug

NTx	Amino-terminal collagen crosslink
OB	Osteoblast
OC	Osteoclast
OC-STAMP	Osteoclast-specific transmembrane protein
OPG	Osteoprotegerin
op/op	Osteopetrotic mutation
OSCAR	Osteoclast-associated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
<i>P.g.</i>	<i>Porphyromonas gingivalis</i>
PGE2	Prostaglandin E2
PIGF	Placental growth factor
PPAR γ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycated end-products
RANK	Receptor activator of nuclear factor kappa B
RANK-L	Receptor activator of nuclear factor kappa B ligand
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RF	Rheumatoid factor
RGD	Arginine-glycine-asparagine motif
ROS	Reactive oxygen species
rpm	Revolutions per minute

RUNX2	Runt-related transcription factor 2
SAV	Streptavidin
SDF-1	Stromal cell-derived factor-1
SIRP α	Signal-regulatory protein-alpha
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPF	Specific pathogen-free
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF- β	Transforming growth factor beta
T _H 17	T helper 17 cell
T _H 2	T helper 2 cell
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase
Treg	Regulatory T cell
TSH	Thyroid-stimulating hormone
UV	Ultraviolet
V-ATPase	Vacuolar proton ATPase
VEGF	Vascular endothelial growth factor
VNTR	Variable number tandem repeats

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Inflammation perturbs bone homeostasis by inducing bone loss. Thus, inflammatory diseases such as diabetes mellitus are epidemiologically linked to bone pathologies. However, the mechanisms of bone loss in these inflammatory diseases are complex where disturbances in the control of bone remodeling are involved. Two major cell types are involved in the regulation of bone remodeling: osteoclasts are responsible for removal of bone and osteoblasts are responsible for formation of new bone. While decreased function by osteoblasts from hosts with diabetes have been implicated in mechanisms of altered bone homeostasis, osteoclasts and their specific contributions were previously unclear.

Bone marrow-derived osteoclasts were isolated from pre-diabetic T1D-prone non-obese diabetic [NOD] mice and hyperglycemic T2D-prone C57BL/6-*Lep^{ob3J}* [db/db], along with peripheral blood mononuclear cell-derived osteoclasts from participants with well-controlled type 2 diabetes [T2D]. Osteoclasts derived from NOD mice had decreased fusion during differentiation, along with elevated RANK-L-induced enzyme production resulting in enhanced bone resorption. Conversely, osteoclasts derived from hyperglycemic db/db mice displayed enhanced fusion along with similar RANK-L-

induced bone resorption compared to controls. Osteoclasts prepared from well-controlled T2D participants displayed no difference in fusion along with similar RANK-L-induced bone resorption compared to controls. All diabetes derived-osteoclasts overcame LPS-induced inhibition whereby diabetes-derived cultures continued to resorb bone in the presence of LPS while control-derived cultures resorbed little to no bone in the presence of LPS. In addition, diabetes-derived cultures also contained elevated levels of pro-osteoclastogenic mediators compared to controls. Importantly, the altered LPS-responsiveness observed was not solely due to soluble mediators produced by the cultures, as a pro-inflammatory cocktail nor conditioned supernatants from diabetes-derived cultures could overcome LPS-induced inactivation in control cultures.

Together these data indicate that diabetes-derived osteoclasts are more resorptive in nature through different mechanisms depending on type and state of disease. In addition, all diabetes-derived osteoclasts are refractory to LPS-induced deactivation whereby enhanced pro-osteoclastic mediator expression is also evident.

CHAPTER 1 LITERATURE REVIEW

Diabetes and Inflammatory Bone Loss

Inflammation perturbs normal bone homeostasis and is known to induce bone loss (1-3). Thus, not surprisingly, inflammatory diseases such as diabetes mellitus are epidemiologically linked to local and general bone pathologies (4-10). However, the mechanisms that target bone loss in these inflammatory diseases are complex and diverse ranging from attack on bone and cartilage by immune cells to disturbances of the systemic control of bone remodeling (6, 11-22).

Diabetes mellitus afflicts over 21 million Americans, including >9% of the adult population (23). Bone and joint abnormalities are frequent co-morbidities of both type 1 [T1D] and type 2 [T2D] diabetes including destruction due to inflammatory diseases such as rheumatoid arthritis [RA] (17, 24, 25) and periodontal disease [PD] (26-29). Both T1D and T2D originate from complex etiology with intrinsic genetic risk factors and extrinsic environmental factors (30-45). Diabetes-associated bone and joint pathologies likewise may originate from shared intrinsic genetic factors or from extrinsic sources of inflammation such as infection (occult or symptomatic) (17, 19, 22, 46-60). Furthermore, bone and joint pathology may develop secondary to autoimmune inflammation [T1D], insulin resistance [T2D], or as a consequence of hyperglycemia [T1D and T2D] (17, 19, 25, 27, 50, 61-65).

Bone Remodeling

Normal process versus inflammatory bone loss: Developmental bone growth as well as post-developmental maintenance and repair of bone are dependent on a dynamic process called bone remodeling. Remodeling of bone is a continuous process

necessary to maintain the integrity of mineralized tissue (66). When bone remodeling is not coupled correctly, either too much bone can be formed or too much can be lost (67). Bone remodeling consists of 5 distinct phases: activation, resorption, reversal, formation, and termination, where two major cell types are involved in the regulation of these phases. Specifically, osteoclasts are responsible for removal of old or damaged bone while osteoblasts are responsible for formation of new bone (68). A proper coupling between bone formation and bone destruction is essential to maintain bone integrity which is regulated at three different levels: 1) locally by a direct interaction between osteoblasts and osteoclasts, 2) by the local interaction between cells of the immune system and bone cells as well as the 3) control by the neuroendocrine systemic on bone metabolism (66, 68-82).

Dysregulation of one or more of these levels of regulation are thought to determine the various skeletal manifestations associated with inflammatory diseases such as diabetes mellitus (6-9, 11, 13, 14, 83, 84). Bone remodeling during inflammation is altered where bone formation ceases and bone resorption takes precedence. Inflammatory bone loss is initiated after an inflammatory stimulus, such as a bacterial product, is sensed by the innate immune cells residing near the bone (70). Pro-inflammatory cytokines such as tumor necrosis factor alpha [TNF- α] and interleukins 1 beta [IL-1 β] and 6 [IL-6] are produced by these cells in response to the stimulus and can act as direct activators of osteoclast differentiation (osteoclastogenesis) as well as initiate the resorptive process (2, 85-91). CD4⁺ T cells activated by the stimulus and cytokines also produce pro-osteoclastic mediators to initiate resorption (92, 93). These cytokines also induce osteoblasts to produce pro-osteoclastic mediators and suppress

bone formation (13, 94-96). Thus, inflammation induces bone loss and suppresses bone healing.

Osteoclasts

Differentiation, activation, and function: While osteoblasts are derived from mesenchymal stem cells of the bone marrow, osteoclasts are derived from the hematopoietic stem cell lineage. Osteoclasts differentiate from monocytic progenitors at the expense of other monocytic lineages such as dendritic cells, granulocytes, macrophages and microglia (97). Osteoclast differentiation is regulated by two essential cytokines: macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa B ligand (RANK-L). M-CSF promotes proliferation and survival of osteoclast precursors while RANK-L induces the commitment and differentiation of the precursors (98-100). Osteoclast differentiation is strictly dependent on the presence of supportive cells that express on their surface or secrete these two essential pro-osteoclastogenic cytokines (101).

The earliest osteoclast progenitor known is the colony-forming unit granulocyte macrophage [CFU-GM] (97). These precursors account for 1-4% of the pool of circulating monocytes and express monocyte/macrophage markers yet do not express many of the osteoclast-specific markers such as tartrate-resistant acid phosphatase [TRAP], the adhesion molecule beta 3 integrin [β 3 integrin], or the calcitonin receptor [CTR] (101-103). Loss of some monocyte/macrophage markers and upregulation of osteoclast markers are the earliest signs that a mononuclear precursor is committed to the osteoclast lineage (104). On the other hand, other macrophage markers such as Mac-1 (CD11b/CD18) are retained on these mononuclear precursors but are lost once multi-nucleation begins. In addition, at this stage osteoclast precursors are post-mitotic

and thus do not replicate once they are become committed to the osteoclast lineage (105).

Osteoclast differentiation is controlled by a highly ordered cascade of osteoclast-specific gene expression. The transcription factor PU.1 expressed in CFU-GM induces the expression of *c-fms*, the receptor for M-CSF (106). This receptor is of the tyrosine kinase super-family and upon ligation, dimerizes and auto-phosphorylates. M-CSF-induced signaling results in the up-regulation of RANK whose ligation by RANK-L is required for the completion of osteoclastogenesis and induction of osteoclast activation (79). Specifically, M-CSF ligation to *c-fms* induces the transcription factor *c-Fos*, whereby if M-CSF ligation is accompanied by RANK ligation, these precursors commit to an osteoclast lineage (107, 108). Microphthalmia-associated transcription factor [*Mitf*] is then activated resulting in the induction of carbonic anhydrase 2 [CA-II], TRAP, and additional *c-fms* (106, 109, 110). Simultaneously, RANK ligation leads to induction of NF- κ B and NFATc1 (111) inducing the expression of β 3 integrin, CTR, cathepsin K, and matrix metalloproteinases [MMPs] (112, 113), completing the commitment to the osteoclast lineage where no further proliferation of these cells occurs (99).

In addition to its roles in differentiation, M-CSF induced signaling enhances pre-osteoclast survival and proliferation while preventing apoptosis and enhancing motility in mature osteoclasts (98, 99, 112). M-CSF is a homodimeric glycoprotein that can be produced in a soluble or membrane-bound form by osteoblasts, fibroblasts, epithelial cells, and activated macrophages (101). Soluble M-CSF is more involved in early osteoclast differentiation while the membrane-bound form is essential for proper cell fusion during subsequent differentiation stages (114). M-CSF also acts as a negative

regulator of its receptor, c-fms, by reducing its transcription which helps to control the resorptive response once it has begun (98, 101, 115).

RANK-L, the ligand for RANK, is a member of the TNF receptor ligand superfamily and is expressed by stromal cells and osteoblasts in bone as well as activated T cells (93, 101), whereby it can be induced by active vitamin D₃, parathyroid hormone [PTH], and TNF- α (101). This molecule is expressed in a membrane-bound form which is later cleaved into a soluble form by MMPs (116). Through the signaling pathways described above, RANK-L, along with M-CSF, promotes the fusion of mononuclear osteoclast precursors and acts to activate the mature osteoclasts to resorb bone.

Only recently have the mechanisms associated with the fusion of mononuclear osteoclast precursors begun to be deciphered where the literature describes several candidates involved in cell-cell recognition and attachment: 1) the Ig superfamily member CD200, 2) signal-regulatory protein-alpha [SIRP α], and 3) dendritic cell-specific transmembrane protein [DC-STAMP] (117). Specifically, during osteoclast differentiation, CD200 and its receptor CD200R are up-regulated just before fusion where their interactions act to enhance fusion promoting RANK signaling (118). Upon ligation with its receptor CD47, SIRP α , another member of the CD200 family, not only induces osteoclast differentiation (117), but also promotes fusion initiation although it is dispensable once the process has begun (119). On the other hand, DC-STAMP has been called a “master fusigen” where it is highly expressed in multinucleated osteoclasts. Interestingly, DC-STAMP only needs to be expressed on one cell and preferably in low levels in order to allow for fusion to occur (120). Similarly, OC-

STAMP, which is similar to DC-STAMP but only expressed on osteoclasts, is also required for fusion where its up-regulation occurs early in the fusion process (121).

Osteoclast mediated resorption requires the following cellular activities: migration of the osteoclast to the resorption site, its attachment to bone, polarization and formation of new membrane domains, dissolution of hydroxyapatite, degradation of organic matrix, and removal of degradation products from the resorption lacuna (97). The reorganization of the actin cytoskeleton and polarization of the plasma membrane results in the development of four compartments in the plasma membrane: 1) sealing zone, 2) ruffled border, 3) functional secretory domain, and 4) basolateral membrane (97). Unpolarized inactive osteoclasts have dispersed podosomes. During osteoclast activation, these podosomes coalesce into a peripheral belt and subsequently into a distinct “actin ring” that forms the sealing zone where the osteoclast adheres tightly to the bone surface (122). The podosomes that attach to the bone utilize the alpha v beta 3 [$\alpha v\beta 3$] integrin which binds to RGD motifs in the bone matrix (123). The resulting actin ring in the sealing zone completely restricts osteoclastic enzyme activity to inside the resorption lacuna. After the formation of the actin ring, trafficking of late endosomes/lysosomes toward the bone surface allow for formation of the ruffled border, the resorptive portion of the osteoclast. Finally, the functional secretory domain forms at the top of the cell, to which transcytotic vesicles formed at the ruffled border are targeted. The functional secretory domain allows for the transcytosis of digested bone matrix from the lacuna to the extracellular milieu (124).

The main physiological function of osteoclasts is to degrade mineralized bone matrix that requires the dissolution of hydroxyapatite and proteolytic cleavage of the

organic matrix that is rich in collagen. Before proteolytic enzymes can reach and degrade collagenous bone matrix, tightly packed hydroxyapatite crystals must be dissolved which occurs by targeted secretion of HCl through the ruffled border into the resorption lacuna (97). Vesicles containing vacuolar proton ATPases [V-ATPases] are transported to the ruffled border along actin filaments and utilize adenosine triphosphate [ATP] to pump hydrogen ions against a concentration gradient into the resorption site (125, 126). Here, the α_3 subunit of the V-ATPase aids in targeting of this normally lysosomal enzyme to the ruffled border (127, 128). Protons are supplied by CA-II, which acts to hydrate carbon dioxide and produce bicarbonate that later dissociates into hydrogen ions and HCO_3^- (129). Chloride channel 7 [ClC-7] aids in pumping chlorine ions, which along with the hydrogen ions, creates hydrochloric acid. This creates an environment with a pH of 3 to 4 that is effective at dissolving hydroxyapatite mineral from the organic bone matrix (130).

After solubilization of the mineral phase, several proteolytic enzymes degrade the organic bone matrix where the acidic environment described above also serves to activate these enzymes (97). Specifically, cathepsin K and MMP-9 are responsible for the degradation of organic bone matrix where both degrade type I collagen into small telopeptides (131, 132). Osteopontin a non-collagenous bone protein is also degraded by cathepsin K (97). In addition, cathepsin K acts to activate TRAP, a hallmark enzyme produced by osteoclasts and their precursors (133, 134). TRAP is a phosphatase that is thought to 1) generate ROS which help degrade collagen 2) aid in degradation of phosphoproteins in the bone matrix such as osteopontin and 3) aid in the release of osteoclasts from the site of resorption so that the cell can migrate (133-136).

As mentioned above, all of these degradation products are transcytosed through the osteoclast and released out into the extracellular milieu via intracellular vesicle trafficking where these vesicles travel to the functional secretory domain, fuse, and empty their contents into the extracellular milieu (124).

Effects of Diabetes on Osteoclast-Mediated Inflammatory Bone Loss

Diabetes mellitus

Although once controversial, the evidence that bone health is compromised in diabetes mellitus is now strong. Bone mineral density is lower, the risk of fractures is increased, and there are strong epidemiological links between inflammatory bone diseases and diabetes mellitus all suggesting significant alterations in bone health (5, 7, 8, 10, 11, 14, 24, 26, 27, 84, 137-139). Several mechanisms have been proposed for diabetes-related alterations in bone health and include the co-morbidities of diabetes and more direct pathophysiological effects of the diseases themselves (5, 7, 8, 11, 14, 15, 72, 140).

Diabetes mellitus is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (141). The two major classifications will be discussed in brief below.

Type 1 diabetes

Type 1 Diabetes [T1D] or insulin-dependent diabetes mellitus [IDDM] is an endocrine disease in humans that, in most cases, results from the autoimmune destruction of the insulin-producing beta cells [β -cells] located in the islets of Langerhans of the pancreas that secrete insulin in response to postprandial elevations in blood glucose (142). Clinical presentation of the symptoms of T1D includes fasting

hyperglycemia, glycosuria and ketoacidosis. These symptoms are usually abrupt in onset and are indicative of destruction of β -cell mass. Thus, over time, individuals with T1D require exogenous insulin to maintain blood glucose homeostasis since insulin production by the pancreas declines (143). The process of β -cell destruction is believed to be chronic in nature and can be detected earlier than the onset of clinical symptoms by the presence of certain autoantibodies including those specific for glutamic acid decarboxylase [GAD], islet cell antibody [ICA], and insulinoma-associated protein 2 antibody [IA-2A], as well as insulin (144). Individuals who have one or more of these antibodies can be sub-classified as having type 1A, or immune-mediated type 1 diabetes (145). Interestingly, the time between the appearance of autoantibodies and full-blown insulinitis (infiltration of the pancreas by immune cells) is highly variable and can range from a few months to even a decade or more (30, 146). It is important to note that T1D can occur in the absence of autoimmune antibodies and without evidence of any autoimmunity. Such individuals are classified as having type 1B, or idiopathic diabetes, where progressive disease marked by hyperglycemia results in the requirement of insulin for the prevention of ketosis and survival much like type 1A (147).

In that type 1A diabetes is of immune etiology, it represents an inherited failure in the maintenance of self-tolerance and thus it is not surprising that individuals with diabetes are at increased risk for a series of autoimmune and inflammatory disorders including those associated with bone loss/destruction (17). Lessons learned from murine models, but not necessarily confirmed in human T1D, reflect a significant lymphoaccumulation that is not restricted to the pancreas suggesting a heightened level of local and systemic inflammation, which could contribute to the manifestation of many

of the inflammatory co-morbidities associated with T1D (148). Similar inflammatory complications also arise from the consequences of hyperglycemia that is characteristic of all classifications of diabetes and thus also could contribute to the manifestation of many of the inflammatory co-morbidities associated with T1D (141, 149-152) including bone loss/destruction (5, 27, 153). Such contributions are discussed in detail below.

Because of the multifaceted nature of diabetes, etiological dissection of diabetes and its co-morbidities in humans has been difficult. Therefore, much of the current understanding of diabetes pathogenesis and its co-morbidities have been learned from spontaneous animal models. Thus, one of the most extensively studied rodent models of T1D, the NOD mouse, will be utilized in the studies presented here. A detailed description of this model can be found in later Chapters.

Type 2 diabetes

Type 2 diabetes [T2D] or non-insulin dependent diabetes is the most common form of diabetes and is characterized by disorders of insulin action and/or insulin secretion where either of which can be the predominant feature but both are usually present at the time of clinical manifestation (154). In many cases, onset of T2D occurs when compensatory mechanisms for insulin resistance drive β -cell failure (155). The risk of developing T2D increases with age, obesity and physical inactivity although T2D does show strong familial aggregation (42, 43). Environmental factors implicated in the triggering of T2D in susceptible individuals include smoking, psychological stress, endocrine-modulating chemicals, aging, and infections (44, 156). Thus, similar to T1D, T2D is a multi-factorial disease where genetic and environmental factors influence its development (156, 157).

T2D patients do not usually require insulin due to their insulin resistance rather than absolute insulin deficiency, although progressive β -cell loss may require insulin therapy for glycemic control. Glucose tolerance first becomes impaired due to the resistance of the insulin receptors to its ligand thereby leading to both higher fasting glucose and hyperinsulinemia (154). Insulin production eventually decreases over time due to the highly toxic, chronic hyperglycemic environment resulting in loss of β -cell function and/or mass (155).

Importantly, there is a relationship between insulin resistance and systemic inflammation in T2D whereby adipose tissue produces many pro-inflammatory molecules including TNF- α , IL-6, transforming growth factor- β [TGF- β], and monocyte chemoattractant protein-1 [MCP-1]. These pro-inflammatory molecules not only induce systemic insulin resistance, but also contribute to the pathogenesis of many inflammatory complications of T2D including inflammatory bone loss (87, 95, 149, 156, 158-162).

As described with T1D, the etiological dissection of T2D and its co-morbidities in humans is difficult. Therefore, much of the current understanding of pathogenesis and its co-morbidities have been learned from animal models. A large array of rodent models exists for the study of T2D where all models manifest hyperglycemia. Importantly, the stresses needed for the onset of diabetes as well as the associated phenotypes are drastically different. In the studies presented here, the B6.BKS(D)-*Lep^{db}*/J mouse model was employed where hyperinsulinemia, hyperglycemia, and obesity are all evident (163, 164). A detailed description of this model and its rationale can be found in later Chapters.

Diabetes mellitus and epidemiological link to inflammatory bone loss

Although many factors influence bone health, the most significant factor is bone mineral density or bone strength. Bone densitometry techniques have become much more sophisticated in the past two decades allowing for the detection of more sensitive differences in both T1D and T2D.

Low bone density or osteopenia was initially described in adolescents with diabetes, where 25-50% were found to have decreased cortical and trabecular bone mineral density (165, 166). Most studies in adults confirm that bone mineral density is lower in patients with T1D than in subjects without diabetes. In contrast, studies in participants with T2D show bone mineral density that is either the same or greater than diabetes-free subjects (138). If the relationship between bone loss and diabetes were related only to hyperglycemia, one would expect a similar incidence of poor bone density in patients with type 1 and type 2 diabetes. Thus, this data suggests that differences between types of diabetes other than glucose control impact bone loss. With that said, hypercalciuria, a marker for bone loss, has been noted in patients with poorly controlled diabetes (type 1 and type 2), which can be reversed upon improved glycated hemoglobin [HbA1c] (167). Thus, metabolic control appears to be at least one major factor in the increased incidence of bone loss in patients with diabetes.

Importantly, excessive osteoclast activity as measured by urine concentrations of bone degradation products have been found to be elevated in both T1D and T2D patients. On the other hand, osteoprotegerin [OPG], the negative regulator of osteoclasts produced by osteoblasts, has been found to be reduced in T1D patients yet increased in individuals with early T2D (7, 11). This may be attributed to the hyperinsulinemia associated with early T2D but not late T2D or T1D discussed above.

Insulin is an anabolic hormone whereby it activates osteoblasts to synthesize proteins necessary for bone formation (168). Similarly, insulin-like growth factors induce the synthesis of bone collagen and deposition of calcium (169). These findings suggest that hyperinsulinemia can stimulate boney overgrowth (170). Similarly, inflammatory bone pathologies such as rheumatoid arthritis [RA] and periodontitis-associated alveolar bone loss are also more prevalent, more severe, and carry with them higher morbidity in patients with diabetes than in those without diabetes (17, 24-29, 171).

Rheumatic diseases such as osteoarthritis and RA have a higher prevalence in patients with both T1D and T2D where damage begins earlier and is more severe than in diabetes-free patients (17, 172). While diabetes is not currently considered a risk factor for developing RA, it is widely known that both conditions do occur simultaneously (24). RA is a chronic inflammatory arthritis which is also classified as an autoimmune disease (46). Thus, similar mechanisms of pathology associated with inflammation in T1D and T2D, as well as those associated with predisposition for T1D autoimmunity, contribute to these epidemiological links (46, 50-52, 55). As with T2D, obesity is a common risk factor for RA and although the mechanism is unknown, one could speculate that the effect of obesity on systemic inflammation discussed above could play a role (55, 61). Rheumatoid arthritis and its co-morbidity with diabetes will be discussed further in the sections below.

Periodontal diseases are inflammatory processes that occur in the tissues surrounding the teeth in response to bacterial accumulations. This chronic and progressive inflammatory response results in the loss of soft tissue stability and alveolar bone destruction (173). Numerous studies have found that patients with both T1D and

T2D have a greater susceptibility to and more severe periodontal disease than diabetes-free patients as measured by increases in clinical attachment loss and alveolar bone resorption (26, 29). Here, the most common view is that periodontal disease development results from complications of diabetes although recent studies present evidence of a bidirectional adverse interrelationship between both T1D and T2D with periodontal disease (20, 22, 26, 28, 60, 64, 171, 174, 175). Specifically, epidemiological studies suggest that the level of glycemic control seems to play a key role whereby poor glycemic control positively correlates with high levels of soft tissue damage and alveolar bone loss (174, 176). In addition, as discussed with RA, similar mechanisms of pathology associated with inflammation in T1D and T2D may also contribute to these epidemiological links (18-20, 22, 60, 177, 178). Mechanisms of association between diabetes and periodontal disease will be discussed in detail below.

Contributions of Intrinsic Defects

While chronic inflammation and hyperglycemia do affect bone metabolism and osteoclast function, as will be discussed later, intrinsic factors such as defects in immune tolerance leading to pro-inflammatory signaling and hyper-responsiveness of osteoclasts to RANK-L stimulation in patients with diabetes are also contributing elements to increased propensity for bone pathology in these individuals (8, 179-181).

Lessons from related cell types: macrophages and dendritic cells:

Hematopoietic stem cells give rise to the earliest precursor of the osteoclast lineage, the CFU-GM. Depending on the growth factors and cytokines given, this cell type can give rise to osteoclasts, dendritic cells, monocytes, and macrophages (97).

Dendritic cells [DCs], major antigen presenting cells, have an altered phenotype and function in patients with T1D where fewer numbers of DCs with decreased

expression of the co-stimulatory molecules B7.1/2 that are necessary for the activation of T cells after antigen presentation, lead to an impaired ability to activate naïve T cells (182, 183). Similarly, the NOD mouse model also has defects in DC differentiation resulting in lower expression of MHC class II and co-stimulatory molecules (184, 185). Interestingly, even with this less mature phenotype, NOD-derived DCs display increased NF- κ B activation concomitant with decreased inhibition of NF- κ B translocation to the nucleus leading to increased production of pro-inflammatory cytokines, along with more efficient activation of naïve antigen-specific CD8+ T cells (186-188).

Similarly, monocytes and macrophages in patients with T1D and T2D have been found to be not only constitutively activated due to constantly circulating toll-like receptor [TLR] ligands, but hyper-responsive to these ligands (20, 41, 179, 181, 189). This again results in increased levels of multiple pro-inflammatory cytokines such as IL-1 β and TNF- α (45, 179). Interestingly, studies of NOD-derived macrophages again demonstrate a less mature phenotype along with this hyper-reactive trait, whereby alterations in NF- κ B activation and translocation play a role as described in the case of DCs above (190-192).

Thus, one could hypothesize that since these related cell types have altered and increased activation, osteoclasts derived from the same hematopoietic lineage may also display aberrant function thereby leading to exacerbated bone destruction. Indeed, osteoclasts utilize the NF- κ B pathway for the up-regulation of critical genes during differentiation and resorption and thus, heightened translocation as described would

therefore lead to increased resorption under both homeostatic and inflammatory conditions (1, 193).

Lack of LPS tolerance: Endotoxin, or LPS, tolerance is defined as a reduced capacity of the host to respond to LPS activation following a first exposure to this stimulus (194). LPS tolerance has also been termed hypo-responsiveness, refractoriness, adaptation, deactivation and desensitization (195). However, LPS tolerance is not a global down-regulation of signaling protein and mediator production, but rather a change in the expression of specific genes and proteins upon second challenge. The purpose of LPS tolerance has been thought to be a mechanism for controlling innate immune responses (inflammation) important for fighting Gram-negative bacteria to prevent host tissue damage and/or to prevent reactivity to commensal flora (196). Lack of tolerance can therefore lead to a constant state of inflammation which could directly exacerbate inflammation-induced bone pathologies (41).

Macrophages derived from diabetes-free mouse models such as C57Bl/6, down-regulate the receptor for LPS, TLR-4, in response to LPS treatment resulting in a tempered response to subsequent challenge (197). On the other hand, T1D-prone NOD mouse-derived macrophages do not down-regulate TLR-4 after LPS treatment suggesting defective tolerance to LPS due to aberrant, constitutive TLR-4 expression (180). Interestingly, tolerance also seems to be defective in monocytes derived from patients with T2D, whereby hyper-secretion of pro-inflammatory mediators is observed when using traditional tolerance-inducing assays (45, 179). As mentioned above, monocytes and macrophages from T2D patients appear to be constitutively activated

apparently due to constantly circulating TLR ligands derived from endogenous and/or pathogenic sources (179).

While TLR responses indirectly modulate osteoclast function through the interplay between the immune system and bone metabolism, TLRs, in particular TLR-4, are capable of directly modulating bone cell metabolism (198-201). Specifically, LPS can act directly on osteoclasts by inhibiting the differentiation of monocytic precursors into bone resorbing osteoclasts and bone resorption by mature osteoclasts (200, 202). Thus, one could hypothesize that similar to the diabetes-specific alterations in LPS responsiveness described above, diabetes-derived osteoclasts could also have alterations in their LPS responsiveness.

RANK-L hyper-responsiveness: As previously mentioned, differentiation and activation of osteoclasts require RANK-L/RANK interactions whereby RANK-L is expressed on a variety of cell types including osteoblasts, T cells, dendritic cells, endothelial cells, and fibroblasts (93, 101). RANK-L/RANK interactions are counterbalanced by osteoprotegerin [OPG], which acts as a soluble decoy receptor for RANK-L, thus regulating the extent of osteoclast formation and resorption (203, 204).

Interestingly, RANK and TLR signaling share common signaling molecules (111, 205), where these molecules play a role in the augmented TLR responses in T1D and T2D discussed above (179-181, 187, 190) suggesting the potential for RANK-L hyper-responsiveness as well. Indeed, Mabileau et al. have demonstrated that osteoclasts derived from the peripheral blood of patients with diabetes suffering from Charcot's arthropathy are more sensitive to RANK-L as indicated by increased differentiation capability and resorption. In addition, these diabetes-derived osteoclasts were less able

to be deactivated by OPG compared to diabetes-free controls suggesting a RANK-L-dependent and RANK-L-independent mechanism for augmented osteoclast function (8). Therefore, patients with diabetes that also suffer from bone pathologies may have intrinsically hyper-responsive osteoclasts to RANK-L stimulation thereby leading to exacerbated bone destruction even under homeostatic conditions which may be further augmented with inflammation.

Presence of chronic inflammation

As highlighted above, both T1D and T2D are accompanied by a chronic state of inflammation (45, 179, 181, 189, 206-209). Recent reviews have highlighted the interactions between bone and immune cells as well as their overlapping mechanisms (3, 69, 70, 210-212). Furthermore, many of the soluble mediators of immune cells including cytokines, chemokines and growth factors regulate the activities of osteoblasts and osteoclasts. Thus, mechanisms governing the interaction between skeletal and immune systems most likely play a significant role in elements leading to increased bone pathology in individuals with diabetes mellitus. Importantly, the examination of the interface between these two systems is by no means complete, thus further examination should contribute to novel therapeutic strategies to treat disease states mediated by both systems as in the case of diabetes-related bone pathologies.

Pro-osteoclastic milieu: As previously mentioned, both M-CSF and RANK-L are indispensable mediators for osteoclast differentiation and activation, yet many other cytokines and local immune cell factors also regulate these processes.

TNF- α can stimulate osteoclast formation and bone resorption where it was demonstrated to be IL-1 dependent (213). In addition, TNF- α inhibits osteoblast differentiation and collagen synthesis as well as induces their apoptosis (214-216). On

the other hand, TNF- α can induce osteolysis in an M-CSF-dependent manner (217). Thus, the osteoclastic milieu of mediators is key in determining osteoclast fate and function as well as the balance of bone remodeling.

IL-1 and IL-6 are both potent stimulators of bone resorption where they act indirectly to augment osteoclast function. Specifically, they both enhance RANK-L production and activity as well as the induction of prostaglandin synthesis in bone cells (74, 96, 218, 219). Interestingly, while TNF- α -enhanced osteoclast activity is dependent on IL-1, IL-1-induced activity is independent of TNF- α (213, 220). Importantly, IL-1-mediated RANK-L production depends on a key signaling molecule also involved in TLR signaling known as MyD88 but is independent of the additional TLR adaptor molecule (TRIF) (221). This may be important in the context of diabetes mellitus where augmented MyD88-dependent TLR signaling is present (20, 45, 179, 181, 208). Excessive IL-6 has been linked to the mediation of multiple diseases associated with increased bone resorption (19, 222-225).

Interestingly, members of the IL-6 family of cytokines including oncostain M and LIF may play inhibitory roles in osteoclast activation although the data is conflicting (226-229). Other inhibitors of osteoclastogenesis include IL-10 and IFN- γ . IL-10 specifically inhibits RANK-L-mediated NFATc1 expression and nuclear translocation (230). In addition, IL-10 inhibits RANK-L expression while inducing OPG expression in supporting cells of the bone microenvironment (231, 232). IFN- γ similarly inhibits RANK signaling in a TRAF6 dependent manner (233). TRAF6 is an additional signaling molecule also involved in TLR signaling, thus alterations in the expression/function of these intermediates in diabetes has the potential to alter the effect of IFN- γ on

osteoclastogenesis. In addition to its direct inhibitory effects on osteoclasts, IFN- γ has indirect effects on osteoclastogenesis by increasing RANK-L expression in T lymphocytes and TNF- α production (234, 235) highlighting the fact that not only is the osteoclastic milieu of mediators key in determining osteoclast fate and function, but the cellular inflammatory milieu is as well.

The immunological conditions associated with T1D and T2D exhibit a chronic pro-inflammatory skewing of the immune system where pro-osteoclastic TNF- α , IL-1, and IL-6 are found in abundance over anti-osteoclast soluble mediators such as IL-10 (30, 144, 236). On the other hand, the anti-osteoclast mediator IFN- γ , is also found at elevated levels under the conditions of diabetes (237, 238). But as eluded to above, in the presence of heavy T-cell infiltrate, also present in diabetes, this cytokine induces a more pro-osteoclastic environment. Importantly, as discussed above, secondary to the chronic inflammatory conditions, diabetes-derived immune cells respond to insulin in an exacerbated fashion, whereby more severe bone loss would be expected given the effects of these soluble mediators on osteoclast activation and function. In addition, the aberrant TLR and cytokine induced responsiveness of these cell types and the phylogenetic relationship of osteoclasts to these hyper-active immune cells suggest that diabetes-derived osteoclasts may also have heightened sensitivity to stimulation further augmenting bone resorption.

Contributions of hyperglycemia

A major clinical symptom of type 1 and type 2 diabetes is hyperglycemia, or high blood sugar. While the mechanisms associated with the development of hyperglycemia differ in the two diseases, the outcome of increased glucose in the extracellular milieu leads to similar bone and joint pathologies (141).

Effects of excess glucose on osteoclast differentiation and function: While many defects in osteoblast differentiation and function have been documented and confirmed in hyperglycemic conditions, findings of aberrant differentiation and/or function in osteoclasts have been controversial (4, 153, 239-245). Hyperglycemic conditions have been shown to increase (246-248) and decrease (240, 249) osteoclast differentiation and activity depending on the model from which the cells were derived, the method of osteoclast derivation, and the experimental conditions utilized (241, 242, 250-252).

This is also the case with studies using rodent models of diabetes mellitus. For instance, studies using the obese Zucker-fatty rat T2D-prone model demonstrated increased osteoclast activity (253) while decreases in osteoclast function were observed in the non-obese T2D Torii models (254). As mentioned above, it is important to note that while both of these models manifest hyperglycemia, the stresses needed for the onset of diabetes as well as the associated phenotypes are drastically different. Here, Zucker-fatty rat models display obesity and insulin resistance, while the Torii model displays only insulin resistance, both of which lead to hyperglycemia (255, 256). Thus, the differences in the effect on osteoclast function are most likely attributed to the effect of obesity on chronic inflammation which directly and indirectly affects osteoclastogenesis as described above.

Drug-induced hyperglycemic models such as the streptozotocin [STZ] treated mouse result from chemical induction of apoptosis of the β cells (257). In these models, decreased osteoclastogenesis and resorption concomitant with lowered urine bone metabolism markers have been observed (258, 259). In addition, defects in the fusion

of osteoclast precursors as a result of decreased DC-STAMP expression was also found in the STZ-treated mouse model, although more osteoclasts were observed (259). However, other markers for osteoclast activation such as RANK-L, M-CSF, and TNF- α were found to be up-regulated in the STZ-treated mouse model suggesting increased osteoclast function in this hyperglycemic environment (260, 261). Therefore, the effects of hyperglycemia in this mouse model on osteoclast differentiation and function are controversial and warrant further study.

Hyperglycemia can directly initiate pro-inflammatory cytokine production by immune cells such as monocytes via NF- κ B activation, specifically TNF- α and IL-1 β , both potent inducers of resorption (74, 86, 87, 152, 262). Therefore, hyperglycemia is in itself an inflammatory stimulus and can lead to a chronic pro-inflammatory state, which is pro-osteoclastic (263).

Long term hyperglycemia leads to increased formation of advanced glycation end-products [AGEs] (264). AGEs are formed by the reaction between free amino groups of proteins, lipids, and nucleic acids with oxo groups of sugars. These converted AGEs have altered confirmation, turnover, and/or function. In addition, AGEs can bind to an additional receptor, aptly called the receptor for advanced glycated end-products [RAGE]. The interaction of AGEs and RAGE on innate immune cells results in the initiation of NF- κ B pathway that leads to the production of pro-inflammatory cytokines thus promoting a pro-osteoclastic environment (264). In addition, AGEs inhibit osteoblast proliferation and activity while promoting osteoblast apoptosis resulting in decreased bone formation (239, 265).

In addition to being the receptor for AGEs, RAGE is actually a pattern recognition receptor which binds a host of endogenous and exogenous proteins (266). RAGE is expressed on osteoclasts where its ligation is required for bone resorption although the ligand is only known to be a serum component (267-271). Thus, hyperglycemia-induced over-activation of RAGE would activate a pro-osteoclastic response as well as perpetuate an already heightened inflammatory environment providing a cyclical enhancement of bone resorption.

Disruption of insulin signaling on bone metabolism: While hyperglycemia is a consequence of both T1D and T2D, T1D results in hypoinsulinemia while early T2D is associated with hyperinsulinemia which can progress to hypoinsulinemia later in disease progression. Insulin has direct and indirect effects on osteoclastogenesis whereby insulin is considered an anabolic bone agent. Specifically, insulin signals osteoblasts to proliferate and function (72, 168), while inhibiting osteoclast pit formation (272). Therefore, increased insulin production in early T2D may lead to increased bone mass partially explaining the high bone mineral density in T2D subjects in the first stages of the disease (138). With declining insulin production during β cell exhaustion in later stages of T2D, bone cell insulin signaling would decrease resulting in decreased bone formation as well as increased osteoclast function thereby setting the stage for increased bone destruction and reduced bone repair.

In support of insulin's proposed effect on bone metabolism, cartilage loss is accelerated during fracture repair in hyperglycemic STZ-treated mice leading to a smaller callus, yet insulin treatment reverses this anomaly by decreasing osteoclast function and increasing bone formation (260, 261, 272). Further, mouse models with

hypoinsulinemia such as the NOD mouse and humans with T1D also display low bone mass with osteopenia and osteoporotic phenotypes (10, 14, 84, 273, 274). Therefore, without proper insulin action as seen in diabetes, bone formation would decrease and destruction would be left unchecked.

Diabetes and Inflammatory Bone Pathologies

Periodontal Disease

Periodontal disease is characterized by a progressive, destructive host response to bacterial accumulations surrounding the teeth. Over time, soft and hard tissue such as the gingiva and alveolar bone, respectively, are destroyed by the chronic inflammation eventually leading to loss of the tooth (173). Periodontal disease is now considered to be the sixth complication of diabetes and is more prevalent and severe in individuals with both T1D and T2D (26, 29, 275). Alveolar bone resorption in periodontal disease, in particular, is caused by over-activation of osteoclasts, yet the exact mechanism for why this occurs to such a great degree in patients with diabetes is unclear (174, 176, 276).

Periodontitis and diabetes co-morbidity

Patients with both type 1 and type 2 diabetes have a greater susceptibility to and more severe periodontal disease than individuals without diabetes as measured by clinical attachment loss and tooth loss (26, 29). Gingivitis, periodontal abscesses, and granulation tissue are also more prevalent in participants with diabetes suggesting an alteration of the host response to the bacterial plaque (277). While this plaque is the necessary initiating factor in periodontal disease, quantity of plaque does not determine progression to periodontitis. In fact, levels of plaque were found to be the same in patients with and without diabetes with periodontal disease, however the severity of

disease was increased in those individuals with diabetes (277). Types of bacterial flora differ between T1D and T2D where T1D individuals carry flora consisting mostly of anaerobic vibrios while T2D flora is comprised more often of gram negative anaerobes such as *Porphyromonas gingivalis* [*P.g.*], although the reason for this skewing is unclear (57, 58).

Periodontal disease is mainly seen as a complication of diabetes, yet there is also evidence that improving periodontal status may also improve glycemic control (20, 22, 26, 28, 60, 64, 171, 174, 175). Here, poor glycemic control is associated with poor periodontal prognosis and thus an individual with well-controlled diabetes displays less severe periodontal disease than their poorly-controlled counterpart (174, 176).

Mortality due to other diabetic complications such as nephropathy and ischemic heart disease is also increased in individuals with diabetes who also suffer from periodontal disease (171). While there is a clear association between diabetes and the severity of periodontal disease and associated alveolar bone loss, the role of osteoclasts derived from hosts with diabetes and their functional status has not been fully elucidated.

Pathology: bacterial colonization, inflammation, and alveolar bone loss

The periodontal tissues, or periodontium, are those tissues that support the teeth including the root cementum, periodontal ligament, alveolar bone, and gingiva surrounding each tooth.

Alveolar bone lines and forms the sockets of the teeth. This type of bone has a high turnover and is constantly remodeled by osteoclasts and osteoblasts. Once a tooth is removed, this alveolar bone is resorbed and will not reform. Alveolar bone is unique in that during tooth movement, bone is lost from one side of the tooth and formed on the opposite side of the tooth leading to asynchronous resorption (173).

While the periodontium does provide an effective barrier against environmental insults such as bacterial colonization, the state of the host immune system and types of bacteria present can lead to breakdown of tissue integrity and inflammation-induced activation of bone resorption. Innate immune cells in the periodontium such as epithelial cells, macrophages, neutrophils, and DCs are the first to respond to pathogenic bacteria and normally keep the infection in check without inducing tissue destruction. However, a shift occurs when the host responds too robustly or is unable to resolve inflammation, as seen in diabetes mellitus, whereby the immune response results in host tissue damage. Specifically, pro-inflammatory cytokines, chemokines, and MMPs serve to activate the innate immune response, mark the site of infection, and recruit adaptive immune cells such as CD4⁺ T_H1 and T_H2 T cells as well as B cells (plasma cells). However, MMPs and pro-inflammatory cytokines such as TNF- α also have the ability to destroy host tissues (278, 279).

These inflammatory processes lead to connective tissue destruction resulting in the migration of the junctional epithelium down along the root surface leading to pocket formation. This free epithelial surface is now extended and therefore exposed to more bacterial biofilm. The connective tissue also migrates deeper towards the root and thus the attachment of the tooth to the alveolar bone is compromised. In addition, the neutrophils once found in the sulcus become displaced near the root surface (173).

The inflammatory response to periodontal pathogens eventually leads to alveolar bone resorption by osteoclasts whereby IL-1 β , IL-6, and TNF- α , principal pro-inflammatory cytokines found in the inflamed periodontium and pro-osteoclastic mediators described above, can lead to the differentiation and activation of osteoclasts

to resorb bone (90, 280). Osteoblasts in the periodontium can also be activated by LPS from invading bacteria and produce RANK-L, IL-1 β , IL-6, and TNF- α (95, 201, 221). Similarly, RANK-L produced by activated CD4+ cells is also in high abundance in the inflamed periodontium, which directly differentiates osteoclast precursors and activates mature osteoclasts to resorb bone (92, 281).

Contributions of diabetes to exacerbation of periodontal disease: The status of the host immune system is a major factor in determining the development and subsequent severity of periodontal disease (278, 281). Patients with diabetes display many innate immune system abnormalities leading to defective recruitment and/or action of leukocytes that result in decreased ability to fight infection, defective wound healing, and exacerbated inflammation (282). Neutrophil chemotaxis to the site of infection and phagocytosis of extracellular pathogens are impaired in a hyperglycemic environment thereby allowing microbes to accumulate that would normally be destroyed, leading to uncontrolled infections (22, 157, 283). Responses to bacterial components are also altered where pro-inflammatory cytokine secretion is augmented, as described in detail earlier. In particular importance to the gingiva, hyper-secretion of pro-inflammatory cytokines such as TNF- α has been documented in T1D patients in response to *P.g.* compared to diabetes-free participants and may explain why the tissue destruction is so severe in these individuals (20). The accumulating bacterial components such as LPS in patients with diabetes can act on activated T cells and osteoblasts to produce RANK-L and indirectly activate osteoclasts (92). LPS can also induce the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α by osteoblasts, monocytes, and macrophages (the latter which are hyperactive in both T1D

and T2D) that can act on osteoclasts directly or indirectly via RANK-L up-regulation (70).

Collagen breakdown is also increased in participants with diabetes via augmented MMP production, which leads to impaired wound healing (282, 284, 285). AGEs, which are more prevalent in periodontal tissues from rodent models of diabetes, lead to increased oxidative stress and tissue destruction while promoting a pro-inflammatory environment that in itself is destructive to host tissues (178, 268). Supporting the rodent model findings, gingival crevicular fluid has been found to contain high levels of glucose in patients with diabetes compared to diabetes-free individuals suggesting diffusion of glucose into the periodontal pocket and possible metabolic changes in the surrounding tissues such as the formation of AGEs that can increase host tissue destruction (286, 287).

While osteoclasts are the cell type responsible for the loss of alveolar bone, the contributions of diabetes and its possible alterations on osteoclast differentiation and function from hosts with diabetes has not been elucidated. Yet with increased bacterial burden and augmented pro-inflammatory cytokine production in patients with diabetes, one can imagine an environment conducive to exacerbated host tissue destruction, especially in the alveolar bone.

Inflammatory Arthritis

Inflammatory arthritis is a general term for a group of bone pathologies in the joint caused by an initial inflammatory stimulus and includes rheumatoid arthritis, psoriatic arthritis, and reactive arthritis. RA, a chronic autoimmune disease, is considered the prototypical inflammatory bone pathology (262). This disease affects an estimated 1.5

million Americans with women being predominantly affected (263) and has been found to be associated with other autoimmune disorders such as T1D (17).

Inflammatory arthritis and diabetes co-morbidity

Diabetes is not considered a risk factor for RA, although RA is positively correlated with T1D and T2D (24, 25). RA is more severe and occurs earlier in patients with T1D than in those without diabetes (17, 172). In addition, obesity as seen in individuals with T2D, can lead to the development of RA (55, 61). Individuals who are morbidly obese (body mass index of 30+) have the highest risk of developing RA compared to normal body weight participants (46, 55). Both RA and diabetes are considered disorders caused by chronic activation of the immune system. Pro-inflammatory cytokines such as IL-6 and TNF- α are up-regulated in both diabetes and RA (264-266). Therefore, the chronic inflammation seen in RA can lead to insulin resistance and thus may predispose one to T2D (25, 55). Insulin resistance in RA may also be caused by the reduction in lean muscle mass and increase in adipose tissue from the sedentary lifestyle of individuals with the disease due to its debilitating nature, which in turn leads to chronic inflammation via adipocyte hyper-secretion of pro-inflammatory soluble mediators (267, 268). Interestingly, medications used to treat RA such as corticosteroids, non-steroidal anti-inflammatory drugs [NSAIDs], TNF- α antagonists, and hydroxychloroquine may improve glycemic control by promoting insulin secretion and insulin sensitivity (25, 63). Thus, improving the chronic inflammation in RA could theoretically improve glycemic control in patients with T2D.

As mentioned previously, osteoclasts can become activated to resorb bone by pro-inflammatory soluble mediators, which are in high abundance in RA. When inflammation runs rampant as in the case of RA, excessive secretion of these mediators

can lead to inappropriate osteoclast activation and exacerbated bone loss. The contributions of diabetes-derived osteoclasts to RA pathogenesis, however, have not been investigated.

Pathology: inflammation and bone destruction

The joint in health consists of articulating bones covered with cartilage to protect against erosion during movement and ligaments and tendons which surround the joint for stabilization contained in a joint capsule. The intra-articular side of the joint capsule is lined with a synovial membrane and is filled with synovial fluid which consists primarily of hyaluronic acid (294). This synovium also consists of extracellular matrix consisting of collagen fibrils, adipose tissue, elastin, and other proteoglycans. The synovial membrane provides nutrients and also lubricates the joint and consists of two types of synoviocytes. Type A synoviocytes are macrophages derived from bone marrow and type B cells are fibroblast-like (295). Synovial fluid and matrix components are produced by type B cells and cleared by type A cells to maintain joint nutrient and fluid homeostasis. Type B synoviocytes also produce MMPs, cathepsins, and serine proteases, all which aid in joint matrix remodeling (296). Few immune cells such as macrophages and mast cells along with fibroblasts and adipocytes also reside in the synovial membrane in the side proximal to the bone (297).

RA is a chronic inflammatory arthritis with features of autoimmunity including autoantibodies to citrullinated protein antigens [ACPA] and immunoglobulin M [IgM] rheumatoid factor [RF]. The joint synovium is the primary site of inflammation where this synovitis leads to joint damage and eventual bone destruction and subsequent deformity (298). Fibroblast-like synoviocytes also produce chemokines along with resident macrophages to recruit immune cells to the inflamed synovium (299, 300).

Leukocytes such as T and B cells, DCs, and macrophages infiltrate the synovium and produce many pro-inflammatory cytokines and chemokines (301). The synovium then thickens and becomes highly vascularized and it is this “pannus” that is thought to invade the cartilage covering the bone. The destruction is mediated by MMPs and aggrecanases, destructive enzymes that target collagen and aggrecan (another proteoglycan found in cartilage), respectively (298). B cells are important in the progression of RA and serve to produce ACPAs and RF as well as present antigen to T cells in the lesion (298, 302). Interestingly, presence of ACPA and RF can be detected up to 10 years before onset of clinical disease (303, 304).

Erosion of the bone underlying cartilage (subchondral bone) is mediated by osteoclasts (288). These cells are found both at the front of the pannus as well as within the subchondral bone and are found early, within the first few days, in the arthritic process (305, 306). However, bone destruction is not detected on conventional x-rays until at least two months after onset of clinical symptoms (307). In c-fos deficient mice that do not develop osteoclasts, bone destruction does not occur, yet synovitis and arthritis are still evident (89). The inflammatory environment of the arthritic joint serves to attract osteoclast precursors and allow them to differentiate into fully resorptive mature osteoclasts. TNF- α expressed by macrophages in the synovium is a potent inducer of osteoclastogenesis and resorption. Other important cytokines that activate osteoclast-mediated resorption that are found in RA joints include IL-1, IL-17, and RANK-L, all produced by CD4+ T cells (T_H1 and T_H17) (288).

Contributions of diabetes to exacerbation of periodontal disease: While the exact etiology is unknown, genetics play a significant role in the development of RA yet

environmental stimuli are necessary for triggering the inflammatory process similar to T1D and T2D (48, 298). Interestingly, some genes associated with RA including *PTPN22* and *CTLA4*, which negatively regulate T cell activation, are also associated with the development of T1D thereby allowing for multiple autoimmunities (50, 51, 308). A loss-of-function variant of the IL-4 receptor was also found to be associated with RA which can lead to skewed Th responses away from the T_H2 lineage (309), as is commonly seen in T1D individuals that exhibit extreme T_H1 polarization (209). Therefore, in a T1D environment where there is chronic T_H1 activation and lack of regulation of T responses, one can imagine an environment suitable for the development of autoimmune RA. Interestingly, periodontal disease, which is a complication of diabetes, is found to be more prevalent in participants with RA with *P.g.* antibodies correlated with ACPA positivity (53, 54). Thus, individuals with diabetes who suffer from periodontal disease may also develop RA, although the exact relationship between the latter two is unclear.

Hyper-responsive monocytes and macrophages, as commonly seen in patients with diabetes (179, 181, 189), may also exacerbate RA. Pro-inflammatory cytokines such as TNF- α secreted in high quantities by these cells would exacerbate the inflammatory response in the joint and thus induce more bone destruction (86, 310). Therefore, genetic predisposition similarities between the two diseases and intrinsic hyper-reactive defects in osteoclast-modulating cell types could set the stage for an even more severe inflammatory arthritis in patients with diabetes.

Hypotheses

Due to the fact that innate immune cells such as macrophages, monocytes, and DCs have been found to be hyperactive in diabetes mellitus to inflammatory stimuli and

that these cells share the same lineage with osteoclasts, it is possible that diabetes-derived osteoclasts are intrinsically more easily activated to resorb bone than those derived from diabetes-free sources. In addition, the chronic inflammatory environment and hyperglycemia present in diabetes may further augment differentiation and/or resorptive function of osteoclasts derived from these hosts compared to diabetes-free sources.

CHAPTER 2 AUGMENTED LPS RESPONSIVENESS IN TYPE 1 DIABETES-DERIVED OSTEOCLASTS

Introduction

Bone and joint abnormalities are frequent co-morbidities of type 1 diabetes [T1D] (140, 311, 312). T1D originates from a complex etiology with intrinsic genetic risk factors and extrinsic environmental factors. T1D-associated bone and joint pathologies likewise may originate from shared intrinsic genetic factors or from extrinsic sources of activation. Furthermore, bone and joint pathology may develop secondary to autoimmune inflammation or as a consequence of hyperglycemia. The complexity of T1D itself and added complexity of bone and joint co-morbidities necessitates well-controlled and innovative approaches to assess the totality of potential causes.

Physiological bone remodeling is a highly coordinated process that orchestrates five sequential phases: activation, resorption, reversal, formation and termination (68). In addition to traditional bone cells including osteoclasts and osteoblasts which are responsible for bone resorption and formation, respectively, immune cells have also been implicated in the regulation of this process. Thus, alterations in immune function have been implicated in many bone diseases (68). While decreased skeletal health in T1D involves alterations in osteoblast maturation and function, the role of osteoclasts in inflammation-induced bone and joint loss is less understood (140).

Osteoclast differentiation is regulated by macrophage colony-stimulating factor [M-CSF] and the receptor activator of nuclear factor kappa B ligand [RANK-L]. Resorption of bone is initiated by binding of the osteoclasts to the mineralized bone surface via alpha v beta 3 [$\alpha\text{v}\beta\text{3}$] integrins forming a sealing zone membrane and a resorption lacuna (313). Vesicles containing osteoclastic enzymes such as tartrate-resistant acid

phosphatase [TRAP], the serine protease cathepsin K, and matrix metalloproteinase-9 [MMP-9] induce collagen degradation after bone demineralization by the vacuolar H⁺-ATPase (133). Osteoclasts are also negatively regulated through soluble mediators including calcitonin and osteoprotegerin [OPG] (69).

Activation of osteoclast-mediated bone resorption can be augmented by infection and inflammation, as well as hormonal alterations (69). Lipopolysaccharide [LPS], a cell wall component of gram negative bacteria, has been found to be highly immunogenic and induces the production of pro-inflammatory cytokines by various immune cells. Osteoclasts and their precursors, which share the same lineage as macrophages and dendritic cells, express many innate immune receptors including toll-like receptors [TLRs] and thus can respond to bacterial components (71, 199, 314). In a co-culture of osteoclasts and osteoblasts, LPS, the ligand for TLR4, augments bone resorption (201). However, when supporting cells such as osteoblasts or other immune cells are absent, LPS inhibits bone resorption in osteoclast pure cultures, although the exact mechanism(s) is not known (200). In addition, pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 stimulate differentiation and activation of osteoclasts with IL-1 β and TNF- α being directly involved in activating resorption (91, 160). In both murine and human T1D, macrophages and dendritic cells have been shown to be hyperactive to TLR stimulation resulting in excessive pro-inflammatory cytokine production (186, 190, 315). In addition, individuals with T1D are less able to clear bacterial infections thereby allowing bacterial components to accumulate further amplifying the inflammatory process (316).

Since individuals with T1D respond to inflammation in such an aberrant fashion, it is entirely possible that these individuals would be more susceptible to severe bone loss than T1D-free individuals given the effects of these soluble mediators on osteoclast activation and function. Similarly, given the phylogenetic relationship of osteoclasts to macrophages and dendritic cells and the aberrant TLR-responsiveness of these cell types in T1D, we postulate that T1D-derived osteoclasts have heightened sensitivity to stimulation resulting in augmented differentiation and activation. Non-obese diabetic [NOD] mice, a model for spontaneous autoimmune diabetes with pathology similar to individuals with T1D, have an osteoporotic phenotype (274). In addition, NOD mice are more susceptible to spontaneous and induced arthritis (317). Therefore in the present study, we characterized T1D-associated osteoclast-specific differentiation, activation and function in the presence and absence of inflammatory stimuli utilizing the NOD mouse model.

Materials and Methods

Mouse Models

NOD/LtJ [NOD], NOR/LtJ [NOR], C57BL/6J [C57BL/6], and BALB/cJ [BALB/c] mice were maintained in a specific pathogen-free [SPF] environment at the breeding facilities of the University of Florida. The NOD mouse spontaneously develops an autoimmune-mediated destruction of the β -cells within the islets of Langerhans of the pancreas producing very similar pathology as seen in individuals with T1D, where by 10 weeks of age severe insulinitis is apparent, but mice are normoglycemic (148). The NOR mouse model has a similar genetic background as the NOD mouse but does not develop insulinitis and diabetes due to the dispersal of C57BL/6 genome within diabetes susceptibility loci (318). Thus, the C57BL/6 strain serves as an additional genetic

background control as well as an immunological control along with the BALB/c strain. C57BL/6 mice are considered T_H1 responders as are NOD mice (148) while BALB/c mice respond predominately in a T_H2-directed manner (319).

Blood glucose levels were measured at time of sacrifice with the Ascensia Contour Blood Glucose Meter (Bayer). Bone marrow was harvested from female mice of all strains at 10-12 weeks [wks] of age. Pancreata were also harvested from NOD and NOR mice. All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

Osteoclast Differentiation

Femora and tibiae were surgically isolated, excess tissue removed, and marrow expelled from bones using a syringe with α -MEM complete media (Sigma-Aldrich) [10% fetal bovine serum (Mediatech), 1% L-glutamine (Thermo Scientific), 1% penicillin/streptomycin/amphotericin B (Fisher)]. Cells were seeded in T75 flasks at a concentration of 1.5×10^6 cells/mL supplemented with 5ng/mL recombinant murine M-CSF [rmM-CSF] (Peprotech) and allowed to culture for 24 h at 37°C and 5% CO₂. Non-adherent cells were removed and 5.9×10^5 cells/mL of adherent cells were seeded in 24-well plates on either glass coverslips (Fisher) or 1 cm² bovine bone slices cut with an Isomet Low Speed Saw (Buehler). All cultures were supplemented with 10ng/mL rmM-CSF and 50ng/mL recombinant murine soluble RANK-L [rmsRANK-L] (Peprotech) and allowed to culture for 6 d with complete media refreshed every 3 d.

TRAP Staining

After 6 d or 9 d of differentiation, cells plated on glass coverslips were fixed with 2% paraformaldehyde/PBS (Fisher). Cells were washed with PBS and permeablized in 0.5% Triton X-100/PBS (Fisher). Cells were washed and probed for leukocyte acid

phosphatase (TRAP) [1:1:1:2:4 Fast Garnet GBC Base Solution:Sodium Nitrite Solution:Napthol AS-BI Phosphate Solution:Tartrate Solution:Acetate Solution] (Sigma Aldrich) after which cells were washed and mounted on glass slides with MOWIOL 4-88 solution (Calbiochem). TRAP positive cells [purple in color] were counted according to number of nuclei present: mononuclear cells [1 nucleus], multinucleated osteoclasts [2-10 nuclei], and giant osteoclasts [11+ nuclei] using light microscopy at 40x magnification. Whole coverslips were counted for each cell type. Percentage of total nuclei was calculated by the number of nuclei in each cell type divided by total number of nuclei counted.

Osteoclast Stimulation

After 6 d of differentiation, media was refreshed with α -MEM complete media supplemented with 10ng/mL rmM-CSF and 50ng/mL rmsRANK-L. Cells were allowed to resorb bone for 72 h in the presence or absence of the following: 1) 1ug/mL *Escherichia coli* LPS [LPS] (Sigma), 2) pro-inflammatory cytokine cocktail [10ng/mL recombinant human TNF- α [rhTNF- α] (R&D Systems) + 10ng/mL recombinant murine IL-1 β [rmIL-1 β] (Peprotech) + 100ng/mL rmIL-6 (Peprotech)], 3) NOD conditioned media [from M-CSF and RANK-L stimulated bone resorption cultures], or 4) C57BL/6 conditioned media [from M-CSF and RANK-L stimulated bone resorption cultures]. Cultures were permeabilized with 1% Triton X-100 and supernatants stored at -80°C until cathepsin K ELISA, collagen type I telopeptide ELISA, MMP-9 ELISA, and Luminex cyto/chemokine analyses were performed. Bone was made devoid of cells with 10% sodium hypochlorite/PBS after which they were washed with PBS and stored in Trump's fixative (Fisher) at 4°C until scanning electron microscopy [SEM] could be performed. All bone resorption outcome measures were normalized to number of TRAP+ cells

[outcome measure x (total number of TRAP+cells/total number of cells plated)], mononuclear cells [outcome measure x (total number of mononuclear cells/total number of TRAP+cells)] and multi-nucleated cells [outcome measure x (total number of multinuclear cells/total number of TRAP+cells)].

Flow Cytometry for Osteoclast Culture Purity

After 6 d of differentiation media was refreshed with α -MEM complete media supplemented with 10ng/mL rmM-CSF and 50ng/mL rmsRANK-L in the presence or absence of 1ug/mL non-pure *E. coli* LPS for 72 h. Cells were allowed to dissociate from the bottom of UpCell (ThermoScientific) coated plates at room temperature. Suspended cells were washed with FACS Buffer [1x PBS + 5% FBS + 0.372g EDTA] and allowed to incubate with the following primary [1:200] and secondary antibodies [1:200]: goat anti-mouse calcitonin receptor [CTR] (Santa Cruz) with anti-goat Alexa Fluor 647 (Invitrogen) and biotin-conjugated rat anti-mouse RANK (eBioscience) with PerCP-Cy5.5-conjugated streptavidin (eBioscience). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FCS Express (De Novo Software).

Cell Viability Assays

Viability was assessed using a colorimetric MTT Cell Growth Assay (Millipore, Billerica, MA) at 6 d and 9 d post-differentiation described above. MTT assay was performed according to manufacturer's instructions and absorbance was quantified with a spectrophotometer set at a dual wavelength reading of 570nm with a reference of 630nm. Culture media alone was used as a negative control while cells lysed with 1% Triton X-100 were used as a positive control.

Scanning Electron Microscopy

Bone slices were sputter-coated with gold and visualized with S-4000 FE-SEM scanning electron microscope (Hitachi). Three random scanning electron micrographs [8-bit grayscale] of bone slices were acquired at 40x magnification with a 2048x1594 resolution and pits were defined as “scalloped” areas with clearly identifiable borders. Identical procedures were applied to every image from all experimental groups utilizing NIH ImageJ software to quantify the surface area resorbed. Prominent repeating elements in the frequency domain were identified and removed after which the inverse fast Fourier transformation was applied yielding the original image with reduced saw marks. The CLAHE algorithm (320) was used to increase contrast [block size: 256, histogram bins: 256, maximum slope: 8], and a Gaussian blur [σ : 2 pixels] was applied to the result. The rolling ball algorithm (321) was applied [radius: 100 pixels] to achieve background intensity equalization. A threshold value (85) was used to convert the result to a 1-bit image [0: normal bone, 1: region of resorption] used for quantitative analysis. Images which contained prominent artifacts spanning 5% or more of the total area were not included for analysis. Percentage of area resorbed was calculated by dividing square microns resorbed by total square microns.

Collagen Telopeptide ELISA

Collagen carboxy-terminal telopeptides were detected using an ELISA according to manufacturer instructions (Immunodiagnostic Systems). Supernatants were pre-incubated with biotin conjugated anti-telopeptide and horseradish-peroxidase [HRP] conjugated anti-telopeptide and added to an ELISA plate coated with streptavidin [SAV]. Following five washes, tetramethylbenzidine [TMB] substrate was used to develop reactivity followed by quenching with H_2SO_4 . Colorimetric reactions were detected

using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine nM concentrations.

Cathepsin K ELISA

Active cathepsin K was detected using an ELISA according to manufacturer instructions (Alpco). Supernatants pre-incubated with HRP-conjugated anti-cathepsin K were added to an ELISA plate pre-coated with polyclonal sheep anti-cathepsin K. Following five washes, TMB substrate was used to develop reactivity followed by quenching with STOP solution. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine pM/L concentrations of active cathepsin K.

MMP-9 ELISA

Total MMP-9 was detected using an ELISA according to manufacturer instructions (R&D Systems). Supernatants were added to an ELISA plate pre-coated with anti-MMP-9. Following four washes, HRP-anti-MMP-9 was used to detect reactivity. Following five washes, TMB substrate was used to develop reactivity followed by quenching with HCl. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 595nm. Gen5 Software (Biotek) and a standard curve were used to determine ng/mL concentrations.

Soluble Mediator Analysis

Cytokines and chemokines from resorption supernatants were detected and quantified using a mouse 22-cyto/chemokine multiplex (Millipore) according to the

manufacturer's instructions. Supernatant and antibody-coated beads were allowed to incubate overnight at 4°C in a 96-well primed plate. Following three washes, reactivity was probed with biotinylated detection antibodies and SAV-phycoerythrin [PE]. All incubations occurred while gently shaking in the dark. Following three washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system with Xponent software (Millipore). Milliplex analyst software (Viagene), 5-parameter logistics and a standard curve were used to determine pg/ml concentrations. Outcome measures were normalized to number of mononuclear cells [outcome measure x (total number of mononuclear cells/total number of TRAP+cells)] and multinucleated cells [outcome measure x (total number of multinuclear cells/total number of TRAP+cells)].

Histological Analysis of Pancreas

Pancreata were fixed in 10% formalin (Fisher) and embedded in paraffin. 5µm sections were mounted on glass slides and deparaffinized in xylenes and rehydrated using 100% ethanol (EtOH), 95% EtOH, 75% EtOH (Fisher), and dH₂O. Sections were stained with Harris hematoxylin, incubated in a bluing solution [1.5% NH₄OH (EMD Chemicals) in 70% EtOH] and stained with eosin [1% aqueous Eosin Y, 1% aqueous phloxine B (Fisher), 100% EtOH and glacial acetic acid (Mallinckrodt Chemicals)]. Sections were dehydrated with 70% EtOH, 95% EtOH, 100% EtOH, and xylene (2 times), mounted with Permount (Fisher) and observed under light microscopy. Islet infiltration was graded on a 0-2 scale, with 0=no insulinitis, 1=peri-insulinitis, and 2=insulinitis where average insulinitis score per pancreata was determined.

Statistical Analysis

One-way ANOVA with Dunns's multiple comparisons were used to analyze and determine statistical significance ($p < 0.05$) as appropriate.

Results

NOD-derived Osteoclasts Display Altered Differentiation

In order to determine if increased bone resorption observed in multiple T1D complications is due to alterations in osteoclast differentiation, the differentiation of bone marrow derived osteoclasts [BM-OCs] from C57BL/6, BALB/c, NOR and NOD mice was evaluated. BM-OCs were allowed to differentiate for 6d and purity of cultures was evaluated using FACS analysis where the expression of RANK and calcitonin receptor [CTR] was used to define the osteoclast populations (Fig. 2-1). In addition, TRAP staining was used to determine the number of mononuclear cells, multinucleated osteoclasts, and giant osteoclasts (Fig. 2-2).

While BM-OC cultures from all strains generated similar percentages of RANK⁺CTR⁺ where on average cultures were 76.5% pure, NOD BM-OCs consistently had a population of cells expressing lower levels of CTR [RANK⁺CTR^{low}] (Fig. 2-1 arrow). In addition, while no differences in the number of mononuclear cells were observed (Fig. 2-2B), there were significantly fewer multinucleated and giant osteoclasts observed in NOD-derived osteoclast cultures when compared to C57BL/6-, BALB/c-, and NOR-derived cultures (Fig. 2-2C,D). Because osteoclast formation is a result of cell fusion events, we also evaluated the percentage of each osteoclast phenotype type compared to the total nuclei present (Fig 2-2E-G). Here again, NOD-derived osteoclast cultures had a significantly lower percentage of cells which were multi-nucleated compared to those derived from all other strains (Fig. 2-2F,G). In order to determine if

NOD BM-OC cultures simply had a delay in fusion events, differentiation of BM-OCs was also evaluated at 9d of culture, where no significant difference in the number of multi-nucleated or giant cells was observed (Fig. 2-2H). Similarly, to determine if there was a difference in osteoclast survival an MTT assay was performed at 6d and 9d of BM-OC culture. Again, no significant difference in cell survival was observed in NOD derived BM-OCs (Fig. 2-2I). Taken together, these data indicate a possible defect in the differentiation of NOD-derived osteoclasts, most likely involving the fusion of mononuclear cells into multinucleated osteoclasts.

NOD-derived Osteoclasts Have Increased Bone Resorption Capabilities in Response to RANK-L Stimulation

Although our differentiation data suggests overall smaller osteoclast size, this data does not address the bone resorbing function of these cells. In order to determine the bone resorbing capabilities of NOD-derived BM-OCs, these cells were seeded onto bovine bone slices and stimulated to resorb bone with RANK-L. To quantify the amount of bone resorbed, SEM and ImageJ analysis were performed where the total area resorbed and number of resorption pits by TRAP+ cells was determined (Fig. 2-3). NOD-derived BM-OCs were found to resorb more surface area in response to RANK-L stimulation compared to C57BL/6-, BALB/c-, and NOR-derived cultures (Fig. 2-3A, B). In addition, the number of resorption pits was significantly higher in NOD-derived BM-OCs cultures compared to those from all other strains (Fig. 2-3A, E). In order to elucidate activity on a per cell basis, the total area resorbed and number of resorption pit data was stratified based on number of TRAP+ mononuclear cells (Fig. 2-3 C, F) and TRAP+ multinucleated cells (Fig. 2-3 D, G). Here again the total area resorbed and the number of resorption pits was significantly higher in NOD-derived TRAP+ mononuclear

cells. On the other hand only the total area resorbed was significantly higher in NOD-derived TRAP+ multinuclear cells. Together these data suggest that irrespectively of the smaller size, more cells in NOD-derived osteoclast cultures resorbed a greater bone surface area.

NOD-derived Osteoclasts Degrade More Type 1 Collagen than Controls via Enhanced Cathepsin K and MMP-9 Secretion

During bone resorption, the organic portion of bone containing type I collagen is degraded in the resorption lacunae where vesicles containing collagen telopeptides are transcytosed out of the osteoclast and released into the extracellular milieu (322). Therefore, in order to further quantify bone resorption, levels of intra- and extra-cellular collagen telopeptides were evaluated. Similar to our SEM data, RANK-L-stimulated NOD-derived BM-osteoclast cultures had significantly higher levels of collagen telopeptides than C57BL/6-, BALB/c, and NOR-derived cultures (Fig. 2-4A, D, G) confirming higher bone resorption activity. Lysosomal cathepsins, such as cathepsin K, and matrix metalloproteinases such as MMP-9 can degrade type I collagen at an acidic pH created in the resorption lacunae (97). Therefore, to investigate mechanisms associated with the observed increased collagen degradation, the amount of cathepsin K (Fig. 2-4B, E, H) and MMP-9 (Fig. 2-4C, F, I) in the same BM-OC cultures was quantified. Increased levels of cathepsin K and MMP-9 were detected in RANK-L-stimulated NOD-derived BM-OC cultures when compared to C57BL/6-, BALB/c-, and NOR-derived cultures (Fig. 2-4B, C). Again, activity was evaluated based on nucleation, where collagen, cathepsin K and MMP-9 was significantly higher in NOD-derived TRAP+ mononuclear cells (Fig. 2-4D - F) with collagen, and MMP-9, but not cathepsin K being significantly higher in NOD-derived TRAP+ multinuclear cells (Fig. 2-

4G - I). These data indicate that RANK-L stimulation of NOD-derived BM-OC results in increased cathepsin K and MMP-9 release leading to increased collagen degradation, with the largest source of bone degradation being TRAP+ mononuclear osteoclasts.

NOD-derived Osteoclasts Respond Aberrantly to LPS

Inflammatory bone pathologies are often seen in individuals with T1D, including inflammatory arthritis and periodontitis-associated alveolar bone loss, where inflammatory mediators including bacterial components are abundant in these milieus (17, 26, 277, 278, 282, 316). Thus, the effect of LPS on T1D-derived osteoclast function was evaluated. Osteoclasts were seeded onto bovine bone slices and stimulated to resorb bone with RANK-L in the presence of LPS. LPS-induced deactivation of osteoclast function was observed in C57BL/6-, BALB/c-, and NOR-derived cultures indicated by a decrease in the area resorbed and collagen release as well as decreased cathepsin K and MMP-9 secretion (Fig. 2-5). However, this LPS-induced deactivation did not occur in the NOD-derived osteoclast cultures as shown by a lack of decrease in the area resorbed and collagen degradation (Fig. 2-5A, B). Interestingly, NOD-derived BM-osteoclasts also displayed an increase in LPS-responsiveness as measured by increases in cathepsin K and MMP-9 secretion compared to RANK-L stimulation alone (Fig. 2-5C, D). These data suggest that NOD-derived BM-OCs are not only unable to be deactivated by LPS, but more importantly secrete elevated levels of bone resorbing mediators in the presence of LPS.

NOD-derived Osteoclasts Secrete Increased Soluble Osteoclastogenic Mediators in Response to LPS

Because osteoclast function is regulated by many soluble immune mediators, the cytokine and chemokine profile within BM-OC cultures from all strains was evaluated.

Increased amounts of the hematopoietic growth factor GM-CSF and pro-inflammatory cytokines IL-1 β and TNF- α as well as the osteoclast chemoattractants RANTES and IP-10 were elevated in BM-OCs cultures from all strains following LPS stimulation (Fig. 2-6). Similarly, the anti-osteoclastogenic mediator IL-10 was also elevated in BM-OCs following LPS stimulation (Fig. 2-6F, L). Interestingly, NOD-derived BM-OCs cultures presented with significantly higher levels of all mediators, with the exception of IL-10, compared to those found in C57BL/6-, BALB/c-, and NOR-derived cultures when normalized to TRAP+ mononuclear cells (Fig. 2-6A-F). While a similar trend was observed when data was normalized for multinucleation (Fig. 2-6G-L), it was interesting to note that GM-CSF expression was no longer significantly higher (Fig. 2-6G) and IL-10 levels were significantly lower (Fig. 2-6L), in NOD-derived BM-OCs cultures. These data indicate an exacerbated pro-osteoclastic response to LPS by TRAP+ mononuclear NOD derived BM-OCs.

NOD-derived Osteoclasts Respond Aberrantly to Inflammatory Mediators

While pro-inflammatory cytokines such as TNF- α and IL-1 β act on osteoblasts and activated T-cells to indirectly stimulate osteoclast differentiation, they also directly activate osteoclasts to resorb bone (2, 86, 159). Thus, in order to determine if the higher levels of these mediators in NOD derived BM-OC cultures could be responsible for the abrogation of LPS-induced deactivation of osteoclast function, BM-OC cultures from all strains were subjected to a cocktail of pro-inflammatory cytokines and their resorptive function evaluated. As expected, increases in collagen release, cathepsin K and MMP-9 secretion were observed in BM-OC cultures from all strains in both the presence of the pro-inflammatory cytokine cocktail and in the absence of LPS (Fig. 2-7) when compared to those observed in the absence of the pro-inflammatory cytokine

cocktail (Fig. 2-4). In the presence of a pro-inflammatory cytokine cocktail, NOD-derived BM-OCs display significantly increased resorptive function when compared to C57BL/6-, BALB/c-, and NOR- cultures (Fig. 2-7), similar to responsiveness in the absence of the pro-inflammatory cytokine cocktail (Fig. 2-4). In addition, the pro-inflammatory cytokine cocktail did not affect abrogation of LPS-induced deactivation in NOD-derived BM-OCs. On the other hand, C57BL/6-, BALB/c- and NOR-derived cultures had significantly lower levels of collagen, cathepsin K and MMP-9 in the presence of LPS than in its absence even in the presence of a pro-inflammatory cytokine cocktail (Fig. 2-7). These data suggest that the pro-inflammatory milieu in NOD-derived BM-OCs is not solely responsible for the LPS-induced hyper-resorptive response observed.

NOD-derived BM-OC Conditioned Media Leads to Increased Bone Resorption in Control Cultures

In order to determine if a soluble mediator was responsible for the abrogation of LPS-induced deactivation of osteoclast function observed in the NOD BM-OC cultures, conditioned media from LPS-free NOD BM-OC cultures was added to C57BL/6-, BALB/c-, and NOR-derived cultures. As a control, conditioned media from LPS-free C57BL/6 BM-OC cultures was added to BALB/c-, NOR and NOD-derived cultures. Again, collagen release, cathepsin K and MMP-9 secretion were used to evaluate osteoclast function. As expected, conditioned media from NOD BM-OC cultures caused an increase in resorption and osteoclastic enzyme production in C57BL/6-, BALB/c-, and NOR-derived cultures when compared to conditioned media from C57BL/6 BM-OC cultures (Fig. 2-8). Importantly, conditioned media from NOD BM-OC cultures were unable to inhibit LPS-induced deactivation in C57BL/6-, BALB/c-, and NOR-derived

cultures (Fig. 2-8). Similarly, conditioned media from C57BL/6 BM-OC cultures were unable to inhibit LPS-induced deactivation in BALB/c-, and NOR-derived cultures. C57BL/6 BM-OC conditioned media did not affect the inhibition of LPS-induced deactivation in NOD-derived BM-OC cultures (Fig. 2-8). Together these data suggest that inhibition of LPS-induced deactivation in NOD-derived BM-OC cultures is most likely due to NOD osteoclast responsiveness rather than excess or absence of an LPS-induced soluble mediator.

NOD-derived Osteoclasts are from Pre-diabetic/euglycemic Mice

Bone resorption can be influenced by many factors including glucose concentration (247, 249). Thus, in order to determine if hyperglycemia is contributing to the inhibition of LPS-induced deactivation of NOD-derived BM-OCs, the stage of T1D progression and blood glucose was evaluated. All strains had normal blood glucose at time of bone marrow harvest (Fig. 2-9A). Similarly, the histology of pancreata from NOD and NOR mice revealed little insulinitis with approximately 75% of islets free of infiltration in the NOD model versus 90% in the NOR (Fig. 2-9B, C). Therefore, NOD mice were considered euglycemic and pre-diabetic at the time of marrow harvest, indicating that the events observed are not due to hyperglycemia or conditions associated with fulminant disease.

Discussion

Individuals with T1D have increased incidence of inflammatory arthritis as well as osteoporosis, two diseases principally mediated by a dysregulation in bone remodeling (17, 72). Bone formation by osteoblasts is decreased in individuals with T1D which tips the balance of bone remodeling towards that of less bone deposition (72). Here we demonstrate an osteoclast-specific contribution to altered bone remodeling, where T1D-

derived osteoclasts are more osteoclastic in nature than T1D-free derived osteoclasts suggesting an additional tip in the balance to that of increased bone resorption.

While NOD BM-OC resorptive capability was heightened in response to RANK-L stimulation, NOD-derived BM-OCs were found to be smaller in size. In addition, NOD-derived BM-OCs cultures consistently contained a population of RANK⁺CTR^{lo} cells. Together these data indicate an aberrant maturation process. The fusion of mononuclear cells into multinucleated and giant cells was decreased suggesting an alteration in cell-fusion mechanism(s). Many surface receptors including DC-STAMP and MFR/SIRP α , are critical for fusion from mononucleated precursors into multinucleated cells. In addition, ADAM8 and ADAM12 which are disintegrins/metalloproteinases secreted prior to fusion, may be affected in this model (117). Calcitonin, the ligand for CTR, is responsible for lowering calcium levels in the blood by inhibiting bone resorption by inducing morphological changes leading to osteoclast retraction (78). In addition, while still controversial, some studies have demonstrated an effect of calcitonin in the fusion of osteoclast-precursors (78). Thus, it is plausible that expression or function of one or many of these molecules is altered in the NOD model leading to decreased fusion.

RANK-L stimulation of NOD-derived osteoclasts resulted in increased cathepsin K and MMP-9 secretion leading to increased collagen degradation. This suggests a hyper-responsiveness to RANK-L by NOD BM-OCs during homeostatic conditions, whereby augmented signaling leads to increased resorptive enzyme production (8, 112). In support of this theory, it has been shown by others that peripheral blood derived osteoclasts from diabetic (both type 1 and type 2) individuals were less

sensitive to the soluble decoy receptor for RANK-L, osteoprotegerin [OPG], than those derived from diabetes-free controls resulting in increased bone resorption, again indicating a heightened sensitivity in RANK signaling (8, 323). In addition, stratification of the resorption data indicates that TRAP+ mononuclear cells within the NOD-derived OC cultures are responsible for the augmented resorption.

Bacterial components such as LPS act on osteoblasts and activated T-cells to produce more RANK-L to stimulate osteoclasts to differentiate and resorb bone (105, 278). However, LPS can also act on osteoclasts directly by inhibiting the differentiation of monocytic precursors into bone resorbing osteoclasts and bone resorption by mature osteoclasts (200, 202). In addition, it has recently been demonstrated that osteoclasts have the capacity to phagocytose bacteria and act as a supporting immune cell (324). While T1D-free derived osteoclasts resorb less bone in the presence of high amounts of LPS, they do produce pro-inflammatory cytokines and chemokines. This suggests a shunting of osteoclast precursors to that of an immune cell phenotype to help fight infection rather than towards mobilization to resorb bone. Interestingly, in addition to being more resorptive, NOD-derived osteoclasts were also more inflammatory in nature than the NOR, C57BL/6 or BALB/c models as indicative of increased soluble mediator secretion. Again, stratification of the data indicates that it is the TRAP+ mononuclear cells which have retained this inflammatory function.

Macrophages and monocytes (relatives of the osteoclast) from individuals with T1D secrete elevated levels of pro-inflammatory cytokines in response to LPS (20, 208). Here NOD-derived osteoclasts also secreted elevated levels of the pro-inflammatory and pro-osteoclastic mediators, GM-CSF, RANTES, IP-10, TNF- α and IL-1 β when

stimulated with LPS compared to those observed in osteoclast control cultures. Where GM-CSF can mobilize osteoclast precursor release from the bone marrow, RANTES and IP-10 can act as chemokines to attract these precursors to the area of inflammation. TNF- α and IL-1 β then augment osteoclast differentiation and function (85, 101, 211). In addition, our data describe for the first time that NOD derived BM-osteoclasts are refractory to LPS-induced inhibition of bone resorption. Thus, one can envision that under a T1D environment, augmented secretion of these mediators can lead to a positive feedback loop where more osteoclasts are recruited to the site of inflammation where abrogation of LPS-induced inhibition leads to exacerbated bone resorption.

We found that neither addition of a pro-inflammatory cytokine cocktail nor addition of conditioned media from NOD BM-OC cultures led to inhibition of LPS-induced deactivation in our control cultures suggesting that soluble factors alone are not responsible for the aberrant LPS-responsiveness of NOD BM-OCs. Importantly, C57BL/6 BM-OC conditioned media did not affect the inhibition of LPS-induced deactivation in NOD-derived BM-OC cultures suggesting that there is not an absence of a soluble mediator in NOD-derived BM-OC cultures responsible for LPS-induced deactivation of osteoclasts. Therefore, we hypothesize that it is the response of the NOD BM-OC to LPS and other mediators that is the cause of aberrant function rather than an excess or absence of soluble mediator expression. For instance, hyper-responsiveness in the TLR4 signaling pathway as indicated by increased translocation of NF- κ B has been described in macrophages and dendritic cells isolated from NOD mice (186, 187, 190). In addition, the receptors for TNF- α [TNF-R] and RANK-L

[RANK], which originate from the same super-family of receptors, also utilize the NF- κ B pathway and thus T1D-associated alteration in these signaling pathways could potentially alter the bone resorbing function of osteoclasts (325). These mechanisms are currently under investigation in our laboratory.

In addition to the inflammatory environment, bone resorption can be influenced by many factors including glucose concentration. For instance, Graves and colleagues have elegantly demonstrated in ligature, calvarial and bone fracture models (in the presence and absence of infection) that hyperglycemia and TNF- α affect fibroblast and osteoblast apoptosis which contributes to *in vivo* cartilage and bone loss in models of type 2 diabetes and hyperglycemia (4, 240, 243, 260, 261, 265, 326-332). While our data demonstrates an osteoclast-specific augmented function in the absence of hyperglycemic contributions, it is plausible that hyperglycemia may further contribute to osteoclast hyperactivity in T1D. Indeed, glucose is the primary energy source of the osteoclast and has been shown to augment osteoclast-mediated resorption via increases in V-ATPase expression (248, 333). Furthermore, lack of insulin, now considered to be a bone anabolic agent, leads to decreased bone formation in participants with T1D (72).

While many contributing factors to the inflammatory bone loss in participants with T1D have been surmised, the totality of osteoclast-mediated pathology was previously unknown. Dissecting osteoclast function and its role in T1D-associated bone pathologies can lead to adjunct treatment for participants with T1D that cannot respond to conventional anti-osteoclast therapies. In the present study, we have shown that a T1D genetic background leads to a hyper-reactive osteoclast phenotype which

responds to bacterial components and pro-inflammatory cytokines in an aberrant fashion resulting in excessive bone degradation via enhanced cathepsin K and MMP-9 secretion concomitant with an increased expression of pro-osteoclastic soluble mediators.

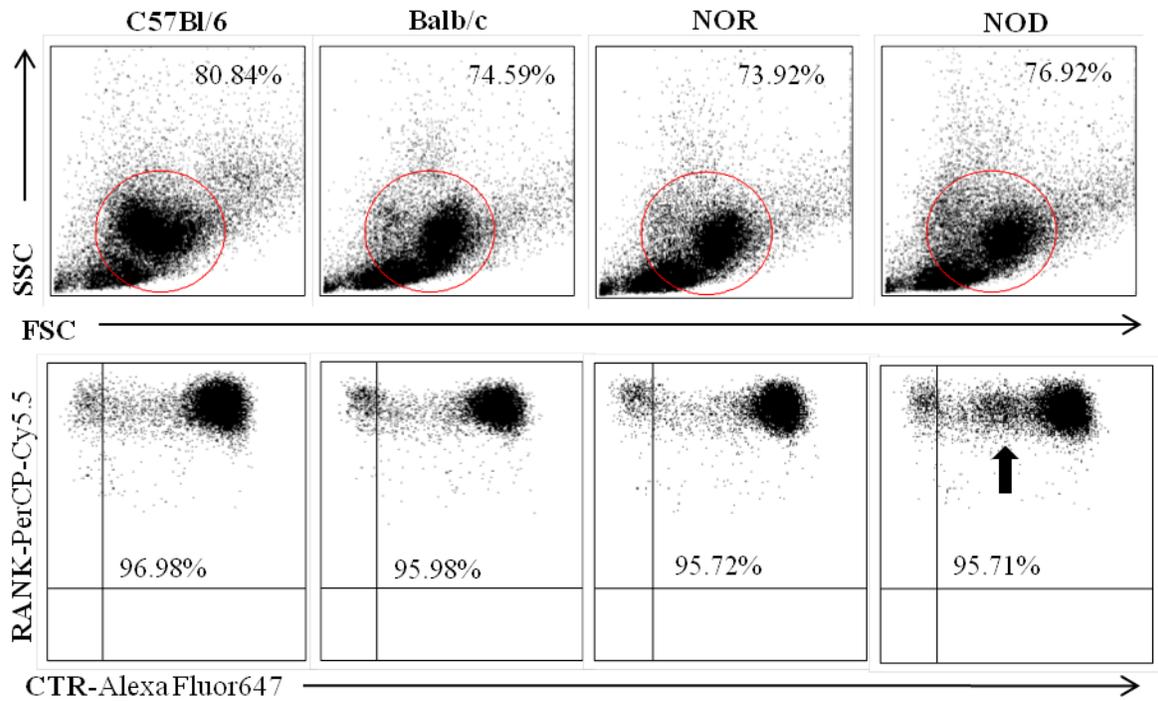


Figure 2-1. Flow cytometric analysis of osteoclast culture purity. BM-OCs were stimulated with RANK-L for 72hrs on UpCell plates. Cells were probed for RANK (PerCP-Cy5.5) and calcitonin receptor [CTR] (AlexaFluor647) and acquired using a FACSCalibur flow cytometer. FCS Express software was used to determine forward and side scatter population of analyzed BM-OCs (circled in upper panel) and percentage of pure RANK+CTR+ BM-OCs (lower panel). Black arrow indicates novel population of RANK+ cells expressing low levels of CTR [RANK+CTR^{lo}] NOD BM-OCs. Data shown as representative scatter plots of each mouse strain.

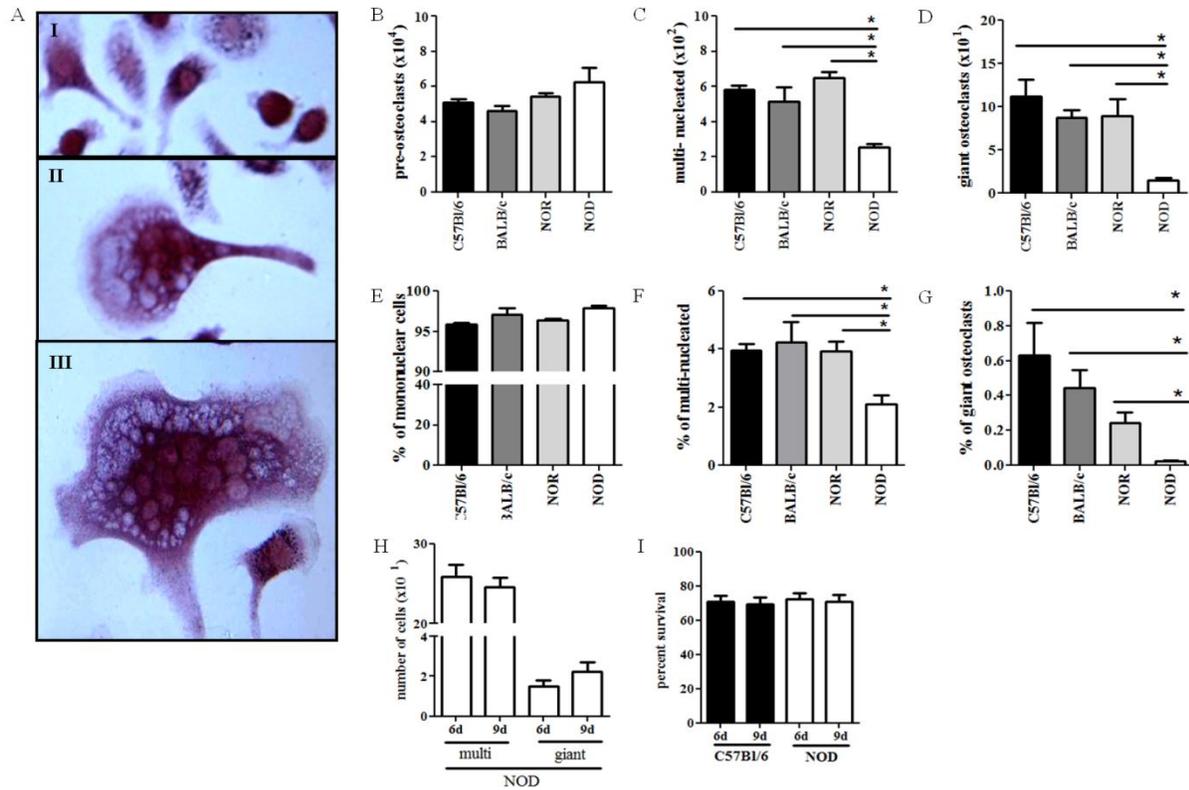


Figure 2-2. NOD-derived osteoclasts display altered differentiation. A) 6d post-differentiation, BM-OCs were imaged with light microscopy at 40x magnification. Representative images of I) mononuclear cells, II) multinucleated OCs and III) giant OCs. B-D) Number of TRAP+ BM-OCs per coverslip were enumerated using 40x magnification. E-G) Data shown as percentage of total nuclei TRAP+ cells. C57BL/6 (black bars, n=18), BALB/c (dark grey bars, n=16), NOR (light grey bars, n=16), NOD (white bars, n=16). H) 6d and 9d post-differentiation, multinucleated and giant BM-OCs NOD mice were quantified. I) 6d and 9d post-differentiation cell-viability of C57BL/6 and NOD BM-OC cultures was assessed by MTT assay. **p* value < 0.05. One-way ANOVA with Dunn's multiple correction.

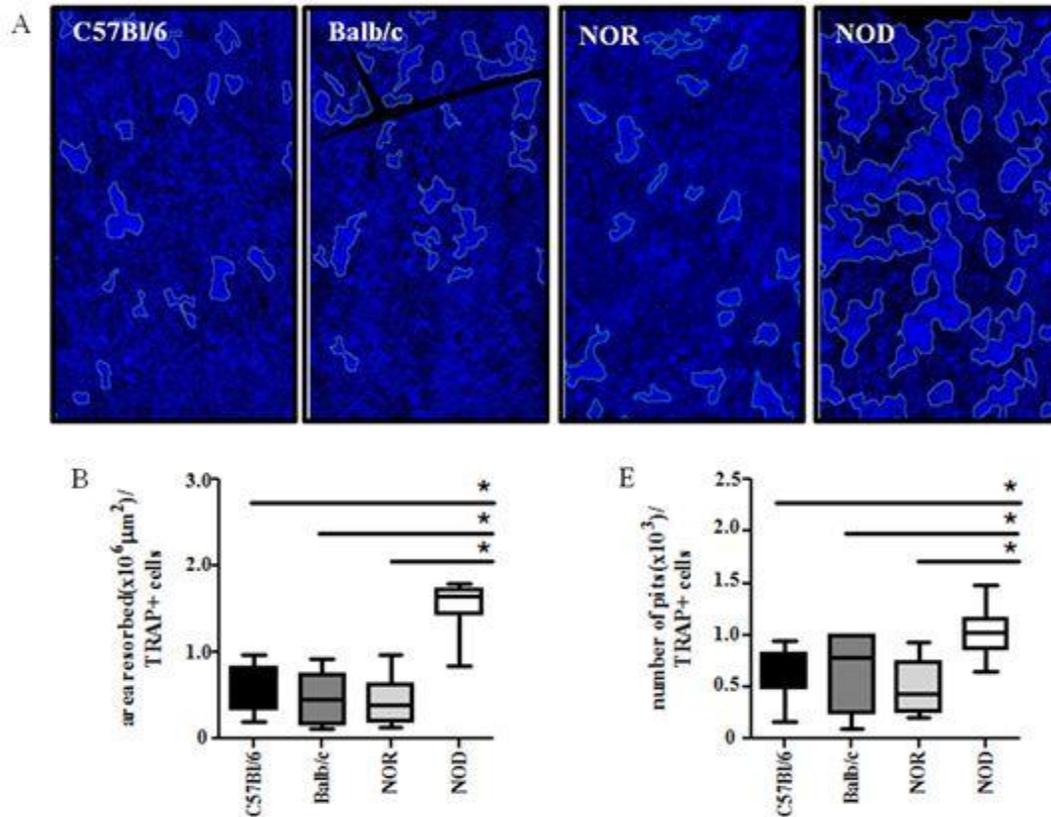


Figure 2-3. NOD-derived osteoclasts have increased bone resorption capabilities in response to RANK-L stimulation. BM-OCs were stimulated with RANK-L for 72hrs on bovine bone slices which were sputter-coated with gold and imaged with SEM. A) Representative SEM and computer-quantified areas of resorption (blue) at 40x magnification. Image J software was used to determine B- D) area of resorption and E-G) number of resorption pits (light blue outlines = borders of resorption areas) normalized to B, E)TRAP+ cells, C, F) TRAP+ mononuclear cells and D, G)TRAP+ multinucleated cells. C57BL/6 (black bars, n=18), BALB/c (dark grey bars, n=16), NOR (light grey bars, n=16), NOD (white bars, n=16). **p* value < 0.05. One-way ANOVA with Dunn's multiple correction.

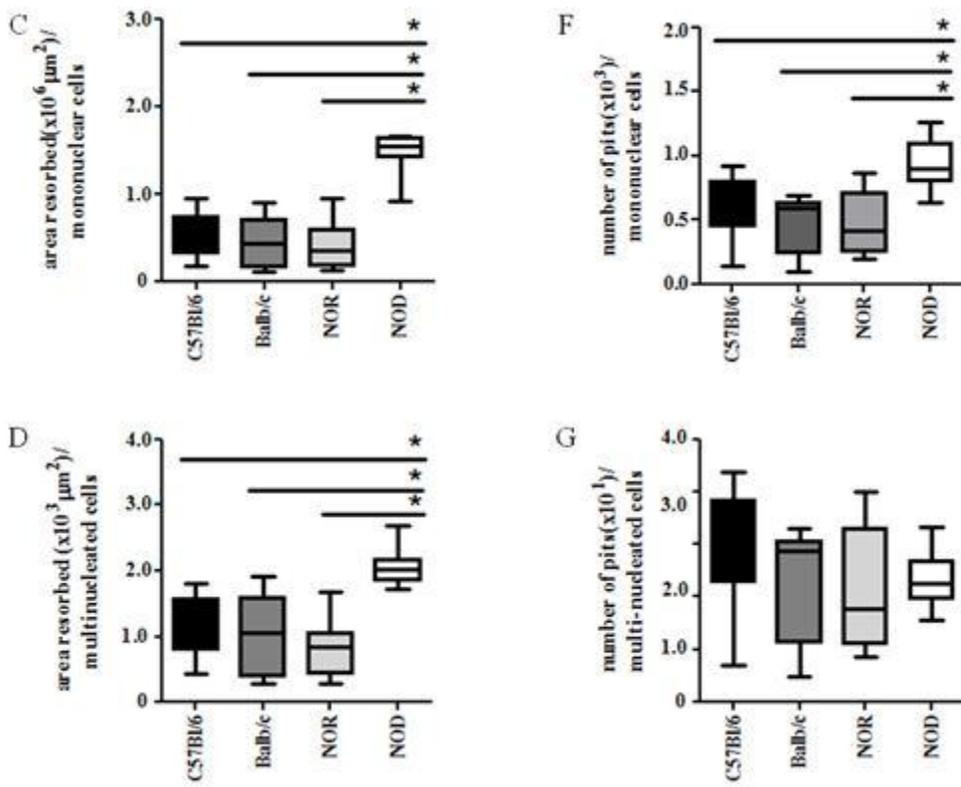


Figure 2-3. Continued

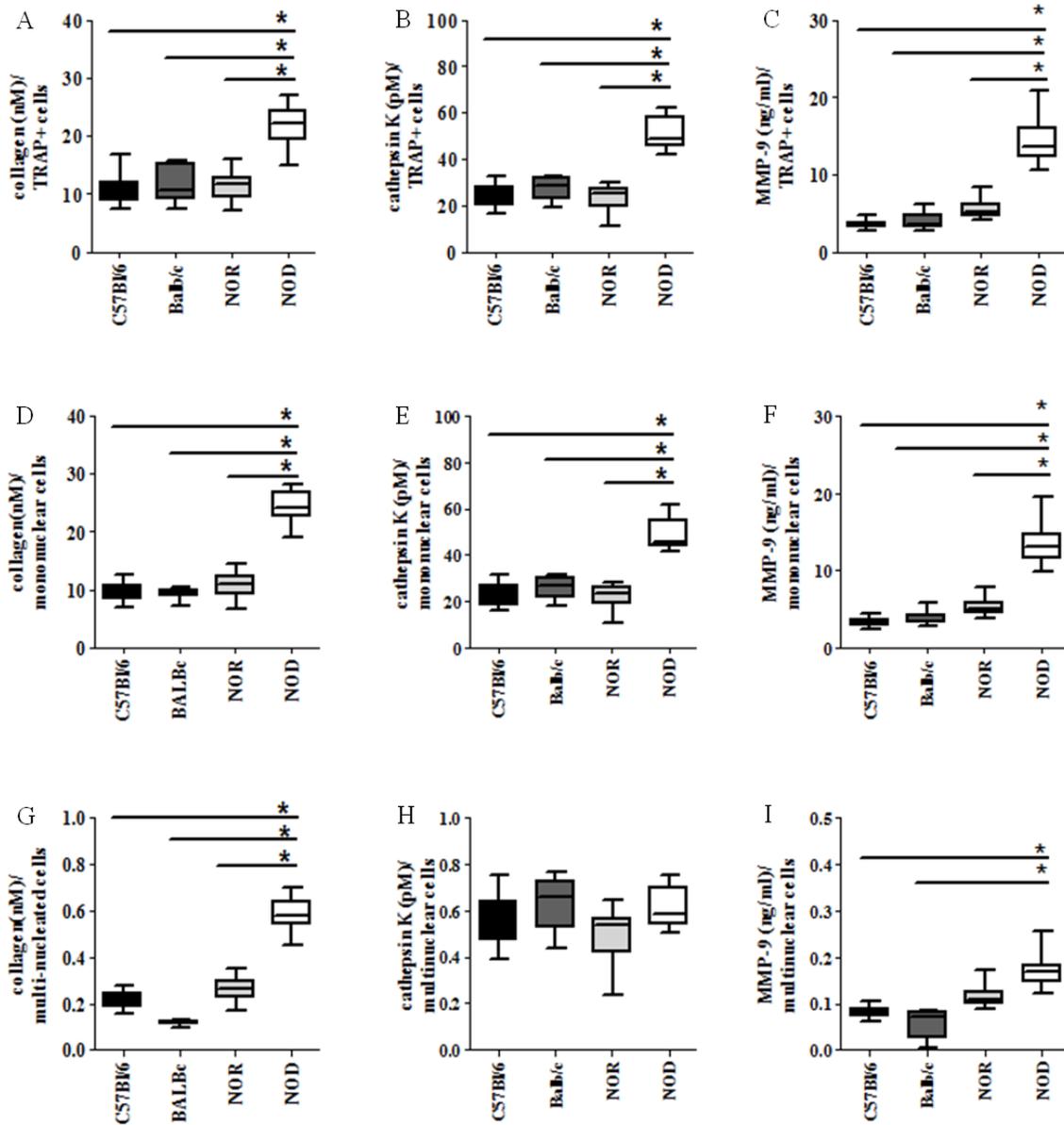


Figure 2-4. NOD-derived osteoclasts degrade more type 1 collagen than controls via enhanced cathepsin K and MMP-9 secretion. BM-OCs were stimulated with RANK-L for 72hrs on bovine bone slices. Supernatants were collected after 1% Triton X-100 solubilization and ELISA used to quantify: A, D, G) collagen I telopeptide, B, E, H) cathepsin K and C, F, I) MMP-9 normalized to A-C) TRAP+ cells, D-F) TRAP+ mononuclear cells and G-I) TRAP+ multinucleated cells. C57BL/6 (black bars, n=18), BALB/c (dark grey bars, n=16), NOR (light grey bars, n=16), NOD (white bars, n=19). **p* value < 0.05. One-way ANOVA with Dunn's multiple correction.

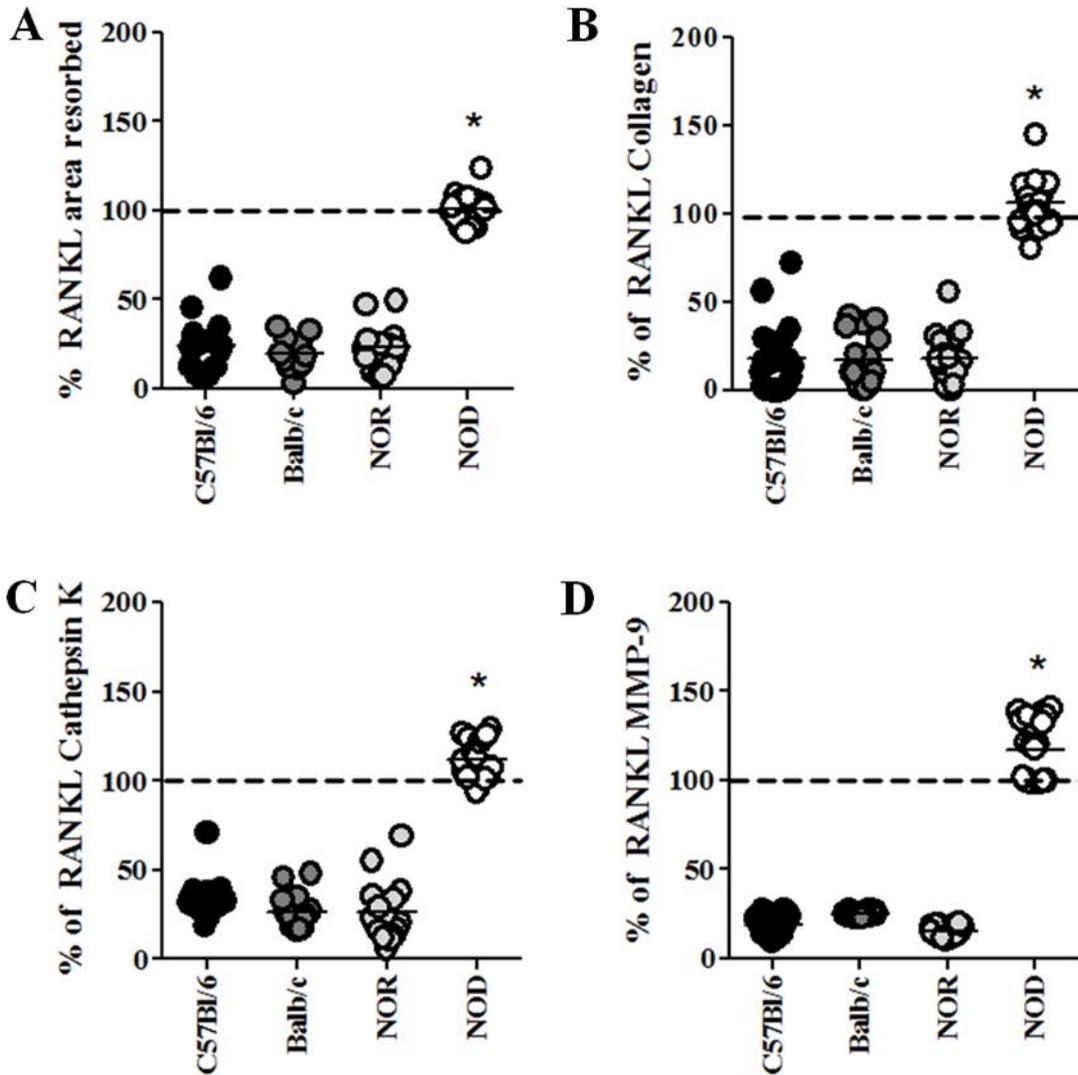


Figure 2-5. NOD-derived osteoclasts respond aberrantly to LPS. BM-OCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS for 72hrs on bovine bone slices. Supernatants were collected after 1% Triton X-100 solubilization and bones were sputter-coated with gold and imaged with SEM. A) Area of resorption, B) collagen I telopeptide, C) cathepsin K and D) MMP-9 were evaluated using A) SEM and B-D) ELISA. Data are expressed as percent expression during RANK-L stimulation calculated by [(value in the presence of LPS/value in the absence of LPS) x 100] C57BL/6 (black circles, n=18), BALB/c (dark grey circles, n=16), NOR (light grey circles, n=16), NOD (white circles, n=19). Dashed line = expression levels in the absence of LPS. **p* value < 0.05. NOD vs. all strains. One-way ANOVA with Dunn's multiple correction.

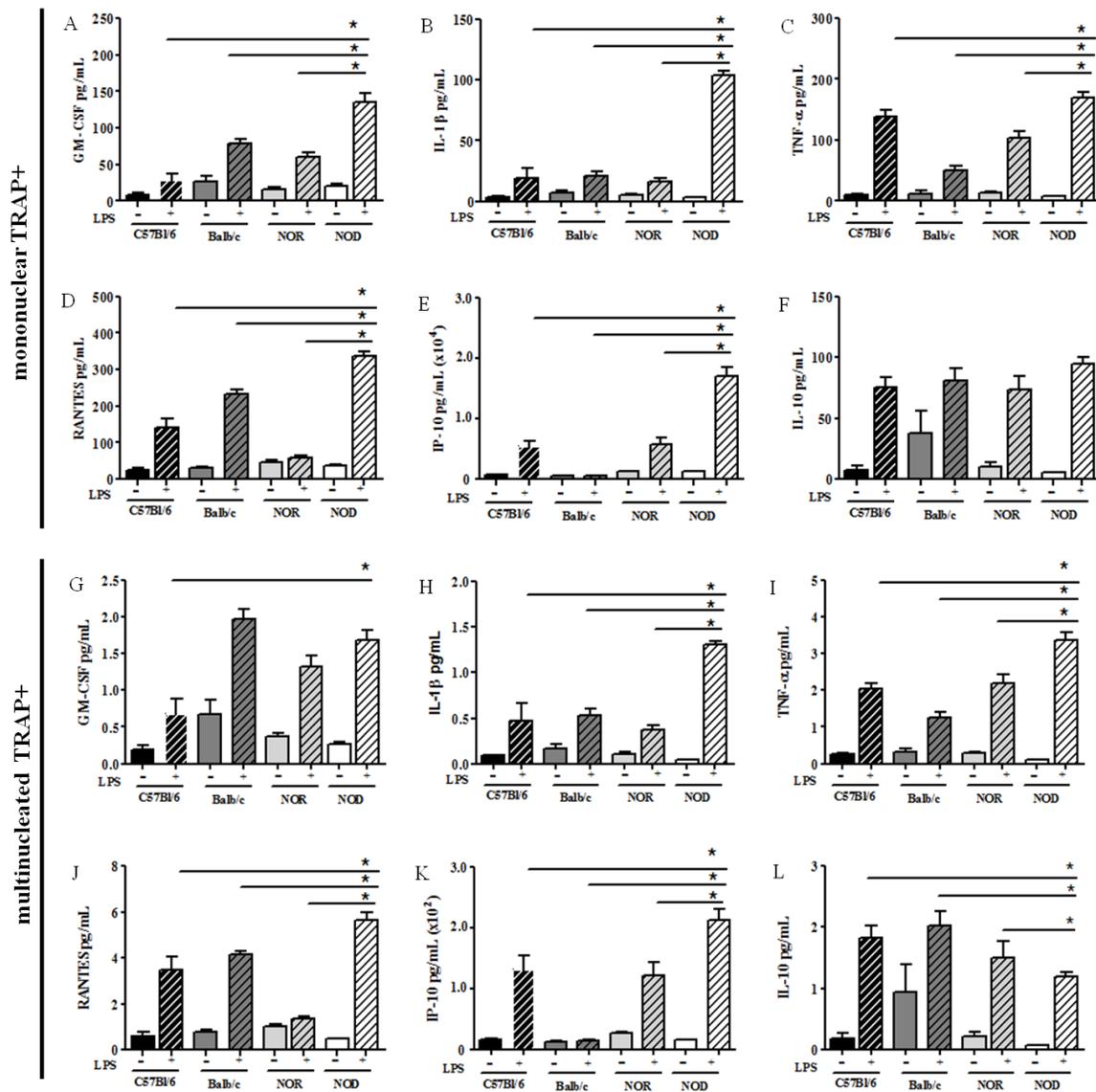


Figure 2-6. NOD-derived osteoclasts secrete increased soluble osteoclastogenic mediators in response to LPS. BM-OCs were stimulated with RANK-L in the presence (+) or absence (-) of *E. coli* LPS for 72hrs on bovine bone slices. A, G) GM-CSF, B, H) IL-1 β , C, I) TNF- α , D, J) RANTES, E, K) IP-10 and F, L) IL-10 levels were evaluated in the permeabilized supernatants using Milliplex technology. A-F) Data shown as pg/mL normalized to TRAP+ mononuclear cells. G-L) Data shown as pg/mL normalized to TRAP+ multinucleated cells. C57BL/6 (black bars, n=13), BALB/c (dark grey bars, n=15), NOR (light grey bars, n=14), NOD (white bars, n=17). Open bars = absence of LPS, hatched bars = presence of LPS. **p* value < 0.05. One-way ANOVA with Dunn's multiple correction.

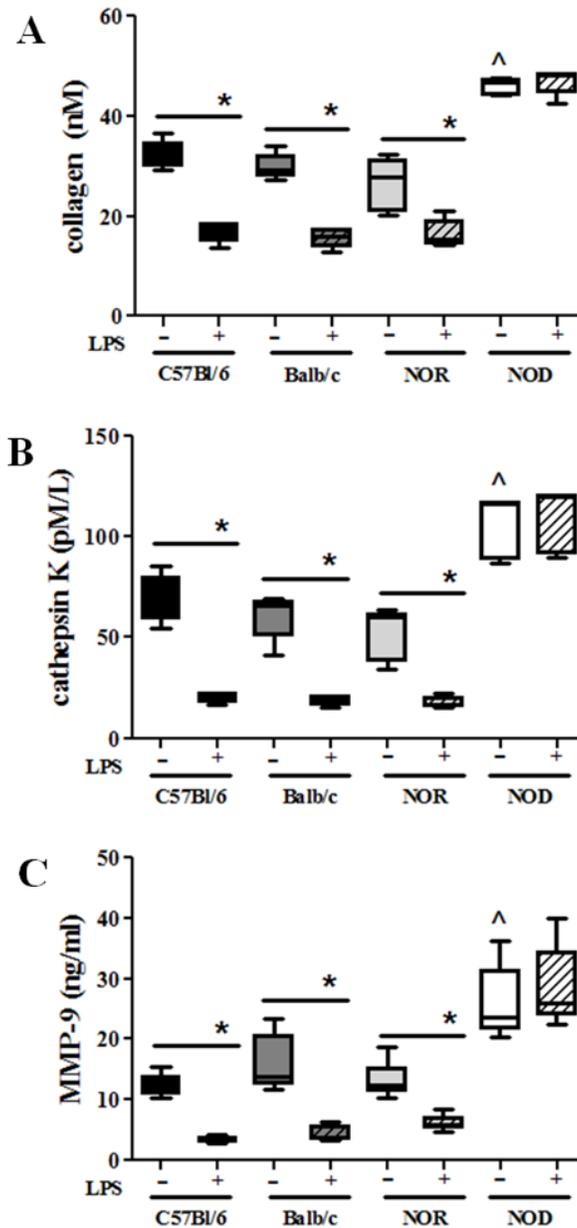


Figure 2-7. Pro-inflammatory cytokines do not inhibit LPS-deactivation of OCs. BM-OCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS and a pro-inflammatory cytokine cocktail [TNF- α , IL-1 β , and IL-6] for 72hrs on bovine bone slices. Supernatants were collected after 1% Triton X-100 solubilization and ELISA used to quantify: A) collagen I telopeptide, B) cathepsin K and C) MMP-9. C57BL/6 (black bars, n=5), BALB/c (dark grey bars, n=5), NOR (light grey bars, n=6), NOD (white bars, n=5). Open bars = absence of LPS, hatched bars = presence of LPS. **p* value < 0.05. ^*p* value indicates < 0.05 NOD vs. all other experimental groups in the absence of LPS. One-way ANOVA with Dunn's multiple correction.

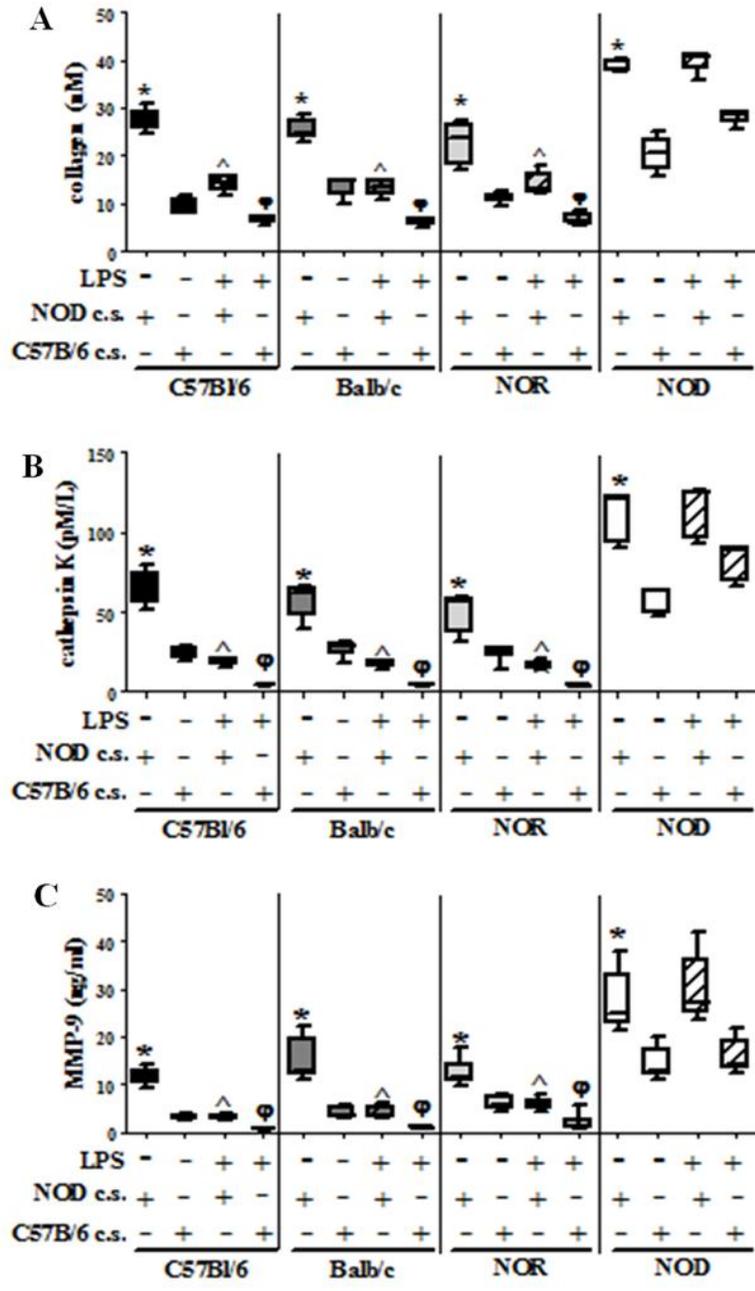


Figure 2-8. NOD-derived soluble mediators do not inhibit LPS-deactivation of OCs. BM-OCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS and either NOD conditioned media [NOD c.s.] or C57BL/6 conditioned media [C57BL/6 c.s.] for 72hrs on bovine bone slices. Supernatants were collected after 1% Triton X-100 solubilization and ELISA used to quantify: A) collagen I telopeptide, B) cathepsin K and C) MMP-9. C57BL/6 (black bars, n=5), BALB/c (dark grey bars, n=5), NOR (light grey bars, n=6), NOD (white bars, n=5). Open bars = absence of LPS, hatched bars = presence of LPS. **p* value < 0.05 NOD c.s vs C57BL/6 c.s.; ^*p* value < 0.05 no LPS vs LPS NOD c.s. ϕ *p* value < 0.05 no LPS vs. LPS C57BL/6 c.s. One-way ANOVA with Dunn's multiple correction.

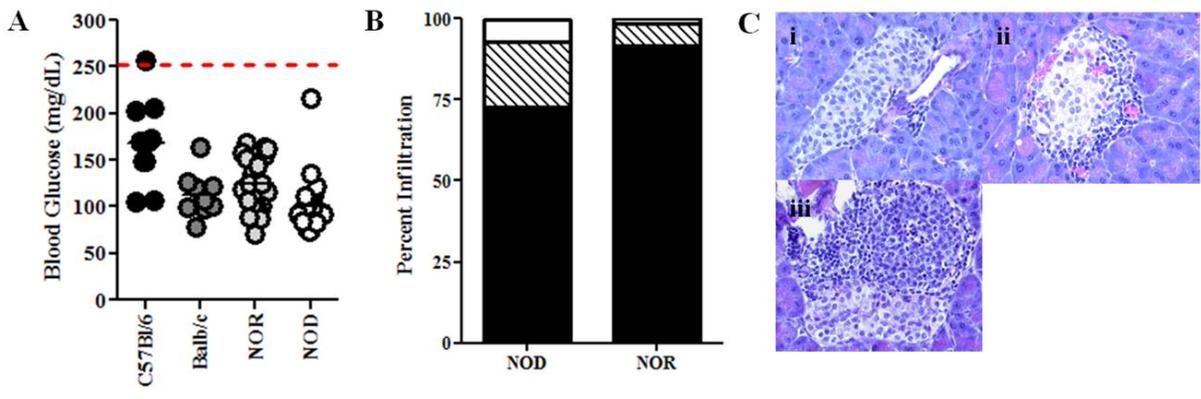


Figure 2-9. NOD-derived BM-OCs are from pre-diabetic/euglycemic mice. A) Blood glucose was measured at time of sacrifice. C57BL/6 (black circles, n=10), BALB/c (dark grey circles, n=10), NOR (light grey circles, n=19), NOD (white circles, n=23) B) Pancreata from NOR (n=5) and NOD (n=5) were fixed, embedded, sectioned, H&E stained and scored using light microscopy based on percentage of lymphocyte infiltration (insulinitis). No insulinitis = black, peri-insulinitis = hatched, intra-insulinitis = white. C) Representative sections of scoring technique. i) no insulinitis ii) peri-insulinitis iii) intra-insulinitis.

CHAPTER 3 EXACERBATED RESPONSE TO MBSA-INDUCED INFLAMMATORY ARTHRITIS IN NOD MICE

Introduction

Inflammatory bone and joint destruction are frequent co-morbidities of type 1 diabetes [T1D]. These pathologies may be caused by intrinsic genetic factors and/or from environmental triggers (24, 46). Inflammation resulting from autoimmune destruction of the pancreas or hyperglycemia as a result of this autoimmunity may also lead to secondary joint pathologies (140, 311, 312). Bone remodeling is normally a tightly regulated process where cells such as osteoclasts and osteoblasts are responsible for bone resorption and formation, respectively (68). Immune cells and soluble mediators secreted by these cells are important regulators of this process. Thus, aberrant immune cell activation and function have been implicated in many bone diseases, including inflammatory arthritis, where the balance of remodeling is tipped towards resorption (69, 288).

Inflammatory arthritis is characterized by abnormal activation of bone resorbing osteoclasts by inflammatory mediators which are produced after an initial stimulus is sensed as foreign by the immune system. This activation leads to proliferation of synoviocytes and infiltration of immune cells into the joint with subsequent erosion of subchondral bone in advanced lesions (288, 298). Injection of methylated BSA [mBSA] induces an acute inflammatory arthritis which is transient in nature. Methylation of BSA causes a change in the molecule rendering it positively-charged which binds the negatively-charged cartilage and other avascular connective tissues in the joint. The retention of mBSA in the joint leads to immune complex deposition and inflammatory

infiltration which creates an environment suitable for progressive cartilage destruction and ultimately bone erosion (334).

Methylated BSA is efficiently processed and presented by antigen presenting cells compared to unmethylated BSA and can persist in the joint for up to 28 days thereby leading to a smoldering arthritis mimicking that seen in humans with inflammatory arthritis. The synovial lining containing synoviocytes also becomes enlarged and a pannus consisting of many cell types including neutrophils and mesenchymal cells then develops and eventually covers the femoral condyle (normally covered in cartilage). Cartilage destruction and subchondral bone erosion occur below this pannus with bone-destroying osteoclasts at the front (335). It is important to note that this inflammatory arthritis is confined to the joint injected and does not progress to systemic inflammation (334). However, with systemic or local challenge with mBSA, a flare of arthritis can be induced again which is principally mediated by antigen-specific T cells, mostly of the T_H17 lineage (334, 336). T_H17 cells are prolific producers of IL-17 which induces RANK-L expression on other cell types to aid in the differentiation of osteoclast precursors into fully functional osteoclasts (69). This T helper type also expresses more RANK-L than the other T helper subsets and thus can activate osteoclasts more efficiently than other T cells (211).

In both murine and human T1D, macrophages and dendritic cells have been shown to be hyperactive to stimulation resulting in excessive pro-inflammatory cytokine production (186, 190, 315). Osteoclasts arise from the same lineage as these innate immune cells (97) and have been shown to respond aberrantly to inflammatory stimuli in the NOD mouse. Pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 have

been known to stimulate differentiation and activation of osteoclasts with IL-1 β and TNF- α being directly involved in activating resorption, (91, 160) and these mediators are found in high abundance in arthritic joints (91, 159).

While osteoblast maturation and function has been long known to be decreased in T1D (140, 239), we have recently shown that osteoclasts are also implicated in inflammation-induced bone loss in the NOD mouse model. In addition, NOD mice are more susceptible to spontaneous and induced arthritis (317). Due to the hyper-responsiveness of NOD-derived osteoclasts to inflammatory stimuli and the susceptibility of this model to induced arthritis, we hypothesized that NOD mice would display more severe inflammatory arthritis than controls due to increased osteoclast-mediated bone resorption. Therefore in the present study, we induced an acute inflammatory arthritis in NOD mice and determined the extent of osteoclast function.

Materials and Methods

Mouse Models

NOD/LtJ [NOD], NOR/LtJ [NOR], and C57BL/6J [C57BL/6] mice were maintained in a specific pathogen-free environment at the breeding facilities of the University of Florida. The NOD mouse spontaneously develops an autoimmune-mediated destruction of the insulin-secreting β -cells of the pancreas producing very similar pathology as seen in individuals with T1D (148). The NOR mouse model has a similar genetic background as the NOD mouse but does not develop insulinitis and diabetes due to the dispersal of C57BL/6 genome within diabetes susceptibility loci (318). Thus, the C57BL/6 strain serves as an additional genetic background control. All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

Arthritis Induction

Methylated BSA (Sigma) was resuspended in sterile phosphate buffered saline [PBS]. 20uL of mBSA (0pg, 300pg, 1ng) in PBS or PBS alone was injected intra-articularly into the knee joints through the infrapatellar ligament with an insulin syringe (0.5cc with 28.5 gauge needle) (BD). Injections were repeated at 7 d. Knee joint diameters were measured using a micrometer caliper (Ajax Scientific) to assess joint swelling at 0, 10, 12, 14, and 16 d post first injection of mBSA. Knee joints were also lavaged with sterile PBS using an insulin syringe at time of sacrifice for collagen ELISAs.

Collagen Telopeptide ELISA

Collagen carboxy-terminal telopeptides were detected using an ELISA according to manufacturer instructions (Immunodiagnostic Systems). Lavages were pre-incubated with biotin conjugated anti-telopeptide and horseradish-peroxidase [HRP] conjugated anti-telopeptide and added to an ELISA plate coated with streptavidin [SAV]. Following five washes, tetramethylbenzidine [TMB] substrate was used to develop reactivity followed by quenching with H_2SO_4 . Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine nM concentrations.

Histological Analysis of Joints

Knee capsules were fixed in 10% formalin (Fisher) and embedded in paraffin. 5µm sections were mounted on glass slides and deparaffinized in xylenes and rehydrated using 100% ethanol (EtOH), 95% EtOH, 75% EtOH (Fisher), and dH₂O. Sections were stained with Harris hematoxylin, incubated in a bluing solution [1.5%

NH₄OH (EMD Chemicals) in 70% EtOH] and stained with eosin [1% aqueous Eosin Y, 1% aqueous phloxine B (Fisher), 100% EtOH and glacial acetic acid (Mallinckrodt Chemicals)]. Sections were dehydrated with 70% EtOH, 95% EtOH, 100% EtOH, and xylene (2 times), mounted with Permount (Fisher) and observed under light microscopy.

Results

Intra-articular Injection of mBSA Causes an Acute Inflammatory Arthritis in NOD, NOR and C57BL/6 Mice

In our model, we observed synovial hyperplasia, extensive pannus formation, and erosion of subchondral bone with 1ng mBSA injections (Figure 3-1). PBS injections alone did not cause cartilage or bone destruction as evidenced by intact cartilage overlying the bone. The synovial lining does appear to be inflamed in the PBS-treated joints; however, this may be due to the trauma caused by intra-articular injections. The disruption of the meniscus in PBS-treated joints may also be an artifact of the sectioning process. While there does not appear to be a difference in the amount of infiltrate or subchondral bone erosion between strains, this may be because of the plane that the joint was sectioned. Further studies would require multiple sections taken per joint to assure that the total infiltrate and joint destruction can be adequately visualized.

NOD Mice do not have Increased Joint Swelling After Induction of Arthritis

Joint swelling is normally an acute process and peaks after 3-5 days post induction of the inflammatory arthritis. Swelling then begins to decline after day 7 but can be reactivated with boosters of antigen (334). We observed no large differences in joint swelling between strains or amount of mBSA injected (Figure 3-2). It is interesting to note that the 300pg group had peak swelling earlier (day 10) than the 1ng group (day 12) which might indicate a more acute and transient arthritis. It may be that we did not

capture the initial edema that is associated with the recruiting of immune cells to the area. Swelling should therefore be measured earlier, at days 3, 5, and 7 to capture the acute inflammation and edema from the initial insult.

NOD Mice Display Increased Bone Destruction via Collagen Degradation in Arthritic Joints

To measure bone destruction, we lavaged the injected joints at time of sacrifice and determined the amount of collagen degradation via ELISA for collagen I telopeptide concentration. As expected, collagen degradation increased with mBSA injections versus PBS alone (Figure 3-3). C57BL/6 and NOR behaved similarly in both the 300pg and 1ng groups with increased degradation at 1ng compared to 300pg suggesting that higher mBSA concentrations lead to increased joint damage. C57BL/6 showed the most pronounced increase with saline versus mBSA with increased collagen breakdown, especially in the 1ng group. NOD displayed more collagen degradation compared to C57BL/6 and NOR in both 300pg and 1ng mBSA treatments. These data suggest that NOD mice are more susceptible to mBSA-induced arthritis and display increased bone destruction via collagen breakdown compared to controls. Moreover, these findings support our *in vitro* bone-marrow derived osteoclast data where NOD-derived osteoclasts degrade more bone than controls with inflammatory stimuli.

Discussion

Individuals with T1D have increased incidence of inflammatory arthritis as well as osteoporosis, two diseases principally mediated by a dysregulation in bone remodeling (17, 72). Bone formation by osteoblasts is notably decreased in individuals with T1D which tips the balance of bone remodeling towards that of less bone deposition (72). We previously demonstrated an osteoclast-specific contribution to altered bone

remodeling, where T1D-derived osteoclasts are more responsive to RANK-L and inflammatory stimuli than T1D-free derived osteoclasts suggesting an additional tip in the balance to that of increased bone resorption. Here we show that the NOD mouse model displays increased collagen degradation by osteoclasts in an arthritic joint compared to NOR and C57BL/6, two T1D-free mouse models.

Our previous study showed that T1D-derived osteoclasts secrete increased levels of cathepsin K and MMP-9, two enzymes necessary for collagen degradation which are directly affected by RANK signaling (8, 112). This heightened function was observed under baseline stimulation with RANK-L and was further augmented in response to a pro-inflammatory cytokine cocktail. The pro-inflammatory environment in the joints treated with mBSA can serve to activate osteoclasts to resorb bone. Since it has already been shown that NOD-derived macrophages and monocytes secrete elevated levels of pro-inflammatory cytokines in response to inflammatory stimuli (190-192), it stands to reason that the osteoclasts in this inflammatory environment where monocytes and macrophages are in high abundance would be activated even further, which indeed is the case in our mBSA-induced arthritis model. NOD mice show increased collagen degradation in the inflammatory environment caused by mBSA intra-articular injections which suggests more bone destruction by osteoclasts.

Inflammatory infiltrates in mBSA-induced arthritis include neutrophils, eosinophils, and mononuclear leukocytes (monocytes and macrophages), all of which secrete pro-inflammatory cytokines and chemokines which serve to recruit other immune cells but also can activate osteoclasts (335). T_H17 cells, the main T cell subset in this arthritis model, also produce RANK-L which increases differentiation of osteoclast precursors

into fully functional osteoclasts (69). Increased expression of RANK-L by T_H17 cells can also serve to over-activate osteoclasts more efficiently than other T cells (211). The hyper-reactive NOD-derived osteoclast, which is already known to respond aberrantly to RANK-L stimulation with further augmented function in response to inflammatory stimuli, is therefore more likely to be over-activated by the pro-inflammatory environment in the arthritic joint and destroy increased amounts of bone compared to T1D-free hosts. Further studies are needed to determine statistical significance in our models along with extensive characterization of the location, amount, and size of osteoclasts in the arthritic joint. Delineating the causative cell type in inflammatory arthritis seen more often in participants with T1D can therefore lead to more targeted therapies to counteract the excessive joint destruction.

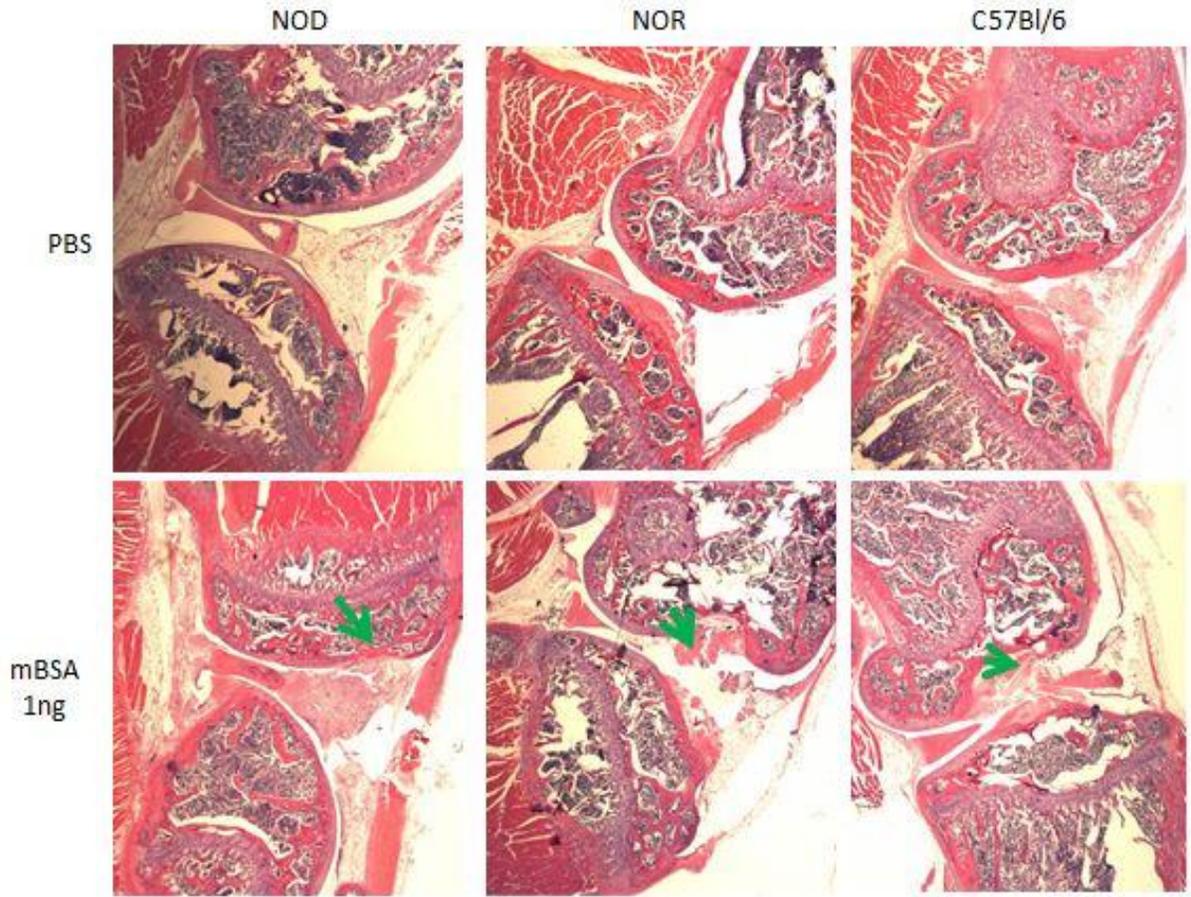


Figure 3-1. Intra-articular injection of mBSA causes an acute inflammatory arthritis in NOD, NOR, and C57BL/6 mice. Knee capsules from C57BL/6 (n=2), NOR (n=2) and NOD (n=2) were fixed, embedded, sectioned, H&E stained and imaged using light microscopy. Arrows indicate bone erosion. Representative sections of joints, shown imaged at 5x magnification.

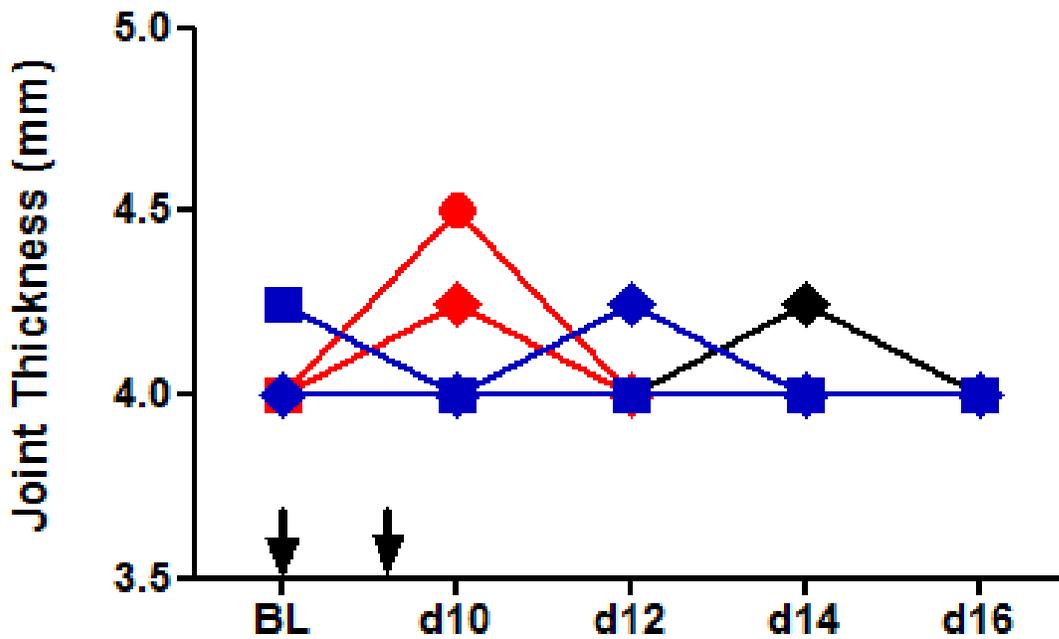


Figure 3-2. NOD mice do not have increased joint swelling after induction of arthritis. Knee diameters were measured by micrometer caliper at day 0, 10, 12, 14, and 16 post injection of mBSA or PBS. C57BL/6 (circles, n=2), NOR (squares, n=2), and NOD (diamonds, n=2). Arrows indicate mBSA injections. Red = 300pg, blue = 1ng, black = 0pg mBSA. Data shown as knee joint thickness in mm.

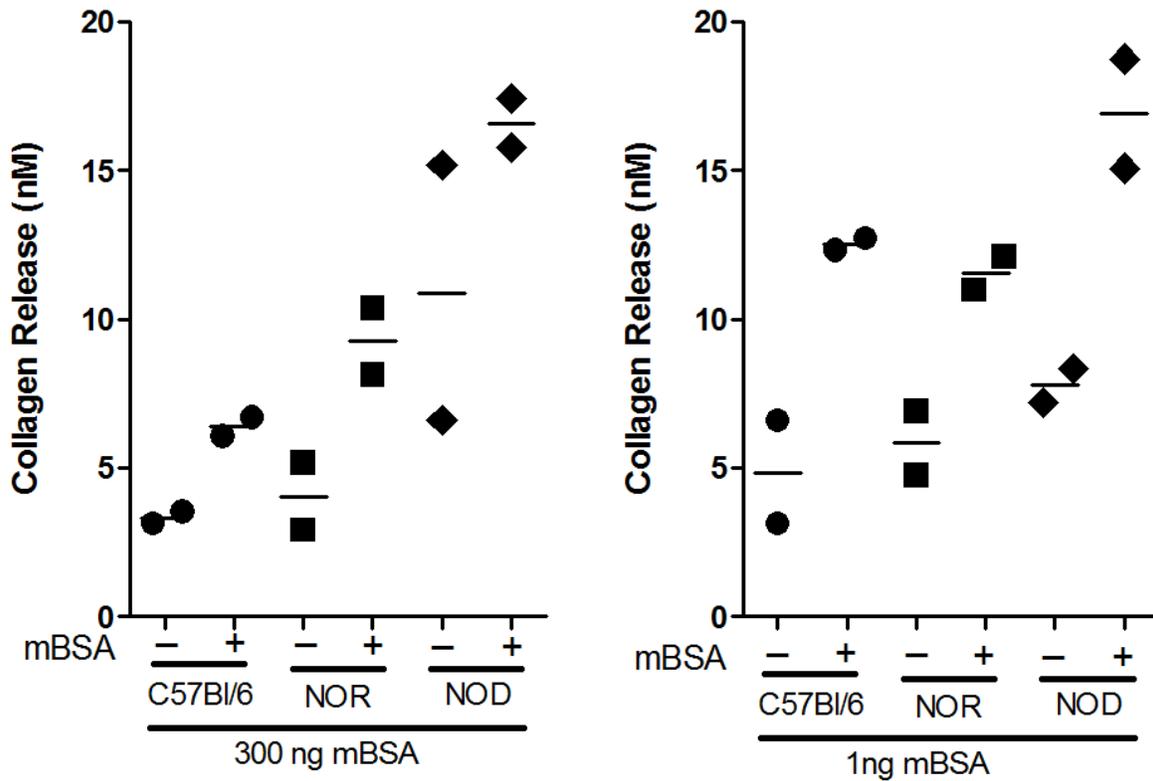


Figure 3-3. NOD mice display increased bone destruction via collagen degradation in arthritic joints. Joint lavages were collected with PBS and ELISA was used to quantify collagen I telopeptide. Data shown in nM concentration in lavages. C57BL/6 (circles, n=2), NOR (squares, n=2), and NOD (diamonds, n=2). (Left) 300pg mBSA group (Right) 1ng mBSA group.

CHAPTER 4 HYPERGLYCEMIA INDUCED AND INTRINSIC ALTERATIONS IN TYPE 2 DIABETES- DERIVED OSTEOCLAST FUNCTION

Introduction

Periodontitis is a cumulative inflammatory condition, initiated by bacteria but perpetuated by the host's immune system which leads to the destruction of the soft and hard tissues of the periodontium and, if left untreated, can lead to tooth loss (276). Susceptibility to periodontitis is enhanced by the interaction between acquired, environmental and genetic factors which modify the host response toward the subgingival biofilm (337). Many systemic diseases including type-2-diabetes [T2D] modify the progression of periodontal disease contributing to increased prevalence, incidence and/or severity of disease (173, 277, 282, 316, 338). T2D, previously referred to as non-insulin-dependent diabetes or adult-onset diabetes, accounts for 90-95% of diabetes cases, where the development of insulin resistance in target tissues leads to hyperglycemia and if left unchecked can result in the loss of insulin secretion by the islets of Langerhans in the pancreas (156). Just as T2D is a major risk factor for periodontitis, periodontitis is now classified as the sixth complication of diabetes where the level of glycemic control is an important determinant in the relationship (175, 339, 340).

The periodontium is comprised of gingiva, periodontal ligament, and alveolar bone which work together to anchor the teeth where the alveolar bone lines the tooth socket and serves as an attachment site of the periodontal ligament fibers (173). Increased alveolar bone loss is observed in periodontitis participants with T2D when compared to diabetes-free individuals (Nelson, Shlossman 1990). This progressive periodontal bone loss in T2D is related to changes that alter the balance between resorption and

deposition phases of bone metabolism (90, 281). Traditional bone cells including osteoclasts [OC] and osteoblasts [OB] are responsible for bone resorption and formation, respectively, although immune cells have also been implicated in their regulation (68). While changes in impaired osseous healing, OB proliferation and function as well as collagen function and deposition have been described in T2D-associated periodontitis, the effect of T2D on OC differentiation and function has not been reported (341).

OC differentiation is regulated by macrophage colony-stimulating factor [M-CSF] and the receptor activator of nuclear factor kappa B ligand [RANK-L]. Resorption of bone is initiated by binding of the OCs to the mineralized bone surface via alpha v beta 3 [$\alpha v\beta 3$] integrins forming a sealing zone membrane and a resorption lacuna (313). Vesicles containing osteoclastic enzymes such as tartrate-resistant acid phosphatase [TRAP], the serine protease cathepsin K, and matrix metalloproteinase-9 [MMP-9] induce collagen degradation after bone demineralization through acidification of the lacunae by the vacuolar H⁺-ATPase (126, 133).

Activation of OC-mediated bone resorption can be augmented by infection and inflammation, as well as hormonal alterations (69). Lipopolysaccharide [LPS], a cell wall component of gram negative bacteria, has been found to be highly immunogenic and induces the production of pro-inflammatory cytokines by various immune cells. OCs and their precursors, which share the same lineage as macrophages and dendritic cells, express many innate immune receptors including toll-like receptors [TLRs] and thus can respond to bacterial components (71, 199, 314). In a co-culture of osteoclasts and osteoblasts, LPS, the ligand for TLR4, augments bone resorption (199). However,

when supporting cells such as osteoblasts or other immune cells are absent, LPS inhibits bone resorption in OC pure cultures, although the exact mechanism(s) is not known (200). In addition, pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 stimulate differentiation and activation of OCs with IL-1 β and TNF- α being directly involved in activating resorption (91, 160). Importantly, inflammatory cytokines and bacterial components are found in abundance within the periodontal lesion where they can directly affect OC differentiation and function thereby shaping the bone remodeling process.

T2D leads to alterations of the function of innate immune cells such as monocytes and macrophages resulting in the production of increased levels of pro-inflammatory cytokines, namely IL-1 β and TNF- α , which are also prolonged in duration (20, 21, 149, 316, 342, 343). In addition, T2D-associated neutrophil defects lead to decreased clearance of microbes, further amplifying the inflammatory process (22, 150) (207).

Thus, since a similar heightened inflammatory response is evident in the periodontium of T2D individuals which is exacerbated in poorly-controlled diabetics (60), it is entirely possible that these individuals would be more susceptible to severe alveolar bone loss than T2D-free individuals given the effects of these soluble mediators on OC activation and function. Similarly, given the phylogenetic relationship of OCs to macrophages and dendritic cells and the aberrant TLR-responsiveness of these cell types in T2D, we postulate that T2D-derived OCs have heightened sensitivity to stimulation resulting in augmented differentiation and activation. Therefore in the present study, we characterized T2D-associated OC-specific differentiation, activation and function in the presence and absence of inflammatory stimuli utilizing both a murine

model of T2D, namely the db/db mouse model, and human primary osteoclasts derived from T2D individuals.

Materials and Methods

Participant Population

The Institutional Review Board [IRB] for protection of human subjects at the University of Florida approved this protocol in accordance with the World Medical Association Declaration of Helsinki. All data and samples were obtained under informed consent. Participants were recruited from the University of Florida, College of Dentistry from January 2009 until January 2012. Inclusion criteria: aged 13 to 75 years old, diagnosed with type 2 diabetes [T2D] without diabetic ketoacidosis, currently under the care of a physician or diabetes-free. Exclusion criteria: diabetic complications that would affect the safety or compliance or that could influence the course of either periodontal disease or diabetes care and glucose control; received immunosuppressive, antibiotic, glucocorticoid, or bisphosphonate therapy over the last 6 months; and current tobacco use. A single venous blood sample of 30mL was collected from all participants, where non-fasting blood glucose levels and glycated hemoglobin [HbA1c] were measured with the Ascensia Contour blood glucose meter (Bayer, Tarry Town, NY) and A1CNow meter (Bayer), respectively.

Peripheral Mononuclear Cell [PBMC] Isolation, Osteoclast [OC] Differentiation and Activation

Whole blood samples were divided equally and diluted 1:1 with balanced working salt solution [1 volume salt solution A:9 volumes salt solution B]. Salt solution A: 1g/L anhydrous D-glucose, 0.0074g/L CaCl₂, 0.1992g/L MgCl₂, 0.4026g/L KCl and 17.565g/L Tris in dH₂O. Salt solution B: 8.19g/L NaCl in dH₂O. Diluted blood was overlaid onto

15mL of Ficoll-Paque (GE Healthcare Life Sciences, Waukesha, WI) and centrifuged for 40 min at 1200rpm. The interface layer containing PBMCs was removed and washed 3 times with 6mL of working salt solution. The resulting PBMC cell pellet was resuspended in α -MEM complete media. and seeded in T25 flasks containing 10mL α -MEM complete media (Sigma-Aldrich, St. Louis, MO) [α -MEM with 10% fetal bovine serum (Mediatech Inc., Manassas, VA), 1% L-glutamine (Thermo Fisher Scientific Inc., Waltham, MA), and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher Scientific Inc.)] supplemented with 10ng/mL recombinant human M-CSF [rhM-CSF] (Peprotech, Rocky Hill, NJ)] at 37°C and 5% CO₂ for 14 d with media refreshed every 3 d. Non-adherent cells were removed and 5.9×10^5 cells/mL of adherent cells were seeded in 24-well plates on either glass coverslips (Thermo Fisher Scientific Inc.) or 1 cm² bovine bone slices cut with an Isomet Low Speed Saw (Buehler, Lake Bluff, IL). All cultures were supplemented with 10ng/mL rhM-CSF and 50ng/mL recombinant human soluble RANK-L [rhsRANK-L] (Peprotech) and allowed to culture for 12 d with complete media refreshed every 3 d. After 12 d of differentiation, media was refreshed with α -MEM complete media supplemented with 10ng/mL rhM-CSF and 50ng/mL rhsRANK-L. Cells were allowed to resorb bone for 72 h in the presence or absence of 1ug/mL *Escherichia coli* LPS [LPS] (Sigma-Aldrich). Cultures were permeablized with 1% Triton X-100 for 10 min. Supernatants were stored at -80°C until cathepsin K ELISA, collagen type I telopeptide ELISA, and cyto/chemokine multiplex analysis were performed. All outcome measures were normalized to total number of TRAP+ cells.

Mouse Models

All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee [IACUC] where

measures were taken to minimize pain or discomfort. B6.BKS(D)-Lepr^{db}/J [db/db] and C57BL/6J [B6] mice were maintained in a specific pathogen-free environment at the breeding facilities of the University of Florida. Non-fasting blood glucose levels and glycated hemoglobin [HbA1c] were measured with the Ascensia Contour blood glucose meter and A1CNow meter, respectively, at the time of sacrifice.

Bone Marrow Osteoclast [BMOC] Differentiation and Activation

Bone marrow was harvested from 10-12 wk old db/db and B6 mice. Femora and tibiae were surgically isolated, and marrow expelled from bones with α -MEM complete media (Sigma-Aldrich) [α -MEM with 10% fetal bovine serum (Mediatech Inc., 1% L-glutamine (Thermo Fisher Scientific Inc.), and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher Scientific Inc.)]. Cells were seeded in T75 flasks at a concentration of 1.5×10^6 cells/mL supplemented with 5ng/mL recombinant murine M-CSF [rmM-CSF] (Peprotech) and allowed to culture for 24 h at 37°C and 5% CO₂. Non-adherent cells were removed and 5.9×10^5 cells/mL of adherent cells were seeded in 24-well plates on either glass coverslips (Thermo Fisher Scientific) or 1 cm² bovine bone slices cut with an Isomet Low Speed Saw (Buehler). All cultures were supplemented with 10ng/mL rmM-CSF and 50ng/mL recombinant murine soluble RANK-L [rmsRANK-L] (Peprotech) and allowed to culture for 6 d with complete media refreshed every 3 d. After 6 d of differentiation, media was refreshed with α -MEM complete media supplemented with 10ng/mL rmM-CSF and 50ng/mL rmsRANK-L. Cells were allowed to resorb bone for 72 h in the presence or absence of 1ug/mL *Escherichia coli* LPS (Sigma-Aldrich). Cultures were permeabilized with 1% Triton X-100 for 10mins. Supernatants were stored at -80°C until cathepsin K ELISA, collagen type I telopeptide ELISA, and cyto/chemokine

multiplex analysis were performed. All outcome measures were normalized to total number of TRAP+ cells.

TRAP Staining

After differentiation, human and murine OCs [hOCs and mOCs, respectively] plated on glass coverslips were fixed with 2% paraformaldehyde/PBS (Thermo Fisher Scientific Inc.). Cells were washed with PBS and permeabilized in 0.5% Triton X-100/PBS (Thermo Fisher Scientific Inc.). Cells were washed and probed for leukocyte acid phosphatase [TRAP] [1:1:1:2:4 Fast Garnet GBC Base Solution:Sodium Nitrite Solution:Napthol AS-BI Phosphate Solution:Tartrate Solution:Acetate Solution] (Sigma-Aldrich) after which cells were washed and mounted on glass slides with MOWIOL 4-88 solution (Calbiochem, San Diego, CA). TRAP positive cells [purple in color] were counted according to number of nuclei present: mononuclear cells [1 nucleus], multinucleated osteoclasts [2-10 nuclei], and giant osteoclasts [11+ nuclei] using light microscopy at 40x magnification. Whole coverslips were counted for each cell type.

Collagen Telopeptide ELISA

Collagen carboxy-terminal telopeptides were detected using an ELISA according to manufacturer instructions (Immunodiagnostic Systems, Scottsdale, AZ). Supernatants were pre-incubated with biotin conjugated anti-telopeptide and horseradish-peroxidase [HRP] conjugated anti-telopeptide and added to an ELISA plate coated with streptavidin [SAV]. Following five washes, tetramethylbenzidine [TMB] substrate was used to develop reactivity followed by quenching with H₂SO₄. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek, Broadview, IL) set at a dual wavelength reading of 450nm with a reference of

655nm. Gen5 Software (Biotek) and a standard curve were used to determine nM concentrations.

Cathepsin K ELISA

Active cathepsin K was detected using an ELISA according to manufacturer instructions (Alpco Diagnostics, Salem, NH). Supernatants pre-incubated with HRP-conjugated anti-cathepsin K were added to an ELISA plate pre-coated with polyclonal sheep anti-cathepsin K. Following five washes, TMB substrate was used to develop reactivity followed by quenching with STOP solution. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine pM/L concentrations of active cathepsin K.

Soluble Mediator Analysis

Cytokines and chemokines from resorption supernatants were detected and quantified using a mouse 22-cyto/chemokine multiplex or a human 14-cyto/chemokine multiplex (Millipore, Billerica, MA) according to the manufacturer's instructions. Supernatant and antibody-coated beads were allowed to incubate overnight at 4°C in a 96-well primed plate. Following two washes, reactivity was probed with biotinylated detection antibodies and SAV-phycoerythrin [PE]. All incubations occurred while gently shaking in the dark. Following two washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system with Xponent software (Millipore). Milliplex analyst software (Viagene, Beverly Hills, CA), 5-parameter logistics and a standard curve were used to determine pg/ml concentrations.

Statistical Analysis

One-way ANOVA with Bonferroni's multiple comparisons were used to analyze and determine statistical significance ($p < 0.05$).

Results

Hyperglycemia Enhances Differentiation of T2D-derived OCs

Db/db mice on the C57BL/6 genetic background provide an animal model of obese, insulin-resistant type 2 diabetes (344, 345) whereby plasma insulin begins to rise around 10 to 14 days of age resulting in obvious obesity around 3 to 4 weeks after which hyperglycemia sets in around four to eight weeks of age. Osteoclasts [mOCs] were derived from the bone marrow of 10-12 week old db/db and C57BL/6 mice and the differentiation potential evaluated. TRAP staining was used to determine the number of multinucleated osteoclasts defined as having 2-10 nuclei and giant cells containing ≥ 11 nuclei (Fig. 4-2A). As expected, at the time of bone marrow harvest, db/db mice had significantly higher non-fasting glucose and %HbA1c compared to C57BL/6 control mice (Fig. 4-1A, B). Here following long term and severe hyperglycemia, a significantly higher number of multinucleated and giant mOCs in db/db-derived cultures were observed compared to C57BL/6 controls (Fig. 4-2C, D).

In order to determine if similar phenomenon occurred in human disease, osteoclasts [hOCs] were derived from peripheral blood monocytes of individuals with and without T2D (Fig. 4-2D). Although the T2D cohort presented with a statistically higher non-fasting blood glucose and %HbA1c, when compared to the diabetes-free cohort (Figure 4-1C, D), the levels of non-fasting glucose and %HbA1c present within the T2D cohort indicate that the cohort was under glycemic control as described by the American Diabetes Association (346, 347). Here in the absence of long term

hyperglycemia, similar numbers of multinucleated and giant hOCs were observed in T2D-derived cultures compared to those derived from diabetes-free individuals (Fig 4-2E, F).

Enhanced Differentiation Results in Exacerbated Bone Resorption

Although the differentiation data suggests that hyperglycemia and not the metabolic disease of T2D results in enhanced differentiation with an overall larger osteoclast size, this data does not address the bone resorbing function of these cells. In order to determine the bone resorbing capabilities of T2D-derived osteoclasts, mOCs and hOCs were seeded onto bovine bone slices and stimulated with RANK-L to promote bone resorption. During bone resorption, after demineralization by the H⁺-ATPase, serine proteases such as cathepsin K in the resorption lacunae degrade the organic portion of bone containing type I collagen, where vesicles containing collagen telopeptides are transcytosed out of the osteoclast and released into the extracellular milieu (322). Therefore, in order to quantify bone resorption, levels of intra- and extra-cellular cathepsin K and collagen telopeptides were evaluated (Fig. 4-3). While mOCs derived from db/db mice produced significantly more cathepsin K and resorbed significantly more bone than control mice (Fig. 4-3A, B), no significant difference in cathepsin K expression nor bone resorption was observed in hOC derived cultures (Fig. 4-3E, F). Due to the increased numbers of multinucleated and giant mOCs in db/db cultures, it would be expected for these cultures to have both increased cathepsin K and bone resorption capabilities. Thus, cathepsin K expression and collagen degradation was normalized on a per cell basis where the bone resorption was now similar between db/db and C57BL/6 mOCs (Fig. 4-3C, D), suggesting that the increased resorption observed in mOC cultures was simply due to increased numbers of multinucleated and

giant mOCs in the culture (Fig. 4-2), rather than enhanced function due to the metabolic disease.

T2D-derived Osteoclasts Respond Aberrantly to LPS

The inflammatory pathology of periodontal diseases includes a large bacterial burden, where activation of bone resorption can be induced by bacterial components. Bacterial components such as LPS act on osteoblasts and activated T-cells to produce more RANK-L to stimulate osteoclasts to differentiate and resorb bone (105, 278). However, LPS can also act on osteoclasts directly by inhibiting resorption by mature osteoclasts (200, 202). Thus, the affect of LPS on T2D-derived osteoclast bone resorbing capacity was evaluated, whereby mOCs and hOCs were seeded onto bovine bone slices and stimulated to resorb bone with RANK-L in the presence of LPS. LPS-induced deactivation of OC function was observed in C57BL/6 mOCs and diabetes-free hOC-derived cultures indicated by a decrease in the cathepsin K secretion and collagen degradation (Fig. 4-3). However, this LPS-induced deactivation did not occur in the db/db-derived mOCs cultures nor the T2D-derived hOC cultures (Fig. 4-3). Interestingly, while db/db-derived mOCs also displayed an increase in collagen degradation and cathepsin K secretion in the presence of LPS compared to RANK-L stimulation alone (Fig. 4-3C, D), T2D-derived hOCs did not (Fig. 4-3E, F).

Elevated Pro-Osteoclastic Milieu in T2D-derived OC Cultures is Augmented by LPS

Because osteoclast function is regulated by many soluble immune mediators, the cytokine and chemokine profile within mOC and hOC cultures were evaluated. Following RANK-L stimulation, db/db mOC-derived cultures had elevated levels of the chemokines IL-6, IP-10, MIP-1 α and TNF- α as well as the anti-osteoclastic mediator IL-

10 when compared to C57BL/6 mOC-derived cultures (Fig. 4-4). Similarly, T2D hOC-derived cultures had elevated levels of IL-6, MIP-1 α and TNF- α , but not IP-10 nor the anti-osteoclastic mediator IL-10 when compared to those derived from the diabetes-free cohort (Fig. 4-5).

LPS stimulation of OC cultures resulted in an upregulation of all cyto/chemokines measured in all mOC and hOC cultures. Significantly higher levels of MCP-1, IP-10 and MIP-1 α were observed in T2D-derived mOC and hOC cultures (Fig. 4-4, 4-5), while hOC cultures also had significantly higher levels of IL-6 and TNF- α (Fig. 4-5). Interestingly, both T2D-derived mOC and hOC cultures presented with a significantly lower amount of the anti-osteoclastic cytokine IL-10 (Fig. 4-4C, 4-5C).

Discussion

Individuals with T2D have a greater risk of developing periodontal disease, where the degree of hyperglycemia is a confounding factor (175, 339, 340). Specifically, gingival inflammation as well as soft and hard periodontal tissue destruction are more severe in individuals with T2D, where the risk of progressive alveolar bone loss increases over time although the exact mechanism is unclear (12, 28, 174, 282, 316, 338). Importantly, other bone metabolism pathologies are prevalent in individuals with T2D, including osteopenia and delayed fracture healing (239).

Previous studies have demonstrated both defective bone formation and osteoblast maturation under hyperglycemic conditions in murine and rat models of T2D. Specifically, Graves and colleagues have demonstrated increased osteoblast apoptosis and less osteoid formation in the Goto-Kakizaki rat model of periodontal disease in T2D (12). This result was also observed by Liu and colleagues in the Zucker diabetic fatty rat, where by bone formation did not occur after resolution of inflammation as was

observed in T2D-free controls (4). Hyperglycemia also affects osteoblast differentiation and mineralization capabilities whereby the production of osteocalcin and calcium deposition are known to be decreased in the presence of high blood glucose levels (239), thus shunting mesenchymal stem cells [MSCs], which serve as precursors for osteoblasts, towards the adipocyte lineage leading to adipose tissue formation in bone marrow (274).

Conversely, the role of osteoclasts in T2D-associated bone loss are less defined, although literature suggests that hyperglycemia may lead to augmented OC function (4, 7, 8, 13, 223, 253). For instance, bone mineral density was found to be decreased in individuals with uncontrolled T2D compared to T2D-free controls, whereby urinary levels of collagen degradation products from bone resorption such as CTx, DPD, and NTx as well as serum TRAP levels were found to be significantly elevated (7). In addition, augmented osteoclastogenesis and resorptive capabilities from peripheral blood mononuclear cells were found in a subset of individuals with diabetes suffering from Charcot's osteoarthopathy, a rare osteolytic complication characterized by neuropathy and bone degradation in the foot (8).

To date, the literature describing the direct effect of hyperglycemia on osteoclast differentiation and function vary with some models suggesting high glucose leads to increased function (247, 248, 333) while others suggest decreased resorptive capabilities under hyperglycemic conditions (249). Here we report that in a murine model of T2D, recent long term hyperglycemia enhances the differentiation/fusion of osteoclast precursors resulting in more resorptive cultures. On the other hand, human osteoclasts from participants with well-controlled T2D, differentiated similarly to those

derived from diabetes-free participants. Cell surface receptors such as DC-STAMP are critical for fusion from mononuclear precursors into multinucleated osteoclasts. Thus, it is plausible that the expression of these fusion receptors is altered by hyperglycemia allowing for more efficient fusion (117). Mechanisms associated with hyperglycemia-induced augmented differentiation are currently being investigated in our laboratory.

While initially our data also suggested a hyper-responsiveness of db/db-derived osteoclasts to RANK-L, when resorption data was normalized to cell number, the observed increased resorption could only be attributed to a significant increase in larger osteoclasts. Interestingly, both human and murine osteoclasts derived from T2D sources displayed altered responses to LPS where deactivation did not occur as was observed in T2D-free cultures. This alteration in function appears to be independent of hyperglycemia considering that hOCs derived from well-controlled hyperglycemic environments also displayed this phenotype. Aberrant LPS-responsiveness in innate immune cells such as monocytes and macrophages, relatives of osteoclasts, have already been described in both type 1 and type 2 diabetes independent of hyperglycemia, resulting in augmented pro-inflammatory cytokine secretion (41).

In conjunction with a lack of inactivation, osteoclasts derived from T2D individuals and db/db mice displayed LPS-induced augmented pro-inflammatory cytokine and chemokine secretion compared to T2D-free controls. Here chemokines such as MCP-1, IP-10, and MIP-1 α which recruit osteoclast precursors (85, 101, 211) were elevated. Similarly, the cytokines IL-6 and TNF- α which are innate and adaptive immune cell activators and can aid in osteoclastogenesis (85, 101, 211) were also elevated. On the other hand, the anti-osteoclastic cytokine IL-10 (85, 101, 211) was significantly lower in

T2D-derived cultures, suggesting a lack of regulatory function in T2D-derived osteoclasts. Indeed, others have demonstrated that peripheral blood-derived osteoclasts from T2D individuals are less sensitive to the soluble decoy receptor for RANK-L, osteoprotegrin [OPG], than those derived from diabetes-free controls again resulting in increased bone resorption (8, 323).

Data presented here describe environmental and intrinsic mechanisms associated with the increased alveolar bone loss observed in periodontal participants with T2D. Specifically, hyperglycemia augmented osteoclast differentiation/fusion resulting in a more resorptive environment. In addition, independent of hyperglycemia, T2D-derived osteoclasts were less responsive to regulation whereby they were refractive to LPS-induced deactivation and secreted lower anti-osteoclastic soluble mediators. Finally, T2D-derived osteoclast cultures were more osteoclastic in response to LPS perpetuating the uncontrolled bone resorption. Thus, one can imagine that the presence of LPS during infections such as periodontal disease would lead to a vicious cycle of uncontrolled osteoclast differentiation and activation that would lead to excessive alveolar bone loss in periodontal participants with T2D.

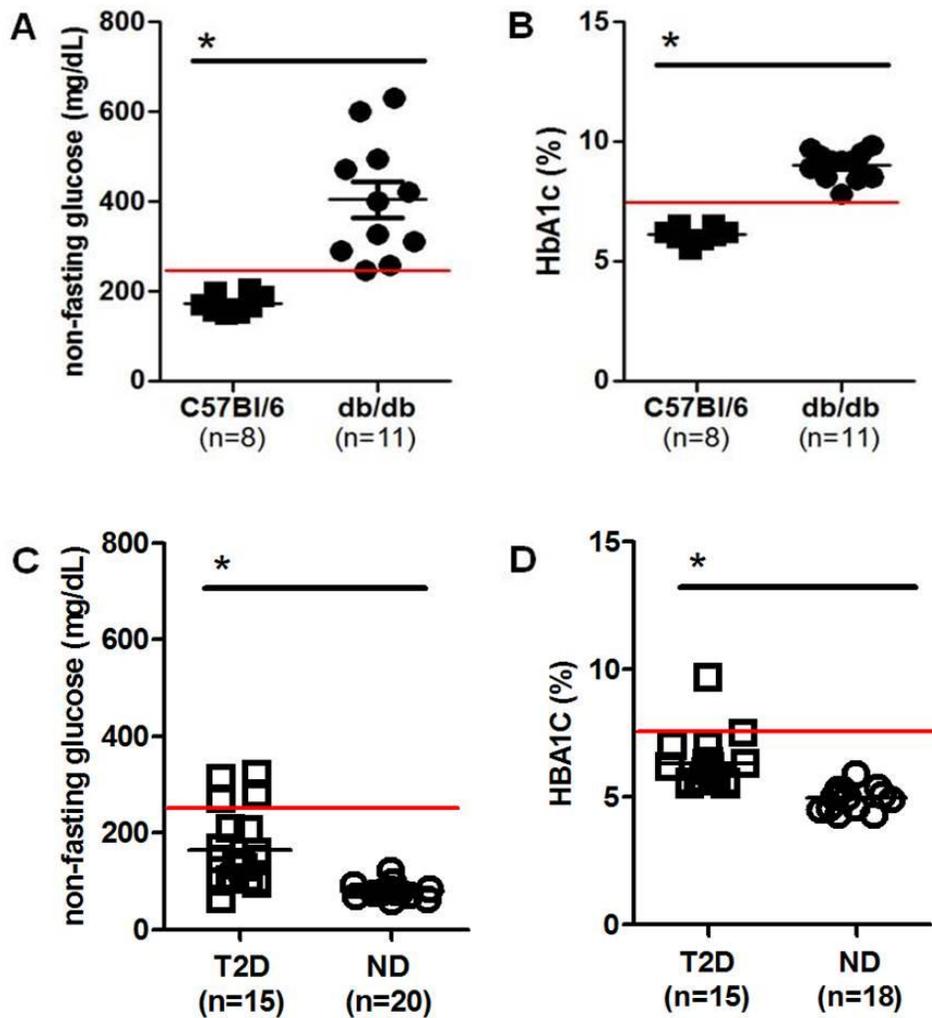


Figure 4-1. Glycemic indices of murine and human cohorts. A, C) Blood glucose and B, D) HbA1c were measured at time of A, B) bone marrow harvest or C, D) peripheral blood collection. Red line is indicative of the high limit of normal indices. Non-fasting blood glucose = 250mg/dL; glycated hemoglobin (HbA1c) = 7.5% *p value < 0.05. Unpaired t-test with Welch's correction. ND = diabetes-free participants, T2D = participants with type 2 diabetes.

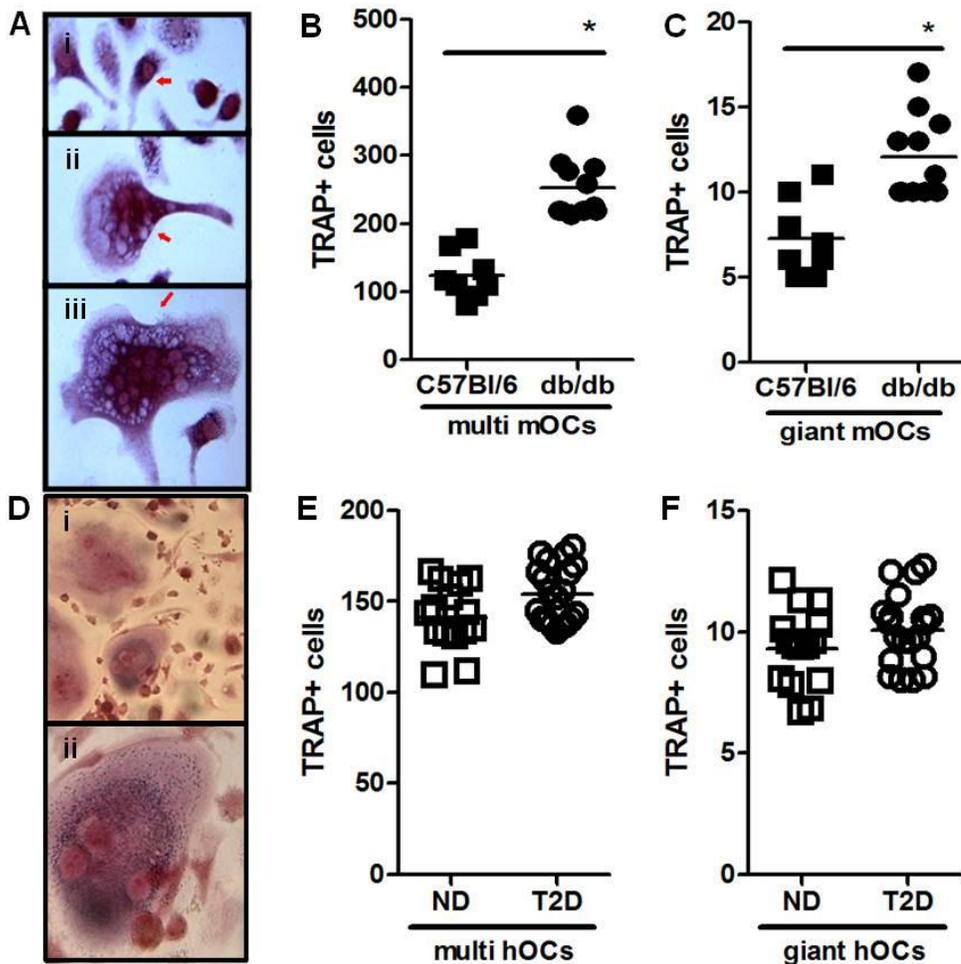


Figure 4-2. Db/db bone marrow derives increased numbers of larger osteoclasts. A-C) 6d post-differentiation, bone marrow-derived mOCs were imaged with light microscopy at 10x magnification. A) Representative images of TRAP positive [purple in color] i) mononuclear osteoclasts, ii) multinucleated osteoclasts and iii) giant osteoclasts. Number of B) multinucleated and C) giant mOCs per coverslip were enumerated using 40x magnification. D-F) 12d post differentiation, peripheral blood-derived hOCs were imaged with light microscopy at i) 20x and ii) 40x magnification. TRAP positive [purple in color] E) multinucleated and F) giant hOCs were enumerated using 40x magnification. **p* value < 0.05. Unpaired t-test with Welch's correction. ND = diabetes-free participants, T2D = participants with type 2 diabetes.

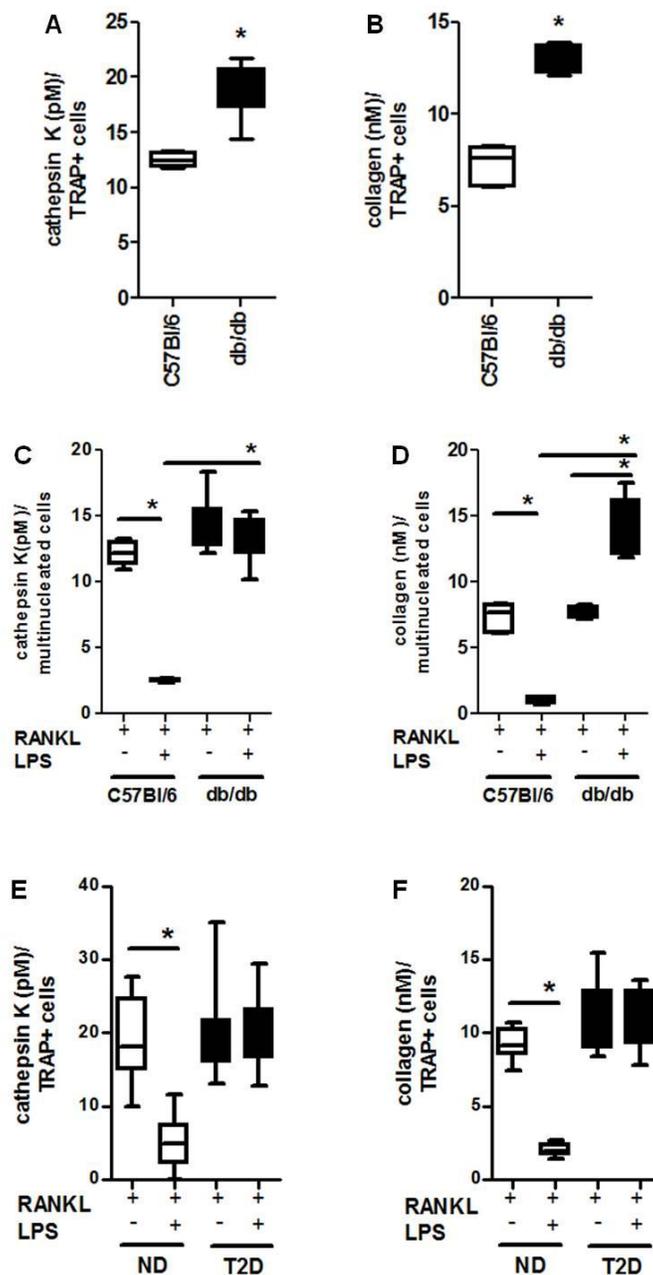


Figure 4-3. Type 2 diabetes-derived osteoclasts are less responsive to LPS-induced deactivation. A-D) mOCs and E, F) hOCs were stimulated with RANK-L in the presence and absence of *E. coli* LPS for 72hrs on bovine bone slices. Supernatants collected after 1% Triton X-100 solubilization were evaluated for A, C, E) cathepsin K and B, D, F) collagen I telopeptide by ELISA. Data shown as pM and nM respectively. C, D) mOC collagen and cathesin K expression normalized to number of multinucleated cells. Normalized outcome measure = [raw value x cell number corrective ratio]. Cell number corrective ratio = [lowest average cell number within sample set] / [average

cell number for group of interest]. *p value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction. ND = diabetes-free participants, T2D = participants with type 2 diabetes.

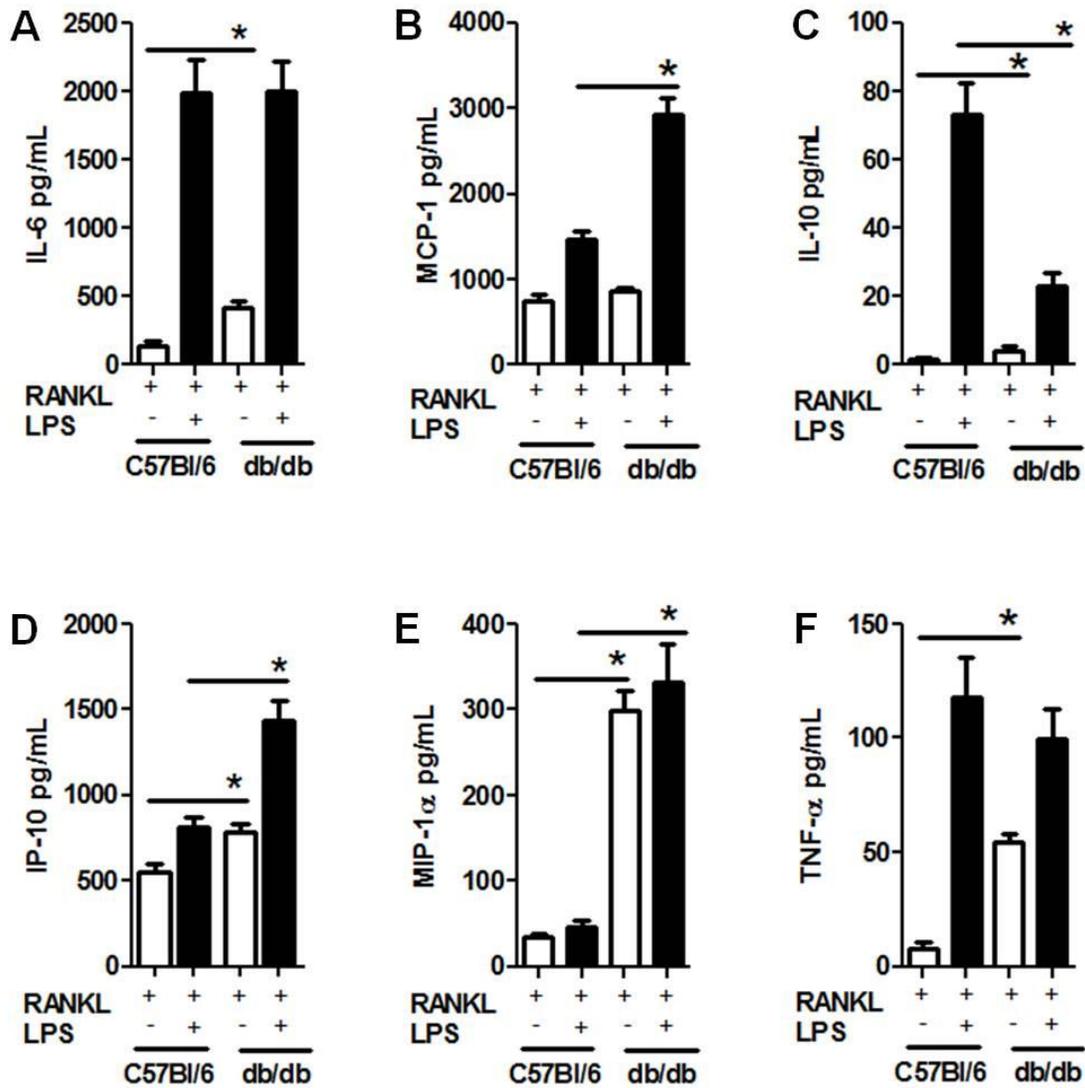


Figure 4-4. LPS-induced elevation of pro-inflammatory and pro-osteoclastic soluble mediators in db/db osteoclast cultures. mOCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS for 72hrs on bovine bone slices. A) IL-6, B) MCP-1, C) IL-10, D) IP-10, E) MIP-1 α and F) TNF- α , levels were evaluated in the supernatants and using Milliplex technology. Data shown as pg/mL. *p value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction.

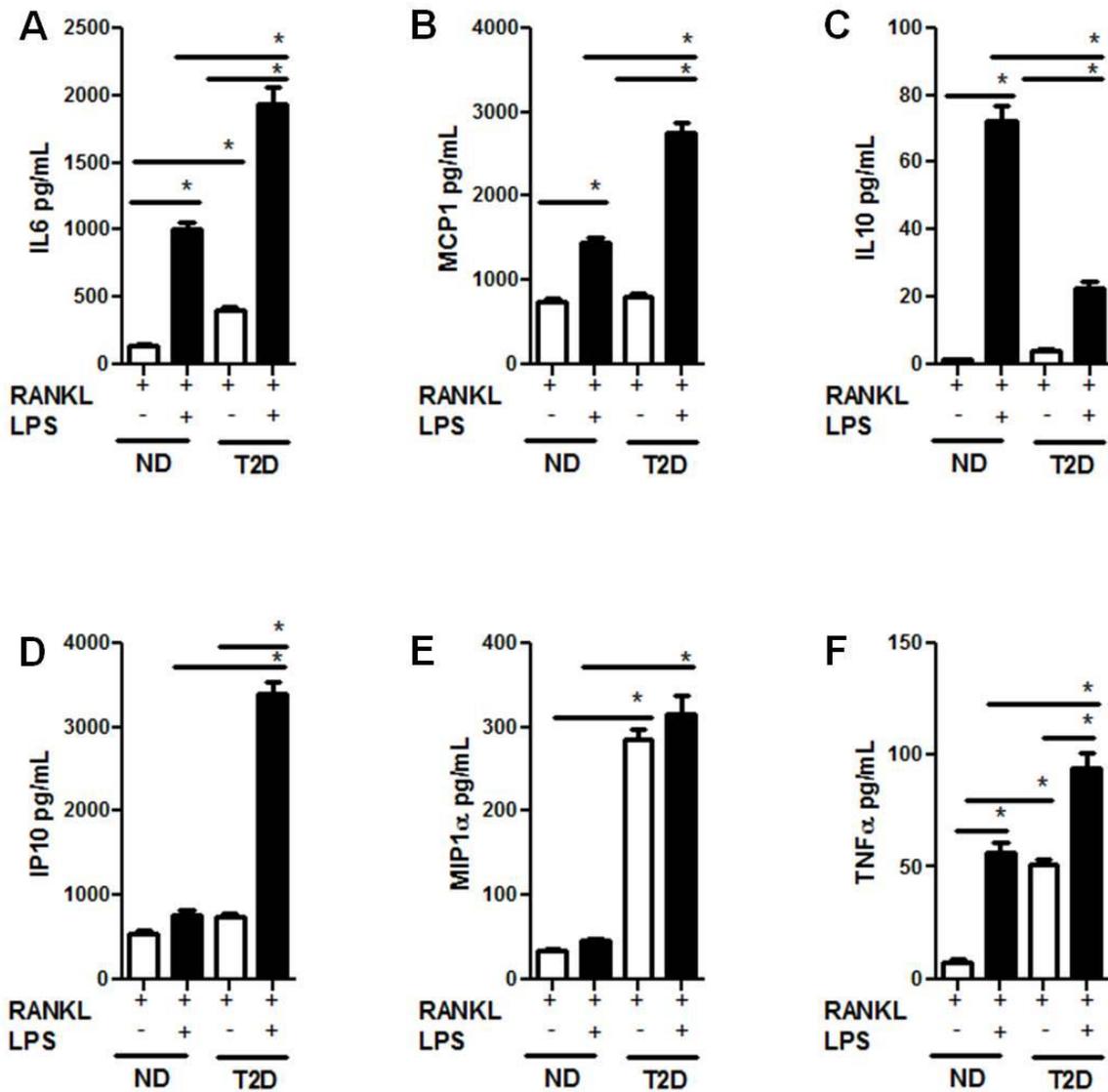


Figure 4-5. LPS-induced elevation of pro-inflammatory and pro-osteoclastic soluble mediators in human type 2 diabetes osteoclast cultures. hOCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS for 72hrs on bovine bone slices. A) IL-6, B) MCP-1, C) IL-10, D) IP-10, E) MIP-1 α and F) TNF- α levels were evaluated in the supernatants and using Milliplex technology. Data shown as pg/mL. *p value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction.

CHAPTER 5 DISCUSSION

Differentiation

Type 1 Diabetes-Derived Osteoclasts

Type 1 diabetes-derived osteoclasts from the NOD mouse model show altered differentiation where fusion is decreased leading to fewer multinucleated and giant cells than the diabetes-free NOR, C57BL/6, and BALB/c strains. This altered differentiation is also seen in NOD-derived cells from the same myeloid lineage as osteoclasts, namely macrophages and DCs. Macrophages derived from NOD mice are more immature in phenotype with lower MHC class I expression than their diabetes-free counterparts yet have increased response to inflammatory stimuli with pro-inflammatory cytokine secretion (190-192). DCs from NOD mice also display lower levels of differentiation markers such as MHC class II and co-stimulatory molecules (184, 185). This finding is recapitulated in humans with T1D where DCs have lowered co-stimulatory molecule B7.1/2 expression and have impaired T cell activation ability (182). It is interesting that while these cells do appear less mature in the NOD mouse, there are more circulating precursors for macrophages and DCs and NOD marrow can derive more of these cell types than other strains (348). However, there is a preference for NOD marrow to shunt these precursors to that of the macrophage lineage (349) which may explain why more NOD osteoclasts resemble mononuclear macrophages than multinucleated osteoclasts in their ability to secrete high levels of pro-inflammatory cytokines. It should also be mentioned that mononuclear cells can have resorptive function (350) which may explain why even with fewer multinucleated cells, T1D-derived mononuclear osteoclasts can still resorb more than diabetes-free cells.

Type 2 Diabetes-Derived Osteoclasts

Type 2 diabetes-derived osteoclasts from the db/db mouse model show enhancement of differentiation contrary to what was seen in T1D-derived osteoclasts from the NOD mouse. The db/db mouse derives more multinucleated and giant osteoclasts and fewer mononucleated precursors compared to the diabetes-free C57BL/6 mouse suggesting augmented fusion capability. This phenomenon may be caused by increased pro-osteoclastic cytokine release from db/db-derived osteoclasts with RANK-L stimulation which can lead to IL-1 and TNF- α release, both important enhancers of osteoclastogenesis (101). Human-derived osteoclasts from participants with T2D, however, do not show this augmented differentiation. In fact, T2D participants derive similar numbers of multinucleated and giant osteoclasts to a diabetes-free cohort. It is important to note that the glycemic state of the human T2D subjects was considered well-controlled while the db/db were severely hyperglycemic at the time of sacrifice. This may account for the differences seen in differentiation where hyperglycemia could enhance osteoclastogenesis.

RANK-L Activation

Normal Osteoclasts

RANK-L will induce differentiation and fusion of M-CSF-dependent osteoclast precursors. The binding of RANK-L to its receptor RANK on these precursors induces NF- κ B and NFATc1 pathways which then turn on osteoclast-specific genes (351, 352). Calcitonin receptor, beta 3 integrin, cathepsin K, and MMP-9, and the transcription factor c-src are all turned on by RANK-L and aid in osteoclast function (112, 113). C-src allows for the fusion of these committed mononuclear precursors to become multinucleated osteoclasts (351). RANK-L will induce the mature cell to start the

resorption process where the cell will attach to the bone matrix, reorganize the actin cytoskeleton to form the actin ring and sealing zone, and eventually form the ruffled border. Targeted acid secretion and subsequent demineralization of the bone then occurs after which the organic portion is degraded by enzymes such as cathepsin K (322).

Diabetic Osteoclasts

Both T1D-derived osteoclasts from the NOD mouse and T2D-derived osteoclasts from the db/db mouse display enhanced bone resorption with RANK-L stimulation. They produce elevated levels of MMP-9 and cathepsin K and subsequently degrade more collagen than non-diabetic controls. Since RANK-L stimulation leads to NF- κ B activation to elicit osteoclast function, it may be that this pathway has defects where the translocation of the transcription factor to the nucleus to turn on osteoclast-specific genes is enhanced. DCs have been found to have defects in the NF- κ B pathway which lead to a hyperactive phenotype whereby they are rendered more sensitive to inflammatory stimuli, release increased amounts of pro-inflammatory cytokines, and have augmented T cell activation capabilities (186-188). Macrophages from NOD mice also display defects similar to DCs where cells are hyper-responsive to inflammatory stimuli and have increased NF- κ B activation (190-192). Monocytes and macrophages in T2D participants are also constitutively activated and respond even more so to inflammatory stimuli (41) which may indicate a similar defect in the NF- κ B pathway. Thus, the diabetes-derived osteoclast may have aberrant NF- κ B translocation after RANK ligation which could explain the increased sensitivity to RANK-L and subsequent increased resorptive capability.

LPS-induced Deactivation

Normal Osteoclasts

While LPS can act on supporting cell types such as stromal cells, osteoblasts, and activated T cells to produce RANK-L which stimulates osteoclastogenesis and initiates resorption (70), LPS stimulation on osteoclasts directly leads to deactivation and inhibits differentiation of precursors. This inhibition of differentiation and function of osteoclasts is thought to be a way to switch the lineage of the cell back to that of a macrophage or immune cell to help clear the supposed infection. Osteoclast precursors in the absence of RANK-L treated with LPS will not differentiate into multinucleated osteoclasts. With co-treatment of LPS and RANK-L, LPS still inhibits osteoclastogenesis. However, once RANK-L has led to the commitment of the precursor to the osteoclast lineage, LPS treatment can enhance differentiation (200, 201). Mature osteoclasts pre-treated with RANK-L stimulated with LPS shut down resorptive processes (200). We have also found that osteoclasts stimulated with LPS instead secrete pro-inflammatory cytokines and chemokines which may serve to recruit other immune cells to the site of infection. Thus, in the presence of LPS, osteoclast-mediated bone resorption is inhibited and cellular function switches to that of an immune cell recruiter.

Diabetic Osteoclasts

Osteoclasts derived from hosts with diabetes do not display LPS-induced deactivation of bone resorption. NOD-derived osteoclasts continue to secrete cathepsin K and MMP-9 to degrade collagen and have similar capability to form resorption pits in the presence of LPS. Osteoclasts derived from the db/db T2D mouse model also show this inhibition of LPS-induced deactivation. Surprisingly, even though the T2D human-derived osteoclasts had similar differentiation potential to diabetes-free humans, LPS-

induced deactivation did not occur. Thus, regardless of the glycemic control of the host at the time of precursor harvest, osteoclasts derived from either T1D or T2D hosts do not respond correctly to LPS and instead continue to resorb bone.

Monocytes and macrophages from T2D participants secrete increased levels of multiple pro-inflammatory cytokines in response to LPS compared to diabetes-free participants. Interestingly, these cells are constitutively activated in T2D hosts and thus are not tolerized to the presence of TLR ligands which are constantly circulating due to poor control of infections. Presence of low levels of TLR ligands such as LPS normally induce tolerance to a second challenge, yet the response seen in T2D participants is exacerbated and never brought under control (41). This phenomenon of hyper-reactivity also seems to be true for osteoclasts derived from T2D hosts, both murine and human. LPS induces an exaggerated response by osteoclasts via increased secretion of pro-inflammatory mediators compared to diabetes-free sources. The continuation of resorption also suggests lack of control of the inflammatory response. Not surprisingly, IL-10, a regulatory cytokine which decreases osteoclast function (212), is secreted in sub-optimal amounts in the db/db and T2D human-derived osteoclast cultures which may account for this lack of control. This defective IL-10 production has also been noted in Breg cells (also of the myeloid lineage) isolated from T2D participants when stimulated with TLR ligands (41). Thus, there seems to be a shared defect in IL-10 secretion in T2D myeloid cells, including osteoclasts, which may lead to exaggerated pro-inflammatory and pro-osteoclastic responses and dampening of IL-10-mediated osteoclast deactivation.

Osteoclast Regulation by Inflammatory Mediators

Precursor Mobilization

Osteoclast precursors from bone marrow can be mobilized out to peripheral sites of bone remodeling by hematopoietic growth factors such as GM-CSF and G-CSF. Chemokines such as SDF-1 induce MMP-9 expression to aid in the mobilization of precursors to bone (212) and are important for precursor survival (353). Interestingly, lower SDF-1 expression leads to precursor cell release from the bone marrow where cells follow the decreasing SDF-1 gradient to bone remodeling sites (2). Pro-inflammatory cytokines such as IL-15 act to increase precursor numbers (212) while chemokines such as MIP-1 α , MIP-3, RANTES, and MCP-3 exert chemotactic effects on these osteoclast precursor populations (353).

GM-CSF and RANTES were found to be elevated in NOD-derived osteoclast cultures treated with LPS suggesting increased capability to recruit T1D osteoclast precursors from bone marrow to the site of resorption. T2D-derived osteoclasts secrete elevated MIP-1 α which would also stimulate osteoclast precursor chemotaxis to the site of resorption where they can differentiate into mature osteoclasts.

Differentiation

Osteoclasts themselves can secrete pro-inflammatory mediators such as TNF- α , IL-6, and IL-1 β which are all potent differentiation factors (324). TNF- α can stimulate osteoclastogenesis even in the absence of RANK-L, yet can augment RANK-L-dependent differentiation. This pro-inflammatory cytokine acts to prime precursor cells in the bone marrow to become osteoclast precursors by up-regulating receptors for M-CSF and RANK-L which can then respond to peripheral TNF- α by becoming osteoclasts (87, 310). TNF- α also induces RANK-L expression on supporting cell types (159).

Chemokines such as IL-8, RANTES, MCP-1, and MIP-1 α stimulate differentiation of osteoclast precursors (212). RANTES, MIP-1 α , and MCP-3 also stimulate chemotaxis of precursors and serve to augment osteoclastogenesis by increasing osteoclast number and size (354). Pro-inflammatory cytokines such as IL-6, IL-11, IL-17, and IL-18 also promote osteoclastogenesis but do so by inducing RANK-L expression on supporting cell types (69). Interestingly, TNF- α , TGF- β , IL-6, IL-11, and IL-18 can substitute for RANK-L when in the presence of M-CSF to promote non-canonical osteoclastogenesis (101).

TNF- α and IL-1 β were found to be secreted in elevated amounts after LPS stimulation in NOD-derived cultures which would indicate a more favorable environment for osteoclastogenesis in a T1D host. Increased TNF- α would also up-regulate the expression of RANK on osteoclast precursors rendering these cells more sensitive to RANK-L stimulation. RANTES was also up-regulated in NOD-derived cultures which would allow for more efficient chemotaxis of osteoclast precursors to the site where they can be activated to differentiate. Alternatively, T2D-derived osteoclasts secrete elevated levels of IL-6, MCP-1, and MIP-1 α which would increase osteoclastogenesis. MCP-1 and MIP-1 α also would lead to augmented precursor chemotaxis and can augment osteoclast size, which is the case in murine T2D-derived osteoclasts.

Activation

TNF- α , IL-1, and IL-6 are important potent stimulators of bone resorption. These pro-inflammatory cytokines activate mature osteoclasts directly by enhancing RANK-L activity and subsequent RANK signaling and indirectly by up-regulating RANK-L on other cells. RANTES, IL-8, and MIP-1 α chemokines serve to stimulate motility of mature osteoclasts (212) yet RANTES and MIP-1 α have no effect on resorption (355).

IL-8, however, is also considered a resorption stimulator and does so independently of RANK-L (212).

Elevated RANTES would allow for increased motility of mature osteoclasts to the site of resorption while TNF- α and IL-1 β , both increased in NOD-derived cultures, would increase osteoclast activation to resorb bone in a T1D environment. This highly pro-resorptive environment seen in NOD-derived cultures would lead to the perfect storm of increased precursor mobilization, osteoclastogenesis, and activation to destroy increased quantities of bone. Decreased anti-osteoclastic IL-10 in NOD-derived cultures would further exacerbate this response. It is also important to remember that with pro-inflammatory cytokine cocktail (TNF- α , IL-1 β , and IL-6) stimulation, NOD-derived osteoclasts resorb almost double the amount of bone than controls which would indicate an increased sensitivity to these inflammatory mediators. Coupled with increased mediator secretion, T1D-derived osteoclasts would therefore be even more sensitive to this inflammatory environment leading to a highly destructive response that cannot be controlled. Similarly, T2D-derived osteoclasts produce elevated MIP-1 α to increase mature osteoclast motility after which increased IL-6 can activate these cells to resorb more bone. Decreased IL-10 secretion by T2D-derived osteoclasts would further amplify this response by lack of this crucial anti-osteoclastic signal. Therefore, T2D-derived osteoclasts would also display increased precursor mobilization, osteoclastogenesis, and resorption induction similar to T1D-derived osteoclasts, albeit by different inflammatory mediators.

Hyperglycemic Effects on Osteoclasts

Differentiation

Hyperglycemic conditions have been shown to have varying effects on osteoclast differentiation (240, 246, 249). Glucose utilization during differentiation is critical for this process to occur and it is assumed that osteoclasts should differentiate more efficiently in hyperglycemic environments. Osteoclasts derived from the murine macrophage immortalized cell line RAW264.7 display optimal differentiation at 5mM glucose yet show similar potential between 2 and 20mM glucose, equivalent to hypoglycemic to hyperglycemic conditions (36-360mg/dL). Differentiation does decrease with 30mM+ glucose concentrations, equivalent to extreme hyperglycemia (540mg/dL), which would most likely be due to toxicity from the hyperosmotic environment (246). Another study found that murine RAW 264.7 osteoclasts in hyperglycemic environments (10 or 25mM equivalent to 180 or 450mg/dL) have decreased differentiation, especially at 25mM glucose (249). While both of these studies do assess osteoclast-specific effects of hyperglycemia, there are performed on immortalized cell lines that may not recapitulate what is seen in primary bone-marrow derived osteoclasts.

Studies performed *in vivo* utilizing a bacterial-induced bone loss model have shown decreased osteoclastogenesis in T2D db/db mice (400-450mg/dL blood glucose levels). Fewer osteoclasts were found in db/db calvarial sections and bone loss was decreased in these animals after *P. gingivalis* inoculation, presumably due to less osteoclasts able to resorb. Baseline resorption capabilities of these osteoclasts in the db/db mouse, however, were not assessed in this study (240). We have shown that after long-term and severe hyperglycemia, osteoclasts derived from db/db mice have augmented differentiation with significantly higher numbers of multinucleated and giant

cells compared to C57BL/6 controls. Conversely, in the absence of long term hyperglycemia, similar numbers of multinucleated and giant osteoclasts were formed in human T2D-derived cultures compared to diabetes-free individuals. These data may suggest augmented RANK signaling in db/db-derived osteoclasts, or a host that has overt hyperglycemia, that may have been caused by irreversible alterations to the precursor population due to a high glucose environment.

Activation

Osteoclasts derived from avian medullary bone utilize glucose as the principal energy source during resorptive function and consume more glucose when cultured on bone. These osteoclasts resorb optimal amounts of bone between 7mM and 25mM glucose, suggesting that hyperglycemia would augment resorptive function (247). Glucose also has been found to up-regulate the expression of V-ATPase which is critical for demineralization of hydroxyapatite (248). High glucose also increases ATP production and increases intracellular calcium levels in osteoclasts, both necessary for the initiation of resorption (333). These findings were recapitulated in mouse RAW 264.7 osteoclasts where maximum cell growth occurred at 20mM glucose (246). However, another study showed that calcitonin receptor, cathepsin K, and MMP-14 were down-regulated in RAW 264.7 osteoclasts while MMP-9 showed little change at 25mM glucose. ROS production, NF- κ B activity, and migration were also decreased in this hyperglycemic environment (249). Again, primary osteoclasts have not been studied in hyperglycemic environments, therefore actual effects of hyperglycemia on function of these cells remains to be determined.

Hyperglycemic T1D NOD mice (~480mg/dL blood glucose) display no change in bone loss measured by serum collagen I telopeptide levels after tibial distraction (273)

nor have increased cathepsin K expression suggesting no increase in osteoclast activity (274). T1D human subjects with hyperglycemia also show no change compared to diabetes-free subjects in regards to plasma collagen telopeptide levels (83) although other studies show increased urine resorption markers in similar T1D subjects compared to controls (9, 137). Conversely, T2D human subjects with hyperglycemia show increased osteoclast function measured by serum TRAP levels and urine bone resorption markers (collagen telopeptides and deoxypyridinoline) (7).

We have found that osteoclasts derived from db/db mice resorb increased amounts of bone via cathepsin K than control mice, however this phenomenon was not observed in human T2D osteoclast cultures. This was found to be due to the increased numbers of multinucleated and giant cells in db/db cultures when cathepsin K expression and collagen degradation was normalized on a per cell basis. Similarly, human T2D-derived osteoclasts do not resorb more bone than diabetes-free controls, which correlates with their similar size distribution. Therefore, increased resorption in db/db osteoclasts is not due to increased activity, but rather to increased numbers of cells able to resorb.

Interestingly, LPS-induced deactivation of osteoclast function is inhibited in both human T2D and murine db/db osteoclast cultures, regardless of the glycemic state of the host. This was also observed in T1D-derived osteoclasts from the NOD mouse, which were normoglycemic at the time of marrow harvest. Pro-inflammatory cytokine and chemokine secretion is also increased in both T1D and T2D-derived osteoclast cultures. While the mechanisms of diabetes differ between these models, it seems that the inflammatory environment that is present in diabetes mellitus alters osteoclast

precursors rendering them unable to deactivate with LPS and more pro-inflammatory in nature.

Possible Adjunct Therapies

Addition of anti-osteoclastic drugs: Anti-RANK-L

Anti-RANK-L, or denosumab, is a high affinity human monoclonal antibody to RANK-L that acts similarly to OPG that is normally secreted by osteoblasts to dampen osteoclastogenesis and bone resorption (356). RANK-L is also important in secondary lymphoid tissue organization and development and when absent, leads to lack of lymph nodes and abnormal spleens (357). Immunodeficiency, however, is not observed in RANK-L deficient mice due to the redundancy of other T cell and DC interactions and administration of anti-RANK-L in humans does not cause immune defects or deficiencies (69). However, reports of cellulitis have been documented in humans undergoing denosumab treatment (358). RANK-L produced by activated T cells can also activate dendritic cells and stimulate autoimmunity as in inflammatory bowel disease (69). Conversely, RANK-L produced by keratinocytes in the skin after UV exposure activates Langerhans cells and may lead to the expansion of Tregs. Therefore, RANK-L not only can activate osteoclasts, but also has effects on the immune system, however these secondary activities may be compensated by other ligands such as CD40 (3). Anti-RANK-L treatment would therefore keep osteoblasts and activated T cells from activating osteoclast precursors to differentiate and mature cells from resorbing bone. Since T1D-derived osteoclasts display augmented function in the presence of RANK-L, it follows that blockade of this osteoclastogenic molecule would dampen this aberrant function and prevent exacerbated bone loss, especially in the case of inflammatory arthritis or periodontal disease.

Addition of anti-osteoclastic drugs: Bisphosphonates

Current anti-resorptive therapies mainly include bisphosphonates such as alendronate (359). Two classes of bisphosphonates are currently used, nitrogen-containing and “simple” bisphosphonates. Nitrogen-containing bisphosphonates such as alendronate and zoledronate are more potent anti-resorptives than the simple class containing drugs such as clodronate. Bisphosphonates act to inhibit osteoclast function either by reducing differentiation, chemotaxis, and resorptive activity or by inducing apoptosis in osteoclasts. Once bound to bone minerals, namely hydroxyapatite, bisphosphonates are then taken up by osteoclasts during the resorption process. Ruffled borders begin to disappear after endocytosis and the cytoskeleton becomes disrupted. Simple bisphosphonates are thought to act as ATP analogues and ultimately lead to cell death due to accumulation of these non-hydrolyzable metabolites. Nitrogen-containing compounds disrupt mevalonate biosynthesis which leads to prenylation of proteins, namely cholesterol and small GTPases. These important signaling proteins regulate cytoskeletal architecture and subsequent cell motility, ruffling of membranes, and transportation of vesicles. Thus, osteoclast differentiation, resorptive function, and survival are all affected by this highly potent class of anti-resorptives (360).

While these drugs are ideal for keeping osteoclasts from resorbing bone, normal wear and tear such as microfractures may not be remodeled and thus healed thereby leading to increased fracture risk. However, low dose administration of these drugs has shown to be efficacious in reducing fracture risk and increasing bone mineralization (360). Therefore, use of bisphosphonates may be a useful adjunct therapy in inflammatory bone pathologies in T1D and T2D, where osteoclasts are aberrantly activated.

Future Directions

Mechanism of inhibition of LPS-induced deactivation

While it is clear that LPS leads to deactivation of resorption by osteoclasts, the exact mechanism of how this occurs is unknown (200, 201). We have shown that IL-10, a regulatory cytokine that leads to inhibition of osteoclasts, is decreased after LPS stimulation in T2D-derived cultures. IL-10 has been known to be produced in decreased quantities by Breg cells derived from participants with T2D (41) as well as macrophages derived from diabetic NOD mice stimulated with LPS (180). It may be that the entire myeloid lineage secretes decreased IL-10 in response to inflammatory stimuli and therefore cannot keep exacerbated responses in check. Determining the response of T1D and T2D-derived osteoclasts to IL-10 is currently being studied in our laboratory where exogenous IL-10 will be added to osteoclast cultures and resorptive capabilities after LPS treatment assessed.

The NF- κ B pathway is also affected in both T1D (186-188) and T2D (41, 206) rendering myeloid lineage cells such as macrophages and dendritic cells inherently more responsive to inflammatory stimuli. I κ B, the inhibitor of NF- κ B translocation, is phosphorylated more efficiently in T1D and thus NF- κ B nuclear translocation is enhanced (186-188). It may be that this enhanced nuclear translocation of NF- κ B leads to increased resorption due to the production of pro-inflammatory cytokines. Further work to determine defects in the NF- κ B pathway are needed to tease out this mechanism.

TLR-4, the receptor for LPS, has also been found to be increased in expression on diabetic NOD bone marrow-derived macrophages. LPS treatment leads to down-regulation of TLR-4 on diabetes-free macrophages however this is not observed on

diabetic NOD macrophages where expression does not change (180). This lack of TLR-4 down-regulation may be a mechanism of aberrant LPS-induced deactivation occurring in our osteoclasts derived from T1D and T2D hosts. Therefore, determining TLR-4 expression before and after LPS treatment on T1D and T2D-derived osteoclasts may lead to a better understanding of the aberrant deactivation.

Mechanism of decreased fusion in T1D osteoclasts

T1D-derived osteoclasts display altered differentiation where fusion is decreased and fewer multinucleated and giant cells are formed. Osteoclast fusion is regulated by many molecules, one of particular interest being DC-STAMP. A T1D mouse model utilizing streptozotocin to destroy β cells showed decreased DC-STAMP expression on osteoclasts along with decreased function yet increased numbers (259). While this model does not correlate as well to human T1D as the NOD mouse, investigation into the expression of this fusigen is warranted and is expected to be decreased on NOD-derived osteoclast precursors.

Regulating receptor expression: Activating (RANK, TNF-R, and IL-1R)

Activating receptors on osteoclasts allow for the initiation as well as propagation of resorption. Because T1D-derived osteoclasts respond to RANK-L and pro-inflammatory cytokines in an exacerbated manner, activating receptors such as RANK, TNF-R, and IL-1R may be altered in expression rendering the cells more easily activated. It has been observed that osteoclasts derived from peripheral blood of diabetic Charcot's participants are also more sensitive to RANK-L-stimulated differentiation, have higher bone resorption capability, and are less able to be deactivated by OPG administration compared to diabetes-free and participants with diabetes without Charcot's (8). These findings suggest increased RANK signaling or increased receptor expression in

diabetes-derived osteoclasts. Another prominent member of the same trimeric cytokine receptor family, the TNF receptor, leads to the activation of the NF- κ B pathway and ultimately activates the cell when bound by its ligand TNF- α (325). TNFR1 is found on osteoclasts, and when stimulated with its ligand, leads to fusion of precursors (87). Similarly, IL-1R bound to its ligands IL-1 α and IL-1 β leads to fusion of osteoclast precursors and activation of resorption (74). Due to the exacerbated response of NOD-derived osteoclasts to the RANK-L with further activation in the presence of a pro-inflammatory cytokine cocktail containing TNF- α and IL-1 β , increased expression of these cytokine receptors or increased intracellular signaling may explain this phenomenon.

Regulating receptor expression: Deactivating (Calcitonin Receptor)

Blood calcium homeostasis is partly regulated by calcitonin which binds directly to osteoclast calcitonin receptors [CTR] and inhibits resorption (350). CTR ligation causes osteoclasts to cease motility and disrupts the ruffled borders leading to cell retraction and loss of resorptive function. Calcitonin also inhibits RANK signaling which leads to decreased osteoclastogenesis and activation (313). While calcitonin is not present in our *ex vivo* osteoclast cultures, determining the true extent of dysregulation in T1D and T2D-derived osteoclasts requires study of both activating and deactivating receptors. Constant CTR ligation over time leads to down-regulation of these receptors where the cell becomes unresponsive to calcitonin treatment (361). Calcitonin levels in participants with diabetes are similar to those without diabetes and therefore would not lead to CTR-ligation-induced down-regulation of CTRs (362). Nonetheless, with decreased CTR expression, one would expect less opportunity for calcitonin to bind and therefore dampened deactivation.

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BIOGRAPHICAL SKETCH

Dana Catalfamo was born and raised in Hammonton, New Jersey and graduated the salutatorian of her class in high school. Shortly after graduation, Dana moved down to Ft. Myers, Florida in 2003 to attend college at Florida Gulf Coast University where she majored in Biology. She graduated magna cum laude and began her graduate career at the University of Florida in August 2007.

Dana originally wished to pursue microbiology during college, however her love for immunology and cell biology shined through after her rotation in Dr. Shannon Wallet's laboratory where she ultimately chose to pursue her dissertation research. Her research interests include innate immunity, autoimmunity, and infectious disease. She plans to attend physician assistant school in the fall to aid in her ultimate career goal of physician scientist specializing in rheumatology.