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Effect of type 2 diabetes on the ability of B and T lymphocytes to stimulate osteoclastogenesis/osteoclastic activity in vitro

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BOSTON UNIVERSITY
HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

**EFFECT OF TYPE 2 DIABETES ON THE ABILITY OF B AND T
LYMPHOCYTES TO STIMULATE
OSTEOCLASTOGENESIS/OSTEOCLASTIC ACTIVITY *IN VITRO***

by

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D.D.S., New York University College of Dentistry, 2016

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Dentistry
In the Department of Periodontology

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DEDICATION

To my mother, father and brother

And all of Hello's Angels

ACKNOWLEDGMENTS

Thank you to Dr. Barbara Nikolajczyk for the opportunity, guidance, and patience for this project. Thank you Dequina, Leena, Basha, and Madhur from the Nikolajczyk lab, and Yang from the Han lab at Forsyth Institute with whom I worked closely with. A special thank you to the soon-to-be Dr. Joseph Lee for the continuous support throughout and friendship while we cared for the cells.

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Lastly, congratulations to Dr. Steven Lin and Dr. Jackie Heath. All the best to your wonderful journey and wishing you a lifetime of love and happiness.

EFFECT OF TYPE 2 DIABETES ON THE ABILITY OF B AND T LYMPHOCYTES
TO STIMULATE OSTEOCLASTOGENESIS/OSTEOCLASTIC ACTIVITY *IN VITRO*

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ABSTRACT

Background

Alveolar bone resorption is more severe in type 2 diabetes-potentiated periodontitis. The key cells responsible for bone resorption are the osteoclast and their quantity and activity are regulated by the RANK/RANKL/OPG system. One of the major RANKL-expressing sources in diseased periodontal tissue are activated B and T lymphocytes, therefore it was hypothesized that type 2 diabetes up-regulates B cell RANKL function.

Objective

The aim of this study was to compare the effect of diabetes on the ability of B and T lymphocytes to stimulate osteoclast activity.

Materials and Methods

Metabolic status (diabetic vs normal) of mice were established using either a low-fat diet (LFD) or high-fat diet (HFD). Spleen lymphocytes were harvested and purified, then cultured in different combinations with adherent mouse leukemic monocyte/macrophage cell line cells over dentin-coated wells and observed for

osteoclastic activity that breaks down the dentin-coating. The area of clearance was used to represent the level of activity.

Results

No difference in osteoclastic activity were noted between the two metabolic statuses, between B or T cells, between lymphocyte stimulated or not.

Conclusion

The findings counters our hypothesis, and are inconsistent with the currently available evidence. Repeat of the experiment is warranted before valid conclusion can be drawn from the data.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DMEM.....	Dulbecco's Modified Eagle Medium High glucose
HFD	High Fat Diet
IL	Interleukin
IPGTT	Intra-Peritoneal Glucose Tolerance Test
LFD.....	Low Fat Diet
LPS	Lipopolysaccharides (LPS)
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor Activator of Nuclear Factor κ B Ligand
RBC	Red Blood Cell
RGB.....	Red Green Blue
TNF.....	Tumor Necrosis Factor
TRAP	Tartrate-Resistant Acid Phosphatase

INTRODUCTION

Periodontitis is a microbially-associated, host-mediated inflammatory disease characterized by loss of periodontal attachment, periodontal pocket formation, and alveolar bone resorption, followed ultimately by tooth loss [1, 2]. Although periodontitis is a highly prevalent disease that affects approximately 46% of the US adult population, understanding of the exact pathogenesis continues to be updated [3]. Current evidence supports the presence of bacterial biofilm, or dental plaque, along the root surface, which initiates gingival inflammation [2]. Overall disease manifestation and progression are further determined by the immune response to the bacterial complexes in the biofilm [4]. In some patients, the disease may remain confined to the gingival tissue or be slowly progressing and thus does not endanger tooth stability. On the other hand, the disease progresses in susceptible patients in whom host mechanistic pathways that were originally programmed to protect against the microbial challenge spiral into an uncontrolled catabolic process [5]. Host-derived enzymes capable of destroying periodontal attachment apparatus become activated, which subsequently results in apical migration of the junctional epithelium, i.e. periodontal pocket formation, and allows further apical spread of the bacterial biofilm along the root surface, advancing disease severity [2, 3].

Severity of periodontitis is measured as clinical attachment loss or radiographic bone loss [6]. Gingivitis is the mildest and entirely reversible form of periodontal disease. While signs of gingivitis is almost ubiquitous in all populations, and a minor loss of periodontal tissue support is frequent in adults, between 5-15% of the global population

suffers severe forms of periodontitis [6]. According to the American Academy of Periodontology, a variety of factors can influence the wide spectrum of periodontal disease severity, including genetic susceptibility, social habits, home care, medications, and more. Among these, diabetes mellitus has been recognized as a bona fide risk factor [7]. Individuals with diabetes are more likely to be affected by higher severity and extent of periodontitis at an earlier age, and experience smaller degrees of improvements following treatment and higher rates of tooth loss [8].

Diabetes mellitus is a common metabolic disease with 6.4% or 285 million adults afflicted globally. Prevalence and incidence are expected to continue rising in the coming decades [9, 10]. This disease, commonly called type 2 diabetes, is characterized by impaired glucose homeostasis resulting from defects in insulin secretion and/or insulin action [11]. In poorly managed diabetes, hyperglycemia predisposes patients to a range of complications, including retinopathy, nephropathy, neuropathy, macrovascular diseases and poor wound healing [12]. Consistent evidence shows that diabetes acts as a modifying factor for periodontal disease [4], particularly in people with poor glycemic control [13]. Individuals with diabetes develop an earlier and stronger gingival inflammatory response than do those without diabetes, and these affected individuals experience greater severity of periodontitis [4, 14].

There are several proposed mechanisms for the role of diabetes in periodontal disease and these can be considered as alterations in the vascular, cellular, and repair processes of the host. However, the exact mechanisms underlying the relationship between periodontitis and type 2 diabetes remains unclear.

One demonstrated link between periodontitis and type 2 diabetes is altered B cell functions [15, 16]. It has recently been shown that type 2 diabetes-associated changes in B cells are required for the osteoclastogenesis and pathologic bone resorption in a pathogen-induced periodontitis mice model [17]. Furthermore, under the same experimental condition, T cells cannot drive the alveolar bone loss in the absence of B cells [17]. These findings are consistent with a model in which B cells play both direct and indirect roles in type 2 diabetes-potentiated periodontitis, particularly in the resorption of bone.

Bone is a dynamic and active tissue that is constantly undergoing renewal in response to mechanical, nutritional, and hormonal influences [18]. Under physiologic conditions, there is a balance between the carefully regulated and coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts [19]. As osteoclasts are the prime bone resorptive cells, their activity is the determinant for alveolar bone loss. The rate of bone resorption is regulated at the level of differentiation of osteoclasts from their hematopoietic precursors [18, 20, 21]. The activation and differentiation of osteoclasts are regulated by three members of the TNF ligand and receptor superfamilies: RANKL (Receptor Activator of Nuclear Kappa-B Ligand), RANK, and Osteoprotegerin (OPG) [18]. RANKL (redundancy deleted), binds to its receptor RANK on osteoclast or pre-osteoclast cell surfaces to promote proliferation and differentiation [1].

Given that RANKL determines the rate of osteoclastogenesis and thereby the rate of bone resorption, higher level of RANKL may be associated with higher severity of

periodontitis according to numerous recent studies. RANKL-expressing sites had deeper periodontal pockets compared with the RANKL-negative sites [22]. Conversely, sites of advanced periodontitis have the highest level of RANKL mRNA, expressed mainly in lymphocytes and macrophages, compared to healthy or less severe periodontitis [23]. . These results suggested that enhanced immune cell RANKL production (“might be” is redundant) associated with alveolar bone resorption. Moreover, not only is RANKL upregulated, the decoy receptor that inhibits RANKL activity, OPG, is also down-regulated in periodontitis tissue [24]. The higher RANKL:OPG ratio favors bone resorption. This ratio has been reported to be further upregulated in smokers and diabetics [25], consistent with the observation that in type 2 diabetes-potentiated periodontitis, the alveolar bone loss is more severe.

Finally, given that 1. Activated B and T lymphocytes are one of the major RANKL-expressing sources in diseased periodontal tissue [1] [24]; 2. Alveolar bone loss is more severe in type 2 diabetes-potentiated periodontitis; and 3. B cells play critical roles in periodontitis, I hypothesized that type 2 diabetes up-regulates B cell RANKL function, which uniquely potentiates periodontal complications of type 2 diabetes (Figure 1) through mechanisms that are alternatively independent and dependent on T cell. The long-term goals of the Nikolajczyk lab is to define the mechanisms that potentiate periodontitis in type 2 diabetes and to use this knowledge to develop new methods to control inflammatory type 2 diabetes complications. Within this scope, the specific goal of my thesis was to study the effect of diabetes on the ability of B and T lymphocytes to stimulate osteoclast activity.



Figure 1. Model of B cell function in type 2 diabetes potentiated periodontitis

METHODS

Animal model with fat-induced diabetes

Five-week old male mice (DIO-B6-M; C57BL/6J) were used as the model animal. Two metabolic status groups were established via control of fat content in diet for a total of sixteen weeks: 1. HFD (High Fat Diet) group, consisting of mice fed ad libitum with chow where 60% of calories were from fat (Research Diets D12492i); 2. LFD (Low Fat Diet) group, consisting of mice fed ad libitum with chow where 10% of calories from fat (Research Diets). Metabolic measurements were performed on the 9th week and 15th week of diet and included intra-peritoneal glucose tolerance tests (IPGTT). Blood glucose levels were measured by glucometer by scoring the tip of the tail and measuring at 0, 10, 20, 30, 60, 90, and 120 minutes. The body weight of mice was measured weekly and just prior to sacrifice at 16th week (or 21 weeks of age).

Preparation of Splenocytes

Animals were sacrificed by CO₂ narcosis and skin was sterilized with 70% alcohol. An incision was made on the left abdomen to expose the abdominal cavity. The spleen was harvested and placed in RPMI medium 1640 (Gibco Life Technologies).

The specimen was minced using the plunger end of a syringe through a 70- μ m cell strainer submerged in RPMI medium in a 3-cm petri dish. The mixture was transferred to a 15ml conical tube and centrifuged at 1,200 rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 3ml of RBC lysis buffer (eBiosciences) at 37°C and incubated at room temperature for 6 minutes. 12ml of RPMI

was added to the suspension then centrifuged at 1,200 rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended in PBS (Corning Incorporated) with 1% Bovine Serum Albumin (BSA – Fraction V) for cell sorting.

Fluorescence-activated cell sorting (FACS) Cell Sorting

The prepared splenocytes suspension was stained with an antibody cocktail containing live/dead indicator (Zombie aqua), hematopoietic cell marker CD45, B cell-specific markers CD19 and CD20, and the T cell-specific marker CD4. The cells were then washed and resuspended in 3ml of FACS buffer (PBS with 1% BSA). A total of 400,000 B and T lymphocytes were sorted into 10ml of RPMI medium. The sorted cells were centrifuged at 1,200 rpm for 10 minutes, and the pellets were resuspended in RPMI medium at 50,000 cells per 50 μ l (10^6 cells/ml) for osteoclastogenesis assays.

RAW cell preparation

An adherent mouse leukemic monocyte/macrophage cell line, RAW 264.7, was harvested from culture by aspirating media and adding 500 μ l of PBS-based non-enzymatic cell detaching buffer. The cell suspension was incubated for 5 minutes at 37°C. The cells were transferred to a 15ml conical tube with 4.5ml Dulbecco's Modified Eagle Medium High Glucose (4.5g/L) (DMEM) with 10% FBS, 1% penicillin/streptomycin and 4.0mM L-glutamate, and centrifuged at 1,200 RPM for 10 minutes. The supernatant was removed and the cell pellet was resuspended in Minimum Essential Medium Eagle – Alpha Modification (Modified MEM) with 10% FBS, 1% penicillin/streptomycin and

4.0mM L-glutamate. The cells were counted using a hemocytometer. The cell suspension was diluted to a concentration of 1×10^4 cells/ml using Modified MEM. The cell suspension was transferred to a 96-well flat bottom plate at 100 cells/well. Recombinant RANKL was added for pre-stimulation to the RAW cells after 6 hours of incubation.

B and T cell Stimulation

For single culture experiments, 1000 of each unstimulated B and T cells were added to each well of RANKL-primed RAW cells. For B and T co-culture experiments, B or T cells were added at 10:1 ratio according to the experimental setting.

RAW cells only were the negative control and RAW cells primed with recombinant RANKL was the positive control. For cultures containing stimulated B cells, the B cells were treated with 10 μ g/ml of E. coli lipopolysaccharides (Invivogen) for 24 hours then washed with RPMI three times prior to transfer to remove LPS prior to addition to RAW cultures. For cultures containing stimulated T cells, the T cells were treated with α CD3/28 antibody-coated Dynabeads (Life Technology) for 24 hours prior to addition to RAW cultures. Supernatants from all cultures were collected for RANKL enzyme-linked immunosorbent assay (ELISA) after 24hrs incubation. Direct RANKL protein measurements in cell supernatants by ELISA were below the level of detection.

Pit formation on Dentin Plates

A second independent set of assays were performed on 96-well format dentin plates for pit formation analysis, a functional measure of the ability of immune cells to

support osteoclastogenesis. 96-well flat bottom plates were used for cell culture for both single and co-culture of B and T lymphocytes. Cells were incubated in these plates (stimulated or unstimulated) then transferred to a second assay performed on 96-well dentin plates (Corning, CLS3988). Anti-RANKL antibody was added to inhibit the interaction between RANK and RANKL therefore osteoclastic activity/osteoclastogenesis which serves as the negative control. The dentin plates serve as a mineralized surface that osteoclasts, if present, would degrade the dentin coating and create a zone of clearance. RAW cells were allowed to adhere to the plates during an incubation period of at least 6 hours. Pre-stimulation of the seeded cells were completed by incubating with RANKL for two days to generate pre-osteoclasts. The medium was changed to remove the added RANKL. B and T cells were introduced as outlined below and incubated for three days to determine the development of functional osteoclasts from the pre-osteoclasts. These plates were used to quantify osteoclastic activity.

Tartrate-resistant acid phosphatase (TRAP) staining for osteoclast formation

After 1000 RAW cells were plated on the 96-well flat bottom plate, 100 μ l of murine RANKL in modified MEM(100ng/ml) was added to each well (final concentration of RANKL was 50ng/ml). Different combinations of B and T cells were added to each well according design (table 1). Media was replenished every other day by removing 100 μ l medium and adding 100 μ l fresh medium containing the same concentration of murine RANKL. After 5 to 7 days of incubation, the cells were stained

using a Tartrate-resistant acid phosphatase (TRAP) stain to quantify the presence of osteoclasts

Pit Assay Analysis

Dentin wells were treated with 1:4 bleach and von Kossa stain to show pit surface. The cleared areas that indicate osteoclastogenic function show up as dark under bright field. Digital images were taken with a Nikon camera. All dentin plate images were taken at a standardized size. The cleared areas were marked with red (RGB 255, 0, 0) using Adobe Photoshop by two operators blinded of the experimental groups. The resulting labeled images were processed through a customized software written in Java that counts the number of RGB (255, 0, 0) pixels (Jerry Chiu, unpublished).

Statistical analysis of the data was performed by GraphPad Prism using 1-way ANOVA with Tukey post-hoc test for corrections for multiple comparisons. A p-value of ≤ 0.05 was considered statistically different.

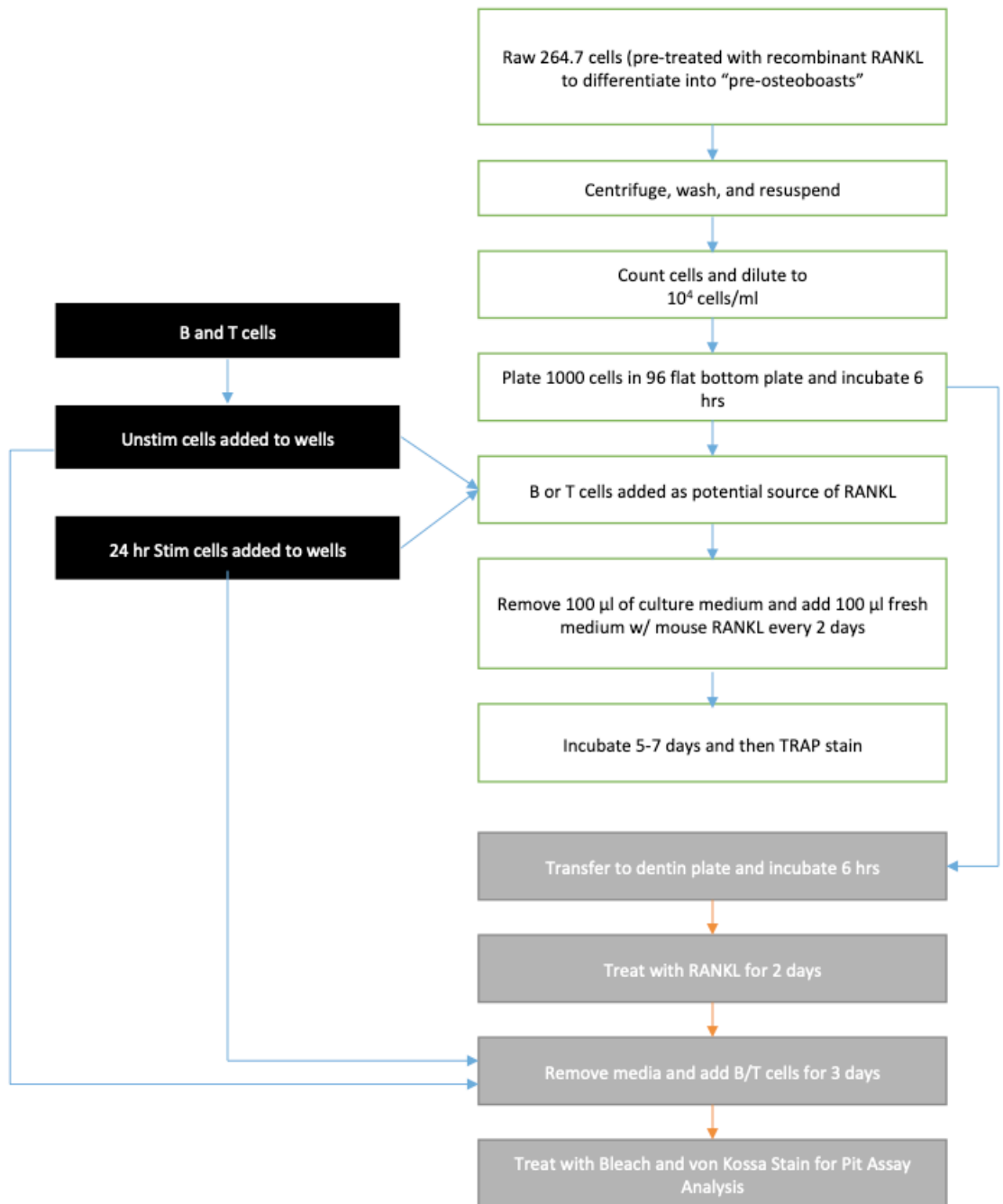


Figure 2. Summary of experimental design for TRAP stain and Pit assay. White boxes show TRAP protocol, gray boxes show PIT assay protocol, and Black boxes show addition of B and T cells in both experiments.

RESULTS

Metabolic status of model animal

Mice were fed with either low fat diet (LFD) or high fat diet (HFD) for 16 weeks. Body weight and fasting glucose level were measured and intra-peritoneal glucose tolerance test (IPGTT) was performed at the 15th week of diet. HFD mice was significantly heavier and had higher fast glucose level. IPGTT showed a slower glucose clearance over 120 minutes in HFD compared to LFD mice. These results indicate impaired glucose homeostasis, which in turn confirms the diabetes-like phenotype in HFD mice.

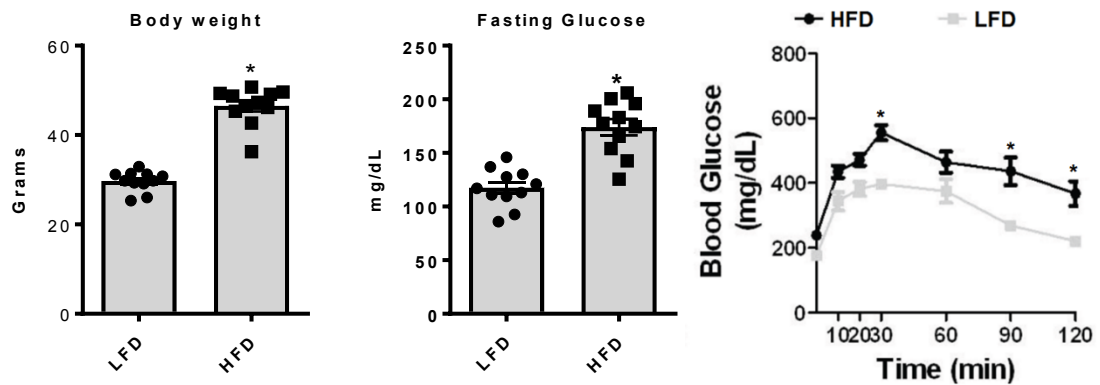


Figure 3. Body weight, fasting blood glucose levels, and intra-peritoneal glucose tolerance test of mice on LFD and HFD.
Time = 15th week of diet (n = 11 *p<0.05).

Splenocyte sorting

Splenocytes harvested from LFD and HFD mice were sorted for into four groups of cells: B lymphocytes from LFD mice, B lymphocytes from HFD mice, T lymphocytes from LFD mice, and T lymphocytes from HFD mice (Figure 4).

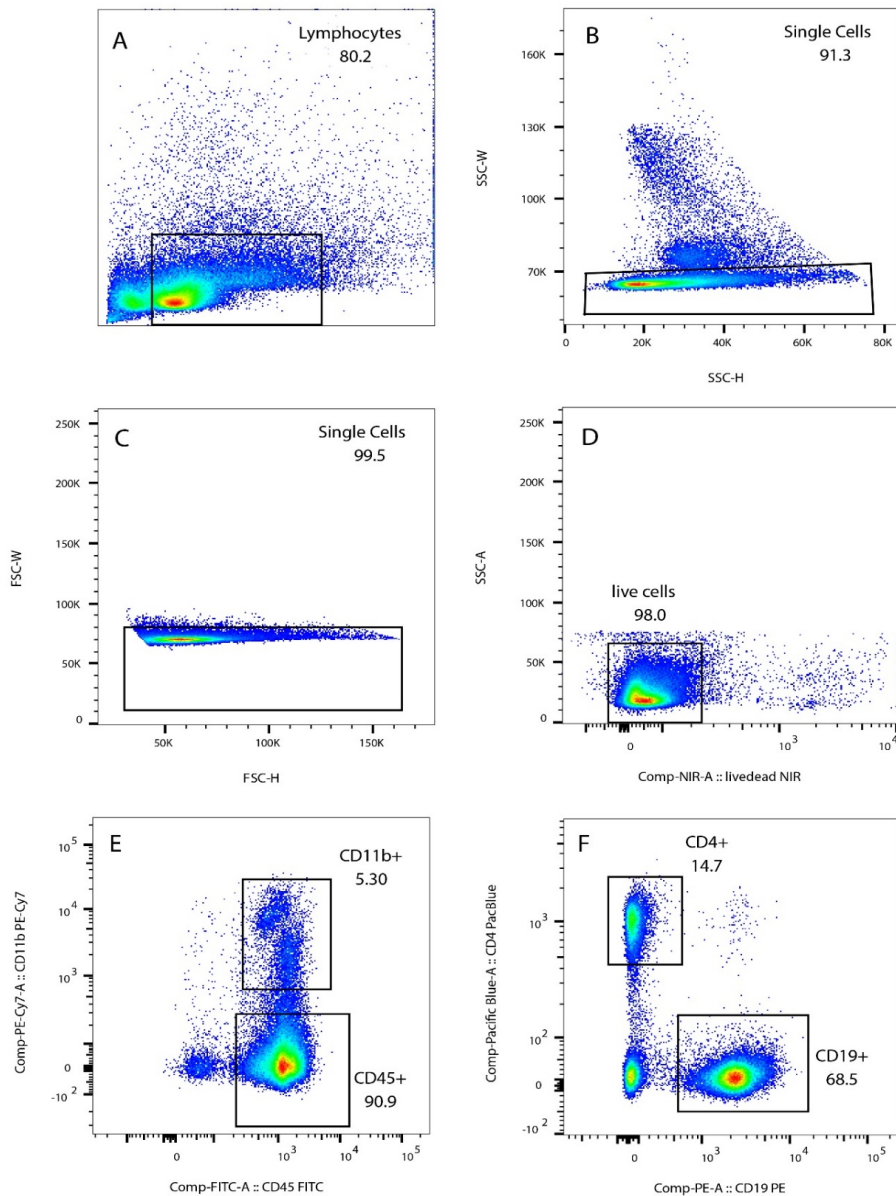


Figure 4. Flow cytometry gating.

(A) Lymphocytes. Bottom left cluster represents debris and top right scattered points represent dead cells. (B, C) Single cells. Double discrimination based on side scatter and forward scatter excluded cells stuck together. (D) Live cells. Zombie NIR live-dead stain used to exclude dead cells. (E) CD45+ Leukocytes. Macrophages were excluded using CD11b+ surface marker. (F) CD4+ T helper cells and CD19+ B cells were purified from gates shown. We obtained the expected B:T ratio of approximately 1:4.5 from C57BL/6 mouse splenocytes

Tartrate-Resistant Acid Phosphatase (TRAP) staining of osteoclasts

Cells were plated in single culture and co-culture combinations as summarized (Table 1). Number of osteoclasts were measured following TRAP stain. RAW cells alone was used as control and background for TRAP staining (Figure 5A). Positive control was represented with RAW cells stimulated with RANKL (Figure 5B). Negative control was represented with RAW cells treated with RANKL and anti-RANKL (Figure 5C). TRAP seeding conditions which show osteoclastic activity in stimulated B cells from LFD mice(Figure 5D). Direct RANKL protein measurements in cell supernatants by ELISA were below the level of detection.

RAW cells	RANKL	Anti-RANKL	B cells	T cells
<input type="checkbox"/>				
<input type="checkbox"/>	<input type="checkbox"/>			
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
<input type="checkbox"/>	<input type="checkbox"/>		Unstimulated, Lean	
<input type="checkbox"/>	<input type="checkbox"/>		Unstimulated, Obese	
<input type="checkbox"/>	<input type="checkbox"/>			Unstimulated, Lean
<input type="checkbox"/>	<input type="checkbox"/>			Unstimulated, Obese
<input type="checkbox"/>	<input type="checkbox"/>		Stimulated, Obese	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Stimulated, Obese	
<input type="checkbox"/>	<input type="checkbox"/>		Stimulated, Lean	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Stimulated, Lean	
<input type="checkbox"/>	<input type="checkbox"/>			Stimulated, Obese
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		Stimulated, Obese
<input type="checkbox"/>	<input type="checkbox"/>			Stimulated, Lean

□	□	□		Stimulated, Lean
□	□		Stimulated, Lean	Stimulated, Lean
□	□		Stimulated, Lean	Unstimulated, Lean
□	□		Unstimulated, Lean	Stimulated, Lean
□	□		Stimulated, Obese	Stimulated, Obese
□	□		Stimulated, Obese	Unstimulated, Obese
□	□		Unstimulated, Obese	Stimulated, Obese
□	□		Stimulated, Lean	Stimulated, Obese
□	□		Stimulated, Obese	Stimulated, Lean

Table 1. All culture combinations for B and T cells.

White cells indicate cultures of single purified cell types. Shaded cells indicate co-cultures of purified B and T cells from mice fed as indicated.

TRAP staining analysis was able to demonstrate presence of osteoclastic activity. However, quantifying the osteoclastic activity was difficult because of high background noise due to some non-specificity in our staining. Analysis of the measurements based on TRAP staining showed no significant differences in stimulated and unstimulated B and T

cells (Figure 6). We therefore pursued pit forming assays on dentin plates with von Kossa staining as a more accurate measurement of osteoclastic activity.

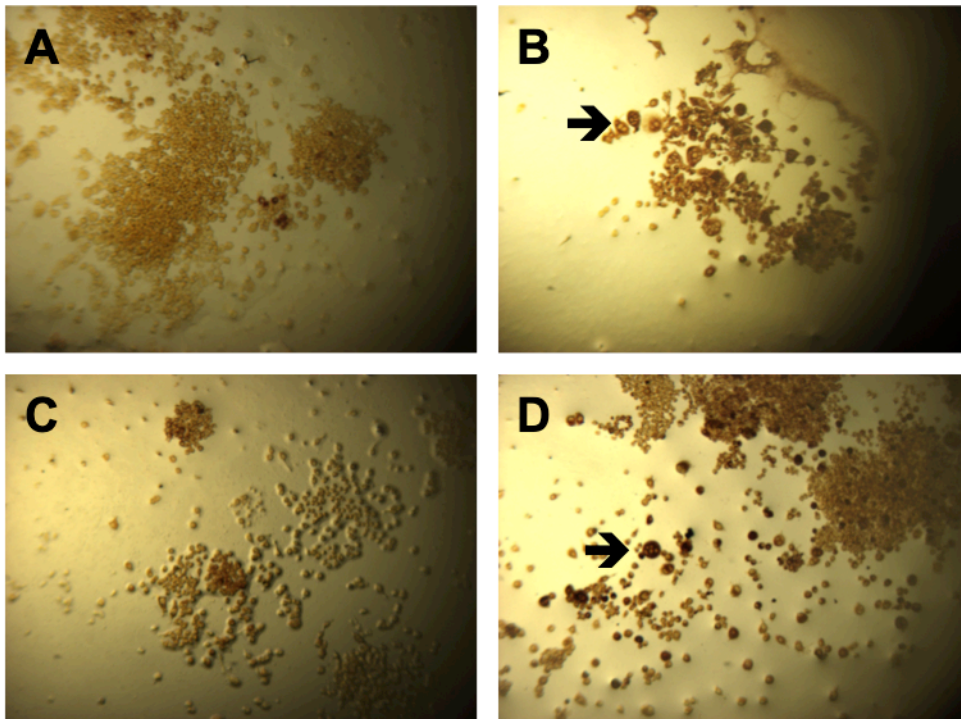


Figure 5. Magnified images of TRAP staining.

(A) Background represented by RAW cells. (B) Positive control, represented by RAW cells stimulated with RANKL twice: once to form pre-osteoclasts, and a second time for full differentiation to osteoclasts. (C) Negative control represented by RAW cells treated with RANKL and anti-RANKL. (D) TRAP staining of stimulated B cells from lean (LFD) mice. Arrow indicates multinucleate osteoclasts.

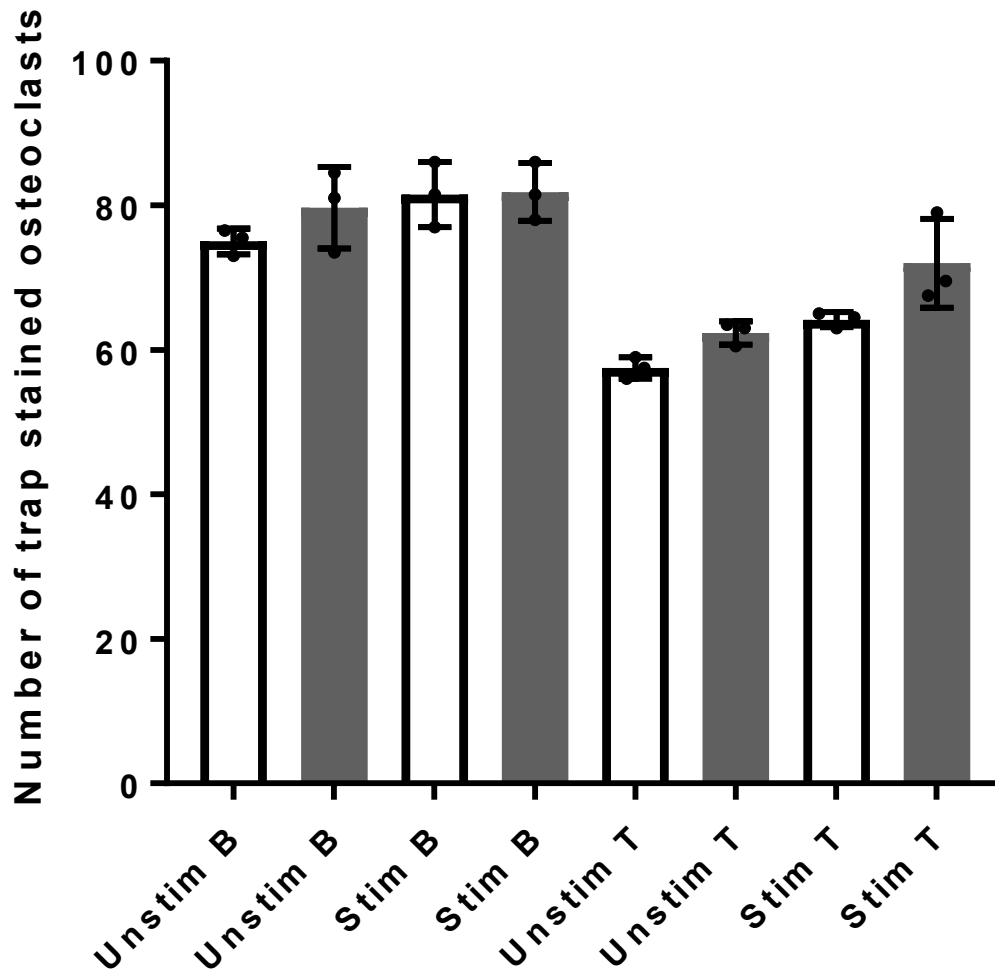


Figure 6. TRAP stained osteoclasts in test groups.

White bars show LFD groups and gray bars show HFD groups. Significant differences were calculated using a one-way ANOVA. Data presented as mean \pm standard error, $p < 0.05$. Analysis of trap data showed no significant differences in stimulated and unstimulated B and T cells

Application of digital tools in routine laboratory tests

Previous protocol for measuring dentin plate pit formation area was primarily manual and labor-intensive. I developed a new approach to processing such data as part of my project to improve the efficiency for the repetitive and mechanical tasks and compiling measurements without affecting the quality of measurements. The unprocessed digital micro-photographs of the dentin plates were analyzed manually to identify the area of clearance and labeled using Adobe Photoshop. Taking advantage of the image processing software I developed offered a much higher user-friendliness than common research lab tools such as ImageJ from National Institute of Health (NIH). This software allowed the simple tasks of identifying area of clearance and coloring the area with an arbitrary color to be completed with ease.

I created a custom-made software designed to process the resulting digital photographs in the Java programming language. The central algorithm used processed the digital image as a two-dimensional array of pixels. Each pixel was tested for its RGB (Red, Green, Blue) value. As the algorithm cycles through the array, each pixel with our specified RGB will contribute to size count of the clearance area. At the end of the algorithm, the output will consist of two numbers: the total number of pixels, and the number of pixels of the specified RGB value. Then, by combining these two numbers and the condition of the digital photograph, the actual area of clearance can easily be calculated. The software automatically process the entire collection of digital photos, obtains the data, then further compiles the collected information into Microsoft Excel format for later statistical analysis.

Pit Assay on Dentin Plate

Representative sample for analysis of pit formation area is shown in Figure 7.

Results from pit assay on dentin plate for osteoclastogenesis are shown in Figure 8.

Single cultures yielded no difference between any of the test groups. No difference in the promotion of osteoclastogenesis, as measured by pit formation area was among B and T cells from LFD and HFD mice, regardless of stimulation. The only statistically significant difference was noted between RAW cells (pre-osteoclast negative control) and RAW pre-osteoclasts with RANKL added (positive control). There is a trend of unstimulated lymphocytes producing higher pit area formation compared to stimulated lymphocytes, which is inconsistent with the current understanding that stimulation will increase RANKL production.

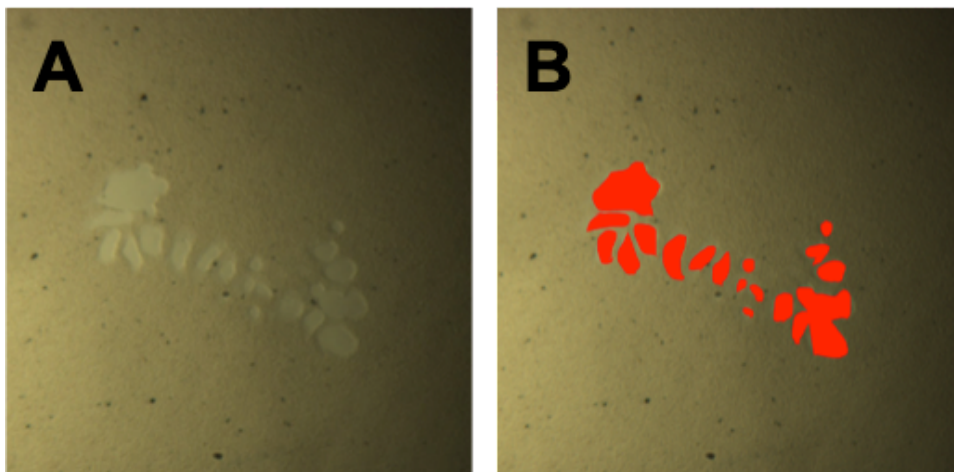
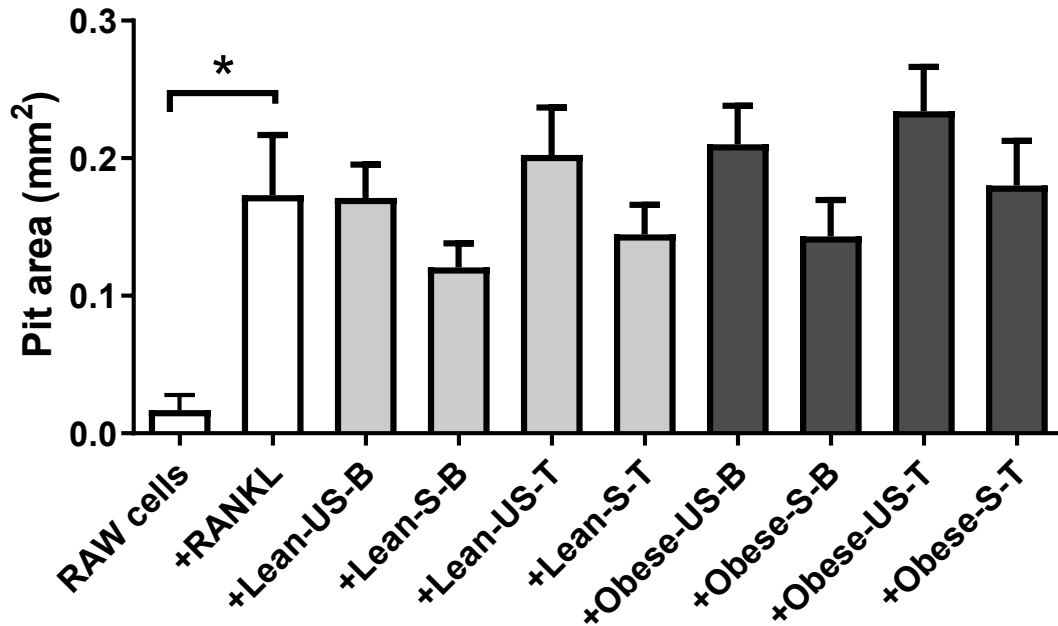


Figure 7. Representative image of pit formation on dentin plate analysis. (A) Original digital photograph of 96-well coated with dentin. Cleared areas indicate osteoclastic activity. (B) Cleared areas labeled using Photoshop (Adobe) with RGB value (255, 0, 0) then measured by customized software (Jerry Chiu).

Pit assay data with Stim and Unstim cells



*P < 0.05; N=11; RANKL = 50 ng/ml

Figure 8. Pit areas from pit assay on dentin plate for osteoclastogenesis.

RAW cells only (Control), pre-osteoclast RAW cells stimulated with RANKL (Positive control). Pit area represents osteoclastic activity which indicates osteoclastogenesis. Stimulation of lymphocytes as described in materials and methods. No significant differences noted test groups, via two-way ANOVA. Data presented as mean \pm SEM; *P < 0.05.

Requirement of RANKL for Osteoclastogenesis

RAW cells in pit assay are pre-stimulated with RANKL to differentiate into pre-osteoclasts. Anti-RANKL antibody was added to inhibit the interaction between RANK and RANKL to determine the requirement of RANKL for osteoclastogenesis. Significant reduction in osteoclastic activity/osteoclastogenesis was noted compared to the corresponding group treated with a negative control isotype control antibody instead of

anti-RANKL (Figure. 9) . Furthermore, we detected no differences between RAW pre-osteoclast cells stimulated with RANKL only and RAW pre-osteoclast cells stimulated with RANKL and isotype antibodies (Figure. 9).

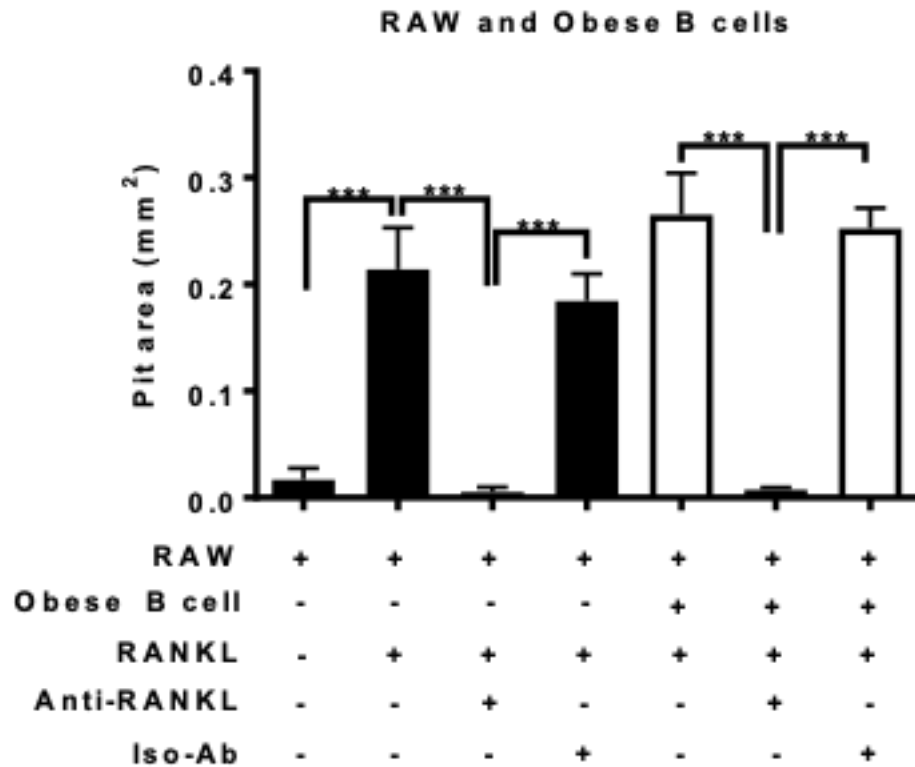


Figure 9. Inhibition of RANKL by anti-RANKL antibody.

Treatment with anti-RANKL significantly reduces pit area compared to corresponding groups. Significant difference calculated by one-way ANOVA. Data presented as mean \pm SEM; *P < 0.05.

Single and Co-cultures of stimulated and unstimulated B and T lymphocytes

Previous work from our lab showed B cells support T cell inflammation in T2D [26]. To test whether lymphocyte cross-talk also potentiates osteoclastogenesis in T2D, we co-cultured B and T cells in the presence or absence of stimulation prior to adding them to RAW pre-osteoclast cultures. We determined osteoclastic activity through RANKL production and the pit areas in these co-cultures (Figure 10).

Stimulated B cells from LFD mice produced the highest osteoclastic activity when co-cultured with stimulated T cells from LFD mice. This level of activity was significantly higher than the osteoclastic activity produced from the co-culture of their counterparts from HFD mice. Co-cultures of B cells and T cells from lean mice significantly increased osteoclastogenesis relative to other cell combinations, as measured by an increase in pit area. No significant difference amongst the unstimulated co-cultures was noted. The data suggest that T lymphocytes have some effect on osteoclastic activity when co-cultured with B lymphocytes (or vice versa) within lean hosts suggesting non-redundant RANKL production relative to single cell type cultures.

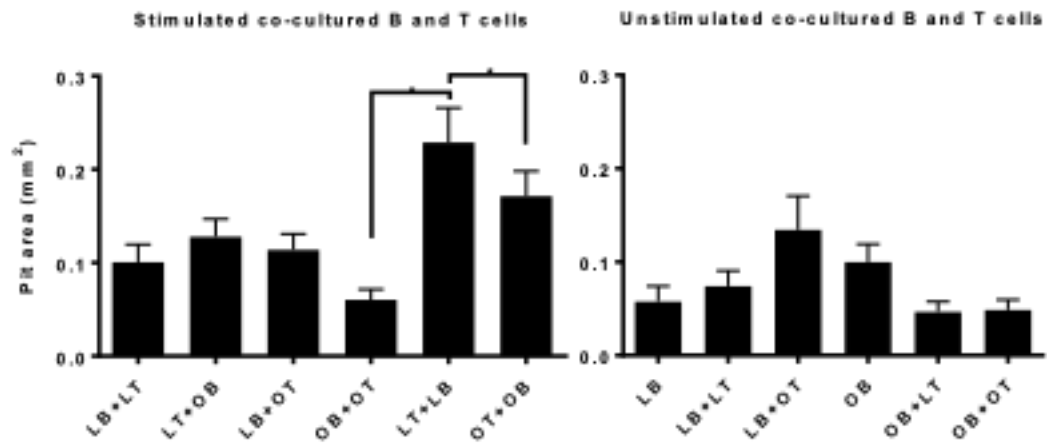


Figure 10. Single and co-cultures of stimulated and unstimulated B and T lymphocytes.

Co-culture ratios are 1000:100 cells shown as written. Significant difference calculated by one-way ANOVA. Data presented as mean \pm SEM; *P < 0.05. “L” in LB and LT groups indicate “lean”, as in the hosts were fed with low fat diet. “O” in OB and OT groups indicate “obese”, as in the hosts were fed with high fat diet. “B” and “T” indicates B or T cells.

DISCUSSION

Type 2 diabetes and Periodontitis

Our long-term goals are to define the mechanisms that potentiate periodontitis in type 2 diabetes and to use this knowledge to develop methods to control inflammatory complications in patients with type 2 diabetes. B cells have a critical role in experimental type 2 diabetes-associated periodontitis in our animal model: the type 2 diabetes-associated changes in B cells were shown to be required for osteoclastogenesis and therefore periodontal bone loss in a diabetic host. Furthermore, without B cells, T cells cannot drive periodontal bone loss in diabetic hosts [17]. Our published findings in obese/T2D mice contrasted with evidence showing T cells promote periodontal bone loss in lean hosts [27]. To further explore this relationship, we tested the hypothesis that type 2 diabetes-associated changes in B and/or T lymphocytes leads to up-regulation of osteoclastogenesis and thereby periodontal bone loss.

The rationale for studying the level of bone resorptive osteoclasts stems from using periodontal bone loss as the primary parameter for determining the severity of periodontitis. Osteoclasts are the primary cells known for bone resorption. Their level of activity depends on their quantity, and osteoclastogenesis is regulated through the RANK/RANKL/OPG system. With upregulated level of RANKL, the system favors osteoclastogenesis and thereby increases the osteoclastic activity. Both B and T cells are major sources of RANKL [24]. These cells' expression of RANKL can be altered by inflammatory mediators which is altered in the diabetic conditions via the accumulation of AGE and their interaction with RAGE [28]. Therefore, by comparing B and T cells

from diabetic and metabolically normal mice and studying the downstream effect on osteoclastic activity, we may gain further insight into the relationship between diabetes and periodontal bone loss.

From this study, when the groups of lymphocytes were tested individually, i.e. single cell cultures where RAW cells were cultured with B cells only or with T cells only, no difference was detected among the experimental groups. No difference was measured in terms of osteoclastogenesis regardless of metabolic status (HFD vs LFD), stimulation vs not, or between B and T cells. This lack of difference may be interpreted as diabetes does not impact the severity of bone resorption. This finding is inconsistent with the current consensus that the periodontal destruction is more severe in diabetic individuals compared to a metabolically healthy individuals, which would have predicted cells from HFD hosts to have increased level of osteoclastic activity. Furthermore, comparing the levels of osteoclastic activity of the experimental groups to the positive control, showed no statistical difference. Given that the level of RANKL directly affects osteoclastogenesis, I conclude that the concentrations of bioactive RANKL are similar among all the groups, excepting expected results from the controls.

Our data clearly show that metabolic status and/or stimulation with lipopolysaccharide or α CD3/28 antibody do not affect RANKL production as measured by downstream osteoclastogenesis. However, this conclusion countered our original model where the latter group would have been predicted to have the highest activity, and also counters the current understanding of changes associated with diabetes regarding inflammation[7] and osteoimmunology[1]. This discrepancy may be attributed to our ex

vivo approach that may not have accurately recapitulate the biology of the in vivo system- our tissue culture well and dentin plates are quite different compared to the oral cavity, and the cellular components and interplay in vivo are more complex in the oral cavity. Although it is formally possible that lymphocyte RANKL play no role in T2D-associated periodontal disease, the abundance of evidence supporting this idea indicate other approaches may be needed to test our hypothesis. Therefore, it may be prudent to repeat the single culture experiments before drawing conclusions.

B cells were previously shown to play a significant role in periodontal bone resorption in vivo[17]. In the current experiment, B cells were co-cultured with T cells to observe for any changes. The co-culture with stimulated B and T cells from HFD mice demonstrated higher osteoclastogenesis compared to most other groups, second only to the co-culture with stimulated B and T cells from LFD mice. The higher osteoclastogenesis from cells from HFD mice was consistent with current evidence and in agreement with our proposed model, but the highest osteoclastogenesis from cells from LFD mice was unexpected. While evidence suggests T cells to promote osteoclastogenesis in lean hosts, which is consistent with this finding, diabetic individuals have an exaggerated inflammatory reaction and more severe bone resorption, therefore osteoclastogenesis is expected to be greater in diabetic hosts than in lean hosts. This may suggest unknown interactions from T cells within metabolically normal hosts.

In a recent review article examining the relationship and interactions between diabetes mellitus and periodontitis, the ‘two-hit’ model was suggested [5]. In this model, which offers one explanation for the pathogenesis of accelerated periodontal destruction

in patients with diabetes mellitus, both exaggerated inflammatory response and destruction and impaired repair are considered. Hyperglycemia from poorly managed diabetes results in accumulation of advanced glycation end-products (AGE) which interact with their receptors (RAGE). This interaction leads to exaggerated inflammatory response to the bacterial challenge such as the dental plaque in the gingival sulcus. Then, increased destruction results from the upregulated expression of RANKL that contribute to osteoclastogenesis and therefore bone resorption [29]. Our findings from the current study is consistent with this proposal and adds to it with further details into the roles of B and T lymphocytes in this interactions depending on the host's diabetes condition. Future focus on impacts of the AGE/RAGE system in lymphocytes will be important towards testing this possibility.

The second 'hit' in the 2-hit model involves impaired repair. This stems from the increased apoptosis of bone-lining cells, which result in impaired bone formation following bone resorption and contribute to the net loss of bone [30] [29]. This effect on bone-lining cells may be attributed to the activity from RAGE. In diabetic mice, inhibition of RAGE can improve healing of excisional wounds [31]. Also, it was shown in a mice skull model with cultured osteoblasts that AGE-RAGE interactions contribute to delayed bone healing and RAGE mediates apoptosis of osteoblasts [32] [33]. Therefore, it may be reasonable to anticipate inhibition of AGE-RAGE interaction to improve the periodontitis severity. A study on rats treated with aminoguanidine which inhibits the AGE-RAGE interaction [34] showed reduced periodontal bone loss during experimental periodontitis induction, this treatment did not result in a different bone level

at the conclusion of the study when compared with treatment using normal saline.

However, it is unclear whether the model animals were in a condition that resembled uncontrolled diabetes, which leaves a possible opportunity to further apply our existing animal model, as we did not test the importance of B cells in repair of periodontal lesions.

Concluding the above information, possible future opportunities to explore are:

1. Does AGE-RAGE interaction affect the level of RANKL and thus osteoclastogenesis in our HFD/LFD mice models?
2. Does inhibition of AGE-RAGE interaction affect the level of RANKL and thus osteoclastogenesis in our HFD/LFD mice models?
3. Does inhibition of AGE-RAGE interaction reduce the severity of periodontal bone loss in our HFD/LFD mice model?

Application of digital tools in routine laboratory tests

By automating pit formation quantification, not only were we able to greatly improve the efficiency and reduce the man-hours greatly, the concerns for human errors in entering and compiling numbers were completely eliminated (assuming that all the samples were labeled correctly). Such digital tool has demonstrated its potential to improve the research laboratory workflow and quality of work in our study. Future development may be warranted. A possible development for similar applications that is within reach is to include an edge-detection algorithm to automatically distinguish the area of interest from the background (such as dentin clearance) and create the accurate

border, which can further improve the efficiency of data analysis by removing the need to color-code manually using other image processing software.

The implication is that it is possible to remove human involvement or at least greatly reduce human labor in the repetitive and mechanical tasks, and at the same time minimizing human bias and error. The value of human labor is in creating and critical thinking, such as synthesizing evidence, designing experiments and interpreting the data, and the greater utilization of our computer tools will allow scientists to maximize these irreplaceable abilities.

REFERENCES

List of Abbreviated Journal Titles

Am J Pathol	American Journal of Pathology
Clin Exp Immunol	Clinical and Experimental Immunology
Community Dent Oral Epidemiol.....	Community Dentistry and Oral Epidemiology
Curr Opin Periodontol	Current Opinion in Periodontology
Diabetes Res Clin Pract	Diabetes Research and Clinical Practice
Int J Mol Med	International Journal of Molecular Medicine
J Am Dent Assoc	Journal of American Dental Association
J Cell Biochem.....	Journal of Cellular Biochemistry
J Clin Periodontol	Journal of Clinical Periodontology
J Immunol	Journal of Immunology
J Leukoc Biol.....	Journal of Leukocyte Biology
J Periodontal Res	Journal of Periodontal Research
J Periodontol	Journal of Periodontology
Nat Rev Endocrinol	Nature Reviews Endocrinology
Periodontol 2000.....	Periodontology 2000
Rev Endocr Metab Disord	Reviews in Endocrine and Metabolic Disorders

References

1. Nagasawa, T., et al., *Roles of receptor activator of nuclear factor-kappaB ligand (RANKL) and osteoprotegerin in periodontal health and disease*. Periodontol 2000, 2007. **43**: p. 65-84.
2. Tonetti, M.S., H. Greenwell, and K.S. Kornman, *Staging and grading of periodontitis: Framework and proposal of a new classification and case definition*. J Periodontol, 2018. **89 Suppl 1**: p. S159-S172.
3. Eke, P.I., et al., *Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012*. J Periodontol, 2015. **86**(5): p. 611-22.
4. Knight, E.T., et al., *Risk factors that may modify the innate and adaptive immune responses in periodontal diseases*. Periodontol 2000, 2016. **71**(1): p. 22-51.
5. Lalla, E. and P.N. Papapanou, *Diabetes mellitus and periodontitis: a tale of two common interrelated diseases*. Nat Rev Endocrinol, 2011. **7**(12): p. 738-48.
6. Demmer, R.T. and P.N. Papapanou, *Epidemiologic patterns of chronic and aggressive periodontitis*. Periodontol 2000, 2010. **53**: p. 28-44.

7. Mealey, B.L., T.W. Oates, and P. American Academy of, *Diabetes mellitus and periodontal diseases*. J Periodontol, 2006. **77**(8): p. 1289-303.
8. Chambrone, L., et al., *Predictors of tooth loss during long-term periodontal maintenance: a systematic review of observational studies*. J Clin Periodontol, 2010. **37**(7): p. 675-84.
9. Shaw, J.E., R.A. Sicree, and P.Z. Zimmet, *Global estimates of the prevalence of diabetes for 2010 and 2030*. Diabetes Res Clin Pract, 2010. **87**(1): p. 4-14.
10. Onkamo, P., et al., *Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends*. Diabetologia, 1999. **42**(12): p. 1395-403.
11. American Diabetes, A., *Diagnosis and classification of diabetes mellitus*. Diabetes Care, 2005. **28 Suppl 1**: p. S37-42.
12. Lamster, I.B., et al., *The relationship between oral health and diabetes mellitus*. J Am Dent Assoc, 2008. **139 Suppl**: p. 19S-24S.
13. Tsai, C., C. Hayes, and G.W. Taylor, *Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population*. Community Dent Oral Epidemiol, 2002. **30**(3): p. 182-92.
14. Salvi, G.E., et al., *Experimental gingivitis in type I diabetics: a controlled clinical and microbiological study*. J Clin Periodontol, 2005. **32**(3): p. 310-6.
15. Jagannathan, M., et al., *Toll-like receptors regulate B cell cytokine production in patients with diabetes*. Diabetologia, 2010. **53**(7): p. 1461-71.
16. Jagannathan, M., et al., *TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients*. J Immunol, 2009. **183**(11): p. 7461-70.
17. Zhu, M., et al., *B cells promote obesity-associated periodontitis and oral pathogen-associated inflammation*. J Leukoc Biol, 2014. **96**(2): p. 349-57.
18. Hienz, S.A., S. Paliwal, and S. Ivanovski, *Mechanisms of Bone Resorption in Periodontitis*. J Immunol Res, 2015. **2015**: p. 615486.
19. Oates, T.W. and D.L. Cochran, *Bone cell interactions and regulation by inflammatory mediators*. Curr Opin Periodontol, 1996. **3**: p. 34-44.
20. Saffar, J.L., J.J. Lasfargues, and M. Cherruau, *Alveolar bone and the alveolar process: the socket that is never stable*. Periodontol 2000, 1997. **13**: p. 76-90.

21. Bruzzaniti, A. and R. Baron, *Molecular regulation of osteoclast activity*. Rev Endocr Metab Disord, 2006. **7**(1-2): p. 123-39.
22. Nagasawa, T., et al., *LPS-stimulated human gingival fibroblasts inhibit the differentiation of monocytes into osteoclasts through the production of osteoprotegerin*. Clin Exp Immunol, 2002. **130**(2): p. 338-44.
23. Liu, D., et al., *Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction*. Int J Mol Med, 2003. **11**(1): p. 17-21.
24. Crotti, T., et al., *Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis*. J Periodontal Res, 2003. **38**(4): p. 380-7.
25. Belibasakis, G.N. and N. Bostanci, *The RANKL-OPG system in clinical periodontology*. J Clin Periodontol, 2012. **39**(3): p. 239-48.
26. Ip, B., et al., *Th17 cytokines differentiate obesity from obesity-associated type 2 diabetes and promote TNFalpha production*. Obesity (Silver Spring), 2016. **24**(1): p. 102-12.
27. Yamaguchi, M., et al., *T cells are able to promote lipopolysaccharide-induced bone resorption in mice in the absence of B cells*. J Periodontal Res, 2008. **43**(5): p. 549-55.
28. Yoshida, T., et al., *Direct inhibitory and indirect stimulatory effects of RAGE ligand S100 on sRANKL-induced osteoclastogenesis*. J Cell Biochem, 2009. **107**(5): p. 917-25.
29. Lalla, E., et al., *Diabetes-related parameters and periodontal conditions in children*. J Periodontal Res, 2007. **42**(4): p. 345-9.
30. He, H., et al., *Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss*. Endocrinology, 2004. **145**(1): p. 447-52.
31. Goova, M.T., et al., *Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice*. Am J Pathol, 2001. **159**(2): p. 513-25.
32. Santana, R.B., et al., *A role for advanced glycation end products in diminished bone healing in type 1 diabetes*. Diabetes, 2003. **52**(6): p. 1502-10.

33. Alikhani, M., et al., *Advanced glycation end products stimulate osteoblast apoptosis via the MAP kinase and cytosolic apoptotic pathways*. *Bone*, 2007. **40**(2): p. 345-53.
34. Chang, P.C., et al., *Aminoguanidine inhibits the AGE-RAGE axis to modulate the induction of periodontitis but has limited effects on the progression and recovery of experimental periodontitis: a preliminary study*. *J Periodontol*, 2014. **85**(5): p. 729-39.

CURRICULUM VITAE

