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### Imidazole Propionate As A Potential Modulator of T-Cell Function

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IMIDAZOLE PROPIONATE AS A POTENTIAL MODULATOR  
OF T-CELL FUNCTION

A Thesis Submitted in Partial Fulfillment of the Requirements  
for the Degree of Master of Science

By: Chinanu G. Gubor  
Major: Nutrition Science

The University of Memphis, College of Health Sciences  
May 2023

## ABSTRACT

Research has shown the metabolic role and therapeutic potential of the gut microbiome in modulating health and disease states. Microbial metabolites are known to be involved in various metabolic functions throughout the body. The purpose of this study was to determine if the microbial metabolite imidazole propionate, shown to be increased in obesity and type II diabetes, has an effect on key human T-cell functions. Two fasted blood samples were collected from 10 human subjects. PBMCs were isolated, and a subset were stimulated in the presence of ImP. After staining for cell-surface antigens and intracellular cytokines, flow cytometry analysis was conducted to assess the effect of ImP on T-cell IFN $\gamma$  production and proliferation. No significant effect of ImP was observed with PMA/ionomycin-induced IFN $\gamma$  levels or CD3/CD28/IL-2-induced proliferation of CD4 $^{+}$  and CD8 $^{+}$  T-cells. These data suggest that ImP does not significantly alter T-cell activation under the experimental conditions used.

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## BACKGROUND

Obesity and type II diabetes are two metabolic diseases that are currently at epidemic levels (Saklayen 2018). As evidenced by the recent COVID-19 pandemic, individuals with these conditions have been shown to have increased susceptibility to infectious disease (Petrakis et al, 2020). Many studies have linked these metabolic conditions to a dysfunctional immune system, and this is thought to be due in part to the state of chronic low-grade inflammation that characterize them (Ellulu 2017; Andersen 2016). When a threat is introduced and an immune response is mounted, there should be a shift from tissue homeostasis to an inflammatory state as the body works to fight off the pathogen, followed by a resolution and return to homeostasis (Andersen 2016). However, with the chronic low-grade inflammation seen in metabolic diseases such as obesity and type II diabetes, this homeostatic set point is altered, which makes it difficult for the body to recognize when to launch an immune response to an actual threat. However, not much is known on the exact pathways or mechanisms by which immunity may be impaired in these individuals.

The gut microbiome is thought to be a mirror to one's health. Studies show that the bacterial composition of the microbiome and its biochemical activity and outcomes can reflect the human's overall health status. For example, in a study by Thingholm et al, microbial composition and functional capacity in obese subjects with type 2 diabetes were shown to differ significantly from that of healthy individuals (Thingholm 2019). Differences were found in specific microbial taxa as well as in serum metabolite concentrations. It is believed that this gut dysbiosis is heavily involved in the development of inflammatory diseases (Li 2017).

Research has already shown the interplay between the gut microbiome and the immune system to play a significant role in disease and health states. Changes in the microbiome affect immune functioning, and vice-versa (Zheng 2020). For example, a study by Mazmanian et al. showed that the bacteria *Bacteroides fragilis* produces polysaccharide A (PSA) molecules that can induce the development of a systemic Th1 immune response (Mazmanian 2005). In a 2014 study, Ling et al. showed an association between specific gut bacteria phylotypes and the development of food allergy in infants (Ling et al., 2014).

## **Microbial Metabolites**

### **Overview of Microbial Metabolites**

When one thinks of the gut microbiome, what often comes to mind are the prebiotics (food for the bacteria) and the probiotics (the bacteria themselves that have been consumed). An underappreciated player in the activity of the microbiome is actually the “postbiotics”, also known as microbial metabolites, which are the byproducts of the bacteria’s consumption of prebiotics, amino acids, and other substances (Wegh 2019). Once produced, these bioactive compounds continue on into the body to carry out various functions that contribute to metabolic functions. These include immune functions in the body; for example, the metabolite butyrate has been shown to play a role in T-cell polarization in mice with colitis (Chen et al, 2019). The most studied microbial metabolites are the short-chain fatty acids acetate, propionate, and butyrate, and their derivatives. Studies have suggested acetate to play key roles in body weight control and insulin sensitivity (Hernandez 2019), propionate to be involved in beta-cell function (Pingitore 2017), inflammation (Tian et al, 2019), lipogenesis, and cholesterol control (Hosseini 2011), and butyrate to help modulate intestinal barrier function and inflammation (Bach Khudsen 2018).

## **Imidazole Propionate- Chemistry and Functions**

Imidazole propionate (ImP), formal name 1H-imidazole-5-propanoic acid, is a metabolite of the gut microbiome formed from the amino acid histidine. Production of imidazole propionate is mediated by urocanate reductase (UrdA), a bacterial enzyme that has been linked to disease states in humans (Venskutonyte 2021). Imidazole propionate levels in the blood are correlated with a *Bacteroides* 2 enterotype and reduced gene richness (Molinaro et al, 2020). Studies in murine models have suggested that ImP impairs insulin signaling by decreasing levels of insulin receptor substrates 1 and 2 and phosphorylated Akt (Koh 2018) and increasing mTORC1 activation marker p70S6K in liver cells.

The foundational paper for this thesis proposal, published by Koh et al. in 2018, was the first to connect the gut microbiome metabolite ImP to impaired metabolic functioning. Bacterial metabolites produced by gut bacteria travel through the portal vein to the liver before entering the systemic circulation (Koh 2018). Using untargeted metabolomic assays to identify amino-acid derived metabolites that may be involved in impairing insulin function and contribute to type 2 diabetes, the investigators found imidazole propionate, as well as 3 other metabolites, to be increased in obese human patients with type 2 diabetes.

The global metabolomics profile of plasma from germ free and conventionally raised mice with a focus on microbial metabolites was also analyzed. Imidazole propionate was the only metabolite found to be increased in the portal vein and plasma collected from the vena cava from the conventionally raised mice. Further studies in these mice showed antibiotic treatment can reduce ImP levels in the circulation, confirming that the microbiota is responsible for its production. Analyzing plasma from an initial human cohort revealed concentrations of ImP to



not only be higher in portal plasma of individuals with type 2 diabetes, but also be higher in their peripheral plasma compared to individuals without type 2 diabetes.

To determine the effect of ImP on glucose tolerance and insulin signaling, germ-free mice were injected with either vehicle or ImP for 3 days. In the ImP-injected mice, this increased ImP levels in the circulation and in the liver, and induced glucose intolerance as shown by intraperitoneal glucose tolerance tests. These results were also replicated in conventionally raised mice. It was also found that hepatic levels of insulin receptor substrates 1 and 2 (IRS1 and IRS2) in germ-free mice were reduced with 3-day treatment with ImP. Incubation of primary hepatocytes with ImP was similarly found to reduce IRS1 and IRS2. In human embryonic kidney cell line HEK293, IRS1 levels were also shown to be decreased with 8-hour treatment with ImP. This suggests that the pathway ImP acts on is consistent across various cell types.

Further studies showed imidazole propionate to activate mTORC1. mTORC1 activation is known to impair insulin signaling by inhibiting IRS tyrosine phosphorylation and proteasomal degradation (Ozes 2001). Rapamycin was also shown to inhibit this effect of imidazole propionate (Koh 2018).

A study by Molinaro et al. further shows the connection between ImP and obesity and type 2 diabetes (Molinaro 2020). Imidazole propionate levels were found to be associated with the *Bacteroides 2* enterotype and reduced bacterial gene richness, which have also been associated with obesity. Molinaro et. al. also found this *Bacteroides 2* enterotype to be associated with an increase in the genes involved in ImP biosynthesis from histidine. These findings further confirm the role of the gut microbiome in producing ImP and thus affecting host metabolism.

A more recent study by van Son et al showed the association between ImP and cardiovascular disease risk factors in obese and overweight individuals without type 2 diabetes

(van Son 2021). Plasma ImP concentrations were positively correlated with blood pressure in overweight and obese humans.

Taken together, these studies show the involvement of imidazole propionate in the pathogenesis of metabolic diseases.

### *How Imidazole Propionate Signals in the Liver*

Imidazole propionate has been shown to impair insulin signaling via insulin receptor substrate in hepatocytes (Koh 2018).

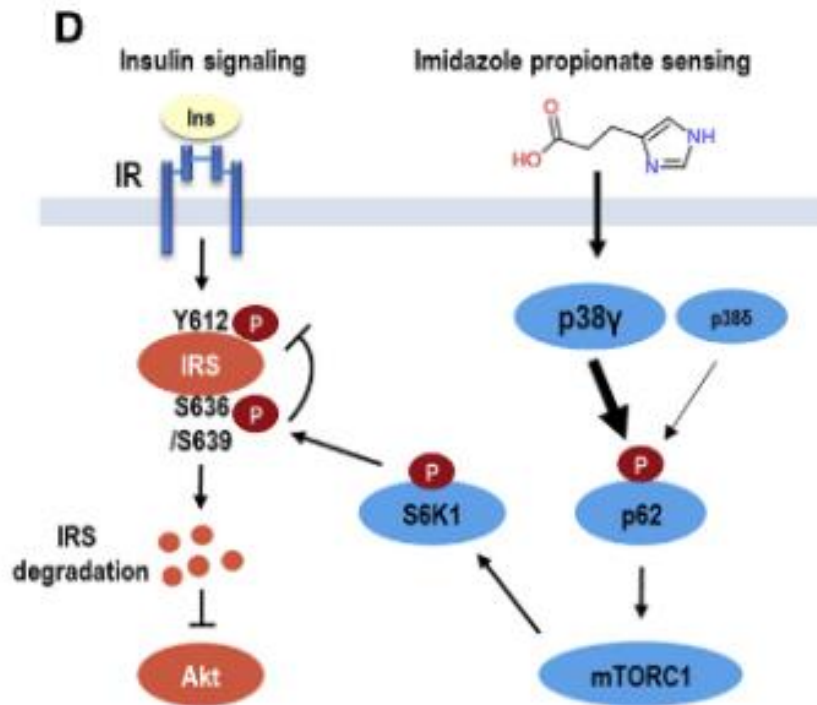


Figure 1. Imidazole propionate-induced signaling in hepatocytes.

Koh et al. (2018). Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. *Cell*, 175(4), 947–961.e17. <https://doi.org/10.1016/j.cell.2018.09.055>

Imidazole propionate's ability to impair insulin signaling starts with the activation of p38γ MAPK. P38γ mitogen-activated protein kinase promotes the phosphorylation of p62, also

known as ubiquitin-binding protein p62, which functions to degrade ubiquitinated proteins. (Liu 2016) This phosphorylation leads to the activation of mechanistic target of rapamycin complex 1 (mTORC1) which is involved in processes such as cell growth and autophagy and has been implicated in many disease processes.

The involvement of mTORC1 in this process is further demonstrated by the ability of rapamycin to inhibit the effects of imidazole propionate on the insulin receptor substrate. p62 regulates the nutrient sensing function of mTORC1 (Duran 2012). Increased activation of both of these in the liver has been observed in individuals with type 2 diabetes (Koh 2018). The result of these processes is a decrease in levels of the insulin receptor substrates 1 and 2 and of insulin-stimulated Akt phosphorylation. mTORC1 activation causes degradation and impaired functioning of insulin receptor substrate, which is important for normal insulin signaling (Koh 2018).

### **T-Cells and Immunity**

T-cells, a subset of white blood cells known as lymphocytes, are part of the adaptive immune system (Kumar 2018). The adaptive immune response refers to the ability of specialized cells to mount a response to antigens in a highly specific manner, and to “remember” these invaders in case of future attacks (Alberts 2002). T-cells, as part of the adaptive immune system, help protect against infection throughout different stages of life.

### **T-Cell Types and Functions**

T-cells develop from hematopoietic stem cells in the bone marrow, then mature and differentiate in the thymus (Kumar 2018). The three most common types are, CD4<sup>+</sup> helper T-cells, CD8<sup>+</sup> cytotoxic T-cells and regulatory T-cells (Tregs). Helper T-cells aid the function of other immune cells by secreting cytokines for their activation. This activation process enables B-

cells to produce antibodies and cytotoxic T-cells to carry out their function, which is to directly destroy virally infected cells (Sauls 2022). Cytotoxic T-cells have a specialized selection process that allows them to directly identify and kill off infected cells. Upon recognizing antigen-specific target cells, these cytotoxic T-cells are able to induce apoptosis. Regulatory T-cells function to maintain homeostasis by controlling the immune response when no longer needed. These Tregs play a key role in preventing autoimmunity in the body (Sauls 2022).

### **T-Cell Signaling Cascades**

The activation of T-cells is key to a successful immune response and is dependent on the function of T-cell receptor (TCR) complexes on their surface (Hwang 2020). Together with other co-stimulatory receptors (e.g. CD4, CD8, CD28), TCRs ligate antigens presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs), which then sets off different signaling pathways resulting in activities such as increased or decreased cell differentiation, proliferation, and cytokine production. This allows for an efficient, precise, and individualized response to specific pathogenic threats (Hwang 2020).

One result of T-cell activation is cellular proliferation. Antigen stimulated T-cell proliferation is dependent on the functioning of  $Ca^{2+}$  as a second messenger in the IP3- $Ca^{2+}$ -NFAT pathway (Hwang 2020). T-cell proliferation is necessary in the process of fighting off infection or disease as it allows for the formation of a large pool of antigen-specific cells to carry out the different cellular functions.

The secretion of certain cytokines can also be considered to be a marker of T-cell activation. Interferon  $\gamma$  ( $IFN\gamma$ ) in particular is produced by activated CD4 and CD8 T-cells and natural killer (NK) cells, and works to enhance pro-inflammatory signaling (Schroeder 2004).  $IFN\gamma$  goes on to potentiate macrophage activation, antigen presentation, CD8 T-cell expansion,

and other immune functions. These functions first require the interaction of IFN $\gamma$  with its receptor. This binding sets off the JAK-STAT pathway which then activates transcription factors and other genes involved in these processes.

### ***Effect of Insulin Signaling on T-Cells***

The insulin receptor has been shown to play a key role in T-cell functions and thus, in adaptive immunity (Fischer 2017). According to the Warburg effect, T-cells at rest utilize oxidative phosphorylation to meet their energy needs. However, upon activation, there is a switch to aerobic glycolysis. T-cell proliferation, differentiation, and cytokine production, which are all indicators of activation, are energy-requiring processes that require increased uptake of glucose, which is accompanied by upregulation of the insulin receptor. Reduced expression of the insulin receptor has been shown to impair glucose uptake and the aerobic glycolysis it requires, and the result is weakened T-cell function. For example, in a study by Fischer et al., where the insulin receptor were silenced using an inducible knockdown system in cytokine production, proliferation, and migration of CD4<sup>+</sup> T-cells were significantly compromised, as well as increased their apoptosis, and also reduced the cytotoxic capacity of CD8<sup>+</sup> cells (Fischer 2017).

## THE PROJECT

### **Justification for Looking at Imidazole Propionate**

In type 2 diabetes, insulin signaling is impaired which prevents the body's cells from efficiently taking up glucose, resulting in high glucose levels in the blood. Imidazole has been shown to impair insulin signaling through mTORC1 in both liver and kidney cells. Impaired signaling through mTORC1 reduces levels of insulin receptor substrates 1 and 2 and inhibits the

phosphorylation of Akt, a protein stimulated by insulin signaling. Akt signaling is necessary for the uptake of glucose by cells, which is a key function of insulin (Mackenzie 2014).

Normal insulin signaling is important to the function and metabolism of T-cells. T-cells require energy in the form of glucose to carry out their functions, and glucose uptake has been shown to be limiting in the activation of T-cells (Jacobs 2008). T-cell activation, which results in proliferation and interferon gamma expression, depends on normal insulin signaling (Tsai 2018). By disrupting Akt signaling and decreasing insulin receptor substrate levels, imidazole propionate may be indirectly impairing the glucose uptake by T-cells, and thus impairing their functioning.

People with conditions such as obesity and type 2 diabetes are shown to have decreased immunity (Frydrych 2018). The hypothesis in this project is that imidazole propionate will reduce T-cell activation and proliferation, as T-cells are dependent on insulin for these functions.

### **Study Question**

The study question for this project was whether imidazole propionate directly affects T-cell functioning. This was explored in human peripheral blood mononuclear cells. Our hypothesis was that imidazole propionate would impair T-cell function.

### **Outcome Measures**

In this project, we measured T-cell proliferation and IFN $\gamma$  levels as markers of T-cell activation following the treatment of cells with ImP.

## **METHODS**

### **Human Subjects**

10 healthy adults (aged 18-35; male 5, female 5) were recruited from the University of Memphis student population. Individuals who were pregnant or breastfeeding, taking

immunomodulatory medications, had a BMI below 18 or above 30, smokers, diagnosed with any autoimmune disease, immune deficiency disease, chronic infection, or any non-infectious chronic disease (e.g. diabetes mellitus, thyroid disease), were ineligible for participation in the study. Subjects provided informed consent, and approval for human studies was obtained from the University of Memphis Institutional Review Board (IRB # PRO-FY2023-194).

### **Materials and Reagents**

1. 1X Phosphate Buffered Saline (Thermo Fisher Scientific, Gibco™, catalog number: 10010-023)
3. Cytiva Ficoll-Paque™ PLUS endotoxin tested (<0.12 EU/mL) (catalog number: 17144002)
4. Gibco Trypan Blue Solution, 0.4% (catalog number: 15250061)
5. SantaCruz Biotechnology Deamino-histidine (CAS: 1074-59-5)
6. BD Cytotfix/Cytoperm™ Plus Fixation/Permeabilization Solution Kit with BD GolgiPlug™(catalog number: 555028)
7. 1X RPMI-1640 Medium- L-Glutamine (catalog number: SH30096.01)
8. Cytiva F2442 Fetal Bovine Serum
9. BioLegend Cell Activation Cocktail (without Brefeldin A) (catalog number: 423302)
10. 5,000 U/mL Penicillin-Streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15070-063)
11. MP Biomedicals Human Recombinant Insulin (catalog number: 193900)
12. BioLegend Pacific Blue anti-human CD8 antibody (clone: SK1, catalog number: 344717)
13. Biolegend Pacific Blue anti-human CD4 antibody (clone: SK3; catalog number: 344619)
14. BioLegend FITC Mouse IgG1 x Isotype Ctrl (clone: MOPC-21; catalog number: 400110)

15. eBioscience Invitrogen CD3 monoclonal antibody (OKT3), FITC (catalog number: 11-0037-42)
16. BioLegend PE anti-human CD4 antibody (clone: A161A1, catalog number: 357403)
17. eBioscience Invitrogen IFN gamma monoclonal antibody (4S.B3), FITC, (catalog number: 11-7319-81)
18. eBioscience Invitrogen Anti-Human CD28 (clone: CD28.2, ref: 16-0289-81)
19. BioLegend Recombinant Human IL-2 (catalog number: 589102)
20. eBioscience Anti-Human CD4 antibody, PerCP-eFluor 710 (clone: SK3; ref: 46-0047-41)
21. Invitrogen CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry (catalog number: C34554)
22. BioLegend Zombie Aqua TM Fixable Viability Kit (DMSO) (catalog number: 423101/423102)

### **Blood Collection**

Blood (9-12 ml) was collected after an overnight fast by venipuncture and collection into Greener Bio-One K2-EDTA tubes. All samples were labeled with sample identification number only. Universal precautions were observed to minimize the risk of infection or injury to the subject and the phlebotomist.

### **Isolation of Peripheral Blood Mononuclear Cells**

Whole blood was diluted with equal parts of Phosphate Buffered Solution (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated via density gradient centrifugation utilizing Ficoll-Paque (Cytiva, catalog number: 17144002) by carefully layering the diluted blood over an equal volume of Ficoll and centrifuging for 30 minutes at 2300rpm at 25 °C without brakes. After centrifugation, cell type separated into various layers based on density (see



figure 2 below). The “buffy coat”, containing the PBMCs, was removed and added to a separate tube. PBMCs were washed twice with PBS. An aliquot of cells was stained with trypan blue (Gibco, catalog number: 15250061) and counted using a hemocytometer to determine cell number.

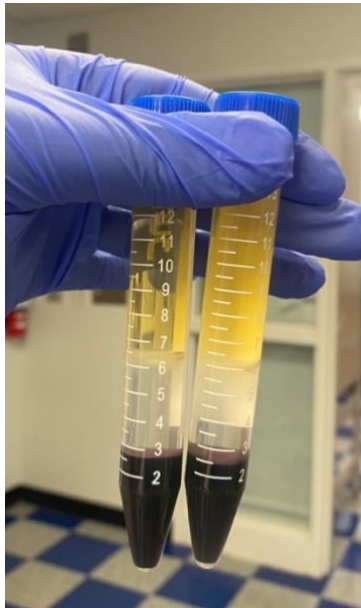


Figure 2. Layers of blood separated using Ficoll-Paque density gradient centrifugation.

### **PMA and Ionomycin Stimulation**

Approximately 800,000 PBMCs were plated into a 96-well round bottom plate in Complete Media (RPMI 1640, 1x glutamine, 1x penicillin/streptomycin, 10% FBS). Cells were stimulated with a stimulation cocktail (BioLegend, catalog number: 423302) that contained phorbol 12-myristate 13 acetate (PMA) and ionomycin in the presence of Brefeldin, a protein transport inhibitor (GolgiPlug, BD, catalog number: 555028) for six hours. Prior to stimulation, cells were cultured in the presence or absence of imidazole propionate (SantaCruz Biotechnology, CAS: 1074-59-5) for 12 hours. Time of incubation and concentration of imidazole propionate was based on studies performed in hepatocytes.

### **PMA and Ionomycin Stimulation in the Presence of Insulin**

Approximately 800,000 PBMCs were plated into a 96-well round bottom plate in complete Media. Cells were stimulated with a stimulation cocktail in the presence of GolgiPlug for six hours in the presence of human recombinant insulin (MP Biomedicals, catalog number: 193900) at 10mg/mL. Prior to stimulation, cells were cultured in the presence or absence of imidazole propionate (SantaCruz Biotechnology, CAS: 1074-59-5) for 12 hours.

### **CD3/CD28/IL-2 Stimulation**

For cell proliferation experiments, PBMCs were isolated as described above and cells were stained with 5uM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, catalog number: C34554) according to manufacturer's instructions. Briefly, cells were incubated in warm complete media containing CFSE for 20 minutes at 37°C in the dark, with shaking every 2 minutes. This was followed by the addition of cold media and incubation on ice for 5 minutes to inhibit the cellular uptake of CFSE. 700,000 to 800,000 CFSE-labeled PBMCs were plated into a 96-well round-bottom plate. Prior to addition of cells, wells were coated with 1mg/mL CD3 (eBioscience, clone: OKT3, catalog number: 11-0037-42) for 2 hours according to the ThermoFisher Scientific protocol. Plated cells were further stimulated with anti-CD28 (eBioscience, clone: CD28.2, ref: 16-0289-81) at 5ug/ml and IL-2 at 0.2 mg/mL. 500uM imidazole propionate was added at the time of stimulation. Cells were incubated at 37°C in a 5% CO2 incubator for 72 hours prior to antibody staining.

### **Staining for Cell Surface Antigens**

For both the IFN $\gamma$  and proliferation assay, cells were removed from the 96-well plate after incubation and transferred to Epindorf tubes and washed twice using PBS, followed by Zombie Aqua (BioLegend, catalog number: 423101/423102) staining for 15 minutes. Zombie

Aqua staining is used to identify dead cells. For the IFN $\gamma$  assay, cells were then stained with the following antibodies in staining media (PBS + 2% FBS): anti-CD4 PE (BioLegend, clone: A161A1, catalog number: 357403) and anti-CD8 PacBlue (BioLegend, clone: SK1, catalog number: 344717). Staining was done at room temperature for 20 minutes, followed by two washes with staining media. The cells were then all fixed with FixPerm solution (BD, catalog number: 555028) for 20 minutes, then washed again with staining media for overnight storage or immediate permeabilization with 1x PermWash solution (BD, catalog number: 555028). For proliferation assays, the following antibodies were used: anti-CD4 PerCP (eBioscience, clone: SK3; ref: 46-0047-41) and anti-CD8 PacBlue (BioLegend, clone: SK1, catalog number: 344717). For 20 minutes, followed by two more washes with staining media, at which point cells were immediately analyzed by flow cytometry (Life Technologies, Attune NxT Acoustic Focusing Cytometer).

### **Permeabilization of Fixed Cells and Staining for Intracellular Cytokines**

For intracellular staining, fixed cells were resuspended in 1x PermWash for 15 minutes, then pelleted. Cells were then resuspended in PermWash and either anti-IFN $\gamma$  FITC (eBioscience, catalog number: 11-7319-81) or an isotype control (BioLegend, catalog number: 400110) (negative control) and incubated for 30 minutes, followed by two washes with PermWash and resuspension in staining buffer for flow cytometry.

### **Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 9. Data are presented as mean $\pm$ SD. Statistical significance was assessed using the Wilcoxon signed-rank T test between two groups. For analysis of sex and treatment effect, the two-way analysis of variance (ANOVA) was used. For all tests,  $p < 0.05$  was considered significant.

## RESULTS

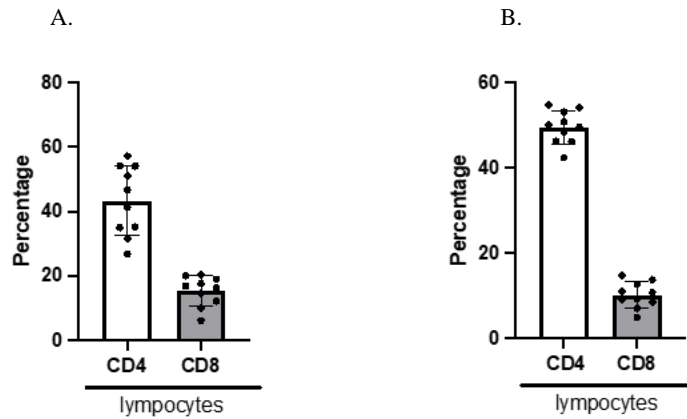


Figure 3. Percentage of unstimulated CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in mixed human PBMC population isolated using Ficoll density gradient, assessed for IFN $\gamma$  expression (A) and T-cell proliferation (B). Cells for IFN $\gamma$  expression (A) were in culture for 18 hours, and for T-cell proliferation (B) for 72 hours.

### **IFN $\gamma$ Expression**

The production of IFN $\gamma$  by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells was analyzed as a marker of T-cell activation. Figure 4 demonstrates the gating strategy to identify IFN $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. In the gating strategy, dead cells were excluded based on a dead cell marker (ZombieAqua) and lymphocytes included based on physical parameters of lymphocytes.

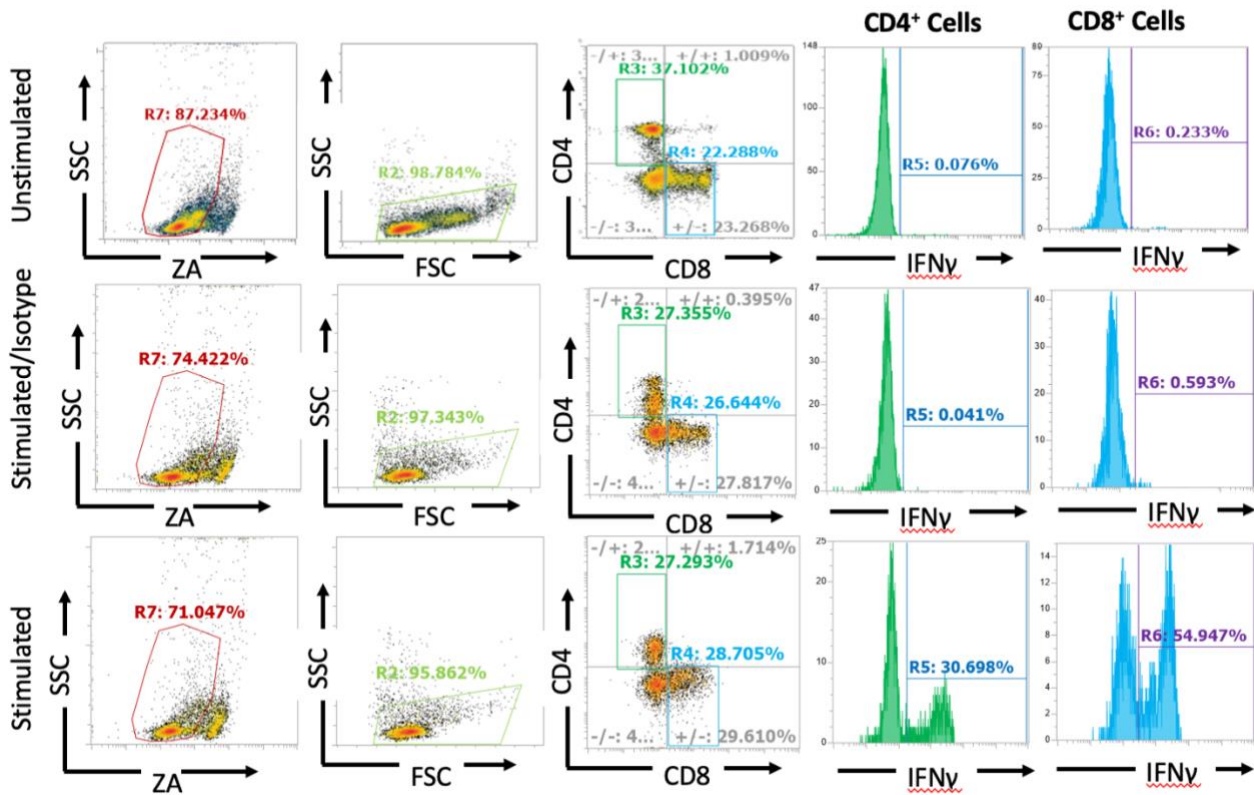


Figure 4. Representative flow cytometry plots and histograms demonstrating gating strategy for identification of IFN $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> cells.

Figure 5 shows a representative image CD4<sup>+</sup> and CD8<sup>+</sup> T-cell IFN $\gamma$  production in response to PMA and ionomycin stimulation in the presence of imidazole propionate among individual subjects.

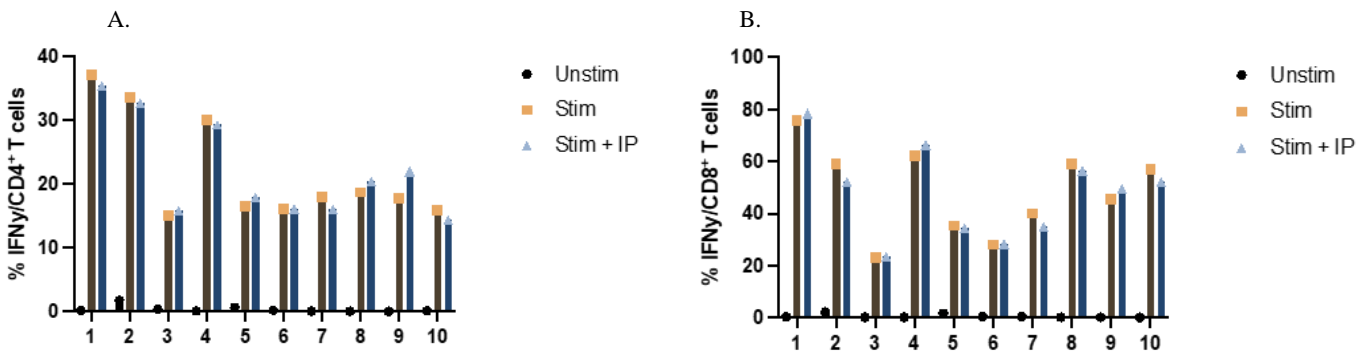


Figure 5. IP effect on interferon gamma expression by CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T-cells stimulated via PMA/ionomycin for 6 hours in individual subjects.

**Imidazole propionate does not reduce IFN $\gamma$  synthesis in response to PMA and Ionomycin stimulation.**

Human PBMCs were treated with 500uM imidazole propionate for 12 hours prior to PMA/Ionomycin stimulation. Six hours after stimulation, intracellular levels of IFN $\gamma$  were quantified in CD4<sup>+</sup> and CD8<sup>+</sup> T cells via flow cytometry. Results are given as percentage IFN $\gamma$ <sup>+</sup>/CD4<sup>+</sup> cells (Figure 6A) and IFN $\gamma$ <sup>+</sup>/CD8<sup>+</sup> cells (Figure 6B). The fraction of IFN $\gamma$ <sup>+</sup> cells within the CD4<sup>+</sup> population after stimulation is 21.89 $\pm$ 8.34 % while the pretreatment resulted in a IFN $\gamma$ <sup>+</sup> fraction of 22.04  $\pm$ 7.72 %. The fraction of IFN $\gamma$ <sup>+</sup> cells within the CD8<sup>+</sup> population after stimulation was 48.52 $\pm$ 16.85% while pretreatment with imidazole propionate resulted in 47.65 $\pm$ 17.39 % IFN $\gamma$ <sup>+</sup> cells within the CD8<sup>+</sup> population. Cells stimulated with and without imidazole treatment were compared using a Wilcoxon T-test. No significant difference was detected with imidazole propionate pretreatment for CD4<sup>+</sup> T cells (p=0.9999) and CD8<sup>+</sup> T cells (p=0.56).

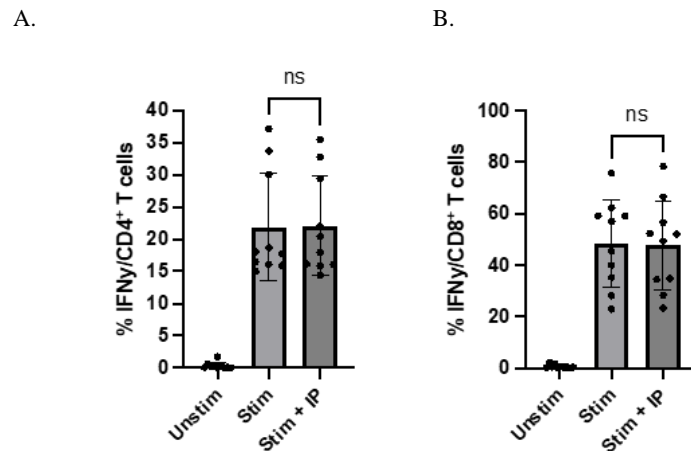


Figure 6. IP effect on interferon gamma expression by CD4<sup>+</sup> T-cells (A) and CD8<sup>+</sup> T-cells (B) stimulated in culture via PMA/ionomycin for 6 hours. N=10, p>0.05

**Imidazole propionate does not reduce IFN $\gamma$  synthesis in response to PMA and Ionomycin stimulation in the presence of insulin.**

The aforementioned experiment was repeated for CD4<sup>+</sup> and CD8<sup>+</sup> T-cells stimulated in the presence of insulin. The fraction of IFN $\gamma$ <sup>+</sup> cells within the CD4<sup>+</sup> population was 22.28 $\pm$ 2.42% vs pretreatment which was 21.46 $\pm$ 2.53%. The fraction of IFN $\gamma$ <sup>+</sup> cells with the CD8<sup>+</sup> population was 49.23 $\pm$ 5.1% vs pretreatment which was 48.63 $\pm$ 5.22%. No significant difference in IFN $\gamma$  levels was detected with imidazole pretreatment for CD4<sup>+</sup> (Figure 7A, p=0.32) and CD8<sup>+</sup> cells (Figure 7B, p=0.49) stimulated in the presence of insulin.

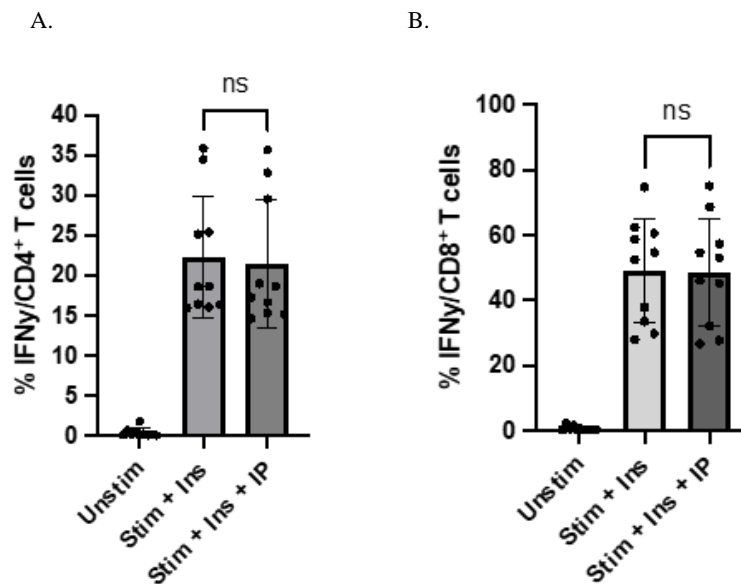


Figure 7. IP effect on interferon gamma expression by CD4<sup>+</sup> T-cells (A) and CD8<sup>+</sup> T-cells (B) stimulated in culture for 6 hours via PMA/ionomycin in the presence of insulin. N=10, p>0.05

To determine the effect of insulin on PMA/ionomycin induced IFN $\gamma$  levels, cells stimulated in the presence and absence of insulin were also compared. Insulin did not increase IFN $\gamma$  in PMA/Ionomycin-activated CD4<sup>+</sup> cells (Figure 8A, p=0.70) or CD8<sup>+</sup> cells (Figure 8B, p=0.92).

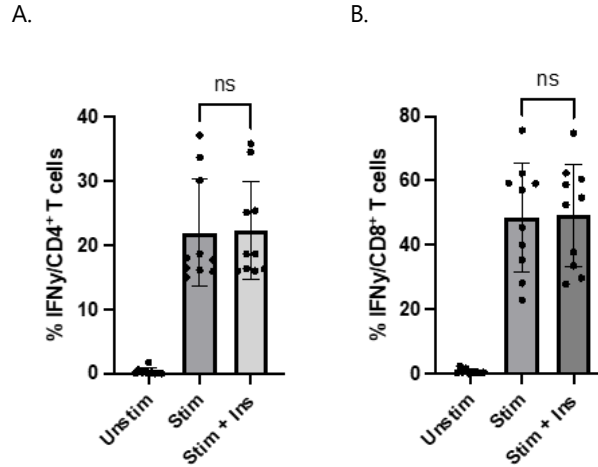


Figure 8. Insulin effect on interferon gamma expression by CD4 $^+$  T-cells (A) and CD8 $^+$  T-cells (B) stimulated via PMA/ionomycin for 6 hours.

To determine whether sex affected IFN $\gamma$  response to imidazole pretreatment before stimulation, a 2-way ANOVA was used to assess differences in IFN $\gamma$  levels in CD4 $^+$  and CD8 $^+$  T-cells. No differences were detected between males and females for IFN $\gamma$  expression in either CD4 $^+$  (Figure 9A,  $p=0.90$ ) and CD8 $^+$  cells (Figure 9B,  $p=0.60$ ).

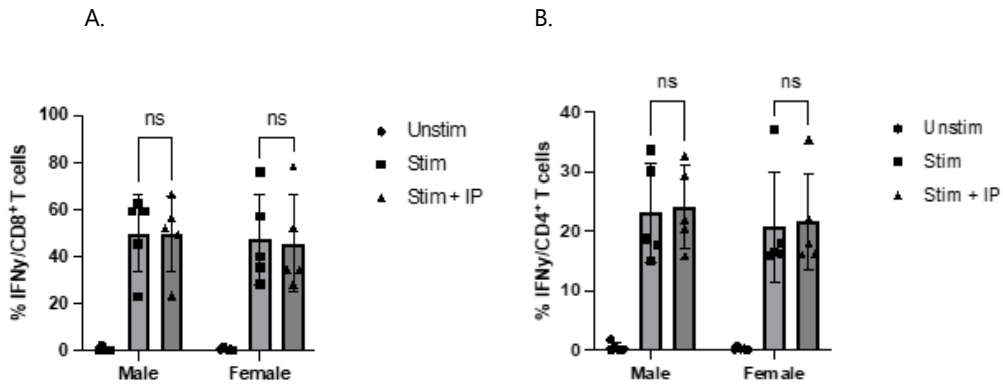


Figure 9. IP effect on interferon gamma expression by CD4 $^+$  T-cells (A) and CD8 $^+$  T-cells (B) stimulated in culture via PMA/ionomycin for 6 hours in males versus females. N=5,  $p>0.05$ .

To assess whether sex made a difference in T-cell activation (assessed via IFN $\gamma$  levels) in the presence and absence of insulin, a 2-way ANOVA was conducted for CD4 $^+$  and CD8 $^+$  T-



cells. No significant difference was found IFN $\gamma$  levels in CD4<sup>+</sup> (Figure 10A, p=0.7) and CD8<sup>+</sup> T-cells (Figure 10B, p=0.63) stimulated in the presence and absence of insulin. Cells stimulated in the presence and absence of insulin were also compared between males and females to assess whether insulin altered T-cell activation. No difference was detected for CD4<sup>+</sup> (Figure 10C, p=0.4490) and CD8<sup>+</sup> (Figure 10D, p=0.2637) T-cells stimulated in the presence and absence of insulin.

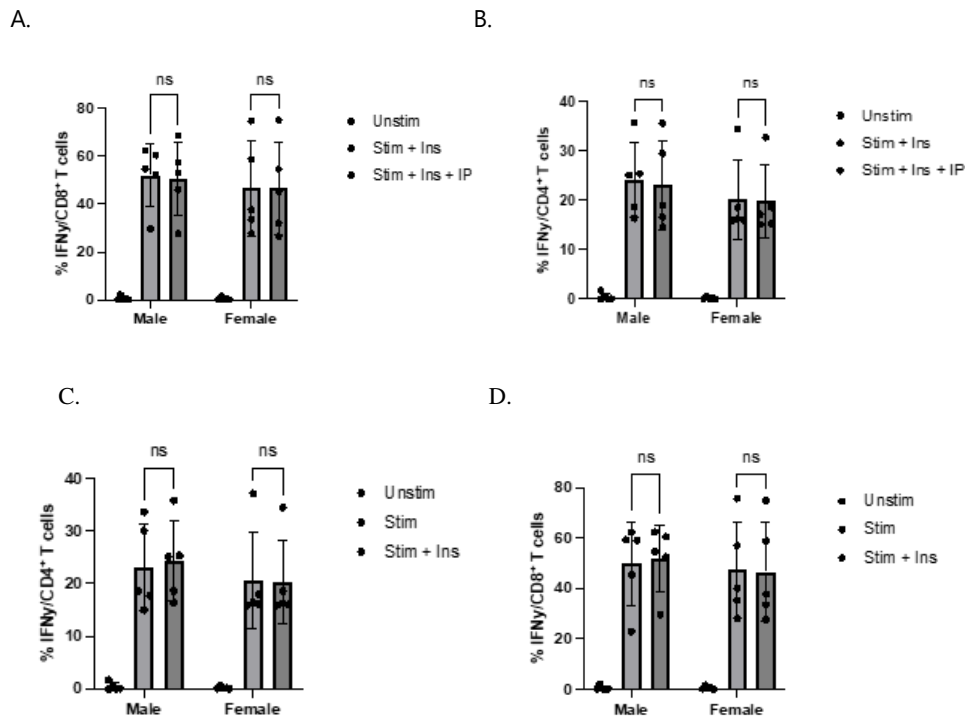


Figure 10. IP effect on interferon gamma expression by CD4<sup>+</sup> T-cells (A) and CD8<sup>+</sup> T-cells (B) stimulated via PMA/ionomycin for 6 hours. Cells were also stimulated in the presence of insulin in males versus female for CD4<sup>+</sup> T-cells (C) and CD8<sup>+</sup> T-cells (D). N=5, p>0.05.

### T-Cell Proliferation

Proliferation by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in response to CD3/CD28/IL-2 stimulation was analyzed as a marker of T-cell activation. Figure 11 demonstrates the gating strategy to identify proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. In the gating strategy, dead cells were excluded based on a

dead cell marker (ZombieAqua) and lymphocytes included based on physical parameters of lymphocytes.

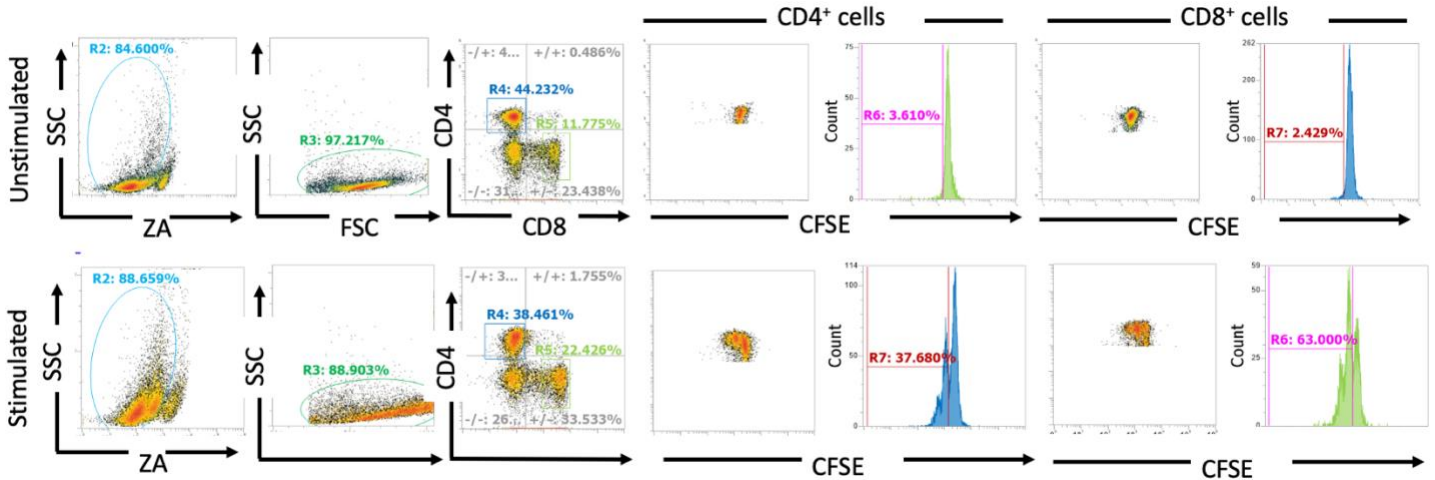


Figure 11. Representative flow cytometry plots and histograms demonstrating gating strategy for identification of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells.

Figure 12 shows a representative image of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation in response to CD3/CD28/IL-2 stimulation in the presence of imidazole propionate among individual subjects.

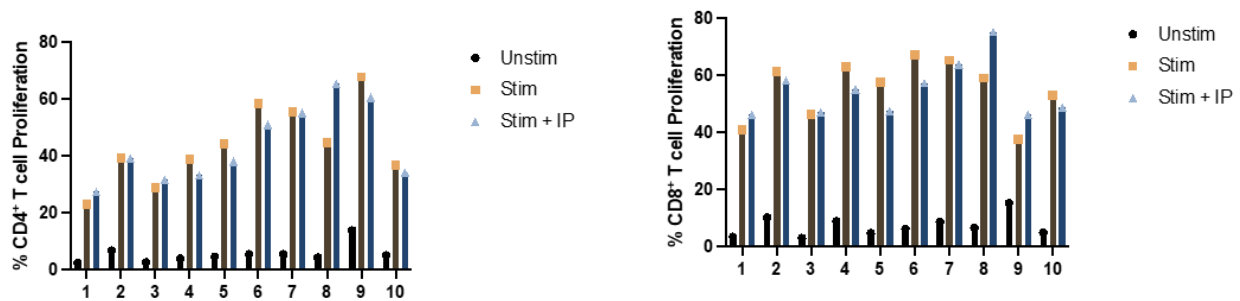


Figure 12. IP effect on proliferation by CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T-cells stimulated via CD3/CD28/IL-2 in individual subjects.

**Imidazole propionate does not reduce T-cell proliferation in response to CCD3/CD28/IL-2 stimulation.**

CFSE-labeled T-cell proliferation was assessed after PBMC stimulation with anti-CD3, anti-CD28, and IL-2. Percentage of proliferating cells were determined by dilution of proliferation dye. The stimulation protocol resulted in the proliferation of CD4<sup>+</sup> cells; 5.48±1.02% (unstimulated) and 43.84±4.3% (stimulated). In the presence of ImP the proliferation fraction for stimulated CD4<sup>+</sup> cells were 43.65±4.22%. The percentage of proliferating CD8<sup>+</sup> cells were as follows: unstimulated 7.22±1.17%, stimulated – 55.21±3.27% and with imidazole propionate pretreatment 54.65±3.01%. The proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells with and without incubation with imidazole propionate was compared using Wilcoxon t-tests. No significant difference was detected for CD4<sup>+</sup> (Figure 13A, p=0.43) or CD8<sup>+</sup> T-cells (Figure 13B, p=0.69).

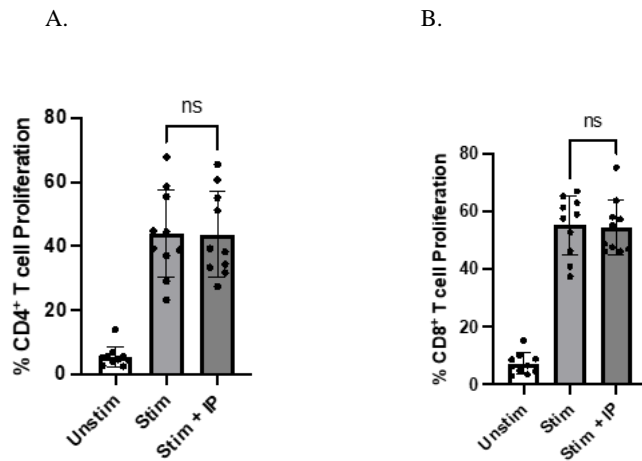


Figure 13. IP effect on proliferation by CD4<sup>+</sup> T-cells (A) and CD8<sup>+</sup> T-cells (B) stimulated via CD3/CD28/IL-2, traced using CFSE labelling. N=10, p>0.05

To determine whether sex affected proliferation response to imidazole propionate, a 2-way ANOVA was used to assess differences in proliferation in CD4<sup>+</sup> and CD8<sup>+</sup> cells. No

differences were detected between males and females for T-cell proliferation in either CD4<sup>+</sup> (Figure 14A, p=0.4132) or CD8<sup>+</sup> (Figure 14B, p=0.2171) cells.

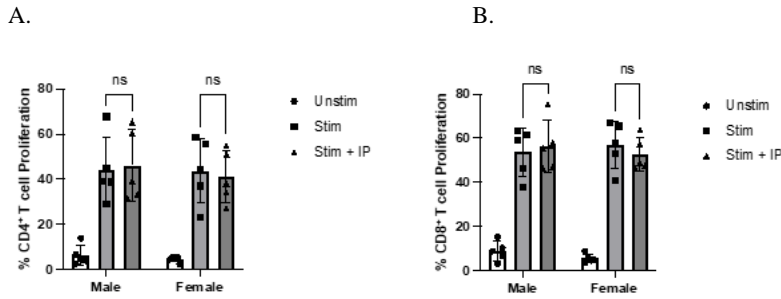


Figure 14. IP effect on proliferation by CD4<sup>+</sup> T-cells (A) and CD8<sup>+</sup> T-cells (B) stimulated via CD3/ CD28/IL-2 in males versus females, traced using CFSE labeling. N=5, p>0.05.

## DISCUSSION

Our hypothesis was that imidazole propionate, which has been shown in human liver and kidney cells to impair the signaling of insulin, could potentially affect human T-cell functioning as well via impaired insulin metabolism. Insulin is one of the human body's major anabolic hormones, responsible for bringing energy into various tissue and cell types (Tokarz et al., 2018). Tsai et al discussed the role of insulin receptor-mediated stimulation in T-cell function (Tsai et al., 2018). Insulin receptor signaling is known to control the production of cytokines by and proliferation of T-cells. In the study, the addition of insulin to a T-cell co-culture was found to increase T-cell proliferation, IFN $\gamma$  production, and IL-2 production. In turn, T-cells with an insulin receptor knockdown had reduced pro-inflammatory cytokine production and proliferation in response to stimulation (Tsai et al., 2018). In a similar study by Fischer et al., silencing of the insulin receptor in knockdown rats decreased glucose transport and glycolysis in activated CD4<sup>+</sup> cells, accompanied by reduced migration, proliferation, and IFN $\gamma$ , IL-4, and IL-10 levels. For CD8<sup>+</sup> cells, cytotoxic capacity in response to doxycycline hyclate administration was

significantly decreased with silencing of the insulin receptor (Fischer et al., 2018). Studies such as these speak to the necessity of insulin and its downstream signaling cascades in the normal functioning of T-cells.

The activation of T-cells is an energy-requiring process that requires an increase in glucose uptake and utilization. This idea is supported by the increase in Glut1 transporter expression that accompanies T-cell activation (MacIver et al, 2008). Glut1 is the major glucose transporter found on T-cells (Palmer et al, 2015). Though Glut1 was once thought to function independently of insulin (Ebeling et al, 1998), recent research in animal models has shown insulin and its receptor to be crucial to the glucose uptake and glycolysis accompanying the activation of T-cells, namely CD4<sup>+</sup> T-cells (Fischer et al, 2017). However, in our experiments, addition of insulin to the cell culture was found to make no significant difference in the stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, as shown through interferon gamma expression and proliferation.

A potential explanation for this lack of an effect is saturation of the insulin receptors on the T-cells. The complete media used for cell culture in the experiments contains insulin, and it is possible that this amount of insulin was already sufficient to promote interferon gamma expression and proliferation to the highest capacity of the T-cells. Thus, for this reason, the addition of more insulin in the cell culture may not have made a difference in the outcome measures used. This hypothesis could be tested by comparing the same outcome measures for cells incubated in insulin-free media.

Research has also shown that Glut1-mediated glucose uptake depends on Akt phosphorylation (Jacobs et al., 2008; Beg et al, 2017), which is a mechanism that imidazole propionate acts on (Koh et al., 2018). However, we did not see any significant difference in IFN $\gamma$

production or proliferation by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in response to imidazole propionate treatment in this study. A potential explanation is that T-cells lack a transporter to uptake imidazole propionate. As a relatively newly identified metabolite, there is limited knowledge on imidazole propionate and how it functions in the body. Furthermore, the first studies to investigate this metabolite did not conduct tests such as Western blot assays to identify potential receptors and transporters, so it is not yet known how IP comes into other cell types to impair insulin signaling. Functional outcomes have been found, but understanding of specific mechanisms is still needed.

## CONCLUSION

The interplay between the immune system and the gut microbiome is an ever-developing field that has garnered much attention especially in recent years. With the high and rising prevalence of metabolic diseases such as type II diabetes and obesity, especially in the western world, there is a need to understand the repercussions of these conditions for human health.

To our knowledge, this study was the first to investigate the potential role of the microbial metabolite imidazole propionate, previously shown to be increased in type II diabetes and obesity and demonstrated to impair insulin signaling in liver and kidney cells, in modulating human T-cell functioning. We have shown that there is no significant effect of imidazole propionate on either interferon gamma expression and proliferation by activated CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. More research is needed to further elucidate the roles of such microbial metabolites in influencing biological functions in the human body.

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