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Inflammatory Cytokine Expression of Immune Cells on Exposure to Invisalign® Material: An In Vitro Pilot Study

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INFLAMMATORY CYTOKINE EXPRESSION OF IMMUNE CELLS ON EXPOSURE TO INVISALIGN® MATERIAL: AN *IN VITRO* PILOT STUDY

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INFLAMMTORY CYTOKINE EXPRESSION OF IMMUNE CELLS ON EXPOSURE TO INVISALIGN® MATERIAL: AN *IN VITRO* PILOT STUDY

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University of Nebraska, 2020

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The purpose of this study is to characterize the inflammatory cytokines that are expressed in peripheral blood mononuclear cells (PBMCs) and THP-1 monocytes exposed to Invisalign® aligner material *in vitro*. Invisalign® material was ground into particles, soaked in artificial saliva, and the eluate was collected. Commercially available cryopreserved pooled and individual human PBMCs and THP-1 monocytes were cultured with Invisalign® material eluate and positive (lipopolysaccharide) and negative controls. The supernatants were collected and used with a BioLegend LEGENDplex™ multi-analyte flow assay, a sandwich enzyme-linked immunosorbent assay (ELISA), to identify the expression of TNF-α, IL-6, IL-8, IL-10, IFN-γ, IL-1β, and IL-12p70. The results of this study show that Invisalign® material stimulates IL-8 expression in certain individual donor PBMCs and in THP-1 monocytes. Invisalign® material also stimulates low levels of TNF-α expression in pooled PBMCs, certain individual donor PBMCs and THP-1 cells. IL-6 was slightly increased in certain individual donor PBMCs. IL-1β was also slightly stimulated by Invisalign® material in THP-1 cells. IL-10, IL-12p70, IFN-γ, and IL-6 were not detected after stimulation with Invisalign® material in THP-1 monocytes. Invisalign ® material stimulated IL-8 expression across experiments, though not significantly, in individual donor PBMCs and THP-1 monocytes when profiled with a BioLegend LEGENDplex™ multi-analyte flow assay. IL-8 is a chemokine responsible for directing neutrophils to the site of inflammation.

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CHAPTER 1: INTRODUCTION

Invisalign® is clear aligner system used in orthodontic treatment to treat malocclusions. It is used worldwide and over 9 million patients have been treated with this product (Invisalign® 2020). The clear trays are a more esthetic option than traditional fixed appliances and have drawn more patients, especially adults, into orthodontic treatment (Kravitz et al. 2009).

The side effects of treatment with Invisalign® may include pain, difficulty with speech and chewing, and mucosal irritations. Many of these side effects decrease with continued wear and acclimation to the appliance. In 2017, Allareddy et al. found other, potentially more serious, side effects associated with Invisalign® material when reviewing the Manufacturer and User Facility Device Experience (MAUDE) database. From 2006-2016 there were 173 reports of side effects after Invisalign® aligner wear, including difficulty of breathing, hives, anaphylaxis, and swelling of the gums and tongue, among others (Allareddy et al. 2017). One case report described a patient who had hives, swelling, and burning of the lips after using Invisalign® material. When the patient was patch tested with Invisalign® material, they were deemed to have contact hypersensitivity (Awosika et al. 2017). Another patient of the University of Nebraska Medical Center College of Dentistry was diagnosed with swollen gingiva after using Invisalign®, with biopsy exhibiting foreign body granulomatous inflammation (Narayana N, personal communication, May 1, 2020).

A previous study on the biologic effect of Invisalign® material showed no cytotoxic effect (Eliades et al. 2009), whereas another found a slight cytotoxic effect (Martina et al. 2019). Invisalign® material does not induce estrogenic effects (Eliades et al. 2009) and does not contain Bisphenol A (BPA) (Kotyk and Wiltshire 2013). Premaraj et al. (2014) found that when particulate Invisalign® material was added to oral keratinocytes, there was an increase in cell death and compromised cell membrane integrity. They postulated that this compromised cell membrane integrity could be from leaching of the material's components.

According to the Material Safety Data Sheet, Invisalign® material is a thermoplastic polyurethane (Material safety data sheet 2015). Although the exact composition is propriety, diisocyanate is one component that could be responsible for some patients' adverse reactions (Premaraj et al. 2014). Isocyanates are known to cause asthma and allergic contact dermatitis (Frick et al. 2003).

Even though immune responses are thought to be the cause of adverse reactions associated with Invisalign® material, mechanisms of the immune reaction-induced inflammation have yet to be studied in detail. Peripheral blood mononuclear cells (PBMCs) and THP-1 monocytes are frequently used in studying inflammation and immune response. PBMCs include lymphocytes (T cells, B cells, and natural killer (NK) cells), monocytes, and dendritic cells (Kleiveland 2015). THP-1 is an immortalized monocyte cell line derived from the peripheral blood of a patient with acute monocytic leukemia (Tsuchiya et al. 1980).

The hypothesis of this study is that immunological cytokines expressed by PBMCs and monocytes after exposure to Invisalign® material drive inflammation. Though there are hundreds of cytokines, this study was limited to the following cytokines: IL-1β, TNF-α, IL-6, IL-8, IL-12, IFN-γ, and IL-10. These cytokines are a mix of pro- and anti-inflammatory agents that are produced by several different immune cell types.

Hypersensitivity immune reactions are classified into four types, according to the Gell-Coombs classification (Coombs and Gell 1968). Type I hypersensitivity reactions are considered immediate hypersensitivity reactions. Symptoms present quickly, within minutes or a few hours and include urticaria, angioedema, anaphylactic shock, and rhinitis. Type II hypersensitivity reactions are antibody-mediated, and examples include thrombocytopenia and hemolytic anemia (Descotes and Choquet-Kastylevsky 2001). Type III hypersensitivity reactions are immune complex-mediated reactions, and examples include serum sickness and Arthus reaction (Justiz Vaillant et al. 2020). Type IV hypersensitivity reactions are delayed reactions and include skin reactions from plant resins, drugs, cosmetics, and environmental chemicals (Descotes and

Choquet-Kastylevsky 2001). Delayed-type reactions take a minimum of 12-24 hours to develop (Krouse et al. 2008), with most developing within 2-14 days after exposure (Descotes and Choquet-Kastylevsky 2001; Warrington et al. 2011).

The side effects noted with Invisalign® material (hives, difficulty breathing, swollen lips and tongue) are most closely related to type I (immediate hypersensitivity) and type IV (cell-mediated delayed-type hypersensitivity). PBMCs stimulated with beta-lactam antibiotics (antibiotics known to cause delayed-type reactions and immediate reactions) had increased expression of IFN-γ (Lochmatter et al. 2009). When stimulated with nickel (a metal known to cause delayed-type reactions), PBMCs did not increase IFN-γ or TNF-α, but there was an increase in IL-4, which are associated more with Th2 cells and humoral immunity (Borg et al. 2000).

The rationale for this project was to document the cytokines expressed by PBMCs and THP-1 monocytes challenged with Invisalign® plastic material. These cytokines are thought to be involved in the Invisalign® material induced-immune response and subsequent side effects. This research is innovative because there has been no research to date on the Invisalign® material's immune response. The primary impact of this study, if successful, would help to understand why some individuals react to Invisalign® material, and the mechanism by which the immune reactions are produced. This knowledge may help prevent unwanted reactions, identify who may be susceptible, and possible treatment options.

The purpose of this study was to examine the expression pattern of cytokines (IL-1, TNF-α, IL-6, IL-8, IL-12, IFN-γ, IL-10) when PBMCs and THP-1 monocytes are exposed to Invisalign® material *in vitro*, using a sandwich enzyme-linked immunosorbent assay (ELISA) technique multiplex flow cytometry.

CHAPTER 2: LITERATURE REVIEW

2.1. Invisalign®

Invisalign® is a clear aligner system used to straighten teeth and has become a popular treatment among orthodontists and patients alike. According to the Invisalign® website, over 8 million patients have been treated worldwide. Invisalign® was introduced in 1997 by Align Technology (Santa Clara, California) as a more esthetic counterpart to traditional fixed appliances. The more esthetic option has drawn more adults into orthodontic treatment (Kravitz et al. 2009), but Invisalign® continues to develop products for even the youngest orthodontic patients.

The Invisalign® clear aligner system is available to orthodontists and dentists to treat a variety of malocclusions. The process starts by taking an impression or digital scan of the patient's teeth and sending it to Align Technology. The computer-aided-design and computer-aided- manufacturing (CAD-CAM) technology creates a treatment sequence that that is reviewed by the doctor (Kravitz et al. 2009; Phan and Ling 2007). The clear aligners are then manufactured and sent to the doctor who oversees patient treatment. Each aligner is designed to fit over the teeth and is programmed to move the teeth about .25-.3 mm. Previously it was recommended that the patient wear each tray for 14 days (Kravitz et al. 2009; Phan and Ling 2007), but currently, many doctors are having their patients wear each tray for a shorter period, even 7 days. The patient is advised to wear their trays for 20-22 hours per day, only taking them out to eat, brush, or drink anything other than water (Phan and Ling 2007).

2.2. Side Effects

As with any treatment, Invisalign® treatment does come with side effects. One side effect of treatment with both traditional fixed appliances and aligners is pain from tooth movement. Although patients undergoing aligner treatment have less pain overall than those with fixed appliances, their pain from aligners peaks during the first 24 hours and decreases until day 7 (White et al 2017). With less pain from treatment, Invisalign® patients also take fewer pain

medications, such as Ibuprofen, than patients with traditional fixed appliances (White et al. 2017). Other common side effects during Invisalign® aligner treatment include difficulty with speech and swallowing, difficulty with chewing, food packing between teeth, mucosal irritation, sleep disturbances, and TMJ problems (Miller et al. 2007; Nedwed and Miethke 2005; Pacheco-Pereira et al. 2018; White et al. 2017). Poor oral hygiene during Invisalign® aligner wear can cause caries and worsened periodontal health; although compared to traditional fixed appliances, patients wearing aligners had better periodontal health status overall (Chhibber et al. 2018; Levrini et al. 2015).

2.2.1. MAUDE Database

To examine more serious adverse effects of Invisalign® aligners, Allareddy et al. (2017) searched the Manufacturer and User Facility Device Experience (MAUDE) database from 2006-2016 for reports on Invisalign®. The MAUDE database is a United States Food and Drug Administration (FDA) program that includes reports from manufacturers, health care professionals, patients, and consumers. From November 1, 2006, to November 30, 2016, there were 173 medical device reports regarding adverse effects from Invisalign® aligners. Difficulty of breathing was the most common event reported (56 events). Other common reports included swollen tongue (31 reports), hives and itchiness (31 events), and anaphylaxis (30 events). Less common events were blisters on the tongue (6 events) and swelling of gums (5 events). In 26% of reported events, the treating doctor thought the event was very serious or life-threatening, with more serious events occurring in the years 2014 through 2016. Because this database is not based on mandatory reporting, the data may not be complete, accurate, or an actual result from the product itself (Allareddy et al. 2017).

2.2.2. Case Reports

In 2017, Awosika et al. (2017) reported that a patient who, two days after starting Invisalign® treatment, developed urticaria (hives) on her extremities, facial swelling, and burning of the lips and oral mucosa. The patient had patch testing performed with an Invisalign® material

sample and, at 96 hours, had a strong positive reaction that they deemed contact hypersensitivity (Awosika et al. 2017).

A patient at the University of Nebraska Medical Center College of Dentistry sought treatment for swelling and bleeding from the gums with no pain. She had been using Invisalign® for the past 18 months and had previously had sores treated with Chlorhexidine. She presented with generalized edematous gingival enlargement. A biopsy showed foreign body granulomatous inflammation. She was treated with oral prednisone for three months to control the inflammation (Narayana N, personal communication, May 1, 2020).

2.2.3. Biological Effects

There are very few studies that report on the effects of Invisalign® material at the cellular level. Eliades et al. (2009) found no cytotoxic effects of Invisalign® material on gingival fibroblasts, although Martina et al. (2019) reported a slight cytotoxic effect induced by the Invisalign® SmartTrack® material (78.8% of cell viability). Invisalign® material does not induce estrogenic effects (Eliades et al. 2009) and does not contain Bisphenol A (BPA) (Kotyk and Wiltshire 2013). Premaraj et al. (2014) exposed oral keratinocytes to ground Invisalign® material and found that cells treated with the material had metabolic inactivity and increased cell death compared to controls. The cells also showed compromised membrane integrity, reduced contact, and reduced mobility. This study demonstrates that Invisalign® material has a cytotoxic effect on keratinocytes and compromises cell membrane integrity. The authors postulate this could be an avenue for Invisalign® material components to enter the system and cause immune reactions (Premaraj et al. 2014).

2.3. SmartTrack® Material

In 2013, Align Technology launched its SmartTrack® material that was later patented in 2017. According to the company, SmartTrack® "delivers more gentle constant force to improve control of tooth movements with Invisalign® clear aligners" (SmartTrack® 2020). The Invisalign® website claims that SmartTrack® material straightens teeth faster and the trays are more

comfortable to take on and off. Compared to the material before SmartTrack®, the patients who had aligners made from SmartTrack® material had less pain for a shorter duration and increased overall comfort (Bräscher et al. 2016).

According to the SmartTrack® Aligner Material safety data sheet, the material is a multilayer aromatic thermoplastic polyurethane/copolyester. Although the specific components and amounts are proprietary, the material safety data sheet contains multiple warnings for diphenylmethane diisocyanate (MDI) as a component of the polyurethane. It warns that although thermoplastic polyurethane is non-hazardous in normal conditions, if heated above the decomposition temperature (492F), the MDI can be released and cause mucous membrane irritation, shortness of breath, and asthma-like symptoms, among others (Material safety data sheet 2015). In 2004, Schuster et al. (2004) found that no byproducts were released from the Invisalign® material when soaked in an ethanol-water solvent. They concluded, though, that because the trays may be abraded within the mouth, this may affect the release of byproducts while being worn. Others have hypothesized that the diphenylmethane diisocyanate (MDI) is the component of the Invisalign® material that causes many of the adverse biologic reactions (Premaraj et al. 2014).

2.4. Isocyanates

Isocyanates are low molecular weight compounds that are used in the production of polyurethanes. They contain one or more N=C=O groups that react with the hydroxyl groups from other compounds to form polymer chains. Polyurethanes that contain MDI are used in many industries, including automotive, clothing, paints, and medical care (Schuster et al. 2004; Wenk and Ehrlich 2012).

2.4.1. Adverse Effects

Isocyanates have been known to cause asthma and allergic contact dermatitis, and many reports have been in workplace settings (Aalto-Korte et al. 2012; Frick et al. 2003). One company that produced laminate boards with an MDI-containing polyurethane lacquer had five employees develop eczema on their hands and forearms that was consistent with allergic contact dermatitis

(Frick et al. 2003). When tested for isocyanate allergy, workers in the motor vehicle, electronic, and paint industries were most likely to react, with MDI being the component most associated with a positive reaction (Aalto-Korte et al. 2012).

2.5. Immunology review

Because the biologic reactions seen in patients treated Invisalign® aligners are immune-related, it is prudent to review basic immunology concepts elaborated in this research. Cells of the immune system are derived from pluripotent stem cells in the bone marrow and differentiate first into myeloid or lymphoid progenitor cells. Cells of the myeloid lineage include erythrocytes (red blood cells), mast cells, thrombocytes (platelets), neutrophils, eosinophils, basophils, and monocytes/macrophages (Figure 2.5). Neutrophils are highly motile cells and are one of the first cells present at the site of inflammation; they work to ingest, kill, and digest pathogens. Eosinophils are important in defending against parasitic infections and are common in cytotoxic hypersensitivity reactions. Basophils are involved in the process of allergic inflammation; when IgE attached to their surface binds with an antigen, basophils release histamine and heparin that is contained within granules in the cytoplasm. Macrophages are derived from monocytes and both are involved in phagocytosis and killing of microorganisms (Goldman and Prabhakar 1996). Monocytes and macrophages also serve as antigen-presenting cells (APCs), which help activate other immune system components, such as T cells, by their cell surface proteins called the major histocompatibility complex (MHC). Other APCs include dendritic cells, B cells, fibroblasts, and epithelial cells (Warrington et al. 2011).

Cells from the lymphoid lineage include T lymphocytes, B lymphocytes, and natural killer cells (Goldman and Prabhakar 1996). T cells have an antigen-binding complex called the T cell receptor (TCR) on its surface and is activated by the MHC-antigen complex on APCs. When activated, T helper (Th) cells secrete cytokines or differentiate into cytotoxic T cells or. Cytokines are proteins that aid in communication and interaction between cells (Warrington et al. 2011).

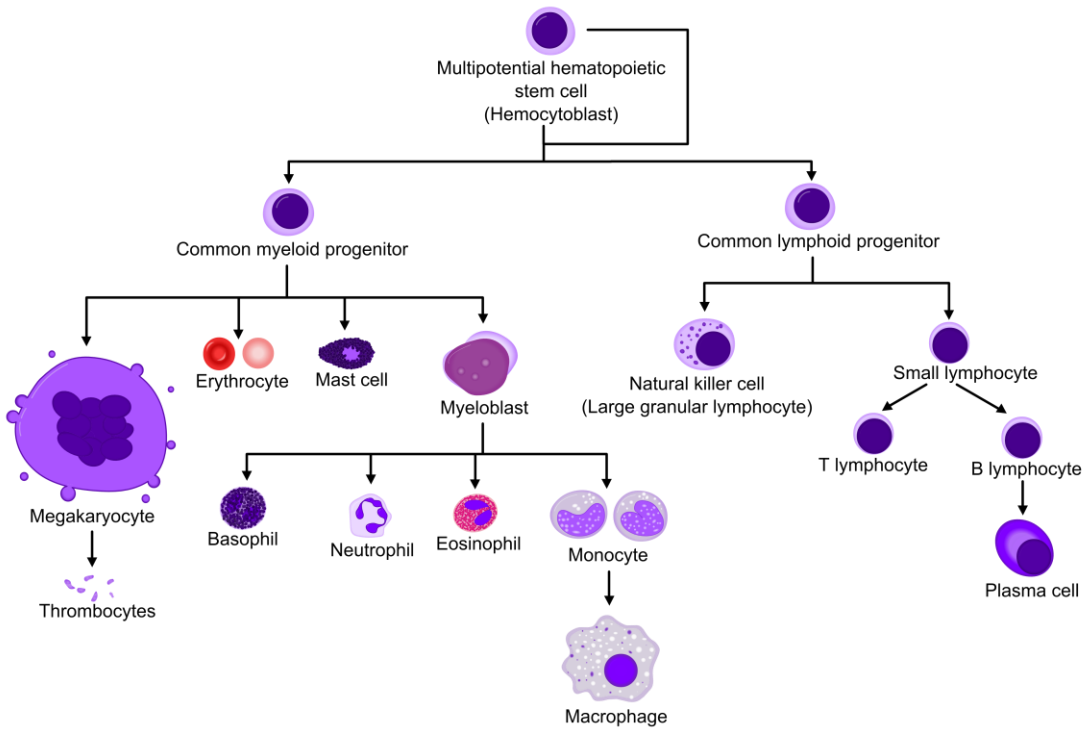


Figure 2.5. Hematopoietic Differentiation of Cells. By A. Rad and M. Häggström. CC-BY-SA 3.0 license.

T helper cells have several subsets, including Th1 and Th2 cells. Th1 cells are important in cell-mediated immunity, and Th2 cells aid B cells to produce antibodies.

The main purpose of B lymphocytes is antibody secretion after differentiation into plasma cells. B lymphocytes also are involved in the processing and presenting of antigens to T cells (Goldman and Prabhakar 1996). Lastly, natural killer (NK) cells kill tumor cells and cells infected with viruses by releasing perforins and granzymes that induce apoptosis (Warrington et al. 2011).

2.6. Inflammation

There have not been any published reports to date to explain exactly the mechanism by which Invisalign® material causes the symptoms previously described in certain individuals. The symptoms are highly variable, from swollen gingiva to anaphylaxis, which leads to speculation as to which mechanisms are at work. Inflammation is the immune system's protective mechanism against tissue injury from many different stimuli, including pathogens or toxic compounds (Chen et al. 2017). Although organ and stimuli specific, the first phase of acute inflammation is the recognition of the stimuli mainly by macrophages and mast cells. This recognition activates pathways that lead to the release of inflammatory mediators, including cytokines. Leukocytes, mainly neutrophils, are extravasated from the blood to the site. Neutrophils are then activated, and the content of their granules is released. Tissue damage can occur as the toxic substance does not differentiate between the cause of the inflammation and the normal tissue (Medzhitov 2008). If the immune system is successful in eliminating the cause, anti-inflammatory agents inhibit neutrophils and recruit monocytes. Monocytes play a role in removing the damaged tissue and repairing the site. When the initial immune response cannot eliminate the cause, macrophages and lymphocytes enter, and the inflammation becomes chronic. If the macrophages cannot engulf the causative agent, a granuloma may form (Medzhitov 2008). Granulomas are a type of chronic inflammation characterized by macrophages, epithelioid cells, and multinucleated giant cells that surround the causative agent to protect the body (Williams and Williams 1983).

2.6.1. Cytokines

The role of cytokines in the inflammatory process can be either pro-inflammatory, anti-inflammatory, or pleiotropic, meaning that they have multiple functions depending on the situation (Borish and Steinke 2003). Each cytokine response varies with different stimuli and can interact in a multitude of ways. Though there are hundreds of recognized cytokines, the following discussion on cytokines is restricted to those most pertinent to the work reported in this thesis.

Interleukin (IL)-1 and tumor necrosis factor (TNF)-α work synergistically as pro-inflammatory cytokines (Dinarello 2000). IL-1 is produced by mononuclear phagocytes and lymphocytes, among others, and contributes to inflammation by increasing PGE2 synthesis and inducing fever, and aiding in T cells' proliferation. IL-1 also stimulates mast cells to release histamine in acute inflammation (Feghali and Wright 1997). IL-1β, in the IL-1 family, is produced mainly by monocytes and triggers fever and acute responses (Cameron and Kelvin 2000-2013), but also plays a role in chronic inflammatory diseases (Dinarello 2000), including periodontitis.

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine produced by mononuclear phagocytes, activated lymphocytes, and natural killer cells. TNF's mechanism of action is known to be similar to IL-1, and their effects together are synergistic. One difference is that TNF can have a direct cytotoxic effect on cells, programming cell death. One of TNF-α's major stimulators is lipopolysaccharide (LPS), an endotoxin from the outer-membrane of gram-negative bacteria (Borish and Steinke 2003; Dinarello 2000).

IL-6 is another pro-inflammatory cytokine with similar fever-inducing functions as IL-1 and is produced mainly by mononuclear phagocytic cells, but also T and B lymphocytes and bone marrow cells. IL-6 helps stimulate the differentiation of B lymphocytes into plasma cells. IL-1 and TNF can induce the synthesis of IL-6, but IL-6 can also inhibit the production of those cytokines, a function that is anti-inflammatory in nature (Borish and Steinke 2003).

IL-8 and IL-12 are also pro-inflammatory cytokines. IL-8 is a chemokine, a chemotactic cytokine, responsible for neutrophil migration to the site of inflammation and activating neutrophil

degranulation. It is produced by mononuclear phagocytes and T cells, among others (Borish and Steinke 2003; Dinarello 2000). Its pro-inflammatory effects are seen in acute inflammation, such as allergy, and also chronic inflammation (Feghali and Wright 1997), such as periodontal disease (Finoti et al. 2017). IL-12 is produced by monocytes and macrophages, B cells, polymorphonuclear neutrophils, and mast cells. It is involved in the differentiation cytotoxic T lymphocytes, natural killer cells, and T helper cells into Th1 cells (Gee et al. 2009). IL-12p70 is the bioactive form when two subunits, p35 and p40, combine (Gee et al. 2009; Kobayashi et al. 1989).

Another cytokine involved with Th1 cells is IFN-γ. IFN-γ, originally called Macrophage activation factor, is produced by Th1 cells, NK cells, and cytotoxic T cells. It has antiviral activity by inhibiting viral replication and also leads to cytotoxic T-cell development. IFN-γ is also proinflammatory in that it can upregulate proinflammatory cytokines, such as TNF-a and IL-12, and increases nitric oxide production from macrophages (Dinarello 2000; Mühl and Pfeilschifter 2003).

The outcomes of diseases can be dependent on the balance of pro-inflammatory vs. anti-inflammatory cytokines. IL-10 is anti-inflammatory by suppressing production of IL-1, TNF, IFN-γ and IL-12 (Dinarello 2007). IL-10 is produced by monocytes, T cells, and B cells (Borish and Steinke 2003; Feghali and Wright 1997).

2.7. Hypersensitivity Reactions

Four types of hypersensitivity reactions were proposed by Coombs and Gell in 1968 (Coombs and Gell 1968). Type I reactions are considered immediate hypersensitivity reactions and are the most common. These are commonly triggered by foods and environmental allergens, such as pollen. Symptoms of these reactions include urticaria, angioedema, anaphylactic shock, and rhinitis. These symptoms usually present quickly, within minutes or a few hours. In type I reactions, IgE antibodies bind to mast cells, and when presented with the allergen, there is degranulation of the mast cell cells and release of mediators, such as histamine (Descotes and

Choquet-Kastylevsky 2001). Common treatments for this type of reaction include avoidance of the allergen, desensitization, and antihistamines (Warrington et al. 2011).

Type II hypersensitivity reactions are antibody-mediated, and examples include thrombocytopenia and hemolytic anemia. Cytotoxic antibodies, IgM and IgG, cause cell damage by multiple mechanisms, including the direct activation of macrophages, neutrophils, and eosinophils and the activation of the complement pathway. Type III hypersensitivity reactions are immune complex-mediated reactions, and examples include serum sickness and Arthus reaction (Justiz Vaillant et al. 2020). This occurs when antibodies such as IgM or IgG react with the antigen and form immune complexes that can be deposited in blood vessels of various parts of the body, including the joints and skin, kidney, lung. When complement is activated, it causes inflammation and tissue injury (Descotes and Choquet-Kastylevsky 2001).

Type IV hypersensitivity reactions are delayed reactions and are unique because they do not involve antibodies, as seen in the previous hypersensitivity reactions. This type of reaction occurs when antigen-presenting cells encounter the antigen and present it to T cells; this causes the T cells to differentiate and release cytokines, typically IFN-gamma and TNF. Type IV reactions include skin reactions from plant resins, drugs, cosmetics, and environmental chemicals (Descotes and Choquet-Kastylevsky 2001). Granulomatous and giant cell reactions are also considered type IV reactions. Delayed-type reactions take a minimum of 12-24 hours to develop (Krouse et al. 2008), with most developing within 2-14 days after exposure (Descotes and Choquet-Kastylevsky 2001; Warrington et al. 2011). Treatment for type IV reactions includes the removal of the stimulus and possibly the use of corticosteroids (Warrington et al. 2011).

The adverse biological effects previously noted from Invisalign® material look most similar to type I and type IV reactions. The reports of anaphylaxis and difficulty breathing are similar to type I immediate hypersensitivity, and the swollen gums/granulomatous inflammation are type IV delayed-type hypersensitivity. Although, some reported side effects could be either type depending on the time of onset of symptoms and the mechanism of the immune response.

For example, difficulty breathing is be associated with type 1 anaphylactic reactions but could also be attributed to inflammation in the oropharynx in a type IV reaction, especially if onset of symptoms is delayed. In this study we looked at a variety of cytokines, none of which will give information on a type 1 reaction mechanism. IL-4 expression would give more information on the possible type 1 reaction but was not included in the Human Inflammation Panel 1 from BioLegend. Therefore, the cytokines in this study focused on possible cytokines in type IV hypersensitivity reactions and those that may be induced with isocyanate stimulation.

2.7.1. Specifics of Type IV Hypersensitivity Reactions

In delayed reactions, the antigen is recognized by an APC, which presents the antigen to the T helper cell, and they bind together. In the tuberculin reaction, a well-known Type-IV reaction, the binding causes a release of cytokines from the APC, including IL-12 and IL-18. These cytokines, along with interferon-gamma (IFN-γ) from NK and Th1 cells, help differentiate the T helper cell into Th1 cells. Th1 cells are known to release IL-2 and IFN-γ. These cytokines can activate macrophages, producing IL-8 IL-1, IL-6, and TNF-α, among others. This contributes to the inflammatory reaction by recruiting neutrophils, monocytes, and lymphocytes to the site and will contribute to granulomatous inflammation if a foreign substance is involved (Kobayashi et al. 2001).

When T cells differentiate into Th2 cells via IL-4 release, the Th2 cells are known to release IL-4, IL-5, IL-6, IL-10 and IL-13. Th2 cells and these cytokines contribute to humoral immunity (compared to cell-mediated with Th1 cells) and allergic responses (Kobayashi et al. 2001). Lochmatter et al. (2009) found that PBMCs exposed to beta lactam and sulfonamide drugs (antibiotics known to cause delayed-type reactions and immediate reactions) had a delayed-type reaction and increased IL-5, IFN-γ, IL-13, and IL-2. Borg et al. (2000) used nickel to stimulate PBMCs, which is a substance known to cause delayed-type contact dermatitis. Nickel ions bind to larger proteins, including MHC protein and the subsequent haptens and modified MHC of dendritic cells. This modification of the binding surface of the MHC activates T cell proliferation

and differentiation. Upon re-exposure to nickel, the hapten-specific T cells are once again activated, leading to the production of inflammatory cytokines and development of the characteristic skin lesion (Saito et al. 2016, Wang and Dai 2013). Interestingly, Borg et al. (2000) did not find an increase in IFN-γ or TNF-α, but did find an increase in IL-4 and IL-5, which are associated more with Th2 cells and humoral immunity.

2.8. Immunologic Reactions from Isocyanates

Mishra et al. (2008) stimulated human lymphocytes with methyl isocyanate and found a dose and time-dependent increase in IL-8, IFN-γ, TNF, IL-1β, IL-6 and IL-12p70. Another study found that PBMCs exposed to diisocyanate antigen had increased production of IL-8 and TNF-α, but no significant increase of typical Th1 or Th2 cytokines IL-4, IL-5, or IFN-γ. They hypothesized that TNF-α is chemotactic for neutrophils and monocytes and that IL-8 can be induced in monocytes by stimuli, including TNF-α (Lummus et al. 1998).

CHAPTER 3: RESEARCH HYPOTHESIS & SPECIFIC AIMS

3.1. Statement of the Problem

Invisalign® clear aligners cause adverse side effects, such as hives, swelling of the lips and tongue, and difficulty breathing, in some patients. The mechanisms underlying these immune-mediated side effects are unknown. The immune response and subsequent expression of cytokines from cells exposed to Invisalign® material have yet to be studied in detail. This information will help better understand how these adverse reactions occur, who might be susceptible, and how they might be treated and/or prevented.

3.2. Central Research Hypothesis

The central research hypothesis is that there will be an increased cytokine expression when cells of the immune system are exposed to Invisalign® material *in vitro*.

3.3. Specific Aims

1) To determine which, if any, cytokines are expressed in immune cells exposed to Invisalign® material *in vitro*.

CHAPTER 4: MATERIALS & METHODS

4.1. Study Design

This descriptive study is an *in vitro* evaluation of the cytokine production of immune cells (PBMCs and THP-1 human monocytes) after cell culture with various conditions and Invisalign® material eluate addition. The cytokines were profiled using a BioLegend LEGENDplex™ sandwich ELISA technique. Four separate cell culture and assays were completed, and the results reported. Because this is a pilot study, the experimental design was changed multiple times after troubleshooting errors from previous experiments.

4.2. Invisalign® Material and Eluate Preparation

The Invisalign® material and eluate preparation protocols were based on the procedures reported by Premaraj et al. (2014). The Invisalign® aligners were ordered in 2017 by the University of Nebraska Medical Center College of Dentistry in Lincoln, NE, to treat an orthodontic patient. The treatment plan was changed mid-treatment, and new aligners were ordered; therefore, the remainder of the aligners were available for this research. The plastic was ground into particles with a 12-inch half-circle, flat-bottom file. The size and distribution of the particles were measured with the technique of dynamic light scattering (DLS) using a Zetasizer system (Malvern Panalytical). The temperature and scattering angle were set at 25°C and 90°, respectively.

To prepare the eluate, 0.1 g of particles were soaked in 1 mL of artificial saliva (Fusayama/Mayer solution, not stabilized, Pickering Laboratories, Mountain View, CA) at 37°C for 24 hours, 48 hours, 72 hours, one week and two weeks. To collect the eluate, the material was centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and stored at 2°C until use. The eluate preparation methods were the same across all experiments.

4.3. Experiment 1: Pooled PBMCs

4.3.1. Cell Culture

Pooled cryopreserved PBMCs were purchased from Zenbio (Research Park Triangle, NC) for this experiment. PBMCs were chosen because they contain many cells of the immune system and are used widely in immunological research (Pourahmad and Salimi 2015; Lummus et al. 1998). There were four Caucasian donors in the purchased PBMC lot, a mix of male/female, with an average age of 61. The percentage of positive cells was 43.7% T cells, 32.2% helper T cells, 8.1% cytotoxic T cells, 11.3% B cells, 28% monocytes, and 9.4% NK cells. The cells were thawed and prepared according to the Zenbio recommended protocol. The cells were plated at $25x10^4$ cells/well in a 96 well plate and cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) and 1% of L-glutamine-penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO). The negative controls contained only cells and media, and the saliva negative control had the addition of 43 μL of artificial saliva (Pickering Laboratories, Mountain View, CA). The saliva control was included so any reaction to the Invisalign® material eluate could be solely attributed to the Invisalign® material. The Invisalign® material sample wells included 43 μL of the 2-week Invisalign® material eluate. The positive controls were stimulated with 500ng/mL *E. coli* lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO). All wells had a total of 250 μL. Cells were cultured in a 5% CO2 incubator at 37 °C for 24 and 72 hours. The samples were collected, transferred to microcentrifuge tubes, and centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and stored at -20°C until use.

4.3.2. LEGENDplex™ Assay

This assay was performed using a LEGENDplex™ Multi-Analyte Flow Assay with a custom 3-plex from the Human Inflammation 1 kit, including IL-8, IL-6, and TNF-α (BioLegend, Cat. No. 740809, San Diego, CA).

4.3.2.1 Assay Preparation

The assay preparation steps, and assay procedures were followed as described in the assay manual. The antibody-immobilized beads were vortexed for 3 minutes to resuspend the beads. The wash buffer was prepared by bringing to room temperature and diluting with 475 mL deionized water.

To prepare the standards, the standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0).

4.3.2.2. Assay Procedure

This assay was performed using a V-bottom plate, and the directions were followed accordingly from the assay manual (Figure 4.3.2.2). All samples and standards were plated in duplicate in a vertical configuration for easy data acquisition and analysis. The standard wells included 25 µL of standard with 25 µL of assay buffer. The sample wells had 25 µL of sample with 25 µL of assay buffer. The samples were not diluted before adding to wells. The mixed beads were vortexed again for 30 seconds before adding 25 µL to each well. The total final volume in each well was 75 µL. The plate was sealed with a plate sealer and covered in aluminum foil before shaking on a plate shaker at 800rpm for 2 h at room temperature.

After the incubation time, the plate was centrifuged at 1050 rpm for 5 minutes, using a swinging bucket rotator with microplate adaptor. After centrifugation, the supernatant was dumped by quickly inverting and flicking the plate in one continuous and forceful motion.

Next, the plate was washed with 200 µL of wash buffer and the centrifugation steps were completed again. Then, 25 µL of detection antibodies were added to each well, and the plate was incubated again for 1 hour. After this, 25 µL of Streptavidin –PE (SA-PE) was added to each well, without prior washing, and incubated for another 30 minutes.

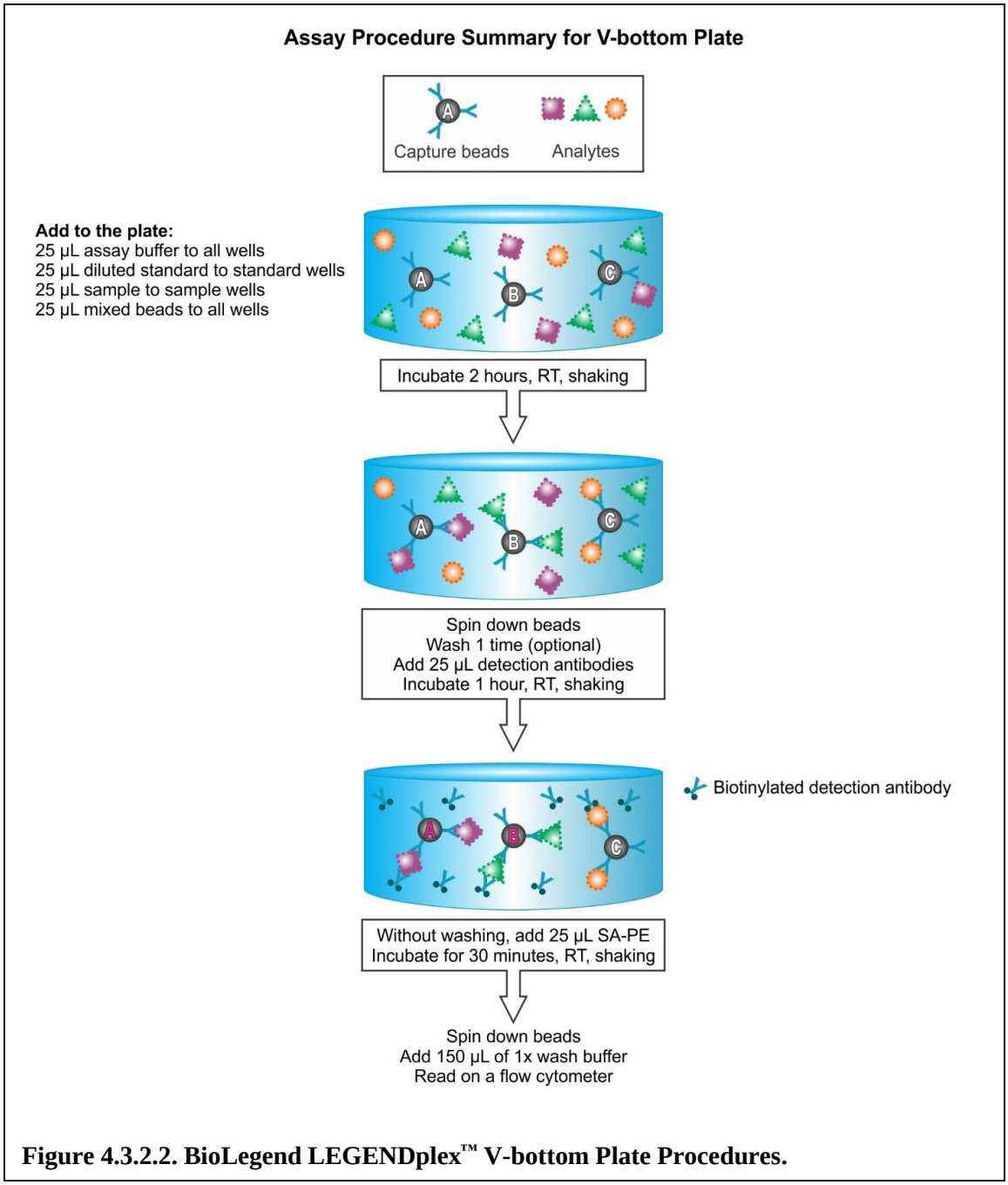


Figure 4.3.2.2. BioLegend LEGENDplex™ V-bottom Plate Procedures.

The plate was then washed two more times. Finally, 150 µL of wash buffer was added to each well, and the beads were resuspended by pipetting.

4.3.2.3 Flow Cytometry and Data Analysis

The samples were read on a flow cytometer on the same day as the assay was performed using a CytoFLEX LX (Beckman Coulter). The settings were configured according to the directions in the LEGENDplex™ manual. The data was analyzed using BioLegend's LEGENDplex™ cloud-based data analysis software, Qognit, found on the BioLegend website.

4.4. Experiment 2: Individual Donor PBMCs

4.4.1. Cell Culture

For this experiment, cryopreserved PBMCs from three separate donors were purchased from Precision for Medicine (Frederick, MD). Donor 1 was a 37-year-old Caucasian male smoker. Donor 2 was a 26-year-old Latino male nonsmoker and donor 3 was a 53-year-old Latino male nonsmoker. The donors were not taking any medications. Cells were thawed and prepared as described by the Precision for Medicine protocols. For this experiment, the FBS was heat-inactivated by heating at 56°C for 30 minutes. The cell culture methods were the same as Experiment 1, with the only change being three separate donors instead of pooled cells.

4.4.2. LEGENDplex™ Assay

The LEGENDplex™ assay for this experiment was performed using the LEGENDplex™ kit, cytokines, procedures, and flow cytometry as previously described.

4.5. Experiment 3: Individual Donor PBMCs

4.5.1. Cell Culture

For this experiment, cryopreserved PBMCs from three different donors were purchased from Precision for Medicine (Frederick, MD). Donor 1 was a 35-year-old Caucasian male, smoker, taking no medications. Donor 2 was a 31-year-old Caucasian woman, nonsmoker, taking Zoloft and Bupropion. Donor 3 was a 35-year-old Caucasian male, nonsmoker, taking Lisinopril

and Lovastatin. Cells were thawed and prepared as described by the Precision for Medicine protocols. The cell culture methods were the same as Experiment 2, with the only change being that the LPS was vortexed for 15 minutes before use, and positive controls included 1 μL/mL of LPS and 500 ng/mL *E. coli* LPS.

4.5.2. LEGENDplex™ Assay

The LEGENDplex™ assay for this experiment was performed using the LEGENDplex™ kit, cytokines, procedures, and flow cytometry as previously described.

4.6. Experiment 4: THP-1 Human Monocyte Cell Line

4.6.1. Cell Culture

The THP-1 human monocyte cell line (ATCC® TIB-202™) was used in this experiment and cultured according to the protocol provided by Dr.Toshihisa Kawai at Nova Southeastern University College of Dental Medicine, where the cells were obtained. THP-1 is a human monocyte cell line from a patient with childhood acute monocytic leukemia (Tsuchiya et al. 1980). This cell line is a model for human monocytes and is used in research as a model for immune reactions and inflammation *in vitro* (Bosshart and Heinzelmann 2016; Yang et al. 2016). The cells were plated at $25x10^4$ cells/well in a 96 well plate and cultured in RPMI-1640 medium (ATCC) with 10% of heat-inactivated FBS (Gibco, ThermoFisher Scientific, Waltham, MA) and 1% of penicillin-streptomycin solution. The negative controls contained only cells and media and the saliva controls had the addition of 50 μL artificial saliva (Pickering Laboratories, Mountain View, CA). The Invisalign® material wells included 50 μL of the 72-hour Invisalign® material eluate. The positive control samples were stimulated with 500 ng/ml *P. gingivalis* LPS. *P. gingivalis* LPS is more relevant to intraoral conditions, although it is atypical and structurally different from *E. coli* LPS (Holden et al. 2014). All wells had a total of 200 μL. Cells were cultured in a 5% CO2 incubator at 37 °C for 24 and 72 hours. The samples were collected, transferred to microcentrifuge tubes, and centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and stored at -20°C until use.

4.6.2. LEGENDplex™ Assay

This assay was performed using a LEGENDplex™ Multi-Analyte Flow Assay Kit with a custom 7-plex from the Human Inflammation 1 kit, including IL-8, IFN-γ, IL-10, IL-12p70, IL-1β, IL-6, TNF-α (BioLegend, Cat. No. 740809, San Diego, CA).

4.6.2.1. Assay Procedure

The assay materials were prepared as previously described. This assay was performed using a filter plate instead of a V-bottom plate, and the directions were followed accordingly (Figure 4.6.2.1.). To pre-wet the filter plate, 100 µL of wash buffer was added to each well and let sit for 1 minute. The vacuum was then applied (not exceeding 10" Hg of vacuum) until wells were drained (about 5-10 seconds) and excess buffer blotted out. All samples and standards were plated in duplicate in a vertical configuration for easy data acquisition and analysis. The standard wells included 25 µL of standard with 25 µL of assay buffer. The sample wells had 25 µL of sample with 25 µL of assay buffer. The samples were not diluted before adding to wells. The mixed beads were vortexed for 30 seconds before adding 25 µL to each well; the total volume in each well was 75 µL. The plate was sealed with a plate sealer and covered in aluminum foil before shaking on a plate shaker at 500 rpm for 2 h at room temperature.

Next, the plate was vacuumed, as previously described. Then 200 µL of wash buffer was added to each well, and another vacuum step was completed to remove the wash buffer. This washing step was repeated. Then, 25 µL of detection antibodies were added and the plate was incubated again on the shaker at 500rpm for 1 h. After this, 25 µL of Streptavidin –PE (SA-PE) was added to each well, without prior vacuuming, and incubated for another 30 minutes on the plate shaker.

The plate was then vacuumed and washed two times. Finally, 150 µL of wash buffer was added to each well, and the beads were resuspended on a plate shaker for 1 minute.

4.6.2.2. Flow Cytometry and Data Analysis

The samples were read on a BD Fortessa X20 (BD Biosciences) flow cytometer on the same day as the assay and configured according to the directions in the LEGENDplex™ manual. The data was analyzed as previously described with the analysis software.

4.7. Statistical Analysis

The data for each duplicate were averaged and the standard error was calculated. The data is reported as the mean concentration in pg/mL with standard error. A Mann-Whitney U test was conducted with the saliva control data and Invisalign® material sample data for IL-8 from experiments 2-4 ($\alpha < 0.05$, two-tailed).

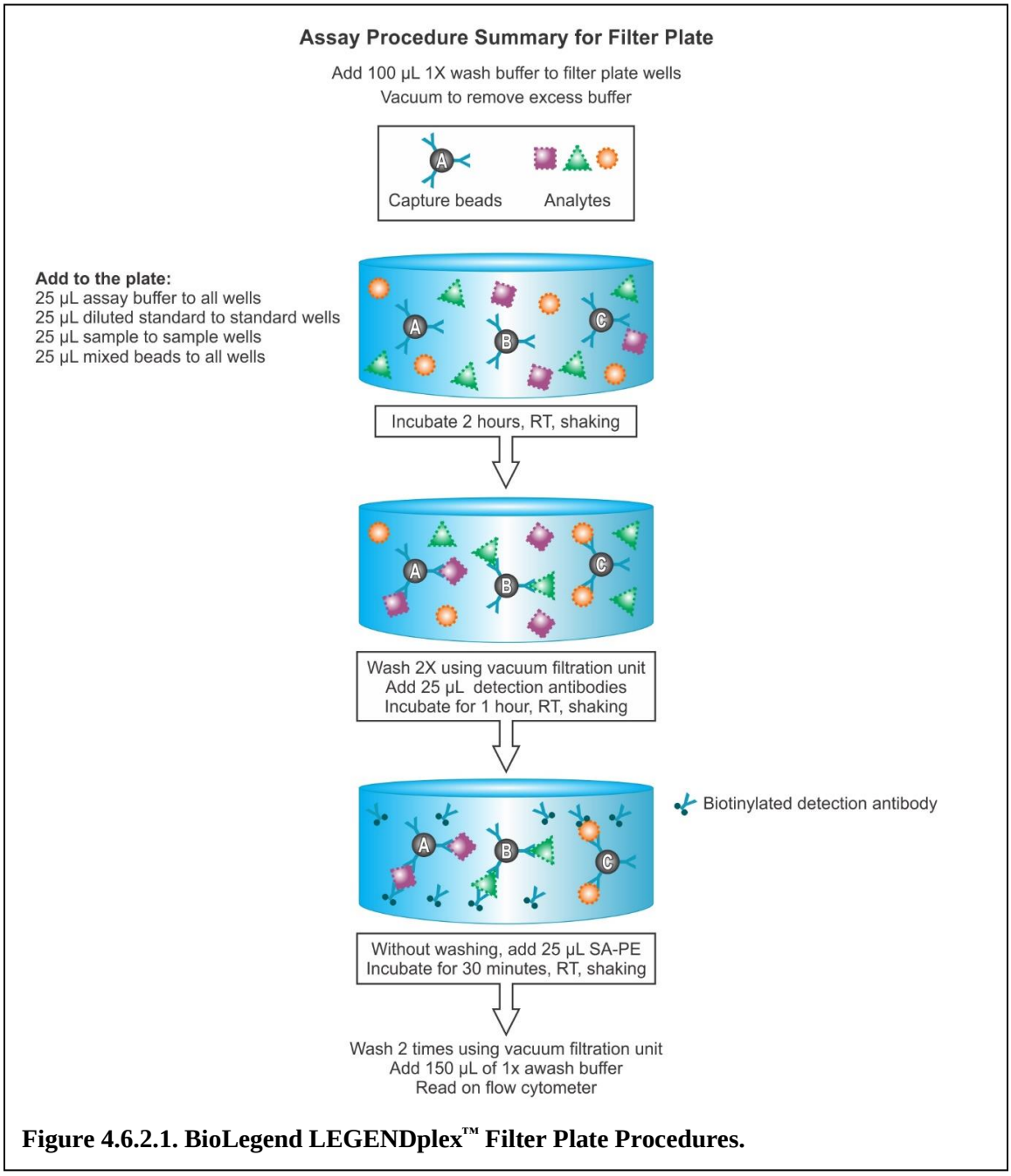


Figure 4.6.2.1. BioLegend LEGENDplex™ Filter Plate Procedures.

CHAPTER 5: RESULTS

5.1. Particle Size

The size and distribution of the Invisalign® material particles used for all experiments in this study were measured with the technique of dynamic light scattering using a Zetasizer system (Malvern Panalytical). The particle report is shown in Table 5.1. and the size distribution graph in Figure 5.1. The Z-average radius of the particles was between 502.9 and 873.5 nm. The smallest particles had a mean radius of 0.71 nm, and the largest detected had a mean radius of 552.6 nm.

5.2. Experiment 1: Pooled PBMCs

The results of the experiment 1 assay are shown in Table 5.2. The standard curves are included in Figure 5.2.

5.2.1. TNF-α

For the samples collected after 24 hours, the negative control (cells alone in media) and saliva control (addition of artificial saliva) samples had mean TNF-α concentrations of 82.56 and 18.52 pg/mL, respectively. The Invisalign® material sample induced slightly higher TNF- α, at 378.71 pg/mL. The LPS positive control stimulated a mean concentration of 1814.27 pg/mL of TNF- α (Figure 5.2.1).

For the samples collected at 72 h culture, the negative control and saliva control had mean TNF-α concentrations of 22.77 and 10.49 pg/mL, respectively. The Invisalign® material sample induced a TNF-α concentration of 128.41 pg/mL, and the LPS positive control sample had TNF-α at 1324.33 pg/mL. The 72 h culture values were less than at 24 h, with the Invisalign® material sample almost 3x lower at 72 h than 24 h (128.41 vs. 378.61 pg/mL TNF- α).

5.2.2. IL-6

The values for IL-6, at 24 h and 72 h, were very high compared to the assay standards (Table 5.2). The negative control at 24 h had 49,0176.53 pg/mL IL-6 and at 72 h had 51,980.48 pg/mL IL-6. Because all values are higher than the highest standard, the values cannot be determined accurately for the various samples.

Sample	Z-Ave (r.nm)	Pk 1 Mean Int (r.nm)	Pk 2 Mean Int (r.nm)	Pk 1 Area Int (%)	Pk 2 Area Int (%)
1	873.5	519	0	100	0
2	545.3	549.1	0.72	90.5	9.5
3	502.9	552.6	2.28	86.8	13.2

Table 5.1. Dynamic Light Scattering Particle Report. The DLS report shows the average radius of the Invisalign® particles ranged from 502.9-873.5 (Z-Ave). Some nanoparticles were found in the sample with a radius of smaller than 3 nm.

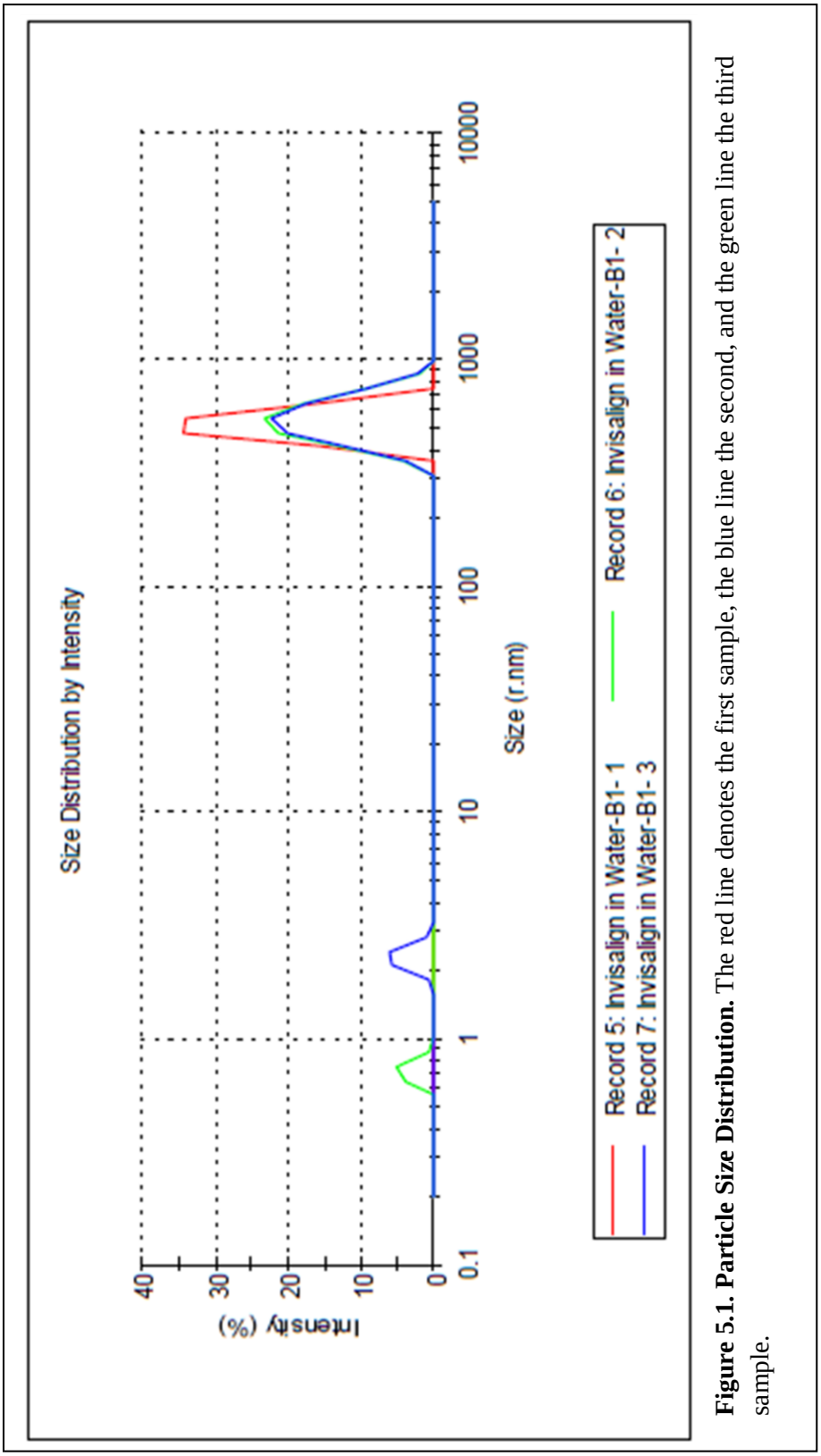


Figure 5.1. Particle Size Distribution. The red line denotes the first sample, the blue line the second, and the green line the third sample.

5.2.3. IL-8

Similar to IL-6, the values for IL-8 were all higher than the highest standard. The negative controls at 24 h and 72 h were 16,054.63 pg/mL IL-8 and 16,007.56 pg/mLIL-8, respectively.

Since higher levels of cytokine expression in the first experiment with pooled PBMCs was due to MHC protein incompatibility, the decision to repeat the experiment with single donor PBMCs was made. In addition, heat inactivation of FBS was also performed to eliminate the unwanted influence of cytokine activation from sources other than Invisalign, such as complement components.

5.3. Experiment 2: Individual Donor PBMCs

The results of the second experiment assay are shown in Table 5.3. The standard curves are included in Figure 5.3.

5.3.1. TNF-α

For donor 1, the TNF- α values for the negative control, saliva control, and Invisalign® material sample were all very low at 0.57 pg/mL. The LPS control induced only slightly higher TNF- α at 1.01 pg/mL. For donor 2, all samples had TNF-α concentrations of 0.57 pg/mL. Donor 3 also had negative control, saliva control, and Invisalign® material samples of 0.57 pg/mL of TNF- α. The LPS control for donor 3 induced 0.68 pg/mL TNF- α. The LPS positive control did not induce TNF- α in any donor, therefore no conclusions can be drawn about Invisalign® material stimulation on the cells.

5.3.2. IL-6

For donor 1, the negative control and saliva control both had IL-6 concentrations of 0.32 pg/mL. The Donor 1 Invisalign® material and LPS induced concentrations of IL-6 at 0.78 and 9.01 pg/mL, respectively. For donor 2, the negative control had 0.32 pg/mL IL-6, saliva control had 0.34 pg/mL IL-6, the Invisalign® material induced 0.38 pg/mL IL-6, and the LPS induced 3.03 pg/mL IL-6.

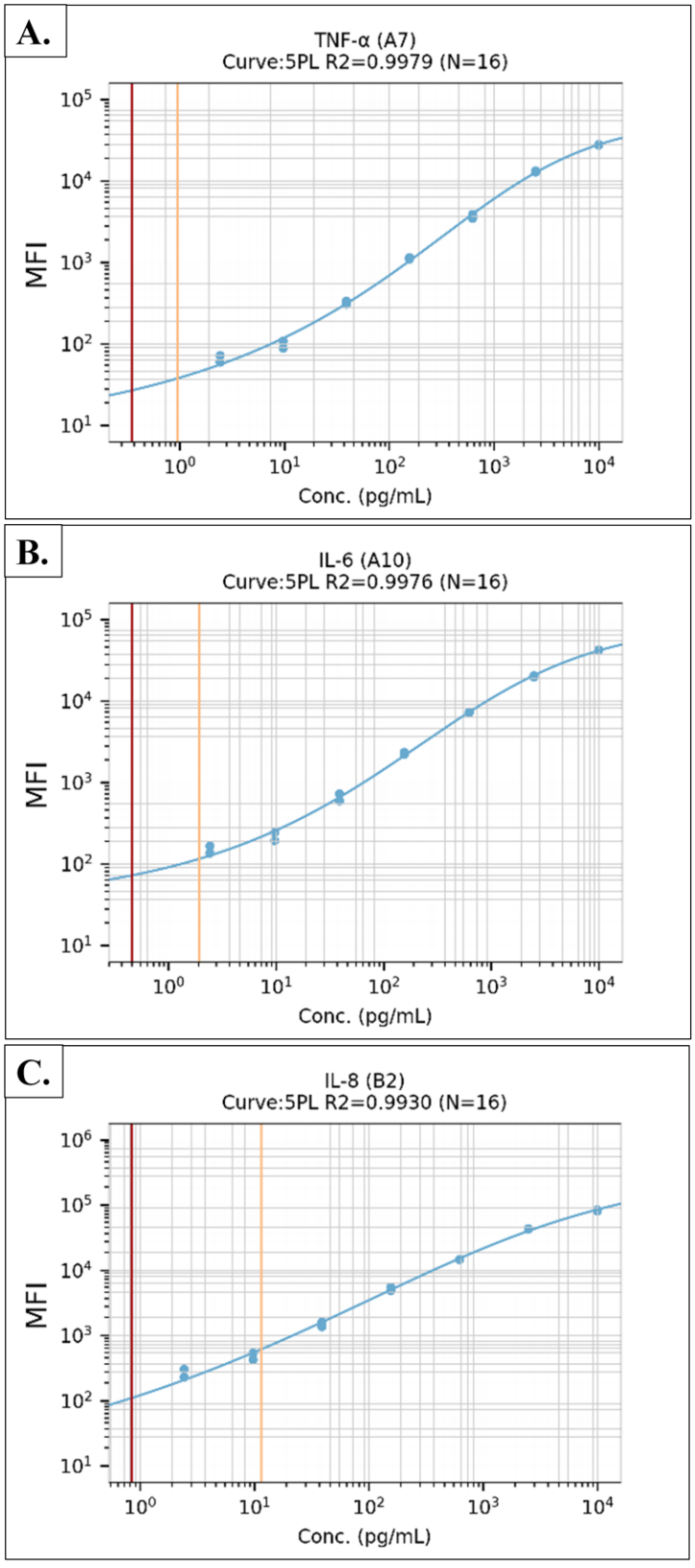


Figure 5.2. Experiment 1 Standard Curves. Concentration (pg/mL) vs. MFI standard curve for **A.** TNF-α. **B.** IL-6. and **C.** IL-8. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

Sample	TNF-α	IL-6	IL-8
24 hours			
Negative control	82.68 ± 2.015	49076.53 ± 1274.48	16054.63 ± 928.285
Saliva control	18.52 ± 3.18	51582.75 ± 19861.315	15766.58 ± 2063.125
Invisalign®	378.61 ± 31.715	261748.17 ± 31186.705	17427.61 ± 857.3
LPS control	1814.27 ± 212.945	288051.99 ± 2268.99	17412.53 ± 1143.06
72 hours			
Negative control	22.77 ± 1.33	51980.48 ± 772.94	16007.56 ± 768.92
Saliva control	10.49 ± 1.015	103471.56 ± 9480.24	20387.32 ± 373.015
Invisalign®	128.41 ± 31.325	337863.20 ± 20851.505	15636.57 ± 904.535
LPS control	1324.33 ± 110.745	210454.90 ± 8369.23	17612.92 ± 285.95

Table 5.2. Mean Cytokine Expression of Pooled PBMCs, Experiment 1. Mean cytokine expression in pg/mL of pooled PBMCs cultured for 24 or 72 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® eluate, and the LPS control has 500 ng/ml LPS. Values shown are the mean of the duplicates reported with standard error.

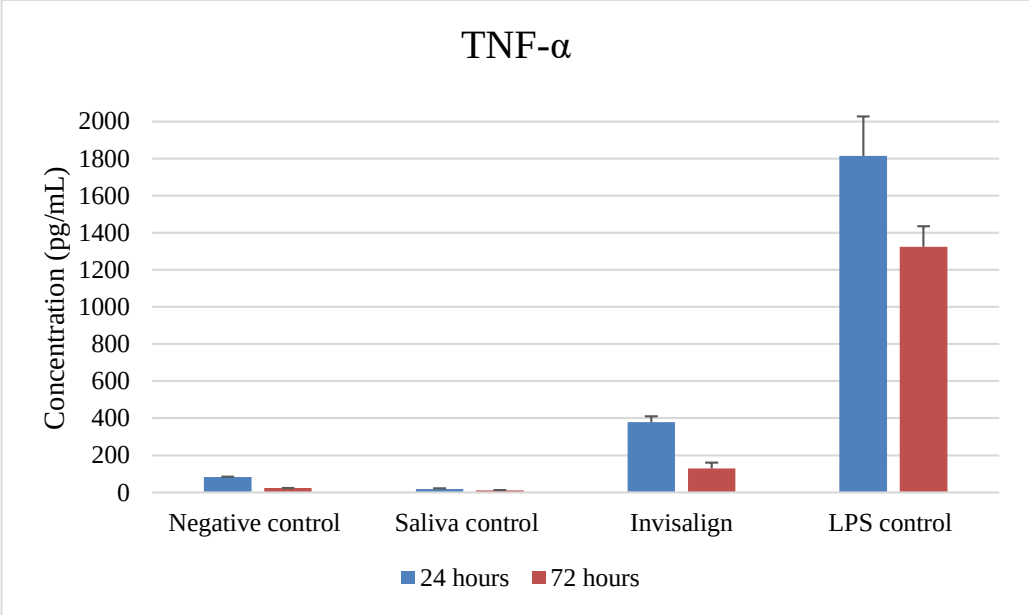


Figure 5.2.1. Mean TNF-α Concentration of Pooled PBMCs, Experiment 1. TNF-alpha concentration in pg/mL of pooled PBMCs cultured for 24 or 72 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® elute, and the LPS control has 500 ng/ml LPS. Values shown are the mean of the duplicates with standard error.

5.3.3. IL-8

For donor 1, the negative control and saliva control were 7.97 pg/mL IL-8 and 6.87 pg/mL IL-8, respectively. The Invisalign® material induced an IL-8 concentration of 63.85 pg/mL, and the LPS induced 138.34 pg/mL IL-8. Donor 2 had a baseline control of 1.56 pg/mL IL-8 and saliva control at 5.20 pg/mL IL-8. The Invisalign® material induced an IL-8 concentration of 2.16 pg/mL, and the LPS positive control induced an IL-8 concentration of 18.21 pg/mL. Donor 3 negative and saliva controls samples had IL-8 concentrations of 4.23 pg/mL and 2.75 pg/mL, respectively. The Invisalign® material induced an IL-8 concentration of 5.37 pg/mL, and LPS an IL-8 concentration of 27.41 pg/mL (Figure 5.3.2).

5.4. Experiment 3: Individual donor PBMCs

The results of the third experiment with individual donor PBMCs are shown in Table 5.4. The standard curves for this assay can be seen in Figure 5.4. The duplicates for the fourth standard, especially for IL-6 and IL-8, are widely varied.

5.4.1. TNF-α

For TNF-α, donor 1 had negative and saliva control sample concentrations of 67.04 pg/mL and 162.95 pg/mL, respectively, of TNF- α. The Invisalign® induced a slightly higher concentration of TNF-α at 224. 11 pg/mL, while LPS-induced TNF-α was very high at 20,104.32 pg/mL. Donor 2 had TNF-α concentrations of 37.01 pg/mL for all samples, except for the saliva control at 55.355 pg/mL TNF- α. For donor 3, the negative control had a TNF-α concentration of 401.99 pg/mL. The Invisalign® material induced 185.10 pg/mL TNF-α and the 1μg/ml and 500ng/mL LPS controls induced TNF-α concentrations of 13,576.61 pg/mL and 17,717.88pg/mL, respectively (Figure 5.4.1).

5.4.2. IL-6

For donor 1, the negative and saliva controls had an IL-6 concentration of 69.37 pg/mL and 117.65 pg/mL, respectively. The Invisalign® material induced an IL-6 concentration of 320.15 pg/mL, and the LPS induced 10,000 pg/mL IL-6. Donor 2 negative and saliva controls

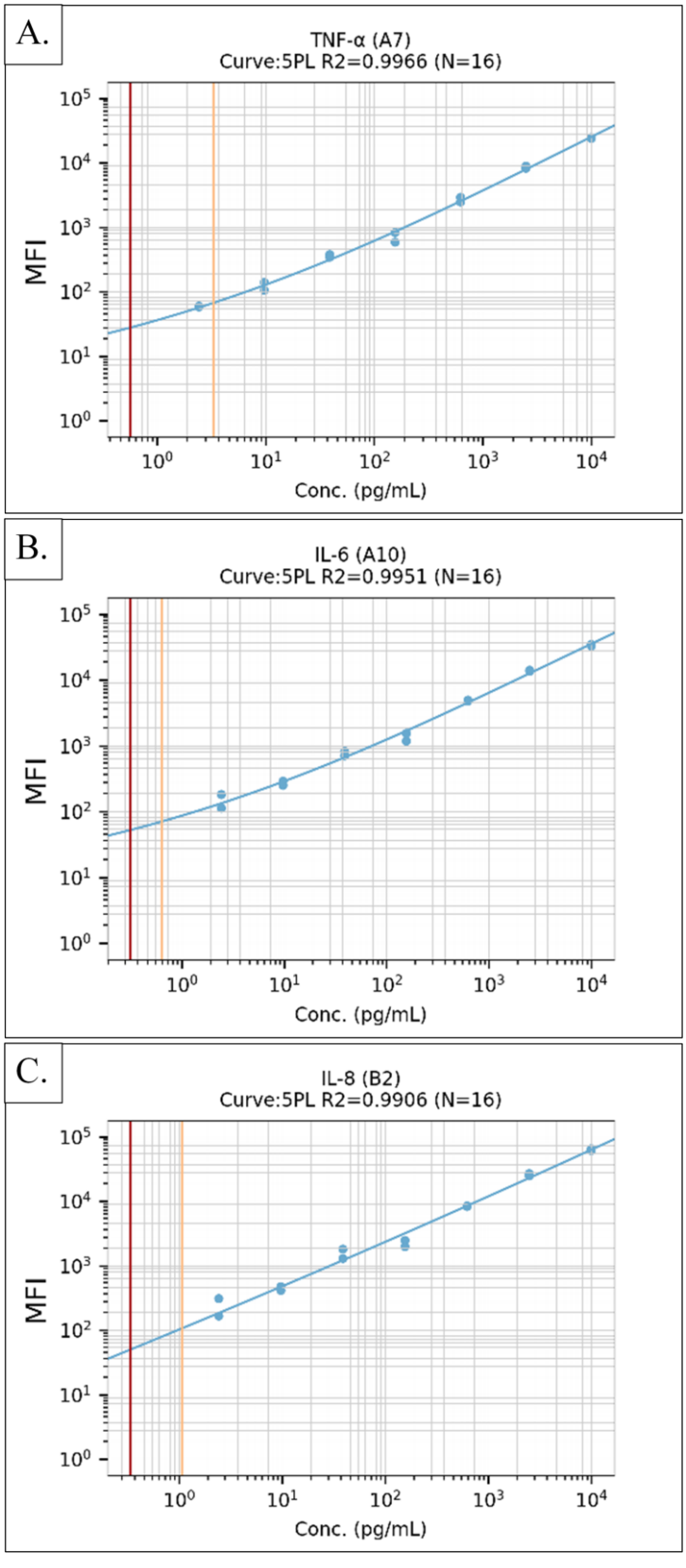


Figure 5.3. Experiment 2 Standard Curves. Concentration (pg/mL) vs. MFI standard curve for **A**. TNF-α. **B.** IL-6. **C.** IL-8. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

Sample	TNF-α	IL-6	IL-8
Donor 1			
Negative control	0.57 ± 0	0.32 ± 0	7.97 ±0
Saliva control	0.57 ± 0	0.32 ± 0	6.87 ± 0.31
Invisalign®	0.57 ± 0	0.78 ± 0.39	63.85 ± 47.25
LPS control	1.01 ± 0.44	9.01 ± 7.27	138.34 ± 109.22
Donor 2			
Negative control	0.57 ± 0	0.32 ± 0	1.56 ± 0.38
Saliva control	0.57 ± 0	0.34 ± 0.02	5.20 ± 3.06
Invisalign®	0.57 ± 0	0.38 ± 0.07	2.16 ± 1.82
LPS Control	0.57 ± 0	3.03 ± 0.49	18.21 ± 5.87
Donor 3			
Negative control	0.57 ± 0	0.435 ± 0.12	4.23 ± 2.73
Saliva control	0.57 ± 0	0.77 ± 0.45	2.75 ± 0.49
Invisalign®	0.57 ± 0	0.65 ± 0.33	5.37 ± 0.18
LPS control	0.68 ± 0.12	1.38 ± 0.95	27.41 ± 15.72

Table 5.3. Mean Cytokine Expression of Three Individual Donor PBMCs, Experiment 2. Mean cytokine expression in pg/mL of three individual donor PBMCs cultured for 24 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® eluate, and the LPS control has 500 ng/ml LPS. Values shown are the mean of the duplicates. Values shown are the mean of the duplicates reported with standard error.

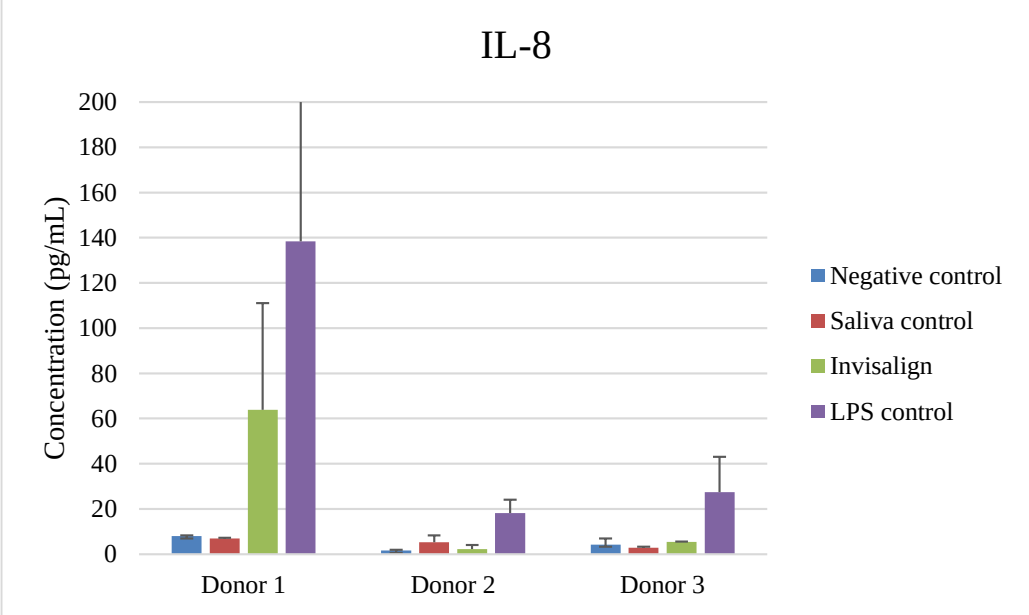


Figure 5.3.2. Mean IL-8 Concentration of Individual Donor PBMCs, Experiment 2. Mean IL-8 concentration in pg/mL of three individual donor PBMCs cultured for 24 or 72 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® elute, and the LPS control has 500 ng/ml LPS. Values shown are the mean of the duplicates with standard error.

were 29.71 pg/mL IL-6 and 45.54 pg/mL IL-6, respectively. The Invisalign® material induced an IL-6 concentration of 40.96 pg/mL and LPS induced 18.85 pg/mL IL-6. Donor 3 negative control concentration of IL-6 was 88.51 pg/mL, and the Invisalign® material induced 345.02 pg/mL IL-6. The LPS positive controls (1μg/ml and 500ng/mL) both induced 10,0000 pg/mL IL-6 (Figure 5.4.1.).

5.4.3. IL-8

For IL-8, Donor 1 had a negative control of 1,626.53 pg/mL IL-8 and a saliva control of 2,580.65 pg/mL IL-8. Donor 1 also had an Invisalign® material- induced IL-8 concentration of 6,463.85 pg/mL. The LPS for Donor 1 induced 14,205.53 pg/mL IL-8. Donor 2 had negative and saliva control IL-8 concentrations of 110.11pg/mL and 125.68 pg/mL, respectively. Donor 2 Invisalign® material induced 122.64 pg/mL IL-8 and LPS induced 71.16 pg/mL IL-8. Donor 3 had a negative control concentration of IL-8 of 2,212.68 pg/mL and a saliva control of 8,256.08 pg/mL IL-8. The 1μg/mL LPS and 500ng/mL LPS induced 44,767.21 pg/mL and 12,319 pg/mL, respectively, of IL-8 (Figure 5.4.3). Therefore, the lower 500ng/mL LPS concentration was not the cause for low positive control in the previous experiment. The negative control samples also had high concentrations for this experiment.

Due to the inconsistent results obtained from the individual donor PBMCs, the decision was made to use the THP-1 monocyte cell line for further experiments.

5.5. Experiment 4: THP-1 cells

The results from the fourth cell culture and assay of THP-1 cells are shown in Table 5.5. and Figure 5.5.1. The standard curves for the assay are shown in Figure 5.5.

5.5.1. IL-1β

At 24 hours, the negative and saliva controls of THP-1 cells had concentrations of IL-1β of 26.16 and 23.02 pg/mL, respectively. The 1:1 Invisalign® material and Invisalign® material samples induced concentrations of IL-1 β at 12.75 pg/mL and 461.83pg/mL, respectively. The LPS positive control induced IL-1β at 14.65 pg/mL.

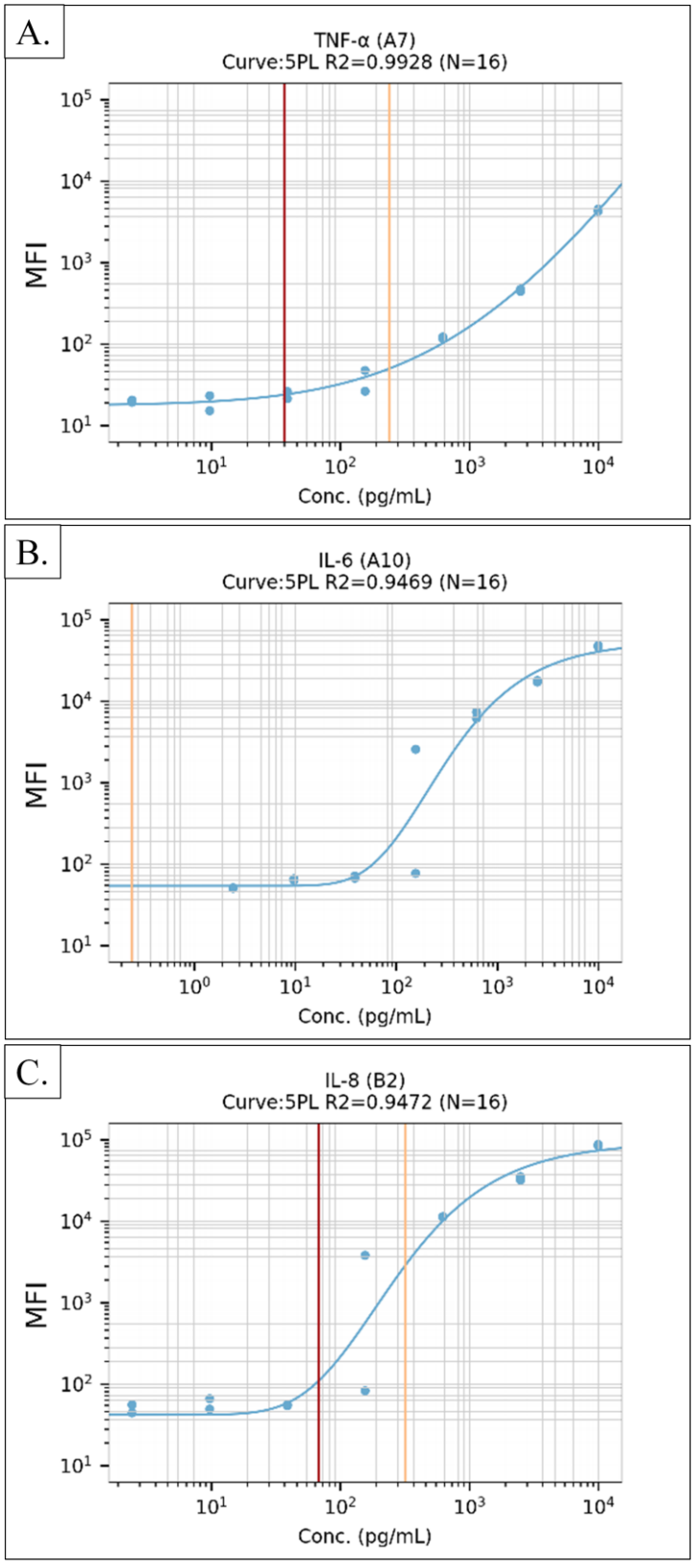
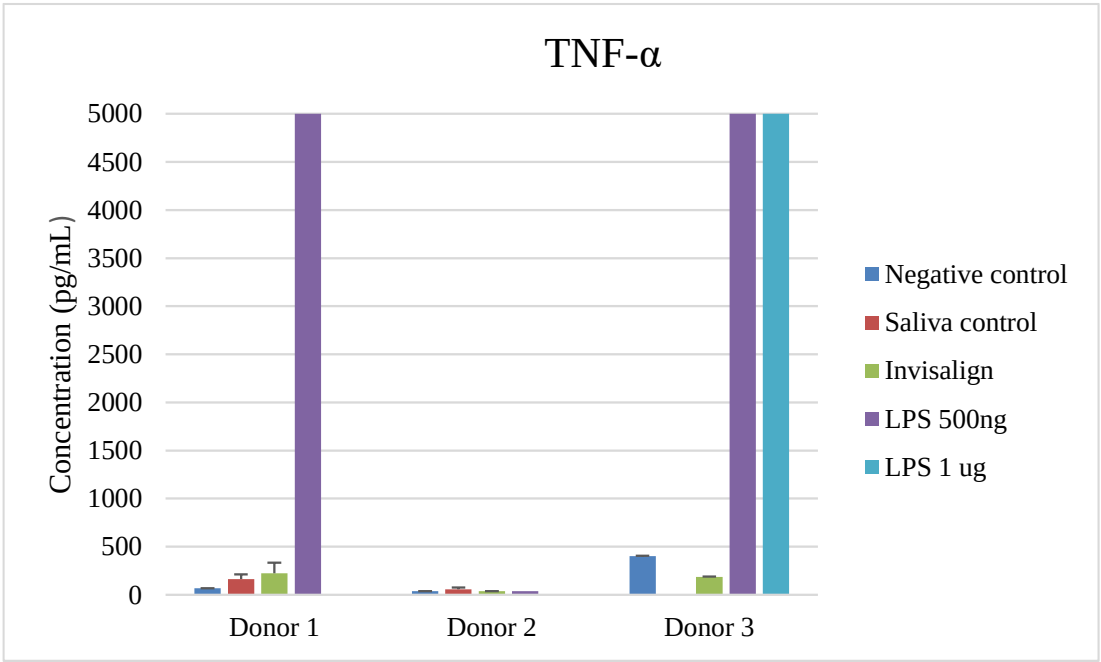


Figure 5.4. Experiment 3 Standard Curves. A. Concentration (pg/mL) vs. MFI standard curve for **A.** TNF-α. **B.** IL-6 and **C.** IL-8. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

Sample	TNF-α	IL-6	IL-8
Donor 1			
Negative control	67.04 ± 1.32	69.37 ± 1.31	1626.53 ± 249.19
Saliva control	162.95 ± 47.85	117.65 ± 22.67	2580.65 ± 961.36
Invisalign®	224.11 ± 108.61	320.15 ± 98.95	6463.85 ± 2318.58
LPS control	20104.32 ± 1697.94	10000.00 ± 0	14205.33 ± 248.20
Donor 2			
Negative control	37.01 ± 0	29.71 ± 5.48	100.11 ± 3.44
Saliva control	55.36 ± 18.34	45.54 ± 13.32	125.68 ± 21.87
Invisalign®	37.01 ± 0	40.95 ± 2.79	122.64 ± 1.04
LPS Control	37.01 ± 0	18.85 ± 0.22	71.16 ± 1.79
Donor 3			
Negative control	401.99 ± 1.30	88.51 ± 0.75	2212.68 ± 84.12
Invisalign®	185.10 ± 3.40	345.02 ± 2.25	8256.08 ± 367.07
LPS 1µg/ml control	13576.61 ± 34.13	10000.00 ± 0	44767.21 ± 24695.57
LPS 500ng/ml control	17717.88 ± 522.9	10000.00 ± 0	12319.75 ± 1911.73

Table 5.4. Mean Cytokine expression of three individual donor PBMCs, Experiment 3. Mean cytokine expression in pg/mL of three individual donor PBMCs cultured for 24 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® eluate, and the LPS control has 500 ng/ml LPS. Values shown are the mean of the duplicates reported with standard error.



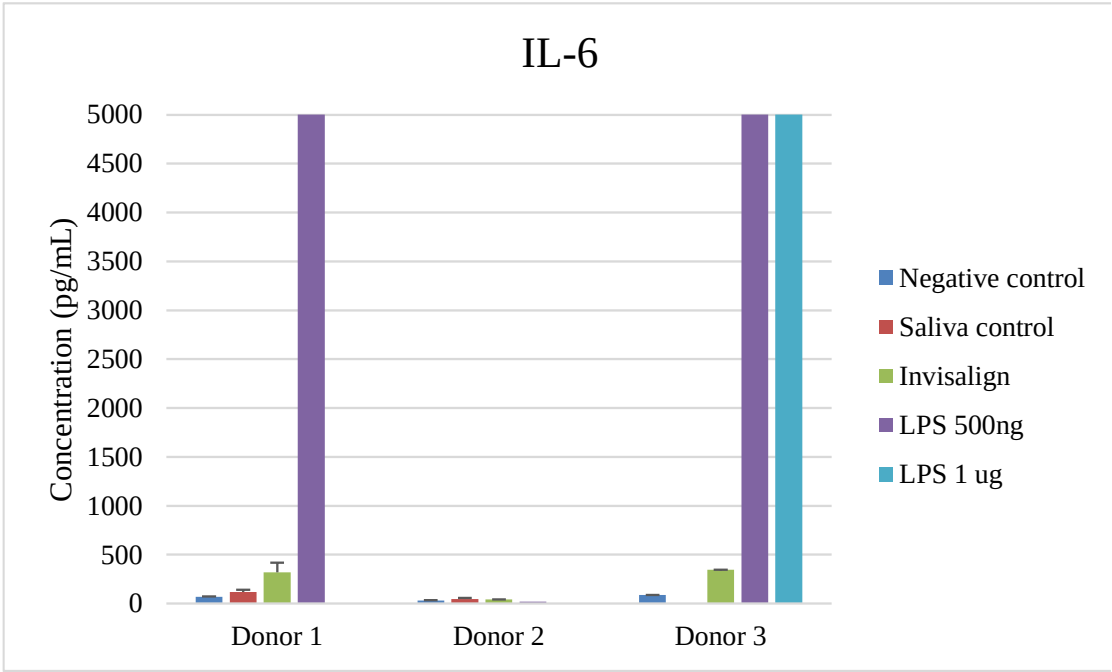


Figure 5.4.1. Mean TFN-α and IL-6 Concentration of Individual Donor PBMCs, Experiment 3. Mean TNF-α and IL-6 concentration in pg/mL of three individual donor PBMCs cultured for 24 or 72 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® elute, and the LPS control has 500 ng/ml LPS or 1µg/mL LPS. Values shown are the mean of the duplicates with standard error.

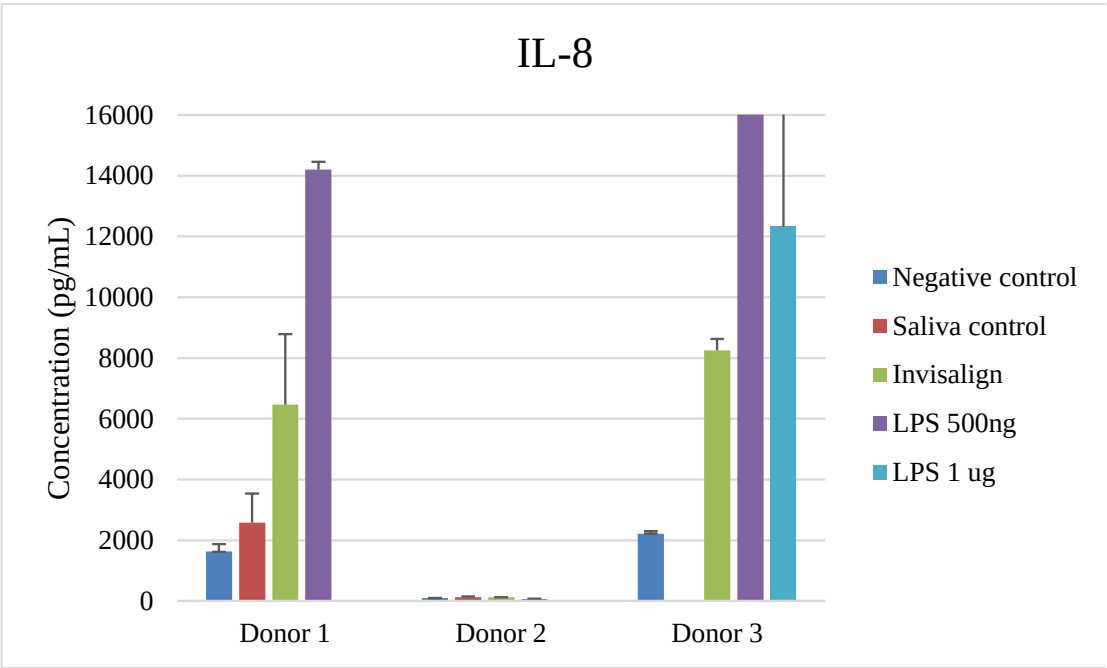


Figure 5.4.3. Mean IL-8 Concentration of Individual Donor PBMCs, Experiment 3. Mean IL-8 concentration in pg/mL of three individual donor PBMCs cultured for 24 or 72 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the Invisalign® sample is the addition of Invisalign® elute, and the LPS control has either 500 ng/ml LPS or 1µg/mL LPS. Values shown are the mean of the duplicates with standard error.

At 48 hours, the negative and saliva controls had 3.27 pg/mL and 4.09 pg/mL concentrations of IL-1β. The 1:1 Invisalign® material sample induced 12.15 pg/mL IL-1β, and the Invisalign® material induced a concentration of 958.42 pg/mL IL-1β. The concentration of IL-1β in the LPS control was 5.06 pg/mL.

5.5.2. IFN-γ

At both 24 and 48 hours, the concentration of IFN-γ from THP-1 cells for all samples was 4.71 pg/mL. There was no variation in samples. The minimum detectible concentration of IFN-γ in the assay is 1.6 pg/mL.

5.5.3. TNF-α

At 24 hours, the concentration of TNF-α for the negative and saliva controls was 6.95 pg/mL and 6.91pg/mL, respectively. The 1:1 Invisalign® material induced a TNF-α concentration of 8.54 pg/mL, and the Invisalign® material induced a concentration of 113.10 pg/mL TNF-α. The LPS induced a concentration of 7.14 pg/mL TNF-α.

At 48 h, the negative and saliva controls had a concentration of 5.58 pg/mL and 3.51 pg/mL of TNF-α, respectively. The 1:1 Invisalign® material and Invisalign® material induced concentrations of 6.90 pg/mL TNF-α and 66.74 pg/mL TNF-α. For the LPS control, it induced a concentration of 3.51 pg/mL TNF-α.

5.5.4. IL-6

All concentration values for IL-6 were 11.91 pg/mL, apart from the the 48 h Invisalign® material that induced 15.57 pg/mL pg/mL.

5.5.5. IL-8

At 24 h, the concentration of IL-8 in the negative and saliva controls was 154.31 pg/mL and 210.69 pg/mL IL-8, respectively. The 1:1 Invisalign® material induced a concentration of 282.85 pg/mL IL-8, and the Invisalign® material induced a much higher concentration of 10,105.59 pg/mL IL-8. The LPS induced an IL-8 concentration of 148.76 pg/mL.

At 48 h, the negative and saliva controls had concentrations of 65.67 pg/mL and 30 .61 pg/mL of IL-8, respectively. The 1:1 Invisalign® material induced 244.575 pg/mL IL-8 and the Invisalign® material induced a very high concentration of 55,037.67 pg/mL IL-8. The LPS control induced a concentration of 94.22 pg/mL IL-8.

5.5.6. IL-10

The concentration value of IL-10 at 24 h was 3.51 pg/mL for the negative control, 1:1 Invisalign® material sample, and the LPS positive control. The saliva control had a concentration of 3.63 pg/mL IL-10, and the Invisalign® material induced a concentration of 9.85 pg/mL IL-10.

At 48 h, the negative and saliva control concentrations were 4.15 pg/mL IL-10 and 3.51 pg/mL IL-10, respectively. The concentration of IL-10 in the 1:1 Invisalign® material sample was 5.5 pg/mL, and the Invisalign® material induced 24.90 pg/mL IL-10. The LPS positive control induced an IL-10 concentration of 7.08 pg/mL.

5.5.7. IL-12p70

The concentration value for IL-12p70 was 3.61 pg/mL for all samples except for 48 h Invisalign® material sample at 3.91 pg/mL IL-12p70, and the 48 h LPS control that induced 4.05 pg/mL IL-12p70. The minimum detectible concentration of IL-12p70 in the assay is 2.0 pg/mL.

5.6. Statistical Analysis: Mann-Whitney U test

Including all donors and time points from experiments 2-4, the IL-8 concentrations of the Invisalign® material samples did not significantly differ from the saliva control concentrations of IL-8 (Mann-Whitney $U=89$, $n_1=n_2=75$, $\alpha < 0.05$ two-tailed).

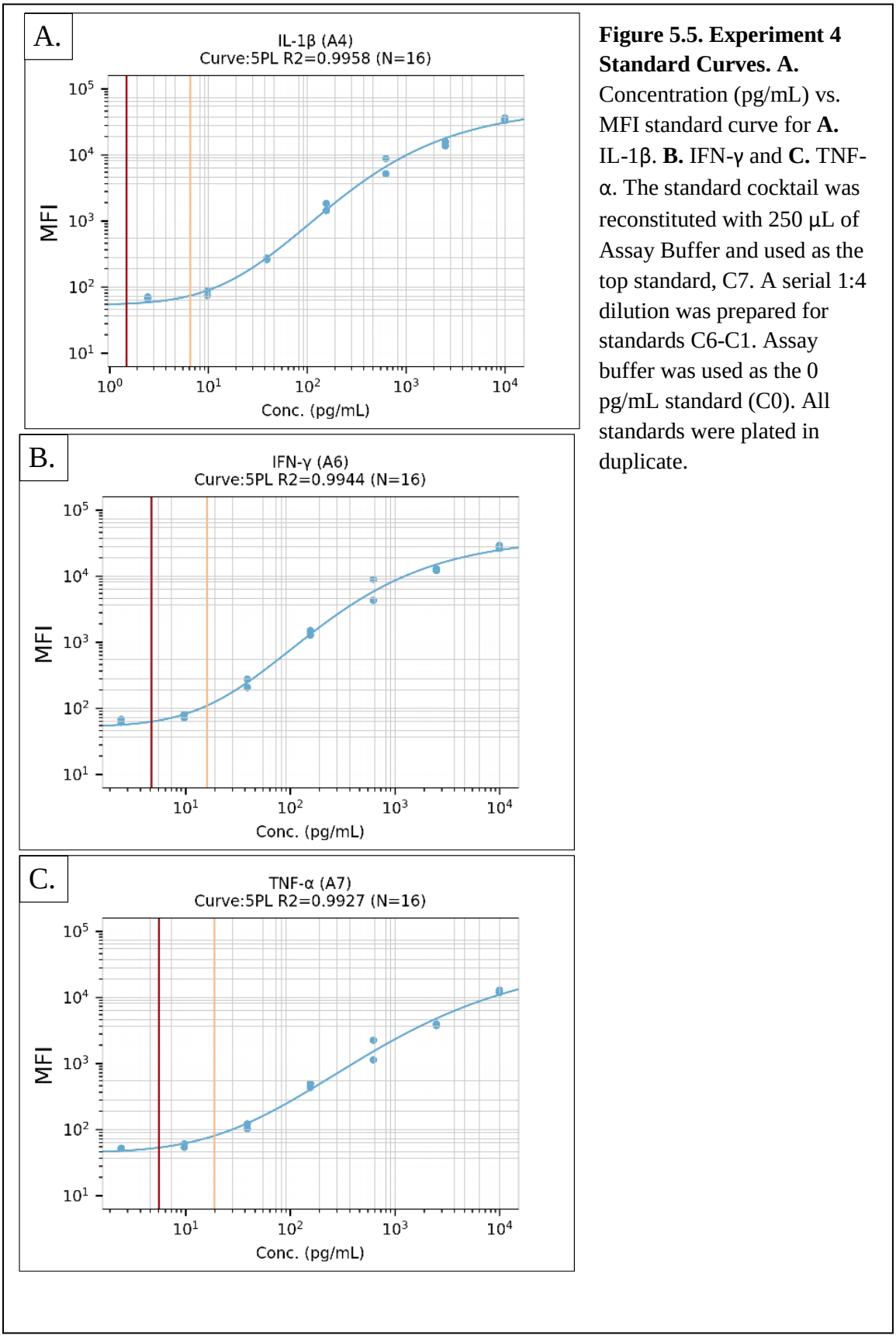


Figure 5.5. Experiment 4 Standard Curves. A. Concentration (pg/mL) vs. MFI standard curve for **A.** IL-1β. **B.** IFN-γ and **C.** TNF-α. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

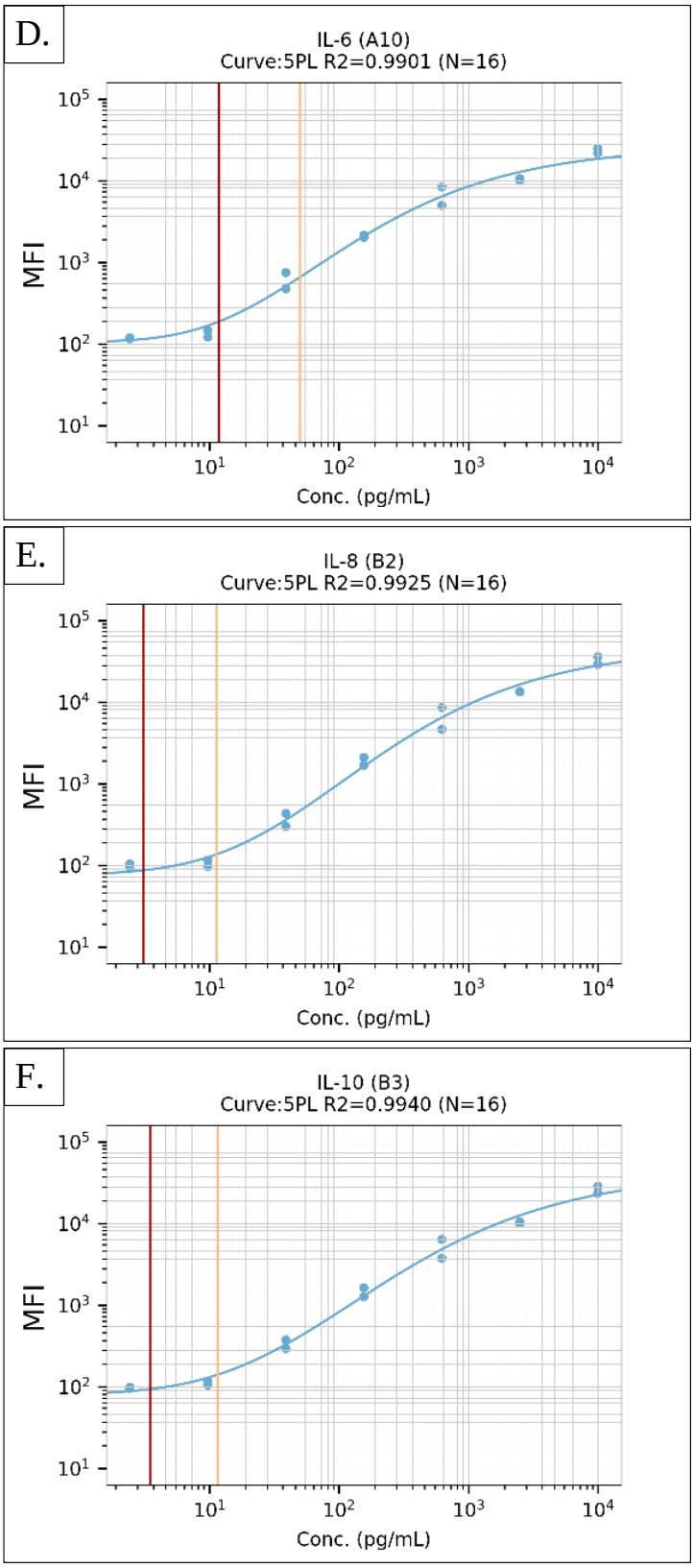


Figure 5.5. (Continued). Experiment 4 Standard Curves. Concentration (pg/mL) vs. MFI standard curve for **D.** IL-6. **E.** IL-8 and **F.** IL-10. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

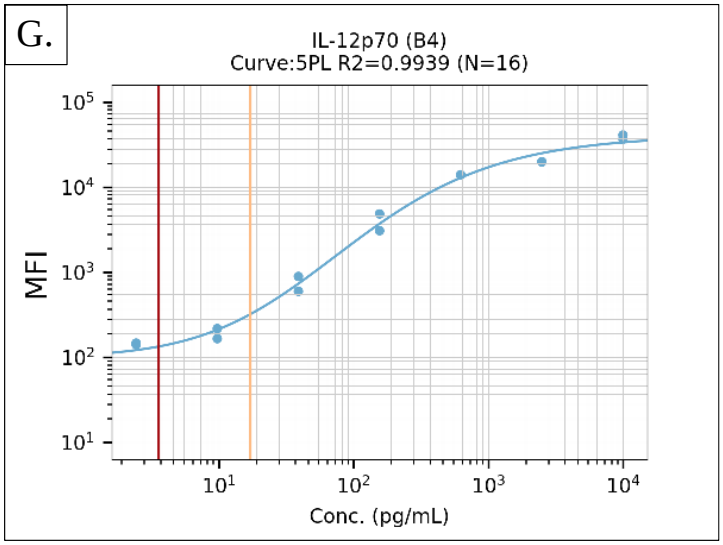


Figure 5.5. (Continued). Experiment 4 Standard Curves. Concentration (pg/mL) vs. MFI standard curve for **G.** IL-12p70. The standard cocktail was reconstituted with 250 µL of Assay Buffer and used as the top standard, C7. A serial 1:4 dilution was prepared for standards C6-C1. Assay buffer was used as the 0 pg/mL standard (C0). All standards were plated in duplicate.

Sample	IL-1β	IFN-γ	TNF-α	IL-6	IL-8	IL-10	IL-12p70
24 hours							
Negative control	26.16 ± 12.99	4.71 ± 0.00	6.95 ± 1.37	11.91 ± 0.00	154.31 ± 92.04	3.51 ± 0.00	3.61 ± 0.00
Saliva control	23.02 ± 14.07	4.71 ± 0.00	6.61 ± 3.10	11.91 ± 0.00	210.69 ± 133.13	3.63 ± 0.12	3.61 ± 0.00
1:1 Invisalign®	12.75 ± 1.12	4.71 ± 0.00	8.54 ± 0.23	11.91 ± 0.00	282.85 ± 72.64	3.51 ± 0.00	3.61 ± 0.00
Invisalign®	461.83 ± 31.39	4.71 ± 0.00	133.10 ± 18.68	11.91 ± 0.00	10105.59 ± 6720.16	9.85 ± 0.23	3.61 ± 0.00
LPS control	14.65 ± 6.40	4.71 ± 0.00	7.14 ± 0.71	11.91 ± 0.00	148.76 ± 2.30	3.51 ± 0.00	3.61 ± 0.00
48 hours							
Negative control	3.27 ± 0.83	4.71 ± 0.00	5.58 ± 0.00	11.91 ± 0.00	65.67 ± 9.84	4.15 ± 0.64	3.61 ± 0.00
Saliva control	4.08 ± 0.77	4.71 ± 0.00	3.51 ± 0.00	11.91 ± 0.00	30.61 ± 15.42	3.51 ± 0.00	3.61 ± 0.00
1:1 Invisalign®	12.15 ± 3.5	4.71 ± 0.00	6.90 ±7 0.5	11.91 ± 0.00	244.575 ± 71.72	5.55 ± 0.58	3.61 ± 0.00
Invisalign®	958.42 ± 49.91	4.71 ± 0.00	66.74 ± 1.80	15.57 ± 0.22	55037.67 ± 45053.01	24.90 ± 10.58	3.91 ± 0.30
LPS control	5.06 ± 1.62	4.71 ± 0.00	3.51 ± 0.00	11.91 ± 0.00	94.22 ± 4.64	7.08 ± 1.78	4.05 ± 0.44

Table 5.5. Mean Cytokine Expression of THP-1 Cells, Experiment 4. Mean cytokine expression in pg/mL of THP-1 cells cultured for 24 or 48 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the 1:1 Invisalign sample has Invisalign® eluate diluted 1:1 with cell media, the Invisalign® sample is the addition of Invisalign eluate (no dilution), and the LPS control has 500 ng/ml *P. gingivalis* LPS. Values shown are the mean of the duplicates reported with standard error.

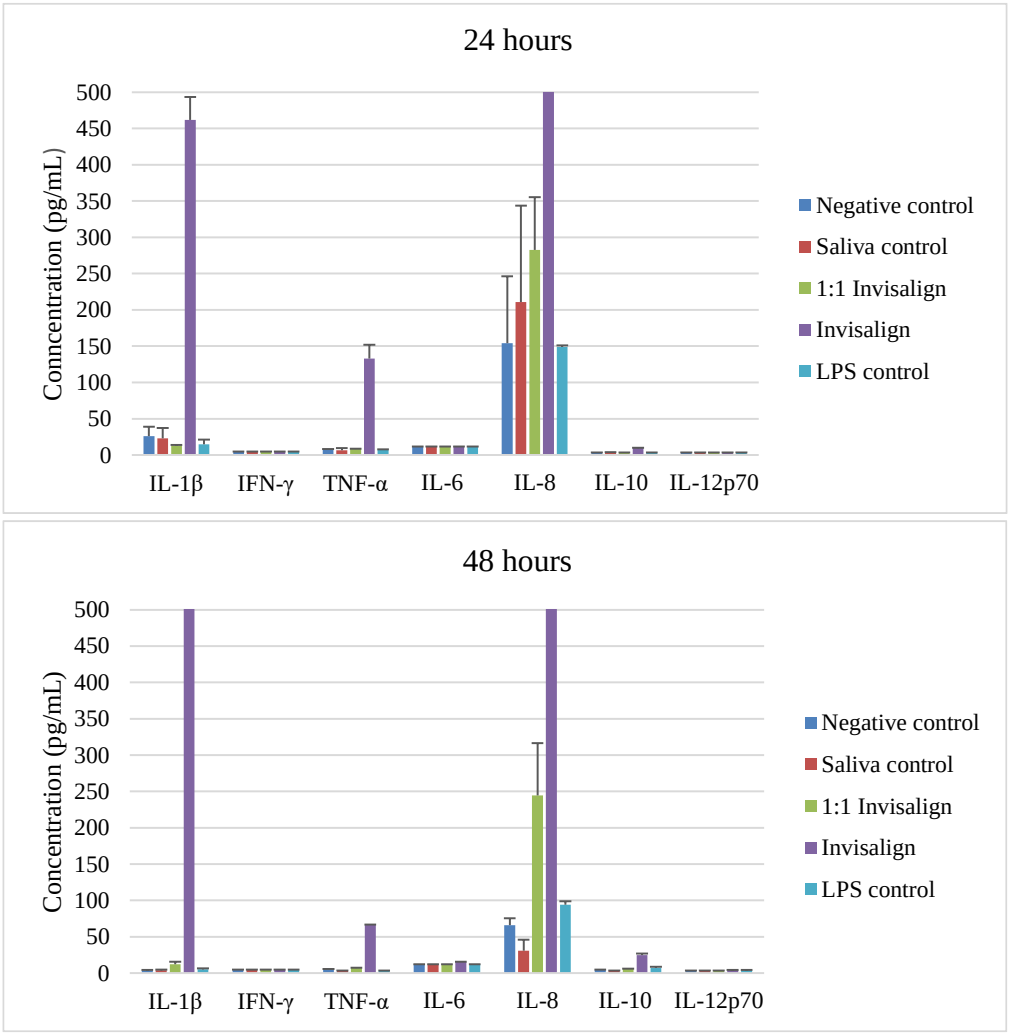


Figure 5.5.1. Mean Cytokine Expression of THP-1 Cells, Experiment 4. Mean cytokine expression in pg/mL of THP-1 cells cultured for 24 or 48 hours with different culture conditions. The negative control has no additive, the saliva control has only artificial saliva added, the 1:1 Invisalign sample has Invisalign® diluted 1:1 with cell media, the Invisalign® sample is the addition of Invisalign (no dilution), and the LPS control has 500 ng/ml *P. gingivalis* LPS. Values shown are the mean of the duplicates with standard error.

CHAPTER 6: DISCUSSION

6.1. Particle Size

The dynamic light scattering (DLS) results indicate that the radius of the Invisalign® material particles was, on average, between 502.9 and 873.5 nm. The smallest particles had a mean radius of 0.71 nm, and the largest detected had a mean radius of 552.6 nm. The Invisalign® material preparation steps were taken from Premaraj et al. (2014), but their values for the ground eluate were much larger when measured with a measure scope. They found particles ranging from 86 x 56 μm to 186 x 161 μm in size. The size difference could be largely attributed to the method used to determine the size. DLS is a technique well-studied in characterizing nanoparticles (Domingos et al. 2009; Souza et al. 2016).

The smallest particles are of most interest because they classify as nanoparticles. A nanoparticle is a particle with a diameter between 1 to 100 nanometers (Lewinski et al. 2008). Nanoparticles can interact with the immune system and become immunotoxic, increasing the expression of pro-inflammatory cytokines (Elsabahy and Wooley 2013). Although it was the supernatant of the Invisalign® material/artificial saliva soakings that was collected for the eluate, there may be nanoparticles remaining in the eluate that could be contributing to the immune reaction. When worn in the mouth, Invisalign® material may be abraded by bruxing of the teeth, which may release nanoparticles, although the size of particles that may be created by bruxing is not known. In addition, routine brushing and cleaning of Invisalign trays at least twice daily could also possibly abrade the plastic and release nanoparticles.

6.2. Experiment 1

The first experiment yielded interesting results for the expression of TNF-α, IL-6, and IL-8. TNF-α concentrations were low in saliva and negative controls at 24 and 72 h. Invisalign® material samples induced approximately 5x higher TNF-α concentrations than the negative controls, and the LPS control induced >1000 pg/mL of TNF-a. These results suggest that Invisalign® material stimulates TNF-α expression from PBMCs. TNF-α is a pro-inflammatory

cytokine produced by lymphocytes, monocytes, and NK cells (Borish and Steinke 2003; Dinarello 2000), which are included in the pooled PBMCs. The TNF-α concentrations at 72 hours were lower than at 24 hours, most likely due to increased cell death with increased culture time. Invisalign® material eluate was shown to have a cytotoxic effect on oral keratinocytes (Premaraj et al. 2014), and TNF-α can also have a direct cytotoxic effect on cells (Borish and Steinke 2003; Dinarello 2000).

Unexpectedly, IL-6 and IL-8 production from PBMC were high without any stimulants, reaching over 49,000 pg/mL for IL-6 and over 16,000 pg/mL for IL-8. Because all values for IL-6 and IL-8 exceeded the highest standard concentration (around 10,000 pg/mL) the values of the different samples cannot be accurately compared. To do this, the samples would need to be diluted to within the range of the assay. The question is why there was such high cytokine expression in negative controls. Bacterial contamination during culture conditions could lead to increased cytokine expression in negative controls, but there would have likely been signs of this with the TNF-α values. Similarly, another possible cause of the high values was that the FBS used in the cell media was not heat-inactivated. Heat-inactivation of complement in FBS is required, which can activate immune cells unwantedly (Heat Inactivation 1996; Gibco fetal bovine serum (FBS) 2014). For the next experiment, FBS was heat-inactivated.

Another possibility for the high IL-8 and IL-6 concentrations was a mixed lymphocyte reaction (MLR). This occurs when the T-cells recognize MHC molecules that are not compatible, causing the T-cells to divide, proliferate, and secrete cytokine, similar to a Graft Versus Host Disease reaction (Janeway et al. 2001). The company from which the cells were purchased claims to test the cells for reactivity, though (Human mononuclear cells 2020), which would mitigate the risk for a MLR. For the next experiment, cells from three individual donors were used to rule out any reactivity between cells.

6.3. Experiment 2

Induction of TNF-α and IL-6 were less than 1 pg/mL for all samples besides the LPS controls, which were still under 10 pg/mL (compared to the first experiment where LPS controls were >1,000 pg/mL for TNF-α). Neither Invisalign® material nor LPS induced expression of TNF-α or IL-6 for any donor. Production of IL-8 was slightly increased in response to Invisalign and more variable than the other two cytokines. In Donor 1, the IL-8 concentration for the Invisalign® material sample was increased from the negative and saliva controls, and the LPS positive control was higher than the Invisalign® material sample. In Donor 1, Invisalign® material may have induced an increase in IL-8 production, although the values are still very low for all samples. Donors 2 and 3 showed slightly increased LPS controls, but the Invisalign® material did not cause increased expression of IL-8. Donor 1 was the only smoker from the three donors and had the highest concentration values for IL-8.

6.4. Experiment 3

To obtain a higher positive control for the next experiment, LPS was vortexed for 15 minutes before use as it is a lipid and needs even distribution. A higher concentration of LPS was also added (1 ug/mL) to obtain increased cytokine expression, though both the 500 ng/mL and 1 µg/mL LPS samples were both >10,000 pg/mL for IL-8, indicating that the LPS addition in experiment 2 (500ng/mL) was not the cause for the low positive control values. The standard curve for experiment 3 showed error between duplicates, especially at the C4 standard point. This was most likely due to an issue with the serial dilution at the C4 standard and/or pipetting error.

The general trend for this experiment showed increased IL-8 values in Donors 1 and 3 for all samples and controls compared to IL-6 and IL-8 concentrations. In Donor 1, the TNF-α concentration in response to Invisalign® material was slightly higher than the saliva control. For IL-6, Invisalign® induced from Donors 1 and 3 almost 4x the negative control concentration. However, the values were still under 400 pg/mL, much less than the positive controls (≥10,000 pg/mL) and the Invisalign® samples for IL-8 in Donors 1 and 3 (>6,000/mL). Donor 2 had low

concentrations for all samples; the LPS control for IL-8 was only 71.16 pg/mL. There is no obvious explanation for the difference in expression in the different donors. Every donor will react differently to stimuli. In Experiment 2, the donor who smoked had the highest concentrations values of IL-8. In this experiment, of the donors with the highest concentration values of IL-8, one was a smoker (Donor 1) and the other was not (Donor 3).

6.5. Experiment 4

Because there was a stark difference in reactivity between donors in the previous experiments, the switch was made to THP-1 cells for this experiment. Using the same cell line in repeated experiments will make the results more predictable and easier to validate. THP-1 cells are monocytic and therefore represent the monocytes of PBMC.

The standard curves for experiment 4 showed some pipetting error between duplicates and the lower values of the standard curves were slightly lower than expected, possibly due to flow cytometer settings. For the next experiment, the voltages and events collected will be increased, as both the MFIs and number of events were lower than recommended for this experiment.

The cytokine showing the most variation in samples, and the highest expression was IL-8. LPS did not induce much IL-8 expression, but the Invisalign® material greatly stimulated expression of IL-8 with values over 10,000 pg/mL at both 24 and 48 hours. The 1:1 diluted Invisalign® material also induced expression of IL-8 but less so than the concentrated eluate. IL-8 is a pro-inflammatory chemokine that directs neutrophils to the site of inflammation (Borish and Steinke 2003; Dinarello 2000). One of the adverse reactions reported from Invisalign® material is swelling of gingival tissues, and neutrophils play a key role in edema. Neutrophils promote inflammation by interacting with the vascular endothelia during extravasation from the blood vessels and by secreting compounds that increase the permeability of the vasculature, such as arachidonic acid (Scott and Krauss 2012).

Invisalign® material also induced expression of IL-1β, but in much lower concentrations than IL-8. TNF-α was also slightly increased from the negative controls, but still less than 100 pg/mL. IL-1β and TNF-α are both pro-inflammatory cytokines, with IL-1β playing a role in acute inflammation/fever and also chronic inflammation (Cameron and Kelvin 2000-2013). The mechanism of TNF is similar to IL-1, and the effects are synergistic (Borish and Steinke 2003; Dinarello 2000).

The positive control values for IL-8, IL-1β, and TNF-α were all low. To obtain higher reactivity to LPS in future experiments, the monocytes should be differentiated to macrophages by phorbol myristate acetate (PMA). In one study, LPS induced TNF-α concentrations 2.5 higher in differentiated THP-1 cells than in undifferentiated cells (Takashiba et al. 1999). When differentiated to macrophages, THP-1 cells expressed high levels of CD14, a macrophage-specific differentiation antigen (Takashiba et al. 1999). CD14 facilitates the LPS activation of cells by transferring the LPS to the Toll-like receptor complex (Plevin et al. 2016).

The use of *P. gingivalis* LPS instead of *E. coli* LPS also may have had an effect on the positive control results. The two different types of LPS activate monocytes in different ways; *P. gingialis* LPS is atypical in that it utilizes mainly TLR2, whereas *E. coli* LPS utilizes TLR4 (Zhang et al. 2008). One study found that *P. gingivalis* LPS induced expression of IL-1β, TNF-α, and IL-6 in THP-1 cells and slightly more so than *E. coli* LPS at some timepoints (Zhang et al. 2008). Another study found that *E. coli* LPS induced the expression of IL-1β and IL-8 in THP-1 cells, more so than *P. gingivalis* LPS (Yiemwattana et al. 2017). Each of these studies used 1μg/mL of *P. gingivalis* LPS instead of the 500ng/mL used in this experiment. Although different strains and extraction methods of LPS can cause different activity levels (Zhang et al. 2017), it is expected that *P. gingivalis* LPS would induce expression of IL-6, IL-8, TNF-α, and IL- β, with proper PMA differentiation. In future experiments, different concentrations of *P. gingivalis* LPS may be used, or the experiment may be conducted with *E. coli* LPS.

The values for IFN-γ, IL-6, IL-10, and IL-12p70 were all very low. The positive LPS controls also showed low values for these cytokines. It was not expected that IFN-γ would be highly expressed as it is produced mainly by natural killer cells and T-cells. Macrophages can also produce IFN-γ, but less commonly (Dinarello 2000; Varma et al. 2002).

IL-10 is an anti-inflammatory cytokine produced by monocytes, macrophages, and T-cells (Couper et al. 2008). In THP-1 cells differentiated to macrophages by phorbol myristate acetate (PMA), one study found there was no significant increase in IL-10 when stimulated with LPS (Liu et al. 2018). Pengal et al. (2006) found that macrophages stimulated with LPS had higher concentrations of IL-10 only when also activated with IgG immune complexes, not LPS alone. These studies show that IL-10 may not be expressed solely by macrophages stimulated with LPS.

IL-12p70 is produced by monocytes, macrophages, B cells, polymorphonuclear neutrophils, and mast cells. In one study, IL-12p70 was stimulated in THP-1 cells by 1μg/mL LPS after PMA differentiation to macrophages (Utsugi et al. 2003). In future experiments, the THP-1 cells should be differentiated to macrophages.

6.6. Limitations of the Study

Many limitations of the experiments were described within the discussion. Each experiment came with its own unique challenges and troubleshooting that needed to be overcome before future experiments.

6.6.1. Reproducibility

To verify the reproducibility of a study and draw statistical conclusions, the experiment needs to be repeated multiple times. One of the main limitations of this study is that each run of the experiment was repeated once, and then the changes needed to be made. Thus, limited conclusions can be drawn. One of the challenges of performing a novel study is the methodology,

and there were multiple attempts to succeed in that regard. Troubleshooting, time, and finances were all limiting factors in repeating the assay.

6.6.2. *In vitro* Research

Another limitation of this study is studying cells under *in vitro* conditions. Although THP-1 cells are commonly used to study a variety of immune responses, the results cannot be fully extrapolated to *in vivo* conditions. THP-1 cells were chosen to standardize cells and for ease of repeatability of the study, but the results cannot be fully extrapolated to *in vivo* conditions as the response of the immortalized cells may be different from primary monocytes in humans (Bosshart and Heinzelmann 2016). The PBMCs from different donors showed highly variable results after stimulation and makes for difficult reproducibility.

The culture conditions used in this study also cannot be extrapolated to what would happen *in vivo* when a patient is wearing Invisalign® aligners. This study's results serve as a jumping-off point for future research that will more closely relate to the patients using this product.

6.7. Conclusions

The results of this are that Invisalign® material stimulates IL-8 expression in certain individual donor PBMCs and THP-1 monocytes, though not significantly (Mann-Whitney $U=89$, $n_1=n_2=75$, $\alpha < 0.05$ two-tailed). Invisalign® material might also stimulate slight expression of TNF-α in pooled PBMCs, certain individual donor PBMCs, and THP-1 cells. IL-6 was slightly increased in certain individual donor PBMCs. IL-1β was also slightly stimulated by Invisalign® material in THP-1 cells. IL-10, IL-12p70, IFN-γ, and IL-6 were not detected after THP-1 monocytes were stimulated with Invisalign® material.

6.8. Future Research

Further research will include reproducing the results in multiple other rounds of the cell culture and assay. Also, adding eluate that has been soaked for different time points will determine whether increased or decreased soaking time of the Invisalign® material produces

different cytokine expression. Other research could include different culture times for the cells to determine whether cytokine production is varied at different points in the cell culture. Another prudent addition to future studies would be adding isocyanate to the samples being studied to determine if the cytokine expression after Invisalign® material addition is similar to that induced by isocyanate. There was a potential trend of PBMCs from smokers reacting more to stimuli than nonsmokers, which could be investigated in future studies, also.

The nanoparticles found in the Invisalign® particulate material is another avenue that could be pursued in further research. To determine if there are any nanoparticles in the eluate, the Invisalign® eluate should be evaluated with DLS.

Other research could look at the effect of this Invisalign® material on other immune cells, such as mast cells. This would give more information on the allergic potential of the material by studying the degranulation of mast cells.

The primary goal of this *in vitro* research was to provide evidence to translate the findings to the patients who are being treated with Invisalign® aligners. One future project will include collecting blood samples from patients who have used Invisalign® aligners and studying the cytokine profile in response to Invisalign. To take it a step further, comparing samples from patients who had adverse reactions from the product from those who did not. This research would begin to elucidate why certain patients have reactions and if there is any way to screen for or prevent those reactions.

BIBLIOGRAPHY

Aalto-Korte K, Suuronen K, Kuuliala O, Henriks-Eckerman M-L, Jolanki R. 2012. Occupational contact allergy to monomeric isocyanates. Contact Dermatitis. 67(2):78-88.

Allareddy V, Nalliah R, Lee MK, Rampa S, Allareddy V. 2017. Adverse clinical events reported during Invisalign® material treatment: Analysis of the maude database. American Journal of Orthodontics and Dentofacial Orthopedics. 152(5):706-710.

Awosika O, Kao S, Rengifo-Pardo M, Ehrlich A. 2017. Angioedema, stomatitis, and urticaria caused by contact allergy to Invisalign. Dermatitis. 28(5):323-324.

Borish LC, Steinke JW. 2003. 2. Cytokines and chemokines. Journal of Allergy and Clinical Immunology. 111(2, Supplement 2):S460-S475.

Borg L, Christensen JM, Kristiansen J, Nielsen NH, MennenT, and Poulsen LK. 2000. Nickel-induced cytokine production from mononuclear cells in nickel-sensitive individuals and controls. Arch. Dermatol. Res. 292(6):285-91

Bosshart H, Heinzelmann M. 2016. Thp-1 cells as a model for human monocytes. Ann Transl Med. 4(21):438-438.

Bräscher A-K, Zuran D, Feldmann RE, Benrath J. 2016. Patient survey on Invisalign® material® treatment comparen the SmartTrack®® material to the previous aligner material. Journal of Orofacial Orthopedics / Fortschritte der Kieferorthopädie. 77(6):432-438.

Cameron MJ, Kelvin DJ. Cytokines, Chemokines and Their Receptors. c2000-2013. In: Madame Curie Bioscience Database. Austin (TX): Landes Bioscience. [accessed 2020 Oct 25]. https://www.ncbi.nlm.nih.gov/books/NBK6294/

Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L. 2017. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 9(6):7204-7218.

Chhibber A, Agarwal S, Yadav S, Kuo CL, Upadhyay M. 2018. Which orthodontic appliance is best for oral hygiene? A randomized clinical trial. Am J Orthod Dentofacial Orthop. 153(2):175-183.

Coombs, RRA, Gell PGH. 1968. Classification of allergic reactions responsible for drug hypersensitivity reactions. In: Coombs, R.R.A., Gells, P.G.H. (Eds.), Clinical Aspects of Immunology. 2nd ed. Philadelphia (PA): Davis. p. 575–596.

Couper KN, Blount DG, Riley EM. 2008. Il-10: The master regulator of immunity to infection. The Journal of Immunology. 180(9):5771.

Descotes J, Choquet-Kastylevsky G. 2001. Gell and coombs's classification: Is it still valid? Toxicology. 158(1-2):43-49.

Dinarello CA. 2000. Pro-inflammatory cytokines. Chest. 118(2):503-508.

Dinarello CA. 2007. Historical insights into cytokines. Eur J Immunol. 37 Suppl 1(Suppl 1):S34-S45.

Domingos RF, Baalousha MA, Ju-Nam Y, Reid MM, Tufenkji N, Lead JR, Leppard GG, Wilkinson KJ. 2009. Characterizing manufactured nanoparticles in the environment: Multimethod determination of particle sizes. Environmental Science & Technology. 43(19):7277-7284.

Eliades T, Pratsinis H, Athanasiou AE, Eliades G, Kletsas D. 2009. Cytotoxicity and estrogenicity of Invisalign® appliances. American Journal of Orthodontics and Dentofacial Orthopedics. 136(1):100-103.

Elsabahy M, Wooley KL. 2013. Cytokines as biomarkers of nanoparticle immunotoxicity. Chem Soc Rev. 42(12):5552-5576.

Feghali CA, Wright TM. 1997. Cytokines in acute and chronic inflammation. Front Biosci. 2:d12-26.

Finoti LS, Nepomuceno R, Pigossi SC, Corbi SC, Secolin R, Scarel-Caminaga RM. 2017. Association between interleukin-8 levels and chronic periodontal disease: A PRISMA-compliant systematic review and meta-analysis. Medicine. 96(22):e6932.

Frick M, Isaksson M, Björkner B, Hindsén M, Pontén A, Bruze M. 2003. Occupational allergic contact dermatitis in a company manufacturing boards coated with isocyanate lacquer. Contact Dermatitis. 48(5):255-260.

Gee K, Guzzo C, Che Mat NF, Ma W, Kumar A. 2009. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. Inflamm Allergy Drug Targets. 8(1):40-52.

Gibco fetal bovine serum (FBS): frequently asked questions. c2014. Thermo Fischer Scientific Inc. [acessed 2020 Nov 19]. https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/CellCultureandTransfection/pdfs/Gibco-FBS-FAQs-Flyer-Global-FLR.pdf

Goldman AS, Prabhakar BS. 1996. Medical Microbiology. 4th edition. Baron S, editor. Galveston (TX): University of Texas Medical Branch at Galveston. Chapter 1, Immunology Overview. [accessed 2020 Oct 25]. https://www.ncbi.nlm.nih.gov/books/NBK7795/

Heat inactivation-are you wasting your time?. 1996. Reprinted from Art to Science 15(1). [accessed 2020 Nov 9]. http://fscimage.fishersci.com/cmsassets/downloads/segment/Scientific/pdf/Cell_Culture/Application_Notes/heat_inactivation.pdf?&storeId=10652#:~:text=Heat%20inactivation%20was%20once%20deemed,C3b%20inactivator%20in%20commercial%20FBS

Holden JA, Attard TJ, Laughton KM, Mansell A, O'Brien-Simpson NM, Reynolds EC. 2014. *Porphyromonas gingivalis* lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces inflammatory cytokines. Infection and Immunity. 82(10): 4190-4203.

Human mononuclear cells (MNCs). c2020. Zenbio. [accessed 2020 Nov 9]. https://www.zen-bio.com/products/cells/pbmcs.php

Invisalign®. c2020. [accessed 2020 Oct 22]. https://www.Invisalign.com/.

Janeway CA Jr, Travers P, Walport M, et al. 2001. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science. Responses to alloantigens and transplant rejection. [accessed 2020 Nov 19]. https://www.ncbi.nlm.nih.gov/books/NBK27163/

Justiz Vaillant AA, Vashisht R, Zito PM. 2020. Immediate Hypersensitivity Reactions. In: StatPearls. Treasure Island (FL): StatPearls Publishing [updated 2020 Sep 29; accessed 2020 Oct 25]. https://www.ncbi.nlm.nih.gov/books/NBK513315/

Kleiveland CR. 2015. Peripheral blood mononuclear cells. In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, editors. The impact of food bioactives on health: In vitro and ex vivo models. Cham: Springer International Publishing. p. 161-167.

Kobayashi K, Kaneda K, Kasama T. 2001. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech. 53(4):241-245.

Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. The Journal of experimental medicine. 170(3): 827–845.

Kotyk MW, Wiltshire WA. 2013. An investigation into bisphenol-a leaching from orthodontic materials. The Angle Orthodontist. 84(3):516-520.

Kravitz ND, Kusnoto B, BeGole E, Obrez A, Agran B. 2009. How well does Invisalign work? A prospective clinical study evaluating the efficacy of tooth movement with Invisalign®. American Journal of Orthodontics and Dentofacial Orthopedics. 135(1):27-35.

Krouse JH, Derebery MJ, Chadwick SJ. 2008. Managing the allergic patient. Elsevier. Chapter 1, Introduction to Allergy. [accessed 2020 Oct 25]. https://www.sciencedirect.com/topics/medicine-and-dentistry/delayed-hypersensitivity

Levrini L, Mangano A, Montanari P, Margherini S, Caprioglio A, Abbate GM. 2015. Periodontal health status in patients treated with the Invisalign® material(®) system and fixed orthodontic appliances: A 3 months clinical and microbiological evaluation. Eur J Dent. 9(3):404-410.

Lewinski N, Colvin V, Drezek R. 2008. Cytotoxicity of nanoparticles. Small. 4(1):26-49.

Liu X, Yin S, Chen Y, Wu Y, Zheng W, Dong H, Bai Y, Qin Y, Li J, Feng S et al. 2018. Lps-induced pro-inflammatory cytokine expression in human airway epithelial cells and macrophages via nf-κb, stat3 or ap-1 activation. Mol Med Rep. 17(4):5484-5491.

Lochmatter P, Beeler A, Kawabata TT, Gerber BO, Pichler WJ. 2009. Drug-specific in vitro release of il-2, il-5, il-13 and ifn-γ in patients with delayed-type drug hypersensitivity. Allergy. 64(9):1269-1278.

Lummus ZL, Alam R, Bernstein JA, Bernstein DI. 1998. Diisocyanate antigen–enhanced production of monocyte chemoattractant protein-1, il-8, and tumor necrosis factor-α by peripheral mononuclear cells of workers with occupational asthma. Journal of Allergy and Clinical Immunology. 102(2):265-274.

Martina S, Rongo R, Bucci R, Razionale AV, Valletta R, D'Antò V. 2019. In vitro cytotoxicity of different thermoplastic materials for clear aligners. The Angle Orthodontist. 89(6):942-945.

Material safety data sheet. 2015. SmartTrack® Aligner Material. Align Technology, Inc. [accessed Nov 16 2020]. https://c3-preview.prosites.com/242082/wy/docs/MSDS_SmartTrack®_Aligner_Material.pdf

Medzhitov R. 2008. Origin and physiological roles of inflammation. Nature. 454(7203):428-435.

Miller KB, McGorray SP, Womack R, Quintero JC, Perelmuter M, Gibson J, Dolan TA, Wheeler TT. 2007. A comparison of treatment impacts between Invisalign® material aligner and fixed appliance therapy during the first week of treatment. American Journal of Orthodontics and Dentofacial Orthopedics. 131(3):302.e301-302.e309.

Mishra PK, Panwar H, Bhargava A, Gorantla VR, Jain SK, Banerjee S, Maudar KK. 2008. Isocyanates induces DNA damage, apoptosis, oxidative stress, and inflammation in cultured human lymphocytes. Journal of Biochemical and Molecular Toxicology. 22(6):429-440.

Mühl H, Pfeilschifter J. 2003. Anti-inflammatory properties of pro-inflammatory interferon-γ. International Immunopharmcology. 3(9):1247-1255.

Nedwed V, Miethke R-R. 2005. Motivation, acceptance and problems of Invisalign ® patients. Journal of Orofacial Orthopedics / Fortschritteder Kieferorthopädie. 66(2):162-173.

Pacheco-Pereira C, Brandelli J, Flores-Mir C. 2018. Patient satisfaction and quality of life changes after Invisalign treatment. American Journal of Orthodontics and Dentofacial Orthopedics. 153(6):834-841.

Pengal RA, Ganesan LP, Wei G, Fang H, Ostrowski MC, Tridandapani S. 2006. Lipopolysaccharide-induced production of interleukin-10 is promoted by the serine/threonine kinase akt. Molecular Immunology. 43(10):1557-1564.

Phan X, Ling PH. 2007. Clinical limitations of Invisalign. J Can Dent Assoc. 73(3):263-266.

Plevin RE, Knoll M, McKay M, Arbabi S, Cuschieri J. 2016. The role of lipopolysaccharide structure in monocyte activation and cytokine secretion. Shock. 45(1):22-27.

Pourahmad J, Salimi A. 2015. Isolated human peripheral blood mononuclear cell (pbmc), a cost effective tool for predicting immunosuppressive effects of drugs and xenobiotics. Iran J Pharm Res. 14(4):979-979.

Premaraj T, Simet S, Beatty M, Premaraj S. 2014. Oral epithelial cell reaction after exposure to Invisalign® material plastic material. American Journal of Orthodontics and Dentofacial Orthopedics. 145(1):64-71.

Saito M, Arakaki R, Yamada A, Tsunematsu T, Kudo Y, Ishimaru N. 2016. Molecular mechanisms of nickel allergy. *Int J Mol Sci*. 17(2):202.

Schuster S, Eliades G, Zinelis S, Eliades T, Bradley TG. 2004. Structural conformation and leaching from in vitro aged and retrieved Invisalign appliances* *part of a thesis submitted by the first author in partial fulfillment of the requirements for the master of science degree at marquette university. American Journal of Orthodontics and Dentofacial Orthopedics. 126(6):725-728.

Scott DA, Krauss J. 2012. Neutrophils in periodontal inflammation. *Front Oral Biol*. 15:56-83.

SmartTrack® material. c2020. [accessed 2020 Oct 22]. https://www.Invisalign.com/the-Invisalign® material-difference/SmartTrack®-aligner-material

Souza TGF, Ciminelli VST, Mohallem NDS. 2016. A comparison of TEM and DLS methods to characterize size distribution of ceramic nanoparticles. Journal of Physics: Conference Series. 733:012039.

Takashiba S, Van Dyke TE, Amar S, Murayama Y, Soskolne AW, Shapira L. 1999. Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kB. Infect Immun. 67(11):5573-5578.

Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 26(2):171-6.

Utsugi M, Dobashi K, Ishizuka T, Endou K, Hamuro J, Murata Y, Nakazawa T, Mori M. 2003. C-jun n-terminal kinase negatively regulates lipopolysaccharide-induced il-12 production in human macrophages: Role of mitogen-activated protein kinase in glutathione redox regulation of il-12 production. The Journal of Immunology. 171(2):628.

Varma TK, Lin CY, Toliver-Kinsky TE, Sherwood ER. 2002. Endotoxin-induced gamma interferon production: Contributing cell types and key regulatory factors. Clin Diagn Lab Immunol. 9(3):530-543.

Wang Y, Dai S. 2013. Structural basis of metal hypersensitivity. Immunol Res. 55: 83–90.

Warrington R, Watson W, Kim HL, Antonetti FR. 2011. An introduction to immunology and immunopathology. Allergy, Asthma & Clinical Immunology. 7(1):S1.

Wenk KS, Ehrlich A. 2012. Isocyanates. Dermatitis. 23(3):130-131.

White DW, Julien KC, Jacob H, Campbell PM, Buschang PH. 2017. Discomfort associated with Invisalign® material and traditional brackets: A randomized, prospective trial. The Angle Orthodontist. 87(6):801-808.

Williams GT, Williams WJ. 1983. Granulomatous inflammation--a review. J Clin Pathol. 36(7):723-733.

Yang L, Guo H, Li Y, Meng X, Yan L, Dan Z, Wu S, Zhou H, Peng L, Xie Q et al. 2016. Oleoylethanolamide exerts anti-inflammatory effects on lps-induced thp-1 cells by enhancing pparα signaling and inhibiting the nf-κb and erk1/2/ap-1/stat3 pathways. Scientific Reports. 6(1):34611.

Yiemwattana I, Chaisomboon N, Yeesibsan J, Pongcharoen S. 2017. Differential induction of MAPK signaling pathways by Porphyromonas gingivalis and Escherichia coli lipopolysaccharide in human monocytes. Journal of international dental and medical research. 10(2):1-5.

Zhang D, Chen L, Shenglai L, Gu Z, Yan J. 2008. Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1β, TNF-α, and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. Innate Immun. 14(2):99-107.