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B lymphocyte RANKL enhances periodontal disease in type 2 diabetes

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B LYMPHOCYTE RANKL ENHANCES PERIODONTAL DISEASE IN TYPE 2 DIABETES

by

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DEDICATION

To my family – Sam, Grace, and Jessica.

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ABSTRACT

Periodontitis (PD) and type 2 diabetes (T2D) are inflammatory diseases, which have B lymphocyte dysfunction in subjects with both conditions. B lymphocytes, when activated, release receptor activator of nuclear factor kappa-B ligand (RANKL), which is a key element for osteoclastogenesis and periodontal related bone loss. Previous studies have looked at PD or T2D but not together within the context of inflammation. To further understand the impact of T2D potentiated periodontal complications, purified B lymphocytes from mice spleen were plated on dentin plates under both stimulated and unstimulated conditions alone and as co-cultures with T lymphocytes to determine the effect on dentin pit formation, a measure of bone loss. Pit area analysis showed that stimulated B lymphocytes from obese mice had significantly higher pit areas when compared to all lean conditions (B/T unstimulated and stimulated). Because obese B lymphocytes had the largest pit areas, this correlated with the highest osteoclastic activity. This supports the hypothesis that T2D contributes to periodontal disease bone loss specifically through B lymphocyte RANKL function. These results suggest that B lymphocytes may be a therapeutic drug target that

can provide new clinical treatments to control T2D potentiated periodontal complications.

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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

LFD.....low fat diet

LPS..... lipopolysaccharides (LPS)

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B lymphocytes

OPG..... Osteoprotegerin

P. gingivalis ..... Porphyromonas gingivalis

PBS..... phosphate buffered saline

PD .....periodontitis

RANK ..... Receptor Activator of Nuclear Factor κB

RANKL ..... Receptor Activator of Nuclear Factor κB Ligand

RBC ..... red blood cell

T2DM..... Type 2 Diabetes mellitus

TNF..... tumor necrosis factor alpha

TRAF ..... TNF receptor associated factors

TRAP ..... Tartrate-resistant acid phosphatase

INTRODUCTION

Periodontitis and type 2 diabetes

Type 2 Diabetes mellitus (T2D) affects populations everywhere with the current number of adults with T2D estimated to be 425 million and projected to increase by more than 50% in the next twenty years [1]. T2D is a metabolic disorder that is characterized by hyperglycemia, hyperinsulinemia, insulin resistance and chronic inflammation. Like T2D, periodontitis (PD) is a common chronic inflammatory disease that affects about 750 million people worldwide. PD has been closely linked to T2D in clinical studies [2, 3], and epidemiological studies have shown that periodontal disease risk within the diabetic population is increased by threefold compared to non-diabetic individuals [3]. Because of the high prevalence of T2D and the subpopulation with PD, it is important to address the risks and comorbidities of these chronic diseases. To further elucidate the dynamic relationship between T2D and PD, many studies have focused on the effect of one disease on the other, yielding evidence that chronic inflammation from PD can negatively affect glycemic control and T2D can increase risk for PD [4, 5]. The combination of PD and diabetes can fuel a feed-forward loop leading to increased inflammatory signaling and elevated levels of cytokines such as TNF-α, IL-6, and IL-8 [4]. Because inflammation is positively correlated amongst obesity, T2D, and PD, it is imperative to understand its connection to the progression of these diseases.

Inflammation in periodontal disease

Periodontal disease starts with gingivitis, which is defined by inflammation limited in the gingiva, and is reversible with proper oral hygiene. If left untreated, gingivitis can lead to PD, in which the inflammation is extended to the soft tissue and can cause alveolar bone resorption, resulting in bone loss of the maxilla and/or mandible. The inflammatory response from PD includes an immune response via activation of B and T lymphocytes, with progressive periodontal lesions characterized by increased prominence of B lymphocytes [7]. B lymphocytes have several functions as part of the adaptive immune system which include secreting antibodies, production of cytokines, and antigen presentation [8, 9]. There is a positive correlation between PD and increased IgG antibody, a generally inflammatory B cell product, in response to oral pathogens in the gingival cavity [10]. Notably, B lymphocytes had similar cytokine production including pro-inflammatory and pro-osteoclastogenic cytokine TNF-α in both PD and T2D subjects, suggesting B lymphocytes play a central role in linking T2D-associated complications that worsen periodontitis [4, 11, 12]. In PD studies independent of T2D, Chen et al (2014) recovered B and T lymphocytes from diseased human periodontal tissues, and showed that more than 90% of B lymphocytes express RANKL whereas 54% of T lymphocytes express RANKL. Additionally, B lymphocytes were not dependent on T lymphocytes to drive bone resorption [13]. In another study, Han et al (2006) injected congenitally athymic donor mice with B lymphocytes specific for *Actinomycetemcomitans* into non-

immune mice and found that increased B cell response lead to increased RANKL expression in actinomycetemcomitans-immunized animals which lead to higher levels of osteoclast differentiation leading to increased periodontal bone resorption in absence of T lymphocytes [7].

Oral inflammation, mainly from immune cells, has important downstream implications for oral bone health as cytokines such as TNF-α and IL-1b which increase differentiation and activity of osteoclasts. Osteoclasts are cells that resorb bone [14]. The TNF-family Receptor Activator of Nuclear Factor κB (RANK) initiates differentiation of osteoclasts and is essential for developing mature osteoclasts [15, 16]. However, there is controversy on the cellular source of RANK-ligand (RANKL). Suda et al, 1999 showed that osteoblasts are the main source of the RANKL that is required for murine osteoclast differentiation under co-culture conditions [17]. However, other studies question this interpretation by showing osteocytes expressed RANKL for regulation of osteoclastogenesis, and that RANKL produced by osteoblasts or their progenitors did not contribute to bone remodeling [18-21]. Furthermore, many studies found B and T lymphocytes to be the major sources of RANKL [7, 10, 22, 23]. Our lab showed B lymphocytes play a major role in PD and osteoclastogenesis in obese T2D hosts, in part due to support of the PD-associated cytokines TNF-α, IL-8, and IL-1B, although RANKL trends failed to reach statistical difference. In contrast, this work found that T lymphocytes appear to be more significant for PD in lean subjects although

T cell RANKL was not specifically measured [12]. Definitive tests on the importance of B or T cell RANKL in PD remain forthcoming.

RANK, the receptor for RANKL, is one of the main pathways that trigger activation of Nuclear factor kappa-light-chain-enhancer of activated B lymphocytes (NF-κB) in osteoclasts [25, 26]. NF-κB is a transcription factor complex that has various functions best understood in immune and inflammatory responses, but also plays roles in osteoclastogenesis [24]. The RANK/RANKL pathway signals by binding to TNF receptor associated factors (TRAF6), which triggers the downstream target NF-κB [29, 30]. Osteoprotegerin (OPG) also regulates RANK signaling-mediated activation, thus the NF-κB required for osteoclastogenesis [27, 28]. OPG acts as a decoy receptor for RANKL and RANKL-OPG binding inhibits the RANK pathway (Figure 1).

Taken together, previous studies suggest that altered B cell functions, including increased proinflammatory cytokines and antibody production may bridge the gap between T2D and PD in vivo [12, 33]. The purpose my work was to specifically test the hypothesis that T2D-linked changes in B cell RANKL activity specifically promotes osteoclastogenesis towards supporting T2D-associated periodontal disease.

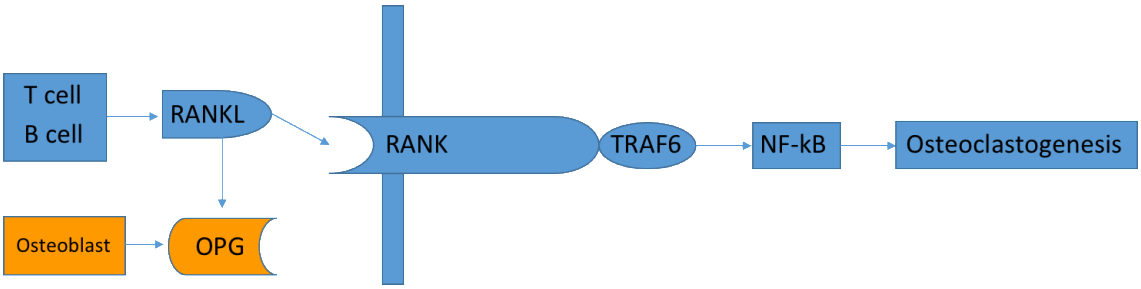


Figure 1. RANKL pathway. RANKL - Receptor activator for NF-κB Ligand, OPG – Osteoprotegerin, RANKL - Receptor activator for NF-κB, TRAF6 - TNF receptor associated factor 6, NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

METHODS AND MATERIALS

Mice

Five-week old male mice (DIO-B6-M; C57BL/6N) were fed ad libitum with a high fat diet (HFD) (N=11, 60% calories from fat; Research Diets D12492i-irradiated) or a comparable low fat diet (LFD) (N=11, 10% calories from fat; Harlan Teklad irradiated) for 10 weeks, except a 6-hr food deprivation period before blood draws for fasting glucose levels. Metabolic measurements were performed on 9 weeks and 15 weeks of diet for glucose tolerance by Intra-peritoneal glucose tolerance test (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 16 weeks diet (21 weeks of age).

Preparation of Splenocytes

Mice were euthanized by CO_2 narcosis and were placed on a clean dissection board and the animal's skin was sterilized with 70% alcohol. The abdominal cavity was exposed by making an incision on the left side of the abdomen and the spleen was removed and placed into a conical tube containing RPMI medium 1640 (Gibco Life Technologies).

Splenocyte suspension was prepared by placing the spleen onto a 70-micron cell strainer that was submerged in RPMI medium in a 3-cm petri dish; spleen was minced through the strainer using the plunger end of a syringe. The

cells were washed through the strainer with RPMI, then transferred to a 15ml conical tube and centrifuged at 1,200 rpm for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in 3 mL of red blood cell (RBC) lysis buffer (eBiosciences) at 37ºC in the same conical tube. The cells were incubated with RBC lysis buffer at room temperature for 6 minutes and then 12 mL of RPMI was added. Cells were centrifuged again at 1,200 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in phosphate buffered saline (PBS) (Corning Incorporated) with 1% Bovine Serum Albumin (BSA – Fraction V) for cell sorting (Figure 2).

Fluorescence-activated cell sorting (FACS) Cell Sorting

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

RAW cell preparation

Adherent mouse LMM (RAW 264.7, Mouse leukemic monocyte-macrophage) cells were removed from plates by aspirating cell line media and adding 500 μl of PBS based non-enzymatic cell detaching buffer. This cell suspension was incubated for 5 minutes at 37℃ and the cells were detached by cell pipetting. Suspended cells were transferred to 15ml conical tube with 4.5ml of Dulbecco's Modified Eagle Medium High glucose (4.5g/L) (DMEM) with 10% FBS, 1% penicillin/streptomycin and L-glutamine (4.0 mM). Cells were centrifuged at 1200 RPM for 10 minutes and the supernatant aspirated before the cells were resuspended in Minimum Essential Medium Eagle - Alpha Modification (modified MEM) with 10% FBS, 1x penicillin/streptomycin and l-glutamine. The cells were counted on a hemocytometer and the cell suspension was diluted to a density of 1×10^4 cells/ml using modified MEM. Diluted cell suspension was transferred to a 96 well flat bottom plate to give a final count of 1000 cells per well. After 6 hours of incubation, recombinant RANKL was added for pre-stimulation to the RAW cells.

B and T cell Stimulation

Unstimulated B and T lymphocytes (1000 cells each) were added to each well of 1000 RANKL-primed RAW cells. For B and T co-cultures, cells were added at a 10:1 ratio. For example, if the co-cultures were labeled “lean B cells with lean T cells”, we added 1000 B cells plus 100 T cells to 1000 RANKL-primed

RAW cells. RAW cells alone served as control for background and RAW cells + RANKL were the positive control. Alternatively, B lymphocytes were stimulated with 10 μg/ml of E. coli lipopolysaccharides (LPS) (Invivogen), or T lymphocytes were stimulated with αCD3/28 antibody coated Dynabeads (Life technology) for 24hrs prior to addition to RAW cultures as detailed below. Supernatants from all lymphocyte cultures were collected for RANKL enzyme-linked immunosorbent assay after 24 hrs incubation. To prevent carry-over of lymphocyte stimuli to RANKL-primed RAW cells, B lymphocytes, T lymphocytes, and B cell/T cell co-cultures were washed with RPMI 3 times prior to transfer. A second independent set of assays were performed on 96 well format dentin plates for pit formation.

Pit formation on Dentin plates

96 well flat bottom plates were used primarily for cell culture for both single and co-culture (Figure 2) of B and T lymphocytes. Cells incubated in standard 96 well plates (stimulated or unstimulated, Figure 2) were then moved to a second assay performed on 96 well dentin plates (Corning, 3988), which have a surface coating that mimic *in vivo* bone. The plate was incubated for at least 6 hrs to ensure cells adhered. The seeded cell were treated with RANKL for two days (pre-stimulation), then the media was changed to remove recombinant RANKL before addition of B and T lymphocytes for three days as below. These plates were used to quantify osteoclastic (bone resorption) activity via pit formation.

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

After 1000 RAW cells were plated on a 96 flat bottom plate, 100 µl of mouse RANKL (100 ng/ml) was prepared in modified MEM. 100 µl of this RANKL solution was added to each well to give a final concentration of RANKL of 50 ng/ml. Then different combinations of B and T cells were added to each well (table 1). Media was changed every 2 days by removing 100 µl of culture medium and then adding 100 µl of fresh media containing the same concentration of mouse RANKL. After 5 to 7 days of incubation, the cells were stained using a Tartrate-resistant acid phosphatase (TRAP) stain to quantify number of osteoclasts per mm^2. Osteoclasts are characterized by their TRAP expression.

Pit Assay Analysis

Dentin-coated wells were analyzed for clearance of dentin by each cell type. Dentin wells were treated with 1:4 bleach and von Kossa stain to show pit surface as dark under bright field. Digital images were taken with a Nikon camera and clearance positive zones in the well were counted and tabulated. All dentin plate images were uniform in size and pit areas were counted as pixels by coloring using Adobe Photoshop CC 2016 to calculate the number of pixels in the red-shaded region in-silico. A standard millimeter ruler was placed under microscope and images were taken at same conditions to calculate number of

pixels/mm^2 (1290614.147 pixels/mm^2) and this was used to convert pit areas from digital images to total area in mm^2.

Statistical analysis of the data was performed by Graphpad Prism (version 7) using 1-way ANOVA with Tukey post-hoc test for corrections for multiple comparisons. A p-value of $\leq$ 0.05 was considered statistically different.

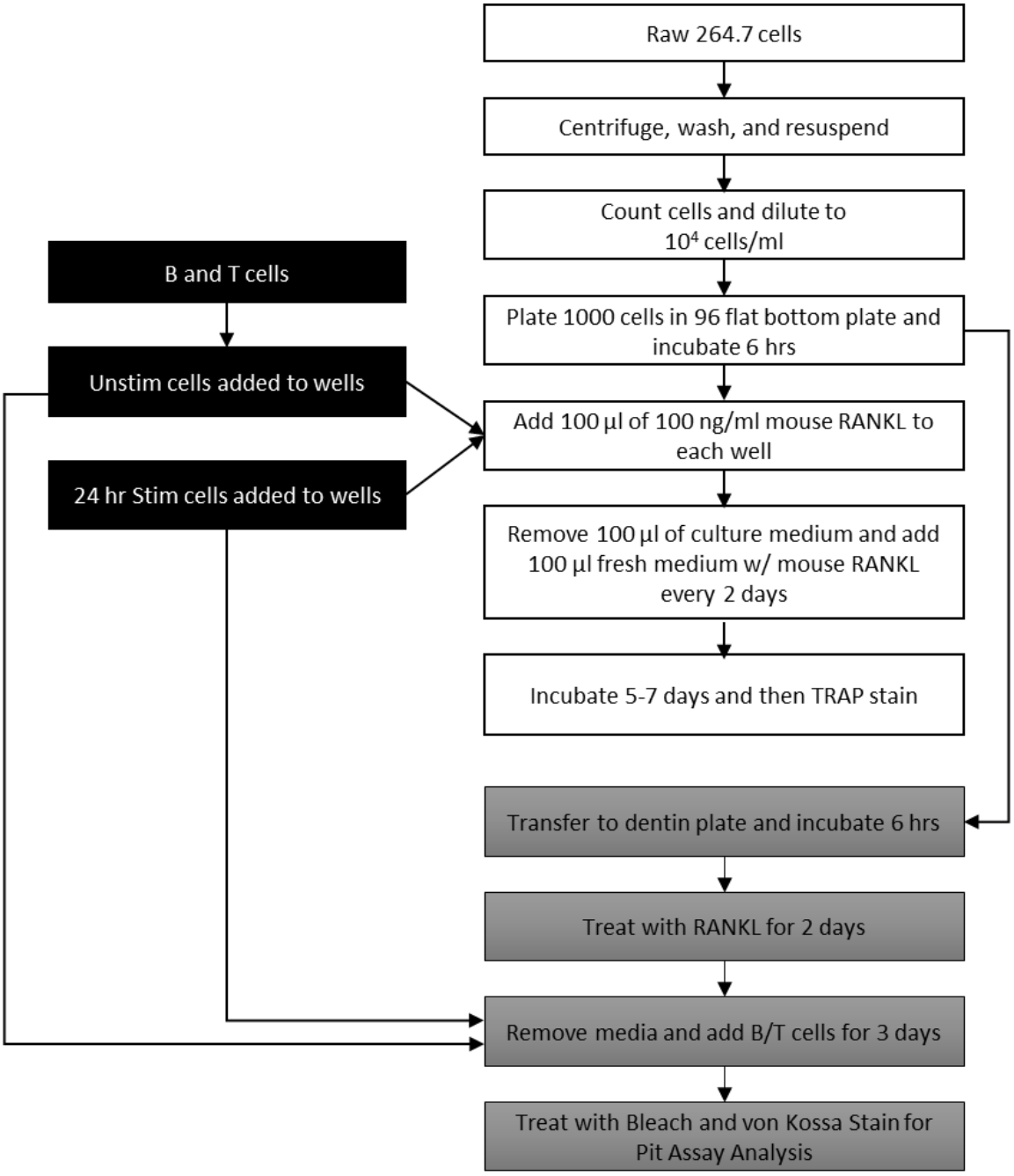


Figure 2. Summary of experimental design for TRAP stain and Pit assay. White boxes show TRAP protocol, gray boxes show PIT assay protocol, and Black boxes show addition of B and T cells in both experiments.

RESULTS

Metabolic measurements of Mice

The HFD mice showed significantly higher body weight and fasting blood glucose compared to LFD mice. Intra-peritoneal glucose tolerance test (IPGTT) showed slower blood glucose clearance from HFD mice compared to LFD mice indicating a diabetic phenotype in the HFD mice (Figure 3).

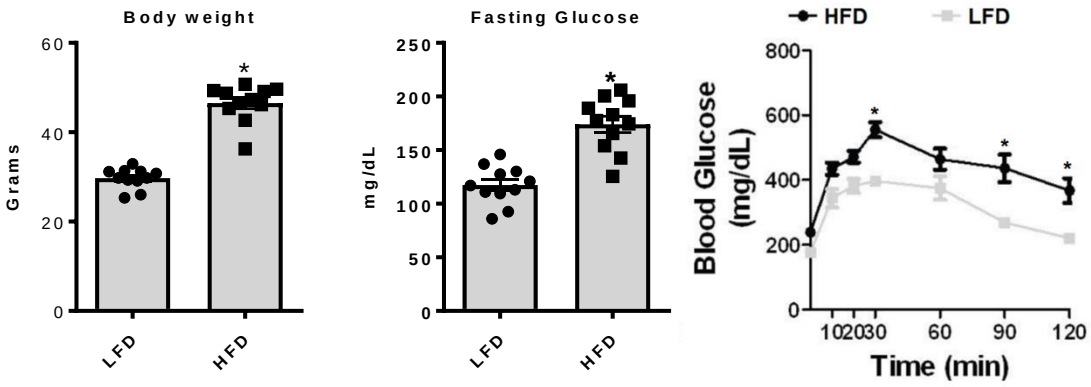


Figure 3. Body weight and fasting blood glucose levels of mice on LFD and HFD at 15 weeks of diet. IPGTT on LFD and HFD mice at 15 weeks of diet Bars show average with points for each individual mouse. N=11 *p<0.05.

Sorting of Splenocytes

There are four distinct groups in this experiment to assess in vitro osteoclastogenesis: lean B lymphocytes, obese B lymphocytes, lean T lymphocytes and obese T lymphocytes. The splenocytes from these mice were sorted for lymphocytes (Figure 4A) with debris shown on the bottom left and the dead cells being the scattered dots on the top right. Double discrimination based on side scatter and forward scatter (Figure 4B and 4C), which will exclude cells that are stuck together, leaving single cells only. Dead cells were excluded by zombie NIR live-dead stain (Figure 4D), macrophages were excluded using Cd11b+ surface marker (Figure 4E), and finally B lymphocytes (CD19+) and T lymphocytes (CD4+) are identified on the bottom left (Figure 4F). Cells were plated in single culture and co-culture combinations on dentin plates (Table 1).

TRAP Single Culture	TRAP Co-Cultures
RAW (control)	RR + Stim B Lean + Stim T Lean
RAW + RANKL (RR)	RR + Stim B Lean + Unstim T Lean
RAW + RANKL + Anti-RANKL	RR + Unstim B Lean + Stim T Lean
RR + Unstim B Lean	RR + Stim B obese + Stim T obese
RR + Unstim B Obese	RR + Stim B obese + Unstim T obese
RR + Unstim T Lean	RR + Unstim B obese + Stim T obese
RR + Unstim T Obese	RR + Stim B Lean + Stim T obese
RR + Stim B Obese	RR + Stim B obese + Stim T Lean
RR + Stim B Obese + Anti-RANKL	
RR + Stim B Lean	
RR + Stim B Lean + Anti-RANKL	
RR + Stim T Obese	
RR + Stim T Obese + Anti-RANKL	
RR + Stim T Lean	
RR + Stim T Lean + Anti-RANKL	

Table 1. All culture combinations for B and T cells

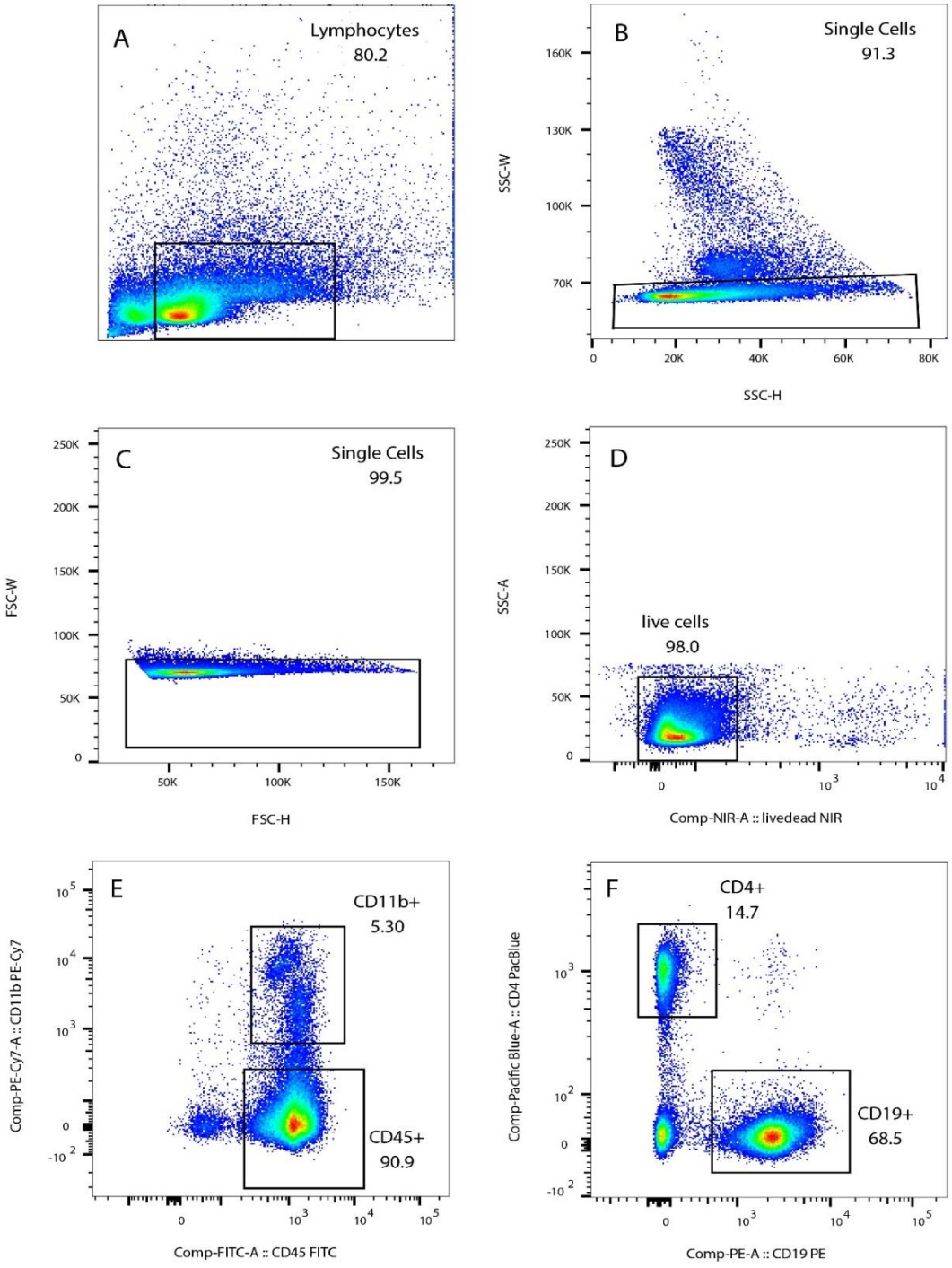


Figure 4. Flow cytometry gating. (A) Lymphocytes, (B) (C) single cells, (D) live cells, (E) $CD45^+$ leukocytes, (F) $CD4^+$ T helper cells and $CD19^+$ B cells

Tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts in different culture combinations (Table 1) was used to visually quantify the number of osteoclasts per mm2 (Figure 5). RAW cells were used as control and background for trap staining (Figure 5a). RAW + RANKL is used as positive control with osteoclasts indicated by the black arrow (Figure 5b), RAW + RANKL + anti-RANKL is used for negative control (Figure 5c), and TRAP seeding conditions (Figure 5d) which show osteoclastic activity in lean stimulated B cells as indicated by black arrow.

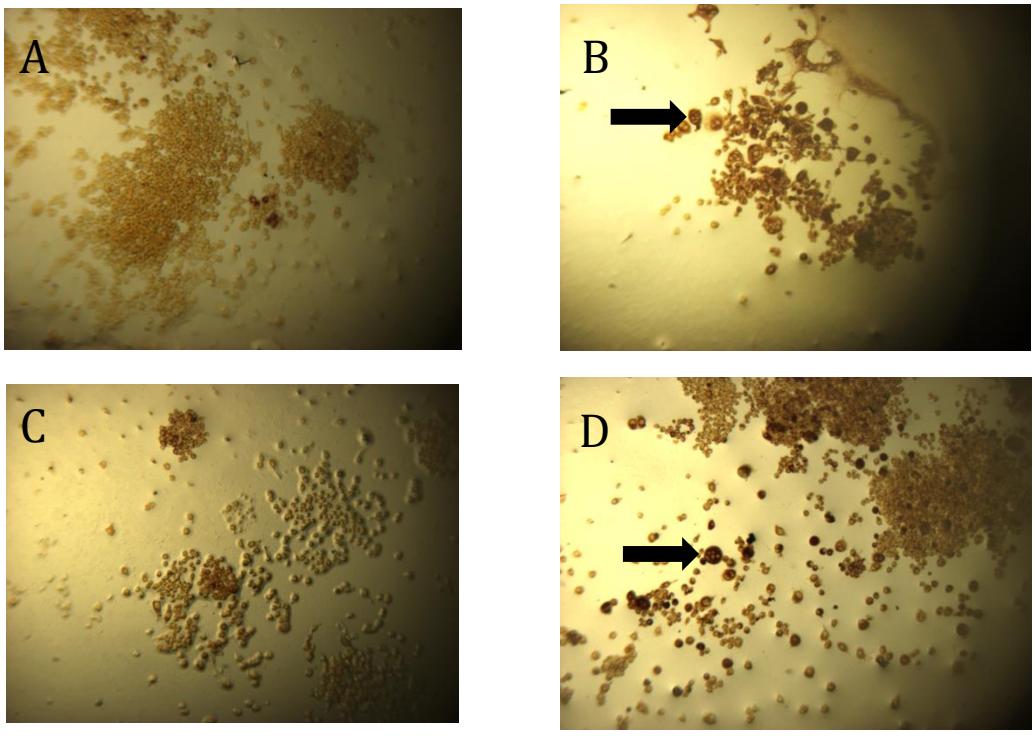


Figure 5. Magnified images of TRAP stained cells in 96-well plates. (A) Control, (B) RAW cells with RANKL, (C) RAW + RANKL + anti-RANKL to show no osteoclasts, (D) RAW + RANKL + stimulated B cells from lean mice with an osteoclast indicated by black arrow.

TRAP staining analysis visually showed osteoclastic activity but proved to be difficult to quantify due to high background noise due to some non-specificity in staining. Analysis of trap data showed no significant differences in stimulated and unstimulated B and T cells (Figure 6).Therefore, pit assay on dentin plates was performed because of the increased sensitivity in staining with von Kossa and allowing more accurate analysis.

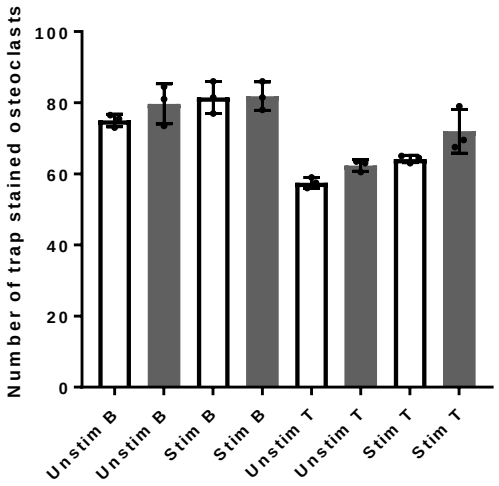


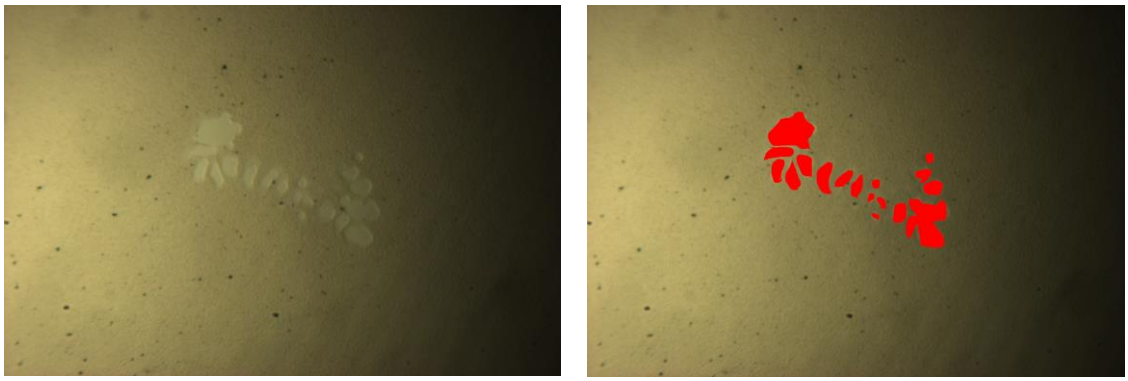
Figure 6. Quantified trap data showed no significant differences. White bars show lean and gray bars show obese. Significance was calculated by one-way ANOVA. Data are presented as mean ± SEM; *P < 0.05.

Dentin plate analysis was blinded and an example of this is shown (Figure 7A). Pits shown as lighter areas in the bright field were colored by h

and to a specific type of red color identified by Photoshop (#ff0000) and the number of shaded pixels were calculated. Dentin plate of single culture showed no significant differences between lean B and lean T lymphocytes but

still induced some osteoclastogenesis in both stimulated and unstimulated conditions when compared to RAW (far left white bar) albeit non-significant (Figure 7B). In both obese B and T cells, stimulated cells were significantly higher than unstimulated cells which was expected. Stimulated obese B cells were significantly higher than lean stimulated B cells which recapitulates T2D associated changes in B cells enhances PD through RANKL as shown by dentin clearance. There was no significant differences between any of the unstimulated conditions between lean and obese.

A.



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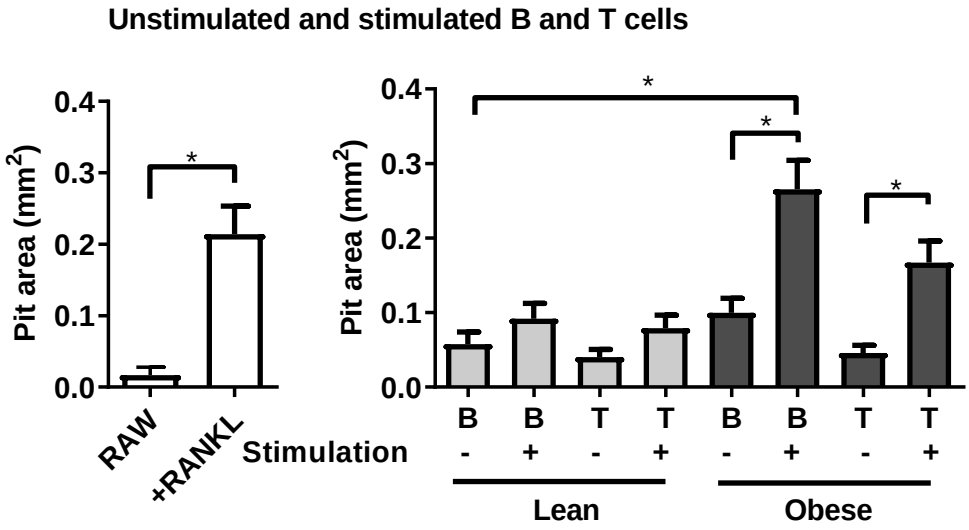

Figure 7. Pit areas from stimulated obese B and T lymphocytes are significantly different form their unstimulated counterparts. (A) Magnified image of a dentin plate with pits (clearance of dentin). Pits were colored red manually and area was calculated by the number of pixels in the shaded area. (1290614.147 pixels per 1 mm^2), (B) Positive control for osteoclastogenesis: RAW (Mouse leukemic monocyte-macrophage), +/-RANKL as labeled. RAW cells -RANKL show background. Pit areas indicate osteoclast differentiation and function in the presence of stimulated and unstimulated B and T lymphocytes from lean and obese mice as indicated. Lymphocytes from lean mice similarly induced osteoclastogenesis, regardless of stimulation conditions (left, gray bars). Significant differences in cultures containing cells from obese mice were calculated by two-way ANOVA. Data are presented as mean ± SEM; *P < 0.05.

In order to determine if RANKL is required for osteoclastogensis, the RANKL pathway was inhibited to see if there would be any expected loss in pit formation. Anti-RANKL antibody inhibits the interaction between RANK and RANKL which suppresses osteoclastic activity which is observed by significant reduction in pit area (Figure 8). Isotype antibody are antibodies that are non-specific to RANKL but match the type and class of the primary antibody (anti-RANKL). This acts as a negative control to differentiate nonspecific antibody signal from the specific antibody signal. As shown in Figure 8, there was no significant differences between RAW+RANKL and RAW+RANKL+ISO-Ab.

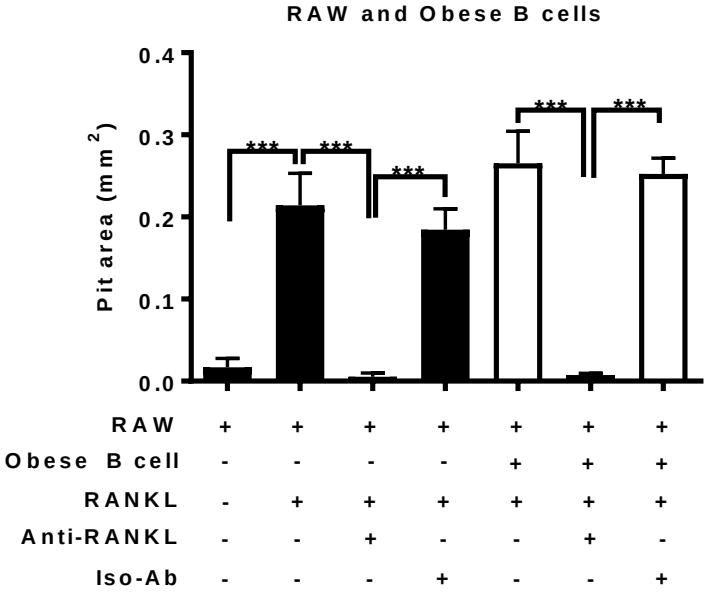

Figure 8. Inhibition of RANKL by anti-RANKL antibody significantly reduced pit area. RAW cells alone (black bars) show pit formation under conditions indicated at the bottom. White bars (on right) show pit formation by RANKL-primed RAW 264.7 cells in the presence of B lymphocytes from obese mice with additional treatments as indicated at bottom. Significant differences in pit formation are calculated by one-way ANOVA. Data are presented as mean ± SEM; ***P < 0.001.

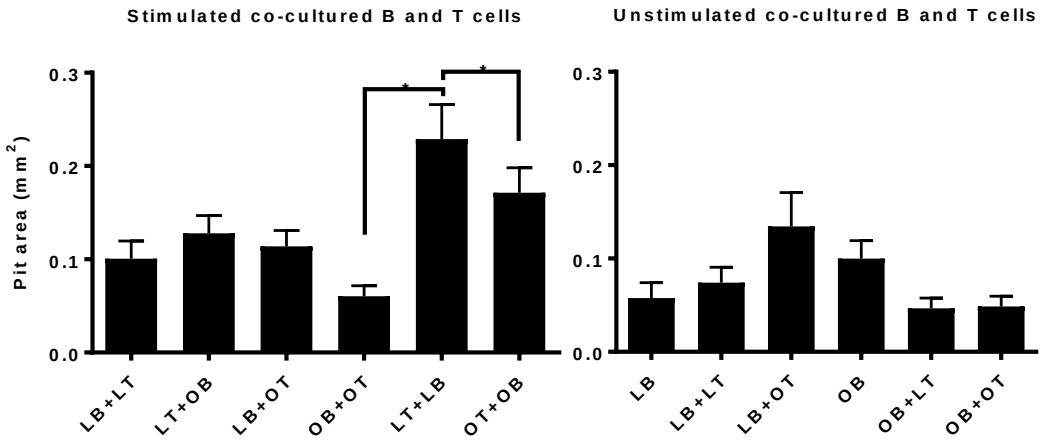

Figure 9. Single and co-cultures of stimulated and unstimulated B and T lymphocytes. Co-culture ratios are 1000:100 cells shown as written. Significantly different groups are calculated by one-way ANOVA. Data are presented as mean ± SEM; *P < 0.05.

Stimulated and unstimulated B and T were co-cultured to determine their relative osteoclastic activity through RANKL production and the pit areas were calculated (Figure 9). Because B lymphocytes were shown previously to have the most RANKL driven osteoclastogenesis, they were cultured along with T lymphocytes to determine if there was any difference in B cell activity. Interestingly, lean stimulated B lymphocytes when co-cultured with lean stimulated T lymphocytes had significantly larger pit areas than the co-culture of obese stimulated B and T lymphocytes. This is contrary to single culture where lean stimulated B lymphocytes had smaller pit areas relative to obese stimulated B lymphocytes (Figure 7b). There was no significant difference amongst the unstimulated co-cultures. This may suggest that T lymphocytes have some effect on osteoclastic activity when co-cultured with B lymphocytes within lean hosts.

DISCUSSION

Both T2D and periodontitis are common, and it is well established that T2D predisposes periodontitis, while the ability of periodontitis to worsen complications of T2D such as glycemic control remaining controversial [3]. It is vital to be able to identify sources of periodontal inflammation or drivers of periodontal bone loss to develop new methods of treatment to reduce periodontitis in patients with T2D. Our data has shown that B lymphocytes from T2D mice significantly increase pit area therefore increase osteoclastogenesis when compared to B lymphocytes from lean mice as determined in the pit area assay. Trends toward increased osteoclastogenesis in T lymphocytes remained statistically insignificant despite replication (N=11). Furthermore, there was no significant difference comparing pit area in the presence of B or T lymphocytes from lean hosts. This data agrees with previous findings which state that T lymphocytes alone do not drive PD in T2D [4]

T2D and PD have also been associated with delayed spontaneous apoptosis of peripheral blood neutrophils (PMN) and Manosudprasit et al (2016) showed that individuals with T2D and PD had relatively longer delays in apoptosis compared to T2D alone [34]. Because delayed neutrophil apoptosis advances organ dysfunction and potential organ failure, it is important to understand that individuals with both T2D and PD are vulnerable to various problems not limited to bone loss shown here [35]. Macrophage dysfunction has been shown to occur in mice fed a high fat diet in conjunction with oral

inoculation and also suggests that immune dysregulation may increase susceptibility of other bacterial infections [39].

Future directions for this study can look into the multiplicity of infection of *P. gingivalis* in both B and T cells to measure the cytokine profile of different intesities of *P. gingivalis infection.* While this current study supports the hypothesis of B cell driven osteoclastogenesis in T2D/PD subjects on dentin plates, it is unclear whether there is a minimum threshold of infection which may cause a domino effect resulting in periodontitis. Furthermore, combinations of bacteria are almost universal in PD so the next step would be looking into other PD related bacteria such as *T. forsythia* and to understand if there is any synergism between different bacteria in driving this disease. The next important step would be to look at a human model of periodontitis as classical pro-inflammatory cytokines in T2D such as TNF-α, IL-1β and IL-6 promote PD [36]. It would also be critical to look into human periodontal tissue to quantitate RANKL expression in B cells and T cells as potential targets for local immunotherapy. This strategy, applied systemically, could prove to be a promising avenue for other bone disorders such as rheumatoid arthritis, osteoporosis, or multiple myeloma [11, 37, 38].

Dentin plate analysis described in this project provides accurate evidence which show obese B lymphocytes can be more osteoclastogenic and pit formation in obese mice is increased compared to their lean counterparts. The data indicates T2D increases B cell function to further advance periodontal bone

loss in a mouse model. Further cytokine profiling can be done through transcriptome arrays of B cells from both lean and T2D. This can look more closely at the mRNA of the cell after treatment of different cytokines to understand the cellular changes from abnormal cytokine levels. This dysfunction in B cells caused by excess cytokine production from T2D complications indicate B cells may be locally targeted through means like slow-release anti-CD20 antibody such as Rituximab. This can target B cells with CD20 surface markers that could be useful in diseases such as PD/T2D, characterized by overactive B cells. Sequestration of the other B cell survival factors may be another method of locally targeting B cells for therapeutic purposes. This can translate our work towards a new clinical treatments be a potential drug target to control periodontitis.

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