Possible Intrinsic adjuvanticity of the Amb a 1(*Ambrosia artemisiifolia* :Ragweed) allergen

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By Andrew Bysice, B.Sc.

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TITLE: Possible Intrinsic Intrinsic adjuvanticity of the Amb a 1(*Ambrosia artemisiifolia* :Ragweed) allergen

AUTHOR: Andrew Bysice, B.Sc. (McMaster University)

SUPERVISOR: Dr. Mark Larché

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ABSTRACT

Amb a 1 is the major allergen found in ragweed. Our observations have suggested that Amb a 1 may bind lipopolysaccharide (LPS), which would likely contribute to the allergenicity of Amb a 1. In order to assess whether Amb a 1 can bind LPS, peptide sequences from Amb a 1 were assayed for their ability to bind to LPS using an ELISA based LPS binding assay. A 15 amino acid sequence in the β- chain of Amb a 1 demonstrated affinity for biotin labeled *E. coli* LPS. The sequence also bound to *P. aeruginosa* LPS, which is structurally disparate in the lipid A region, indicating that the sequence has flexibility in recognizing different lipid A moieties, or that the binding site may not include the lipid A portion of the LPS molecule. An IL-10 ELISA was also used to determine whether the LPS bound to the peptides induced an immunological response in leukocytes. Peptides containing the LPS-binding sequence were able to bind to LPS and induce IL-10 production, suggesting the interaction between Amb a 1 and LPS may have immunological consequences. We have identified a sequence within the major ragweed allergen Amb a 1 that has the potential to bind to LPS. This indicates that the allergen may provide its own adjuvant when encountered by the immune system, leading to an enhanced immunological response to an otherwise innocuous environmental protein.

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ABBREVATIONS

7AAD- 7-aminoactinomycin D

Amb a 1- *Ambrosia artemisiifolia*

Ara h 1- *Arachis hypogaea*

APC- Antigen Presenting Cell

APC- allophycocyanin

BSA- Bovine Serum Albumin

CD- Cluster of Differentiation

DMSO- Dimethyl sulfoxide

EBV- Epstein-Barr Virus

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme-linked immunosorbent assay

FBS- Fetal Bovine Serum

FITC- fluorescein isothiocyanate

IKK- IκB kinase enzyme complex

IL- Interleukin

IRAK1- Interleukin-1 receptor-associated kinase 1

HDM- House dust mite

HPLC- High Performance Liquid Chromatography

LAL- Limulus Amebocyte Lysate

LBP- LPS binding protein

LPS- Lipopolysaccharide

MHC- Major Histocompatibility complex

MyD88- Myeloid differentiation primary response gene (88)

NKT- Natural Killer T cells

OVA- Ovalbumin

PAMP- Pathogen associated molecular patterns

PBMC- Peripheral Blood Mononuclear Cell

PBS- Phosphate buffered saline

PCM- polydimethylcyclosiloxane

PRR- Pattern recognition receptors

rpm – Revolutions per minute

SEM- Standard error of mean

SIT- Specific Immunotherapy

SOCS3- Suppressor of Cytokine Signaling 3

SPR- Surface Plasmon Resonance

STAT3- Signal transducer and activator of transcription 3

TMB- 3,3',5,5'-Tetramethylbenzidine

TCR- T cell Receptor

TIR- Toll/Interleukin-1 receptor

TLR- Toll-like receptors

TNF- α- Tumor necrosis factor

TRAF6- TNF receptor associated factors 6

UBC13- Ubiquitin C 1

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1.0 INTRODUCTION

1.1 The Immune System

The immune system is designed to maintain homeostasis by eliminating pathogens while remaining unresponsive to innocuous proteins found in the environment and in the host. The immune system is composed of two distinct arms; the innate immune system and the adaptive immune system. The innate system responds to conserved molecules on pathogens in a non-specific manner and has no immunological memory, while the adaptive immune system is pathogen specific and has immunological memory.

1.2 Innate Immunity

The innate immune system has two components; intrinsic barriers and cellular barriers. Intrinsic barriers are physical barriers that prevent pathogens from crossing epithelia and colonizing tissue. Epithelia is comprised of skin and lining of the body's mucosal layers. The mucosa also secretes mucus that prevents pathogens from binding to the surface. In addition, the mucosa produces chemical substances and enzymes that have antimicrobial properties.

If a pathogen bypasses these physical barriers, it will be recognized by innate immune cells. Innate leukocytes identify pathogens based on their expression of highly conserved microbial structures or pathogen- associated molecular patterns (PAMPs) that

are essential for the pathogen's survival. Examples of PAMPs are peptidoglycans, bacterial flagellin and bacterial lipopolysaccharide (LPS). The receptors on immune cells that recognize PAMPs are termed pattern recognition receptors (PRR). One group of these receptors are termed Toll-like receptors (TLR). TLRs receptors initiate a downstream signaling event to induce an inflammatory response to enhance the immunoresponsiveness to the pathogen. This inflammatory response, which includes proinflammatory cytokines, initiates cell recruitment into the surrounding tissue and facilitates the activation of the adaptive immune system.

1.3 Adaptive Immunity

The adaptive immune system consists of two distinct arms, cell mediated immunity and antibody. For cell mediated adaptive response, the major histocompatility complex (MHC) II sequesters short peptides of the digested pathogen and presents a peptide on the surface of a professional antigen presenting cell (APC). These professional APCs include dendritic cells, B cells and macrophages. The T cell Receptor (TCR) binds to the MHC complex and recognizes the peptide sequence and the cytokine milieu that will direct the T cell to a certain fate. Depending on the cytokine milieu, naïve T cells differentiate into T helper cell 1 (Th1) cells for bacterial/viral infections or T helper cell 2 (Th2) for helminth infections. These cells have immunological memory and enhance innate effector cells' ability to destroy pathogens. For antibody responses, naïve B cells have IgM antibodies that can bind to a wide variety of structures. Once a pathogen is

bound to an immunoglobulin M (IgM) antibody, it is then internalized into the B cell and digested. Then peptides of the pathogen are presented to the helper T cells in MHC II molecules. The T cell then activates the B cell to differentiate into either memory cells, which harbor immunological memory or plasma cells, which produce antibodies.

1.4 Allergy

Hypersensitivity is defined as an adverse clinical reaction to an innocuous environmental protein, or allergen [1]. Allergic hypersensitivity is an immunological disorder that is increasing in prevalence in the industrialized world [2]. This disorder is characterized by a Th2 polarized response that includes high levels of immunoglobulin E (IgE). Atopic disease is characterized by a group of disease processes in a set of patients that have the capacity to become sensitized to proteins. The clinical manifestation of allergic sensitization include, but are not limited to, asthma, allergic rhinitis and eczema [2]. Atopic diseases affect approximately 1 in 5 people in the United States [3]. The frequency of allergic sensitization is increasing. For example, in 1995, the frequency of asthma in the USA was 5.5% of the population [4], by 2005, it rose to approximately 7% [3]. Although allergic disease is on the rise, the reason for this increase is currently unknown.

1.4.1 Pathology of Hypersensitivity

An allergic reaction typically is broken down into an early phase and late phase reaction. The early phase is mediated by mast cells and basophils via IgE cross-linking. The hallmark reaction is when the IgE antibodies, which are bound to high affinity FcεRI receptors on mast cells, are cross-linked by a particular allergen to which an individual is sensitized. This cross-linking causes downstream signaling and initiates mast-cell degranulation. Degranulation involves the release of histamine, tryptase, lipid mediator leukotrienes, prostaglandins, cytokines and platelet-activating factor. Leukotrienes cause smooth muscle contraction, vasodilatation and increased vascular permeability. This early phase reaction happens within minutes after an individual is exposed to allergen. The clinical symptoms depend on the site of the allergen exposure. If the allergen is inhaled then symptoms include airway constriction, coughing, shortness of breath, tight chest, wheezing, sneezing and a runny nose. However, if the allergen is introduced intradermally, a 'wheal and flare' reaction will take place. If exposure to the allergen is systemic, then manifestations can include angio-edema, urticaria and anaphylaxis. Cytokines and chemokines, such as Interleukin-4 (IL-4) and IL-13, are released during the early phase. A late phase reaction follows the early phase reaction peaking six to nine hours after allergen exposure. The hallmark of the late phase reaction is the recruitment of Th2 cells and eosinophils into the tissue, causing edema. If the allergic reaction occurs in the lungs, the clinical signs of a late phase reaction are airway narrowing and mucus secretion. Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13 are secreted by Th2 cells during the late phase. IL-4 is secreted when professional APCs presents the allergen to

Th2 cells. The presence of IL-4 is thought to recruit and maintain the allergen-specific Th2 cells and along with IL-13 and certain co-stimulatory molecules, plays an important role in promoting the production of IgE by allergen-specific B-cells. As discussed, the IgE then binds to the FcεRI on mast cells and the system is primed for another hypersensitivity reaction.

1.5 Allergy Treatment

Clinical management of allergic diseases are broken down into two branches, either pharmacological or disease modifying. Patients with allergic disease are educated about their disease and are encouraged to avoid their triggering allergen to circumvent allergic symptoms. If avoidance fails, patients have the option of pharmological intervention to alleviate symptoms. Pharmacological treatments include, but are not limited to, corticosteroids, anti-histamines, leukotriene modifiers and anti-IgE monclonal antibodies (Omalizumab) [5]. The mechanisms of the phamacological interventions vary, but all treatments are palative rather than curative. For example the mechanism for corticosteriod steriod treatment is a global immune supression, which is due, in part, to the anti-inflammatory cytokine IL-10 [6]. Since these treatments are not curative, researchers have developed alternative treatments that are disease modifying. One of these treatments is allergen-specific immunotherapy (SIT). SIT has been used for almost a century for treating allergic asthma and rhinitis. SIT consits of repeated administration of an allergen (usually subcutaneously) to which a patient is sensitized. A whole allergen

is administered at low doses to decrease immunoresposiveness, or tolerance, to the allergen. As the patient becomes increasingly tolerant of the allergen, the dose of allergen is increased until the patient is completely tolerant. Importantly, SIT has been shown to be clinically effective in decreasing symptoms, decreasing medication usage and the duration of tolerance can extend up to a year after treatment [8]. The mechanism by which SIT works has become more clear. There is evidence that SIT decreases the Th2 while increasing the Th1 component of the anti-allergen response [7].

Although SIT is effective, the main drawback of SIT is that it exposes individuals to an allergen to which they are sensitized and can result in a potentially fatal adverse allergic reaction. Moreover, conventional SIT does not induce permanent tolerance to the allergen. A therapy derived from SIT is peptide immunotherapy (PIT). Peptide immunotherapy is the use of short synthetic peptides derived from an allergen to induce tolerance to the that allergen [9]. Peptides do not provoke adverse IgE-mediated reactions because they are short, preventing them from cross-linking adjacent IgE receptors. The use of short peptides for PIT is essential as peptides greater than 25 amino acids in length have been associated with adverse allergic events [7]. PIT has shown promise as a vaccine-type therapy [10, 11]. Recent studies with peptides derived from Fel d 1, the major allergen in cats shown that the mechanism of this tolerant effect is through IL-10 [12]. Giving a single dose of mixed epitopes of an allergen shows a sustained protection from that allergen [12].

The mechanism by which peptide immunotherapy induces tolerance is still needs to be elucidated. However, some clues have indicated that the sequences of the allergen can induce antigen-specific populations of immunosupressive cells. Peptides that are presented by nonprofessional APCs to T cells induce 'anergy', which is described as antigen-specific nonresponsiveness [9]. Presentation of antigenic peptides can induce the generation of T cells with imunnosupressive capabilites [9]. These immunosupressive T cells are termed T regulatory cells (Tregs). Specific peptide sequences that are presented in the MHC II binding groove can stimulate an IL-10 producing T reg cell. An example of a peptide sequence inducing Treg- IL-10 production is Epstein Barr Virus (EBV). EBV evades detection by the immune system by expressing a viral homologue of IL-10 to supress MHC expression preventing T cell proliferation to the virus [13]. Also the virus produces an antigen while in its latent stage, called LMP1. Researchers found that a 20 amino acid peptide of LMP1 induced T reg cells to elicit IL-10 [14]. Therefore, it is plausable that allergens could contain sequences that induce Tregs since the majority of individualuls are not be sensitized to that allergen.

1.6 T regulatory cells

To avoid unwanted and unnecessary immune responses, the immune system has regulatory mechanisms to inhibit responses against nonpathogenic antigens. One of the central players in immunological regulation is the regulatory T cell or "Treg". Tregs were first described as $CD4^+$ $CD25^+$ cells. These cells were found to express a transcription factor, FoxP3, making Tregs a functionally distinct T cell subpopulation [15]. $FoxP3^+$

Tregs are generated from the thymus and are considered “naturally arising”, but Tregs can also be formed in the periphery from naïve T cells and are referred to as “adaptive” Tregs. Adaptive Tregs can have a classical $CD4^+CD25^+$ $FoxP3^+$ phenotype or other phenotypes such as production of IL-10: type 1 Treg cells (Tr1). Also, there are CD8+, CD3+ CD4-CD8-, γδ T cells, and natural killer T (NKT) cells that are part of the Treg subsets [15]. Tregs suppress antigen-specific immune responses by a variety of mechanisms, including IL-10.

1.7 Allergens

Allergens are only a tiny fraction of proteins that the population is exposed to via ingestion, inhalation, or direct contact. Interestingly, allergies develop to the same set of allergen sources, such as house dust mite, ragweed and peanut, and reoccur throughout the human population. Therefore, it could be the structural properties of the allergens that are responsible for sensitizing an individual. In 1978, Aas posed the question ‘what makes an allergen an allergen?’, a question which is still a matter of debate amongst immunologists [16].

There is an intimate interaction between allergens and the innate immune system that may explain an allergen’s allergenicity. Allergens as a whole are a very diverse group of proteins. They vary in the environmental source, coming from plants, fungi,

insects, and mammals. Also, they vary in function in their respective hosts, from structural proteins to proteases. There have been attempts to find a unifying theory that defines an allergen's allergic potential, including defining the allergen epitopes recognized by T and B cells. However, attempts to identify common structural epitopes amongst a very diverse T and B cell array have shown little evidence that these epitopes are the reason for an allergen's allergic potential [17]. Comparison of the size, glycosylation status, resistance to proteolysis, enzymatic activity [17, 18], and surface charge [19] of allergen revealed none of these factors have been linked to an allergens allergic potential. However, there has been some recent evidence to support the theory that allergens have intrinsic properties that activate the innate immune system and allow them to promote a Th2 immune response [17, 18]

1.8 Sensitization

PRRs are crucial for regulating antigen presentation to immune cells and therefore are key for initiating effector T-cell responses [20, 21]. Antigen presentation by APCs without a signal from a PRR, such as Toll like recptor 4 (TLR4), can create tolerance to that antigen [20]. TLR4 via its adaptor protein MD2 binds to the PAMP lippopolysaccaride (LPS) and forms a complex with CD14. LPS is part of the outer membrane of Gram-negative bacteria and its function is to maintain structural integrity of the cell and protect the cell from environmental stresses such as antibiotics [22]. LPS is a very potent stimulator of the innate immune system. To induce an adaptive T cell response, a TLR ligand (e.g. LPS) and a antigen must be in the same phagolysosome of the APC when presenting the antigen peptide to the naïve T cell [23]. Association of

LPS to TLR4-MD2-CD14 complex creates a downstream signaling event that initiates transcription of inflammatory cytokines (TNF –α, IL-1β, IL-6) and T cell differentiation. The amount of LPS present during antigen priming of the adaptive immune responses can lead to different T cell differentiation outcomes. Eisenbarth *et al* showed that using a "very low dose" (<1 ng) of LPS with the antigen ovalbumin (OVA), leads to tolerance and using a "low dose" (100 ng) of LPS causes an allergic Th2 response [24]. Conversely, using a "high dose" (100 μg) of LPS causes a Th1 response [24]. These findings opened the door to possibilities of a more intimate link between TLR4 signaling and allergic sensitization.

A molecular link between the house dust mite (HDM) allergen, Der p 2 and the TLR4 complex was recently described [25]. Der p 2 is highly allergenic. This allergen has the highest amount of positive skin prick tests amongst house dust mite positive patients [25]. Interestingly, Der p 2 is structurally homologous to MD-2, which is a lipid binding protein in the TLR4 immune complex [26]. Additionally, it was demonstrated that Der p 2 is functionally homologous to MD-2 [25], since a direct interaction of Der p 2 with CD14 and TLR4 leads to downstream signaling [25]. Also, Der p 2 can facilitate LPS recongtion via TLR4, with or without MD-2. Der p 2 also acts as a MD-2 homolog *in vivo* [25]. It was concluded that Der p 2 and LPS drives Th2 inflammation through TLR4 signaling. Low LPS exposure can shift a naïve T cell to a Th2 cell.

Furthermore, the glycoprotein peanut allergen, Ara h 1, has structural homology with glycan structures of helminths. Ara h 1 can bind to dendritic cells (DC) specifically

via ICAM-grabbing nonintegrin (DC-SIGN) to elicit a Th2-skewed response [27]. This research has opened possibilites that allergens have intrinsic adjuvant capabilites. Since the Der p 2 allergen may have an intrinsic ability to stimulate TLR4, it may be possible for other allergens to interact with the TLR4 antagonist LPS.

1.9 Lipopolysaccharide

The molecular structure of LPS is heterogeneous in nature, composed of hydrophilic and hydrophobic regions, which tends to form aggregates of varying sizes. Therefore, the molecular weight of LPS varies. It is estimated to be between 10 kDa and 1,000 kDa. LPS is composed of three parts; Outer antigenic polysaccharide (O-antigen), core oligosaccharide chain, and lipid A.

The outer antigenic polysaccharide is the hydrophilic outer surface facing the environment. These structures are chains of repeating oligosaccharides. The structural diversity of the sugars is strain specific for a species. For example, O-antigens are used to classify strains of *Salmonella* species [22]. The core oligosaccharide domain of LPS connects the O-antigen polysaccharides to lipid A.

Lipid A is the inner most segment of LPS, consisting of a glucosamine phosphorylated disaccharide with hydrophobic fatty acid chains [22]. This segment of the LPS is the responsible for its toxicity and which is considered an “endotoxin”. If released into the blood stream, lipid A can cause septic shock, a disease process that can be

defined as systemic inflammation. Lipid A causes inflammation by binding to TLR-CD14-MD2 receptor complex on macrophages, mononuclear cells and endothelial cells that initiates downstream signaling that transcribes TNF-α and IL-1-β cytokines. This reaction would be a desirable responses to local infections, however, systemically it can cause death. Lipid A is the region of LPS that binds to the TLR4/MD-2 [28]. Karps' group had found that Der p 2 can bind to LPS and interact with the TLR4 complex [25] and Bottomlys' group had discovered that the amount of LPS could modulate T cell responses [24]. Other allergens' [64] have been found to have LPS binding pockets. This indicates that it is possible that allergens have an intrinsic ability interact with LPS signaling machinery.

1.10 Toll-like receptor 4

The LPS receptor is comprised of a complex, TLR4-MD2-CD14. This complex is present on many cell types [29], which include monocytes and dendritic cells. The complex requires an accessory protein, LPS binding protein (LBP) that isolates a monomeric unit of LPS (lipid A) from its oligomeric micelles. LBP then transfers the monomer to CD14. CD14 concentrates LPS for binding to TLR4-MD2 complex causing downstream signaling for inflammatory cytokine production, such as TNF-α. Once TLR 4 recognizes LPS, the receptor undergoes oligomerization and downstream adaptor proteins bind to the Toll-interkeukin-1 receptor (TIR) domain located on the cystolic region of TLR 4. The TIR domain regions are highly conserved, and single point mutation can render the receptor unresponsive to LPS [30]. These regions mediate

protein-protein interactions. The adaptor protein, myeloid differentiation primary response gene 88 (MyD88) is recruited to the TIR domain and in turn, initiates a cell signaling cascade. MyD88 recruits and activates IL-1 receptor-associated kinase-4 (IRAK-4). The IRAK-4 kinase is responsible for the activation and degradation of IL-1 receptor-associated kinase-1 (IRAK1). IRAK-1 recruits an adaptor protein, TNF receptor-associated factor 6 (TRAF6) which in turn forms a complex with ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 isoform A and activates transforming growth factor-β-activated kinase 1 (TAK 1). TAK1 then phosphorylates both downstream IκB kinase (IKK) and mitogen-activated protein kinase (MAPK). These two proteins have their own pathways. The IKK complex activates the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) which transcribes proinflammatory cytokines, such as TNF- α, IL-1, IL-6. However, these proinflamatory cytokines can be damaging to the host if unregulated. To avoid such damage, IL-10 is produced by macrophages as negative feedback mechanism to dampen excessive inflammation. Transcription factors of proinflammatory cytokines, such as NF-κ B, also transcribe the IL-10 cytokine. Therefore, after a proinflammatory burst of cytokines due to LPS, there is a subsequent IL-10 secretion to dampen damaging inflammation. Harnessing the ability to induce IL-10 production can be valuable for treatment for inflammatory diseases, including allergy.

1. 11 Interleukin-10

IL-10 was identified as a factor that was secreted by Th2 cells that inhibited cytokine production from Th1 cells [31]. Since then, IL-10 has been found to be produced by a large number of cells. It is a well known suppressor of T-cell proliferation, Th1 cytokines IFN-γ, IL-2 and Th2 cytokines IL-4, IL-5. IL-10 inhibits dendritic cell maturation as well as decreasing expression of MHC II and costimulation ligand expression on dendritic cells and monocytes [15]. Additionally, IL-10 has been found to suppress IgE production [15] and can inhibit proinflammatory cytokines, TNF-α, IL-6 and IL-1.

The IL-10 receptor complex is a dimer of heterodimers that contains IL-10R1 and IL-10R2 [32]. IL-10R1 is the principle receptor for IL-10 ; however, the second receptor is required for complete signaling. Most hematopoietic cells express low levels of IL-10R1 but increased expression of the IL-10R1 gene can be induced by various stimuli [32]. The ubiquity of IL-10R expression shows the importance of the use of the IL-10 cytokine as a global immune suppressor.

When IL-10 binds to the IL-10R complex, it initiates a complex signal transduction pathway. When the ligand and receptor complex is formed, the JAK1 and Tyk2, which are associated with IL-10R1 and IL-10R2 respectively, are activated. These kinases phosphorylate the tails of the receptors and recruit STAT3 to the IL-10R1 [33]. STAT3 homodimerizes, then is released from the receptors and translocates into the

nucleus. The STAT3 transcription factor binds to a number of promoters, and to IL-10 itself [34]. This shows that IL-10 production is positively regulated, however this process is self limiting. STAT3 also activates its negative regulator, suppressor of cytokine signaling 3 (SOCS3) [35]. SOCS3 is induced by IL-10 and mostly regulates the downstream signaling events, not the IL-10R genes [35]. Harnessing the ability to induce IL-10 production can be valuable for treatment for inflammatory diseases, including allergy.

Clinical trials using recombinant IL-10 (rIL-10) have been attempted to treat inflammatory diseases such as psoriasis [32], rheumatoid arthritis [32] and Crohns disease [36] . These trials have shown modest clinical improvements, however, delivery of the rIL-10 to the site of inflammation has proved to be a challenge which may be a factor in the results of the clinical trails. One downside of this treatment is the potential risk of overdosing the amount of rIL-10 and actually enhancing inflammation [37]. Therefore, peptide immunotherapy has shown promise in its ability to induce tolerance via IL-10 while being safe for patients [12].

1.12 Ragweed allergen

A peptide immunotherapy was designed to alleviate the problem of ragweed allergy. *Ambrosia artemisiifolia* (ragweed) produces pollen that is one of the most important causes of seasonal allergy in North America. Allergy to ragweed pollen proteins affects up to 36 million individuals [38] and Amb a 1 has been identified as the

major allergen. Amb a 1 is an acidic 38 kDa protein of 397 amino acids [39]. The protein is a pectate lyase, which is responsible for degrading plant sugar material. The Amb a 1 allergen is made up of 2 chains: the α-chain and β-chain [38]. The β-chain mainly contains IgE epitopes while the α-chain mostly contains T-cell epitopes [38]. T cell epitopes of Amb a 1 were predicted using the MHCPred2 program. This algorithm estimates hydrophilicity, surface solvent accessibility, chain flexibility, and number of proteasomal cleavage sites for T cell epitopes that can bind in MHC II. Fifty (50) peptides were selected from the program as possible candidates for eliciting IL-10 production such as the EBV peptide. Peptides were screened for eliciting IL-10 production by human peripheral blood mononuclear cells (PBMC). The sequence Amb a $1^{376\text{-}390}$ was selected as a possible candidate for eliciting IL-10 (M. Larché, unpublished). The peptide elicited IL-10 production from all subjects, which is uncharacteristic of a true T cell epitopes which would be expected to elicit responses in a subset of subjects based on expression of appropriate MHC restriction elements. This observation raised the possibility that IL-10 production from PBMC stimulated with Amb a $1^{376\text{-}390}$ could be due to LPS contamination during peptide synthesis.

2.0 MATERIALS and METHODS

2.1 Peripheral Blood Mononucleocyte (PBMC) Isolation and Separation

A butterfly syringe (BD, New Jersey, USA) and Plus blood collection tubes containing sodium heparin (BD, New Jersey, USA) were used to collect venous blood following donor consent. Blood was pooled in a 50mL Falcon tubes (BD Falcon, New Jersey, USA). Twenty-five mLs of blood was carefully pipetted onto 15mLs of Ficoll-Paque PLUS (GE Healthcare, Montreal, Canada). The blood-Ficoll prepartion was centrifuged (AllergraX-15R, Beckman Couler, Mississauga, Canada) at 400xg for 25 minutes to seperate the blood into erythrocytes, buffy coat (containing PBMCs and platlets) and plasma. PBMCs were recovered and transfered into a 50mL tube. Twenty-five mLs of 2.5% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, USA), 1% Penicillin-streptomycin, and 1 unit of heparin/mL in RPMI 1640 (Invitrogen, New York, USA) (FBS-RPMI) were added to the isolated PBMCs as a wash step to dilute residual Ficoll due to its toxcity to PBMCs. The cells were centrifuged at 250xg for 10 minutes. The supernatant was decanted and the PBMC pellet was resuspended in 5mLs of FBS-RPMI. 20 µl of the suspended cell specimen was diluted with 180 µl of unsupplemented RPMI 1640 and 20 µl of the suspended cell specimen was mixed with 20µl of trypan blue (Invitrogen, New York, USA). 10µl of trypan blue-cell mixture was placed on Cell Countess (Invitrogen, New York, USA) plates. Plates were inserted into the Countess cell counter (Invitrogen, New York, USA) to determine the concentration of viable cells.

Three measurements were made and averaged. The following equation (1) was used to calculate the volume (X) of medium was required achieve the desired cell concentration (2.5×10^6 per mL) for cell culture.

$$X = \frac{\text{(Average of cell count) x (10 (for dilution)) x (5mLs of cell FBS-RPMI solution)}}{(2.5 \times 10^6)} \quad (1)$$

2.5×10^6 per mL was used as this is the standard concentration of PBMC for culture preparation in the Larché Lab (Larché M, Unpublished). The result (X) was the volume of serum-free media (Cellular Technology Ltd, Shaker Heights, USA) required to obtain 2.5×10^6 cells/mL. The 5mL of cells were centrifuged at 225xg for 10 minutes, the supernatant was decanted and the cells were re-suspended in serum-free media at the volume calculated.

2.2 Peptide Production

Allergen peptides were synthesized by F-moc chemistry at the GL Biochem (Shanghai, China) Ltd and purified by High Performance Liquid Chromatography (HPLC). All of the peptides were >95% pure before use in stimulation assays as judged by mass spectrometry. Peptides were received in solid phase and reconstituted in PBS or unsupplemented RPMI 1640 to a concentration 1mg/mL. After dilution, peptides were

stored at -20°C. Peptides were thawed for dilution by placing tubes on ice until the solution was completely thawed.

Peptide sequences were derived from house dust mite allergen Der p 1: HDM. Ragweed allergen peptide was derived from Amb a 1: RGW. Timothy grass allergen peptide was derived from Ph1 p 5: TIM. Peptide sequences and their variants are noted **Table 1**.

2.3 Peptide Stimulation of PBMCs

Peptides were diluted from 1mg/ml to 200 µg/mL, 60 µg/ml, 20 µg/ml, 6 µg/ml or 2 µg/ml in unsupplemented RPMI 1640 medium. 100µl of the diluted peptides were placed in duplicates in 96 well cell culture plates (BD Biosciences, New Jersey, USA) and stored at 4°C or -20°C. If the plate was stored at -20°C, it was thawed on ice. 100µl of PBMCs, at a concentration of 2.5 x 10^6/mL, was added to each well of a 96 well of the cell culture plate containing 100µl of peptide. Cells and peptides were incubated at 37°C in 5% CO_2 for three (3) days and 120µl of the culture supernatants were isolated and frozen at -80°C. For analyses, supernatants were thawed on ice.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

A commercial ELISA kit (eBioscience, San Diego, USA) was purchased and used for quantifying IL-10 in cell culture supernatant. Stock capture antibodies of anti-human IL-10 were diluted from 48µl to 12 mLs with coating buffer as specified by the

manufacturer. 100ul of the diluted capture antibodies were immobilized in each well of a 96 well ELISA plate (Thermoscientific, Watham, USA). Wells were incubated overnight at 4^{o}C. Residual capture antibodies were discarded and wells were washed with wash buffer (1xPBS containing 0.05% Tween-20) five times. 200µl of assay diluent (A.D.) (eBioscience) were added to wells and incubated at room temperature for one (1) hour. Recombinant human IL-10 was diluted in A.D. from 1,000,000 pg/ml to 300 pg/ml, 100 pg/ml, 20 pg/ml, 4 pg/ml, 2 pg/ml for creation of a standard curve. The A.D. was discarded and 100µl of the recombinant IL-10 and culture supernatant were placed in wells, in duplicate, and incubated for two (2) hours at room temperature while shaking on a microplate shaker (VWR, Pennsylvania, USA) at 400 revolutions per minute (rpm). After incubation, the standard and supernatants were discarded and wells were washed five times with wash buffer. Anti-human IL-10 biotin-conjugated antibodies were diluted from 48µl to 12mLs in A.D. as instructed by manufacturer (eBioscience). 100µl of the secondary antibody solution were placed in wells and incubated while shaking (400rpm) at room temperature for one (1) hour. After incubation, the secondary antibody solution was discarded and wells were washed five times with wash buffer. 48µl of streptavidin conjugated-HRP were diluted in 12mL of A.D. as instructed by the manufactuer (eBioscience). 100µl of the streptavidin-conjugated HRP solution were added into wells and incubated while shaking (400 rpm) for one (1) hour at room temperature. After incubation, excess streptavidin-HRP solution was discarded and wells were washed 7 times with wash buffer. 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate were added to the wells and incubated while shaking (400 rpm) for 15 minutes at room temperature. Following incubation, 50µl of H_2SO_4 were added to stop the enzymatic

reaction. The plate was examined with a VICTOR microplate spectrophotometer (Perkin Elmer, Woodbridge) reader at 450nm to determine the optical density of each well.

Analyses of optical densisty results was calculated with Workout 2.0 (Dazdaq Solutions, Brighton, United Kingdom) using a 5-paramter analysis to convert optical density values into concentrations (pg/ml) based on the standard curve optical density measurements.

2.5 LPS Binding Assay

Peptides were diluted in coating buffer to concentrations of 30µg/ml, 10µg/ml, 3µg/ml, or 1µg/ml. 100µl of peptide solution were immobilized in duplicates in each well of a 96 well ELISA plate (Thermo Scientific, Waltham, USA). Plates were incubated overnight at 4^{o}C. Excess peptides were discarded and wells were washed with wash buffer (1xPBS 0.05% Tween-20). This was repeated 5 times. Wells were blocked with 200µl of A.D. and incubated at room temperature for one (1) hour. Blocking A.D. was discarded after the incubation. Biotin-conjugated *E.coli* LPS O111:B4 (Invivogen, San Diego, USA) or *P. aeruginosa* 10 LPS (Sigma Alrich, St. Louis, USA) was diluted to approximately 1.56 µg/ml and 3.75 µg/ml, respectively, in A.D. Diluted biotinylated LPS was added to wells and incubated for two (2) hours at room while shaking (400 rpm). The LPS solution was discarded and wells were washed five times with wash buffer. Streptavidin-conjugated HRP (Invivogen, San Diego, USA) was diluted in A.D to

0.1µg/ml. 100 µl of the streptavidin-conjugated HRP solution were placed in wells and incubated while shaking (400 RPM) for 30 minutes at room temperature. After incubation, streptavidin conjugated- HRP solution was discarded and wells were washed 7 times with wash buffer. 100µl of TMB substrate were added to wells and the plate incubated while shaking (400 rpm) for 15 minutes at room temperature. Following incubation, 50µl of H_2SO_4 were added wells to stop the enzymatic reaction. The plate was examined with a VICTOR microplate spectrophotometer reader at 450nm to determine the optical density of each well

2.6 Limulus Amebocyte Lysates (LAL) Assay

A commercial gel clot assay (Lonza, Switzerland) was used to measure endotoxin in the peptide solutions. Lyophilized lysates were reconstituted by adding 5.2 mL of endotoxin-free (as certified by manufacturer) water to the vial. The vial was swirled for least 30 seconds. The bacterial endotoxin control vial was reconstituted in endotoxin free water. This was vortexed for 15 minutes. The positive control was diluted using endotoxin-free water to a concentration of 1 EU/ml. One endotoxin unit is equivalent to approximately 100 pg of *E. coli* LPS. A serial dilution was made to create a positive standard of *E.coli* O55:B5 LPS (0.5 EU/ml, 0.25 EU/ml, 0.12 EU/ml, 0.06 EU/ml, 0.03 EU/ml). Peptides that were to be measured for the presence of endotoxin were diluted to 100µg/ml, 30µg/ml, 10µg/ml.

Next, 0.10 mL of positive standard, peptide sample, or water (negative control) was transferred into the appropriate 75 mm reaction tube. 0.10 mL of the reconstituted

lysate was added to each tube. Duplicates were created for peptides, positive standard and the negative control preparations. Immediately following the addition of the lysate to each tube, the contents were mixed thoroughly and placed in a 37°C water bath. After 60 minutes, each tube was removed and inverted. A positive reaction was characterized by the formation of a firm gel that remained intact momentarily when the tube was inverted. A negative reaction was characterized by the absence of a solid clot. Some lysate showed an increased turbidity or viscosity. This was considered a negative result.

2.7 Biotinylation of *Pseudomonas aeruginosa* 10 LPS

P. *aeruginosa* 10 LPS was purchased commercially (Sigma, St. Louis, USA). Two standard buffers were made, an oxidation buffer (0.1M of acetic acid, 5.5 pH) and a coupling buffer (1x PBS, 7.2 pH). Powdered LPS (2 mg) was mixed with 2 mL of oxidation buffer and 4.3mg of $NaIO_4$ were measured into an amber vial and placed on ice for 30 minutes. After 30 minutes of on ice, 1 mL $NaIO_4$ and 1 mL of LPS solution were mixed in the amber vial and placed on ice for 30 minutes. To remove excess $NaIO_4$, a Zeba spin desalting column (5 mL) (Thermoscientific, Rockford, USA) was used. The columns were equilibrated with 2.5 mLs of coupling buffer. The buffer was added, centrifuged at 1000xg for two (2) minutes and this was repeated three times. 2 mL of the LPS solution were added to the spin columns and centrifuged at 1000xg for two (2) minutes. Then, 200µl of prepared 50mM hydrazide-biotin (Thermoscientific, Rockford, USA) in Dimethyl sulfoxide (DMSO) was added to 1800µl of the resulting oxidized LPS.

The resulting mixture was left for two (2) hours at room temperature. Biotinylated LPS was separated from non-reacted material by a desalting column that was equilibrated with coupling buffer as previously described. Biotinylated LPS was stored at 4^{o}C.

2.8 Trypsin Digest/ Boiling Peptides

To digest peptides, a trypsin homologue, TryPE (Invitrogen, New York, USA) was used. TryPE cleaves protein at the same sites as trypsin. The cleavage sites on RGW07D are shown: (K^KGEAAIK^LTSSAGVLS^K). 500µl of 1mg/ml of peptide that were diluted with 1000µl of the trypsin homologue. The digest reaction was incubated over night at 37^{o}C. The peptide digests were placed in a beaker of boiling water and were boiled for 15 minutes. The peptide digests were diluted to 200µg/ml in endotoxin-free PBS. Digestion was confirmed by mass spectrometry.

2.9 Mass Spectrometry

Peptides were sent to the Bioanalytical and Mass Spectrometry Lab at McMaster University for analyses. The following procedure was written and carried out by the technician, Inga Kireeva.

Extracts were added to 0.1% formic acid for peptide identification by micro-flow liquid chromatography electrospray tandem mass spectrometry (microLC-ESI-MS/MS) using a ThermoFisher LTQ-XL- Orbitrap Hybrid Mass Spectrometer (ThermoFisher, Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin,

CA). Chromatography was performed using 0.1 % formic acid in both the A solvent (98%water/2% acetonitrile) and B solvent (80% acetonitrile/10% isopropanol/10% water), and a 5%B to 95%B gradient over 30 minutes at 5 µl/min through an Eksigent capillary (CSP-3 C18 -100, 0.3m x 100mm) column.

The instrument method consisted of one MS full scan (200–2000 *m*/*z*) in the Orbitrap mass analyzer, an automatic gain control target of 500,000 with a maximum ion injection of 500 ms, one microscan, and a resolution of 60,000. Three data-dependent MS/MS scans were performed in the linear ion trap using the three most intense ions at 35% normalized collision energy. The MS and MS/MS scans were obtained in parallel fashion. In MS/MS mode automatic gain control targets were 10,000 with a maximum ion injection time of 100 ms. A minimum ion intensity of 1000 was required to trigger an MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 s and exclusion duration of 45 s.

The lock-mass option was enabled for the FT full scans using the ambient air polydimethylcyclosiloxane (PCM) ion of m/z = 445.120024 or a common phthalate ion m/z = 391.284286 for real time internal calibration

2.10 Data Processing, Database Searching for Mass spectrometry

All MS/MS samples were analyzed using FT Programs Software: Protein Calculator (Thermo Fisher Scientific).

2.11 Flow Cytometry

Flow Cytometry experiment was designed and conducted by post doctoral fellow Dr. Tom Mu of the Larché Labratory at McMaster University.

Cell Culture for flow cytometry

For analysis of extracellular IL-10 assay, PBMCs were cultured for 4 hours under the following culture conditions: unstimulated, stimulated with 10 μg/ml peptide (Shanghai, China) and stimulated with LPS (5 μg/ml; Sigma-Aldrich, USA). Cells were mixed, by pipetted up and down carefully, and collected. Wells were rinsed with cold PBS buffer pH 7.2 supplemented with 0.5% bovine serum albumin and 2mM Ethylenediaminetetraacetic acid (EDTA).

For IL-10 intracellular staining assay, PBMCs were incubated for 20 hours with 2μM monensin (BD Pharmingen, Mississauga, Ontario, Canada) under the following culture conditions: unstimulated, stimulated with 10 μg/ml peptide (GL Biochem, Shanghai, China) and stimulated with LPS (5 μg/ml; Sigma-Aldrich, USA). Cells were pipetted up and down carefully and collected. Wells were rinsed with 0.5% BSA/phosphate-buffered saline.

Extracellular IL-10production assay by flow cytometry

IL-10 producing cells were assayed in accordance with the IL-10 secretion assay kit

instructions (Miltenyi Biotec, USA). $5x10^6$ cells per sample were washed with cold PBS buffer, pH 7.2, supplemented with 0.5% BSA and 2mM EDTA. Cells were centrifuged at 300xg for 10 minutes at 5 ^oC and the supernatant was isolated. Cells were suspended in 80µl cold medium (RPMI-1640 supplemented with 100 units/mL penicillin-streptomycin, 0.292 mg/mL L-glutamine and 10% human AB serum as instructed by the manufacturer), and 20µl anti-IL-10 antibodies were added, mixed and incubated for five (5) minutes on ice. 5mLs of warm medium (RPMI-1640 supplemented with 1x Penicillin-streptomycin, L-glutamine and 10% human AB serum) was added to cells and incubated for 45 minutes at 37 ^0C the cell tubes were turned every 5 minutes to resuspend the settled cells.

Cells were washed with cold PBS buffer, pH 7.2, supplemented with 0.5% BSA and 2mM EDTA. Cells were stained in the dark, on ice, for 30 minutes with IL-10 detection antibody (PE) and selected cell surface markers: CD14 fluorescein isothiocyanate (FITC), CD19 allophycocyanin (APC), CD3 APC eFluor 780, CD4 PerCP Cy5.5 (eBioscience, USA), 7-aminoactinomycin D (7AAD) (eBioscience, USA). Cell markers were selected based on cells contained within PBMC and suspected producers of IL-10. Stained cells were analyzed with a LSR II flow cytometer using the Diva software package (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA).500,000 events were collected per sample. IL-10 FMO was used to determine IL-10 gating strategy. FACS data were analyzed with FlowJo Software (Tree Star, Ashland, OR, USA).

*IL-10 intracellular staining assay by flow cytometry***.**

Cells were washed with 0.5% bovine serum albumin/phosphate-buffered saline, and

then stained with the surface antibodies. Cells were then washed, permeabilized (BD Cytofix/Cytoperm solution), and intracellular staining according to the manufacturer's instructions of BD Cytofix/Cytoperm fixation/permeabilization kit (BDbiosciences, Canada). The following fluorescent antibodies were used: CD14 APC, CD19 FITC, CD3 Alexa Fluor 700, and IL-10 PE (eBioscience, USA).

2.12 Surface Plasmon Resonance (SPR) Protein Interaction Array System

SPR protein interaction array system is a biosensor that has the ability to quantify binding capacities between macromolecules. A light source is directed towards a sensor chip and an optical detection unit is used to detect refractive index change by interacting molecules; when macromolecules interact, there is a change in refractive index at the surface layer of the sensor chip. The refractive changes are measured constantly so that the binding events can be captured in real time. When there is a higher affinity between macromolecules, this leads to a greater refraction, indicating higher affinity of binding. This instrument was used as an alternative to quantify binding capacity between peptides and LPS.

The ProteOn XPR36 Protein Interaction Array System (Biorad, Hercules, California, USA) was used to measure the binding capacity between LPS and allergen peptides. A ProteOn NLC Avidin Sensor Chip was used for immobilization of biotinylated E.*coli* LPS O111:B4. The immobilization step was performed in the horizontal orientation for all six channels on the chip. The ProteOn XPR36 system used a

running buffer of 1xPBS 0.05% Tween (PBST). The chip was air initialized as per instructions from the manufacturer. A regenerator step was preformed alternating 1M NaCl and 50 mM NaOH at a flow rate of 30µl/min for 60 seconds. This was repeated 4 times. Biotinylated LPS was diluted in running buffer (PBST) to 0.005 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.03 mg/ml, or 0.04 mg/ml. The LPS solutions were either sonicated in a water bath (Branson Ultrasonic Cleaner, Danbury, Connecticut, USA) for 10 minutes, sonicated with a sonication probe (Misonix, Microscon XL 2000, Farmingdale, New York, USA) for 3 minutes at a frequency of 22.5 kHz or not sonicated. The flow rate for the immobilization step of biotinylated LPS was set to 25 µl/min for 180 seconds. After the immobilization of LPS, two blank washes of PBST were flown over the chip at a rate of 50 µl/min for 60 seconds. A regenerator wash was preformed using a glycine buffer at pH 3 to dissociate any residual LPS molecules. The flow rate of this step was set at 100ul/min for 180 seconds. This step was repeated twice.

The immobilization step of LPS was read in response units (RU). A higher RU indicated a higher capacity of binding to avidin.

2.13 Statistical analysis

For PBMC peptide stimulation assay and LPS peptide binding assay, data were tested for significance using a two way ANOVA, followed by a Duncans post-hoc test. Significance was tested of dose response within peptides by two way ANOVA with a

Students test of the Pearson Correlation. Data were expressed as mean ± SEM for bar graphs. Statistical significance was marked with an '*' when $P \leq 0.05$. Statistical significance within groups was indicated with a '#" when $P \leq 0.05$

Peptide names and respective sequences

Peptide Name	**Sequence**
HDM03D	RNQSLDLAEQELVDSASQH
HDM 202	DEFKNRFLMSAEAFE
TIM07 B1	KIPAGELQIIDKIDA
RGW07	GEAAIKLTSSAGVLS
RGW07A	GEAVLRLTSSAGVLS
RGW07B	GESALSLTSSAGVLS
RGW07 D	KKGEAAIKLTSSAGVLSK
RGW07DS	KK-Scramble-K
RGW07DR	GEAAIRLTSSAGVLS
RGW07D 15.2	GEAAISLTSSAGVLS
RGW07D 15.3	GEAAIALTSSAGVLS

Table 2

3.0 RATIONALE

Amb a 1 is the major ragweed allergen. The structure and immunological nature of the allergen has been studied extensively, but the underlying cause for its allergenicity still needs to be elucidated. A possible explanation for its allergenicity might be due to the fact that the allergen has an intrinsic ability to bind to LPS. LPS could act as an adjuvant, stimulating the innate immune system to cause an aberrant immune response against the protein.

A peptide was identified from Amb a 1, Amb a 1 $^{376\text{-}390}$, as a potential therapeutic for ragweed allergy, however, further investigation lead to a discovery that LPS could be present in the peptide solution.

4.0 HYPOTHESIS

To explain the enhanced allergenicity of Amb a 1, it was hypothesized that:

Amb a 1 $^{376\text{-}390}$ binds specifically to LPS.

An implication if this hypothesis (not tested in this thesis) is that the whole Amb a 1 protein is able to bind LPS via the 376-390 sequence. This intrinsic ability to bind to LPS could be an underlying cause of the enhanced allergenicity of Amb a 1.

5.0 RESULTS

Aim 1

To investigate the relationship between PBMC IL-10 responses and the presence of lipopolysaccharide in allergen-derived peptides.

Rationale

Activation of inflammatory process can occur through LPS stimulation of the TLR-4 pathway, primarily in monocytes/macrophages. LPS induces the inflammatory cytokine TNF-α. IL-10 is produced subsequently to down regulate TNF-α expression. During screening of peptide sequences as candidates for an allergy vaccine, two sequences (RNQSLDLAEQELVDSASQH house dust mite *Dermatophagoides pteronyssinus* allergen Der p $1^{149-167}$; referred to in this document as HDM03D, GEAAIKLTSSAGVLS ragweed *Ambrosia artemisiifolia* allergen Amb a $1^{376-390}$: referred to in this document as RGW07) elicited high levels of IL-10 in all subjects tested. Since the sequences were predicted T cell epitopes, IL-10 production was believed to be due to either (1) the function of T cell epitope or (2) the presence of LPS.

Der p 1$^{149\text{-}167}$ *(HDM03) induces IL-10 secretion from human PBMC*

PBMC were cultured for 3 days with titrated doses of HDM03D. The peptide was diluted to 100 μg/mL, 30 μg/mL or 1 μg/mL to determine the optimal dose for maximal IL-10 production. Human PBMCs were stimulated *in vitro* with the peptide and an ELISA assay was used to measure the presence of IL-10. HDM extract is known to induce IL-10 production in PBMCs and, therefore, was used as a positive control. In **Figure 1**, HDM03D elicited IL-10 production from PBMC in a dose-dependent manner. IL-10 production was also observed with HDM extract stimulation.

Modified Amb a 1 $^{376\text{-}390}$ *(RGW07D) induces IL-10 secretion from PBMC*

Analysis of the solubility of RGW07 (Amb a 1$^{376\text{-}390}$) by others (Circassia Ltd; unpublished data) revealed that the peptide displayed poor solubility at the high concentrations (>2.5mg/mL) required for formulation of a multi-peptide vaccine. In an attempt to improve solubility characteristics of the peptide, lysine residues were added to the carboxy- and amino- termini, creating the RGW07 derivative RGW07D (KKGEAAIKLTSSAGVLSK). The modified peptide displayed improved solubility in aqueous solution (>10mg/mL) and was therefore employed in subsequent experiments. RGW07D was diluted to 100 μg/mL, 30 μg/mL, 10 μg/mL, 3 μg/mL, 1 μg/mL and 0.3 μg/mL to determine a dose response. The positive control was HDM03D which had previously been shown to induce IL-10 (**Figure 1**), and negative controls were TIM07B1 (Timothy grass *Phleum pratense* allergen Phl p 5$^{145\text{-}159}$; KIPAGELQIIDKIDA) and

HDM202 (House dust mite allergen Der p 1^{73-87}; DEFKNRFLMSAEAFE). TIM07B1 and HDM202 were selected because they did not elicit IL-10 production in earlier peptide vaccine screening assays (unpublished data). **Figure 2** demonstrates that RGW07D and HDM03D elicited significant secretion of IL-10 from PBMC in a dose dependent manner. The negative controls TIM07B1 and HDM202 did not induce IL-10 production.

HDM03D and RGW07D were originally selected for screening as potential components for peptide vaccines targeting allergen-specific T cells, based on *in silico* T cell epitope prediction algorithms. The majority of T cell epitopes would be expected to elicit a cytokine response in some, but not all, individuals due to the requirement of the appropriate peptide-binding MHC alleles. In contrast, both peptides elicited IL-10 responses in all subjects tested (n>50). As a potential explanation for IL-10 production in all subjects, it was hypothesized that the response was due to small amounts of LPS in the peptide solution, since LPS can induce IL-10 production in monocytes [40]. Since the peptides were >95% pure after synthesis, as judged by mass spectrometry and HPLC, it was possible that the <5% impurities might include LPS. Therefore, whether LPS was present in the peptide solutions was determined using a *Limulus* amebocyte lysate (LAL) assay.

Limulus amebocyte lysate assay detects the presence of LPS in HDM03D but not RGW07D

To identify the presence of LPS in peptide solutions a LAL gel clot assay was used. Since LPS can vary in molecular weight, due to its ability to aggregate, LPS is measured in endotoxin units (EU). The sensitivity of the gel clot LAL assay is 0.03 EU/ml. Peptides were diluted to 100 μg/mL, 30 μg/mL or 10 μg/mL and mixed with 100 μl of *Limulus* (horseshoe crab) amebocyte lysate to determine the presence of LPS. As shown in **Table 1**, LPS was detected above the 0.03 EU/ml detection limit in HDM03D at the highest concentration of peptide tested (100 μg/mL). A borderline positive result (the LAL gel clot assay is somewhat subjective at the limit of detection of the assay as partial clot formation occurs) was observed at 30 μg/mL. Negative controls (no IL-10 induction in the PBMC assay) HDM202 and TIM07B1, had no detectable LPS within the sensitivity limits of the assay (**Table 1**). Despite strong induction of IL-10 in PBMC cultures, no LPS was detected in RGW07D samples at any of the concentrations measured using this method. However, the results of the LAL gel clot assay were interpreted with caution since some LPS-binding peptides can mask detection of LPS in this assay by preventing LPS-induced amebocyte clotting (e.g. polymixin B) [41].

Highly purified peptides do not elicit IL-10 production in PBMC IL-10 assays.

Since a low level LPS presence in synthetic peptides may occur during manufacturing, the influence of peptide purity was evaluated as a possible explanation for

the results of both the LAL assay and the PBMC IL-10 assay. Different batches of the same peptides were purified in two different facilities (GL Biochem, Shanghai). Batches were synthesized in a standard facility, and in a Good Manufacturing Practice (GMP) facility. The more stringent requirements for purity of reagents and processes that are required for GMP certification were expected to result in peptides with lower levels of contamination, particularly with LPS.

Peptides that were purified in GMP and non-GMP facilities were tested for their ability to elicit IL-10 from PBMCs. Human PBMCs were stimulated *in vitro* with the peptides and an ELISA assay was used to measure the presence of IL-10. HDM extract was used as a positive control for IL-10 production and TIM07B1 and HDM202 GMP peptides were used as negative controls. No IL-10 production was observed following culture of PBMC with GMP peptides HDM03 GMP, RGW07D GMP and HDM202 GMP. In contrast, HDM03D and RGW07D synthesized and purified in the non-GMP facility strongly induced production of IL-10 (**Figure 3**). These results, together with those in **Table 1,** demonstrated that peptides from the non-GMP facility likely contain LPS.

Induction of IL-10 was observed in several independently synthesized batches of HDM03D and RGW07D, whereas similar patterns of global IL-10 production were not observed in over 200 other peptides produced in the same facility (unpublished observations, Larché, M). The recurring induction of IL-10 with different batches of peptide, the global nature of IL-10 induction (atypical of T cell responses) and a positive

LAL assay for HDM03D, led to the conclusion that at least HDM03D and possibly RGW07D (perhaps masking LAL detection of LPS, or the presence of another endotoxin), had an intrinsic affinity for LPS (or perhaps another endotoxin in the case of RGW07D). Since, within PBMC, monocytes are the primary source of IL-10 in response to LPS, it was hypothesized that IL-10 was secreted by monocytes in response to low levels of LPS (or another endotoxin) specifically bound by HDM03D and RGW07D.

Monocytes are the main producers of IL-10 in the PBMC IL-10 assay

To explore the hypothesis that monocytes were responsible for IL-10 production, flow cytometry was used to identify the phenotype of cells secreting IL-10 following culture with RGW07D. RGW07D was chosen because it elicited the greatest amount of IL-10 in PBMC cultures. PBMC were stimulated for 4 hours with 10 μg/mL of the peptide. The 4 hour time point was selected as this is the earliest time at which IL-10 is detected from blood monocytes when exposed to LPS. Cells were stained with IL-10 detection antibody (phycoerythrin; PE), select cell surface markers: CD14, CD19, CD3, CD4, and the viability dye 7-aminoactinomycin D (7AAD). **Figure 4** depicts the gating strategy for the stimulated PBMCs. The live cells were selected based on the absence of 7AAD staining (binds to DNA in dead/dying cells) and the front scatter laser FSC(A) which is used to determine the size of the cells (**Figure 4 A**). Doublets and cell aggregates were excluded (**Figure 4 B**). Then, FSC and the side scatter laser (SSC), which is used to judge internal complexity, were used to gate on lymphocytes and monocytes, excluding any cellular debris (**Figure 4 C**). Cells were identified based on phenotypic markers as described in the **Materials and Methods** (**Figure 4 D, E, F**).

Cells that were stained for phenotypic markers were gated for IL-10 production. The gating strategy for IL-10 was based on fluorescent minus one (FMO) IL-10 antibodies (See **Appendix I**: **Figure 1, Figure 2**).

In **Figure 5,** cellular subpopulations stimulated with media only, TIM07B1, HDM03D, RGW07D, and RGW07D GMP were analyzed for the presence of IL-10. Flow cytometry revealed that RGW07D and HDM03D elicited IL-10 from monocytes but not other cell types in PBMC. The gated areas indicate the subpopulation of cells that were analyzed for IL-10 secretion. In the cell populations of CD4 T cells, B cells, CD8 and NK T cells (which are identified by the marker of CD3), dendritic cells and NK cells displayed IL-10 producing cell percentages similar to media and the negative control TIM07B1. However, 3% of the population with the CD14 marker, which indicates monocytes, produced IL-10 when stimulated with HDM03D compared to 0.202% and 0.204% of cells producing IL-10 in response to peptide TIM07B1 and media only, respectively. Interestingly, RGW07D stimulation elicited IL-10 production in 2.79% of monocytes compared to RGW07D GMP (0.322%). These findings correlated with previous experiments (**Figure 3**) in that RGW07D, but not RGW07D GMP, elicited IL-10 production from PBMC. Since monocytes are the major cell type that responds to LPS, it appeared that LPS, or another endotoxin, was present in peptide samples that elicited IL-10 production in PBMC cultures. However, since the sequences were originally selected as potential T cell epitopes, the possibility could not be excluded that some of the IL-10 elicited by the RGW07D (and HDM03D) peptide, may have been due to the peptide functioning as a T cell epitope.

Destruction of RGW07D with a combination of heat and proteolysis

Since LPS is heat and protease resistant [42] but peptides are not, the RGW07D peptide was boiled and proteolytically digested to determine if IL-10 production was due to the presence of LPS, or the action of the peptide functioning as a T cell epitope. RGW07D was chosen as it elicited the greatest production of IL-10 (**Figure 2**) and also because the LAL assay could not rule out the possibility that the presence of LPS was being masked by peptide binding. Reduction in IL-10 production after digestion and boiling would indicate that the intact peptide sequence was responsible for IL-10 production (i.e. the sequence was acting as an IL-10-inducing T cell epitope). In contrast, maintenance of IL-10 production after digestion and boiling would be indicative of the presence of LPS or another heat- and proteolysis-resistant endotoxin. To digest RGW07D, a trypsin homologue, TryPE was used, which cleaves the same peptide bonds as trypsin (K^KGEAAIK^LTSSAGVLS^K). To verify that RGW07D was successfully digested, the samples were analyzed by mass spectrometry. In **Figure 6** (A) displays an intact peptide before digestion. The *z* number indicates ionization levels of the peptide. The z number is multiplied with the relative abundance value and will give the molecular weight of the peptide. Therefore, all three z values, when multiplied by the relative abundance displayed the peptide was intact by its predicted molecular weight (1788.11 Da). In **Figure 6** (B) and (C), the mass spectrometry trace illustrates that the RGW07D fragments of the longer and shorter peptide, respectively. The longer peptide segment in (B), LTSSAGVLSK, displays high relative abundance at 961.56 Da. This is close it its theoretical value of 962.11 Da. In segment (C) shows the shorter peptide GEAAIK, with

a high relative abundance at 588.34 Da close to the theoretical value 587.67 Da. Thus, it was concluded that the digest of RGW07D was successful.

Digested RGW07D elicited IL-10 in PBMC cultures

To evaluate whether LPS, peptide or both were responsible for IL-10 production in the PBMC assays, native RGW07D, the RGW07D trypsin-digested and boiled + trypsin-digested peptides were incubated with human PBMCs *in vitro*. The presence of IL-10 was measured by ELISA. As shown in **Figure 7**, RGW07D elicited IL-10 as in previous stimulation experiments (**Figure 2**). RGW07D trypsin-digested and RGW07D trypsin-digested + boiled elicited substantially lower IL-10 production (approximately 4-fold reduction). However, neither trypsin digestion alone, nor the combination of trypsin digestion and boiling, resulted in reduction of IL-10 production to background levels. Thus, RGW07D appeared to elicit IL-10 by virtue both of its peptide sequence (i.e. T cell epitope function) and the presence of a heat- and proteolysis-resistant moiety such as LPS. To ensure the digests were eliciting IL-10 production from monocytes within the PBMC preparation, a flow cytometry experiment was conducted to determine which cells were producing IL-10.

Monocytes are the source of IL-10 production in digested RGW07D

To determine which cells were responding to the RGW07D digests, a flow cytometry experiment was performed in which PBMC were incubated for 20 hours with

2μM monensin and stimulated with 10 μg/ml of native RGW07D peptide, RGW07D trypsin-digested, RGW07D trypsin-digested + boiled, or LPS (5 μg/ml). Cells were stained with the following antibody/fluorophore combinations to surface markers: CD14 APC, CD19 FITC, CD3 Alexa Fluor 700, and IL-10 PE. The gating strategy was identical to that in **Figure 4**. LPS was used as a positive control for IL-10 production from monocytes. The majority of the IL-10 detected was produced by CD14+ monocytes (**Figure 8**). In response to culture with LPS, 1.43% of monocytes stained positive for intracellular IL-10. Native RGW07D elicited IL-10 production from 0.967% of the monocyte population, a finding consistent with previous results in **Figure 5**. Trypsin-digested RGW07D and RGW07D that had been trypsin-digested and boiled elicited IL-10 from monocytes (0.77% and 0.692% respectively), but not T cells or B cells. These data complement those shown in **Figure 7**, supporting the notion that the presence LPS (or another heat and proteolysis-resistant endotoxin) stimulates IL-10 in PBMC, more specifically monocytes.

Aim 2

To determine whether peptides RGW07D and HDM03D have an intrinsic capacity to bind LPS.

Rationale

Multiple independently synthesized batches of RGW07D and HDM03D induced IL-10 production in PBMC from all individuals. In contrast, TIM07B1 and over 200 other peptides made at the same facility induced much lower and less frequent IL-10 responses. The presence of low levels of LPS was detected in high concentrations of HDM03D, but not in RGW07D with the caveat that some peptides with a high affinity for LPS can mask its detection in the LAL assay. Some IL-10 inducing activity was retained in RGW07D following proteolytic and heat-mediated destruction of the primary peptide sequence, indicating a heat and proteolysis-resistant moiety within the peptide preparation. If the presence of LPS (or other endotoxin) in peptides were due to random contamination during the synthesis process, all peptides from a single batch should have equivalent levels of LPS. Moreover, no single peptide would be expected to reproducibly induce IL-10 production from batch to batch. Thus it was hypothesized that certain allergen sequences, such as RGW07D and HDM03D, may have an intrinsic, sequence-dependent affinity for LPS that results in the concentration of rare LPS molecules encountered in the synthesis process. Such an ability would result in the allergen being encountered by the immune system in the context of LPS exposure. Indeed, if an allergen were able to bind LPS specifically, it is likely that gram-negative bacteria present within

the allergen source (e.g. ragweed pollen or house dust mite faeces) would be coated in the allergen molecule, perhaps representing an extremely immunogenic complex capable of eliciting strong Th2 responses. In order to determine whether RGW07D and HDM03D sequences have an intrinsic affinity for LPS, an LPS binding assay was developed. For reasons of time and because RGW07D elicited the strongest IL-10 responses in PBMC cultures, the majority of the data generated relate to RGW07D, rather than HDM03D.

Establishment of an LPS binding assay

Before the allergen peptides could be tested for their capacity to bind to LPS, an LPS binding assay needed to be established and validated with a peptide that was known to bind to LPS. Polymixin B, a peptide with anti-microbial activity, is known to bind to LPS with high affinity and was therefore used to establish a 96 well plate-based assay to detect LPS binding activity in peptides. Although Polymixin B is known to bind to LPS, it was not known whether it could bind to biotinylated LPS and, thus, in the first step in the development of the assay was to titrate polymixin B against biotinylated LPS, as described in detail in the **Materials and Methods**. **Figure 9** displays a series of dilution curves to identify the optimal concentration of LPS-biotin to be used in the LPS peptide binding assay. As the concentration of polymixin B decreased at a constant LPS concentration, the OD_{450nm} declined. This dilution response demonstrated that the assay was functional. Similarly, as the concentration of LPS decreased, the OD decreased. LPS-biotin at a concentration 1.56μg/mL was selected as the operational concentration as it was in the middle of the concentration range as it would be sensitive to changes in the

concentration of the immobilized peptides. At lower biotin-LPS concentrations, peptide binding would not be detected. At higher biotin-LPS concentrations, high non-specific background signal could make it difficult to demonstrate dose-dependent peptide binding. To demonstrate that this interaction was reproducible, the experiment was repeated three times with polymixin B concentrations of 30μg/mL, 3μg/mL, 1μg/mL, 0.3μg/mL, 0.1μg/mL and biotin-LPS concentration of 1.56μg/mL (**Figure 10**).

RGW07D peptide binds to E. coli LPS

To evaluate binding to LPS, the following peptides were selected from previous experiments: HDM03D GMP, HDM03D, RGW07D, RGW07D GMP, HDM202 GMP, HDM202, TIM07B1. Peptides were diluted to 30μg/mL, 10μg/mL, 3μg/mL and 1μg/mL and immobilized onto a 96-well plate. The immobilized peptides were incubated with 1.56μg/mL LPS- biotin. RGW07D bound to biotinylated *E. coli* LPS. As shown in **Figure 11,** HDM03D and TIM07 B1 displayed no detectable binding at any concentration. HDM03D GMP displayed low but significant levels of biotin-LPS-binding, which was also observed with HDM202 and HDM202 GMP. RGW07D GMP bound strongly to biotin-LPS, whereas weaker (but dose-dependent) binding was observed with the non-GMP RGW07D. GMP purified peptides bound to LPS with a greater capacity compared to non-GMP purified peptides (RGW07D and HDM03D). This could be due to a greater number of binding sites available for biotin-LPS binding.

In conclusion, these data suggested that peptide RGW07D had an intrinsic capacity to bind to bacterial LPS.

RGW07 (Amb a 1 $^{376-390}$) binds to E.coli LPS

The solubility of the native RGW07 sequence had been enhanced by the addition of lysine "wrappers" to carboxy and amino termini. Binding of polymixin B to LPS is to some extent dependent on positively charged side chains. Since the addition of three lysine residues conferred additional positive charges to RGW07D, the possibility that these charges were responsible for LPS binding activity was investigated. RGW07 was immobilized to 96-well plate to ensure that the sequence, not the positively charged lysines, was facilitating the binding. RGW07DS was created as a negative control. RGW07DS was a "scramble" sequence of RGW07 that was selected randomly with the lysines maintained at each end. This was to account for non-specific binding of the positive lysines to the negatively charged LPS. As demonstrated in **Figure 12**, the scramble peptide RGW07DS did not bind specifically to LPS, whereas both RGW07D and RGW07 did so in a dose-dependent fashion. Thus, the capacity of the peptide to bind to LPS is not based on additional charge provided by the addition of lysine residues.

RGW07 sequence binds to P. aeruginosa *LPS*

Studies were conducted to determine whether the peptide sequences derived from RGW07 were selective in their capacity to bind to diverse LPS structures. *P. aeruginosa* LPS is structurally different than E.*coli* LPS, specifically in the lipid A region [29] (**Figure 17**). However, no commercial biotinylated LPS of *P. aeruginosa* was available for purchase. Therefore, biotinylation of *P. aeruginosa* LPS was performed (See **Materials and Methods Section 2.7**). To determine the success of biotinylation of *P. aeruginosa* LPS, a polymixin B/LPS dose response curve was generated as for *E.coli* LPS. Since the efficiency of the oxidation reaction was unknown, it was assumed that the coupling of biotin to *P. aeruginosa* LPS was 100% efficient and there was no LPS lost in the purification process. Immobilized polymixin B at each concentration was incubated with a concentration of *P. aeruginosa* LPS conjugated to biotin beginning at a concentration of 25μg/mL and diluted two-fold to 0.78μg/mL. **Figure 13** shows that the biotinylation reaction was successful although at low doses of polymixin B the sigmoid nature of the curve was lost in some cases. As the concentration of LPS decreased, the OD decreased. LPS-biotin at a concentration 3.125μg/mL was selected as the operational concentration as it was in the middle of the concentration range and it would serve as an acceptable positive control in the peptide binding assay as it would be sensitive to changes in the concentration of the immobilized peptides. With the operational theoretical concentration selected, the RGW07 peptide and its derivatives were tested to observe their capacity to bind to *P. aeruginosa* LPS. Peptides were immobilized and incubated with 3.125μg/mL of *P. aeruginosa* LPS-biotin. As demonstrated in **Figure 14**

biotinylated *P. aeruginosa* LPS bound both RGW07 and RGW07D, in a similar fashion to *E.coli* LPS., The negative control scramble sequence RGW07DS did not bind to *P. aeruginosa* LPS. These data suggest that the RGW07 sequence can bind to structurally different LPS molecules and that binding is tolerant of significant structural differences in the lipid A region.

RGW07A, an isoform of RGW07, binds to E.coli *and* P. aeruginosa *LPS*

Sequencing of Amb a 1 genes and proteins has identified 4 isoforms of the protein which, although largely homologous, contain polymorphic residues. Inspection of the amino acid sequences of these four isoforms in the RGW07 region revealed some limited heterogeneity (see **Table 2** in **Materials and Methods**). The sequences of RGW07 (1.3 isoform: GEA**AIK**LTSSAGVLS), RGW07A (1.2/1.4 isoform: GEA**VLR**LTSSAGVLS), and RGW07B (1.1 isoform: GEA**ALS**LTSSAGVLS) are conserved except for a sequence of 3 amino acids. It was hypothesized that the positively charged amino acid in RGW07 (K) and RGW07A (R) were crucial for binding to negatively charged LPS. To test this hypothesis, isoform-specific polymorphisms were inserted into the RGW07 sequence. RGW07 with a conservative change (K to R) named RGW07R and non-conservative mutations (K to S, or K to A) named RGW07 15.2 and RGW07 15.3, respectively, were synthesized and evaluated for LPS binding. Peptides were diluted to 30 μg/mL, 10 μg/mL, 3 μg/mL and 1 μg/mL and immobilized to a 96-well plate. Immobilized peptides were incubated with 1.56μg/mL of biotinylated *E. coli* LPS. As demonstrated in **Figure 15**, RGW07 bound to *E. coli* LPS-biotin similar to previous

results (See **Figure 12**). RGW07A bound to LPS more strongly than RGW07, giving an approximately 4-fold increase in optical density reading in the assay. For RGW07B, there was decreased binding capacity, compared to RGW07, although significant binding was observed over background (RGW07DS). As previously shown in **Figure 12**, RGW07D bound to LPS in a dose–dependent fashion. For RGW07 R and RGW07 15.2, low, but significant, binding was observed at the highest peptide concentration evaluated. RGW07 15.3 did not bind to *E. coli* LPS-biotin. The negative control, RGW07DS, did not bind to LPS as seen in previous results (**Figure 12**).

Similar experiments were performed with *P. aeruginosa* LPS. Peptides were diluted to 30µg/mL, 10µg/mL, 3µg/mL and 1µg/mL and immobilized to a 96-well plate. Immobilized peptides were incubated with 3.125µg/mL of *P. aeruginosa* LPS-biotin. Similar to *E.coil* LPS, *P. aeruginosa* LPS has the capacity to bind to the isoform peptides. As shown in **Figure 16**, results were broadly similar to those obtained with *E.coli* LPS. RGW07 bound to *P. aeruginosa* LPS-biotin similarly to previous results (see **Figure 14**). However, these values were slightly lower than those obtained with *E. coli*. This could be due to the approximation of the concentration of *P. aeruginosa* LPS and/or the inefficiencies of the biotin coupling reaction. RGW07A bound to *P. aeruginosa* LPS strongly, similar to *E. coli* LPS. For RGW07B, there was no binding at any of the concentrations. As previously seen with *E. coli* (**Figure 12**), RGW07D bound to *P. aeruginosa* LPS in a dose-dependent fashion. RGW07 R and RGW07 15.2 bound to *P. aeruginosa* LPS weakly. RGW07 15.3 did not display detectable binding to *P. aeruginosa* LPS-biotin. Thus, the presence of a basic, positively charged amino acid in

the polymorphic region of the RGW07 sequences appeared to be critical for LPS binding. Interestingly, having a conservative mutation at the same location (RGW07R) decreased LPS binding. This demonstrated that the sequences are extremely selective and subtle amino acid substitutions can have effects on the peptides ability to bind to LPS. From the data generated it appeared that LPS binding may be controlled by the sequence of three polymorphic amino acids in the RGW07 sequence.

6.0 DISCUSSION

To initiate an adaptive T cell immune response, there are two signals that must be present: an adjuvant (ex. LPS) that stimulates the innate immune system and an antigen that contains a sequence that a naïve CD4 T cell recognizes via its TCR. CD4 T cells then differentiate into Th1 or Th2 cells, among other CD4 T cell subsets. However, if the adjuvant signal is not present when MHC II presents the peptide to the T cell, tolerance develops. The etiology of allergy has become clearer, it has recently become apparent that some allergens have an intrinsic ability to stimulate the innate immune system or have intrinsic adjuvanticity [46, 47]. Therefore, an allergen can provide the antigenic source for T cell recognition and stimulate the innate immune system. Der p 2 can mimic the structurally homologous MD-2 molecule and stimulate the TLR4 pathway to induce a Th2 response [25]. Furthermore, the peanut allergen Ara h 1 can mimic helminth glycans and stimulate dedritic cells via the cell surface pattern receptor DC-SIGN, inducing a Th2 response [27]. In the current thesis project, the serendipitous observation that isolated peptide sequences induced IL-10 production from PBMC of all donors has led to the identification of a sequence within the Amb a 1 allergen (Amb a 1 $^{376\text{-}390}$: RGW07) that has the capacity to bind to LPS. Based on these observations with a peptide fragment of the molecule, it appears likely that the entire allergen protein , Amb a 1, is able to bind to environmental LPS and stimulate the innate immune system to induce an adaptive immune response. Findings from this study could shed light on the mechanism of allergic sensitization to Amb a 1 and improve our understanding of the etiology of allergic disease. Further research may lead to methods that can circumvent the initial sensitization

phase of Amb a 1, or improve current treatment, for example through the use of the non-LPS-binding isoform Amb a 1 [1.1].

Data generated as part of this thesis project have identified potential artifacts associated with the use of PBMC cytokine responses for the screening of synthetic peptides for immunodominant T cell epitopes. Results demonstrated that while peptides may have the ability to induce IL-10 production by virtue of their function as T cell epitopes, the presence of LPS during peptide synthesis may lead to false positives in the screening process. Thus, caution should be exercised when interpreting cytokine responses (particularly those associated with activation of the innate immune system (such as IL-10, TNF-α, IL-6 etc) from PBMC cultures challenged with peptides as LPS or other microbial products may be present.

Peptide immunotherapy is a disease modifying therapy that induces tolerance to allergens via IL-10 production. Various groups [48,49] have screened candidate allergen peptides for immunodominance and subsequently designed peptide vaccines. During a peptide screening study aimed at identifying immunodominant T cell epitopes as candidate vaccine peptides for house dust mite and ragweed vaccines, two peptides (HDM03D and RGW07) were identified that elicited substantial IL-10 responses from PBMC of all individuals tested. Induction of cytokine responses in all subjects is atypical of T cell epitopes and this observation formed the basis for the current study. Since IL-10 can be induced by activation of monocytes through the TLR4 pathway, there was concern that the IL-10 production could be due to the presence of LPS during peptide synthesis. A

LAL assay determined that HDM03D preparations contained small quantities of LPS. However, a variant of the native RGW07 peptide that displayed increased solubility and higher IL-10 production (RGW07D) tested negative for the presence of LPS. The findings from the LAL assay were interpreted with caution as some peptides that bind to LPS with high affinity (i.e. polymixin B) are known to mask the region of LPS that interacts with *Limulus* amebocytes leading to a false negative LAL test [50]. Polymixin B binds specifically to the lipid A component of LPS [51], thereby inhibiting the detection of LPS by a LAL assay. It is possible, therefore, that the RGW07D peptide may behave in a similar fashion.

LPS has been characterized as relatively resistant to heat denaturation whereas proteins and peptides are denatured. Further denaturation/destruction of proteins and peptides can be achieved through proteolysis. A combination of heat and proteolysis was employed to determine whether the IL-10 response elicited in PBMC was dependent on the peptide amino acid sequence acting as a T cell epitope, or whether the cytokine was the response to LPS (or another heat/proteolysis-stable endotoxin-like substance). RGW07D digested with a trypsin analogue and RGW07D subjected to both boiling and trypsin digestion elicited less IL-10 from PBMCs than native RGW07D as quantified by ELISA. A possible explanation is there could be a synergistic effect of the peptide and LPS that enhances the stimulation of PBMCs. Alternatively, part of the IL-10 response may have been derived from the action of the peptide as a T cell epitopes and part of the response may have been derived from LPS. Thus, heating and/or proteolysis resulted in the loss of the T cell epitope activity but not the LPS. Recent studies, however, have

found that boiling can decrease cytokine responses to LPS [43] [52]. However, this does not explain the decrease in IL-10 production in cells cultured with proteolytically cleaved, but not boiled, peptide. A possible explanation of this could be that another bacterial product, lipopeptide, could elicit IL-10 by signaling through the TLR2 pathway on monocytes [53, 54]. These TLR2 agonists can be eliminated by boiling and digestion. Also, these lipopeptide contaminants cannot be detected by the LAL assay [55]. Thus, the decrease of IL-10 production by boiling and digestion could be due to the elimination of lipopeptide. Another possible explanation for this decrease in IL-10 production amongst RGW07D digests is that the peptide itself could be facilitating interaction between the TLR4 receptor complex and LPS. This would be analogous to the innate immune peptide β- defensin 2, which has been proposed, by Medzhitov and colleagues, to bind to trace amounts of LPS and facilitate and enhance interaction between LPS and TLR4 [56], much like LBP.

Flow cytometric analysis of PBMC subpopulations identified by lineage-specific surface markers indicated that RGW07D could elicit IL-10 production from monocytes. Monocytes and B cells are known to respond to LPS stimulation with the production of both inflammatory and anti-inflammatory cytokines including IL-10. However, it should be noted that the flow cytometric assays performed were conducted on a time scale that would preferentially detect IL-10 produced by monocytes (4 hours) but not by T cells (>24 hours). Thus, although the data indicate that monocytes were producing at least some of the IL-10 signal observed from PBMC, they do not exclude the possibility that T

cells were also producing IL-10 in response to the T cell epitope characteristics of the peptide.

In order to determine the ability of peptides to bind LPS an LPS binding assay was developed and validated using polymixin B (an anti-microbial peptide with a known high affinity interaction with LPS). LPS-binding peptides frequently have both cationic and amphipathic characteristics [57, 58] and RGW07D has similar characteristics (See **Materials and Methods: Table 2**). In order to increase the solubility of the native ragweed sequence RGW07, a derivative was made by the addition of positively charged lysine residues at both the amino and carboxy termini (RGW07D). In order to exclude the possibility that binding of LPS to RGW07D was an artifact created by the lysine residues, a scrambled sequence peptide was created with the same lysine terminal residues (RGW07DS). RGW07DS displayed little to no detectable binding in the biotinylated LPS binding assay, whereas RGW07 showed significant binding capacity. Similar results were obtained with RGW peptides binding to P. *aeruginosa* LPS. Thus, it appeared that the amino acid sequence itself was responsible for binding both species of LPS. Furthermore, these data demonstrated that the sequence can accommodate structurally different lipid A regions when binding to LPS (**Figure 18**). This may imply that the interaction between the peptide and the LPS molecule is not dependent on the lipid A moiety of LPS.

LPS binding sites, such as those in LBP [59] and MD-2 [60] , have been identified in proteins through peptide binding studies. Formal demonstration of the binding of the whole Amb a 1 protein to LPS was beyond the scope of the current project,

but such a demonstration forms the next logical step towards definitely identifying this allergen as an LPS binding protein capable of delivering not just the antigenic epitopes of the protein to the adaptive immune system, but also the innate immune triggers such as the TLR4 ligand LPS. One prerequisite for binding of LPS by the whole Amb a 1 molecule would be that the putative binding site (i.e. the RGW07 peptide sequence) would be exposed on a solvent-accessible surface. Interestingly, the sequence itself is contained at the C-terminus of the β- chain Amb a 1 molecule and is exposed to environmental solvent (**Figure 18**). Therefore, the sequence is available for LPS binding.

Short ragweed (*Ambrosia artemisiifolia*) exists in four known isoforms. The isoform-specific sequences of the RGW07 region were compared and assayed for their ability to bind to E. *coli* and P. *aeruginosa* LPS. Closer inspection of the sequences revealed a variable region of 3 amino acids amongst the four isoforms which were captured in three peptide sequences, since the 1.2 and 1.4 isoforms are homologous at this region (RGW07, RGW07A and RGW07B; see **Table 2** in **Materials and Methods**). Binding data revealed that RGW07A, containing an arginine instead of lysine, had increased LPS binding capacity compared to RGW07. RGW07B, which contained a non-polar amino acid and had a lower binding signal compared to RGW07 and RGW07A. However, RGW07B binding capacity was still significant compared to the negative control RGW07DS. This led to the conclusion that the positive charged amino acid contained in the RGW07 sequence was a factor for facilitating binding to LPS. RGW07R, RGW07 15.2 and RGW07 15.3 were created to test the theory that the positive amino acid was critical for binding to LPS (See **Table 2** in **Materials and**

Methods). While RGW07R and RGW07 15.2 did significantly bind to both species of LPS with a significant dose response, it appeared that these amino acid substitutions (which were isolated, single residue changes rather than substitution of the entire polymorphic trimer) diminished the sequences ability to bind to LPS. Therefore, it was concluded that the amino acids are extremely specific for binding LPS and even conservative substitutions can affect the binding capacity.

It has previously been demonstrated in LPS binding peptide studies that slight mutations in conservative and non-conservative amino acids, can significantly decrease or increase the binding capacity of a peptide [61]. These findings indicate a possible area of intervention for treatment of ragweed allergy. If the RGW07 sequence of Amb a 1.3 is responsible for sensitization, ecological intervention could be undertaken to skew the plant population toward the non-sensitizing isoform; Amb a 1.1. Prevention of sensitization would be the ideal form of allergy treatment. The environmental impact of such human intervention should be undertaken with care as it could have unforeseen consequences on ecosystem balance.

With many questions still unanswered, future experiments are needed to elucidate the interaction of Amb a 1 with LPS. While it was demonstrated that the Amb a $1^{376\text{-}390}$ sequence had the capacity to bind to biotinylated LPS of both E. *coli* and P. *aeruginosa* LPS, another method of measurement would be needed to confirm the results from LPS binding experiments. Surface Plasmon Resonance (SPR) protein interaction array system

is a biosensor that has the ability to quantify binding capacities between macromolecules. Other groups have used SPR to measure LPS-peptide interactions by immobilizing the peptide to the chip [62, 63]. SPR was attempted in the current study but the results were inconclusive and, due to time constraints, incomplete (**Appendix II**). LPS-biotin aggregation was a significant problem in establishing the peptide-LPS SPR assay. Preparations are currently under way to biotinylate RGW and HDM03D peptides for use in a revised SPR study in which the peptides, rather than the LPS will be immobilized on the sensor chip. These biotinylated peptides will enable measurement of the LPS binding capacity of the peptides. While peptide binding studies can identify possible LPS binding sites in a protein, it is not yet certain that the Amb a 1 $^{376\text{-}390}$ sequence can bind to LPS while contained in the intact protein. Possible steric hindrance could interfere with LPS binding. Therefore, a whole allergen LPS binding assay would be required to demonstrate that the Amb a 1 $^{376\text{-}390}$ sequence is still operational while being contained in the Amb a 1 allergen. To test this, recombinant Amb a 1 can be immobilized while being incubated with biotin labeled LPS, similar to the LPS binding assay described. Recombinant Amb a 1 allergen will shortly be available from Dr. Fatima Ferreira,a collaborator from the University of Salzburg. Dr. Ferreira is also mutating the Amb a 1 allergen. A mutated recombinant Amb a 1 allergen will have the Amb a 1 $^{376\text{-}390}$ sequence cleaved from the allergen. Other recombinant Amb a 1 mutations will include the different isoforms. This is to test the capacity of each isoform to bind to LPS. If it is possible to demonstrate that the sequence of Amb a 1 $^{376\text{-}390}$ can bind to LPS while part of the whole Amb a 1 allergen, a next logical step would be to attempt to sensitize mice and induce ragweed allergy using different Amb a 1 isoforms that do or do not bind LPS. Ideally, this would

demonstrate that a mouse could be sensitized to Amb a 1 as a whole and fail to be sensitized to Amb a 1 with the Amb a 1 $^{376\text{-}390}$ sequence mutated or cleaved. A prediction based on the isoform binding data obtained in the current project would be that Amb a 1.1 would not sensitize mice whereas Amb a 1.3 would, this would demonstrate the effects of the Amb a 1 $^{376\text{-}390}$ sequence to facilitate sensitization *in vivo*.

7.0 LIST OF FIGURES

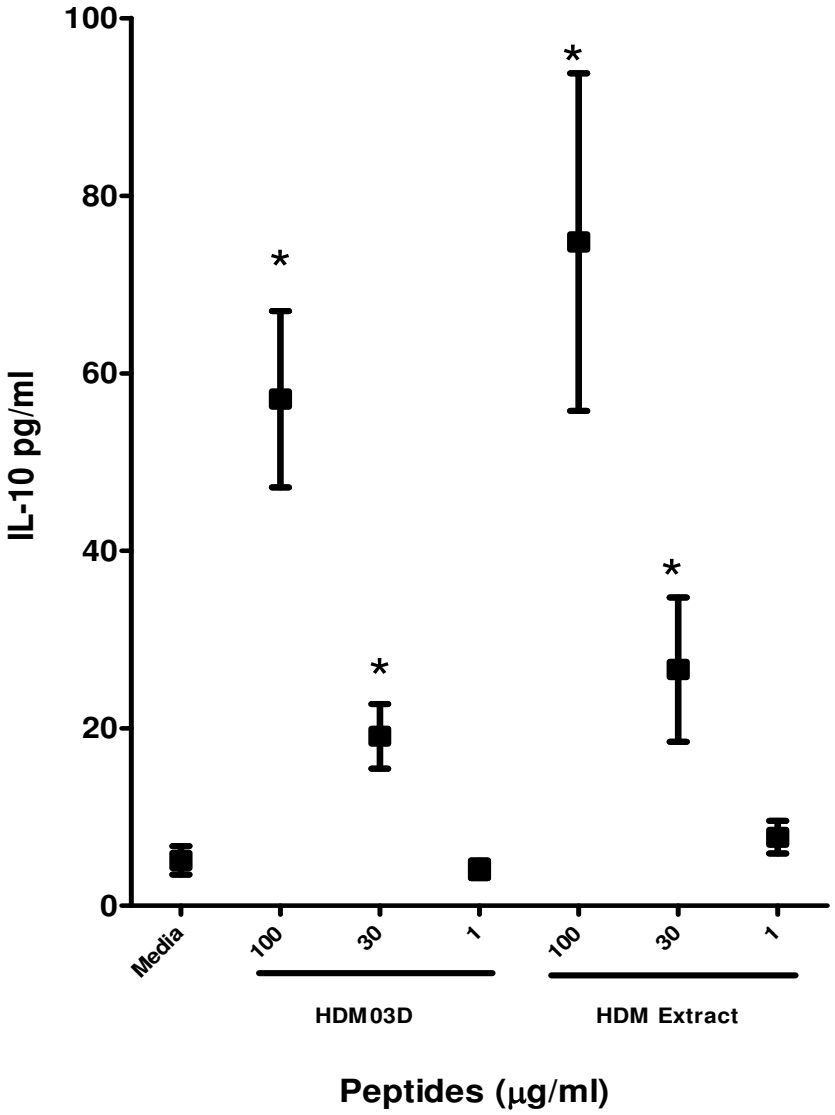

Figure 1. IL-10 production induced by HDM03 variant peptides. Fresh allergic human PBMCs were isolated and diluted to 2.5 million cells per mL in cellular medium and 100μlwere added to peptide solutions at 100μg/ml, 30μg/ml and 1μg/ml. The supernatants were collected after 3 days of incubation and analyzed by ELISA for the presence of IL-10. Data were collected from six different patients (n=6) in duplicate. * indicates $p \leq 0.05$. P values were determined using Duncans post-hoc test. Data is presented as the mean +/- SEM. House dust mite (HDM) extract was used as a positive control.

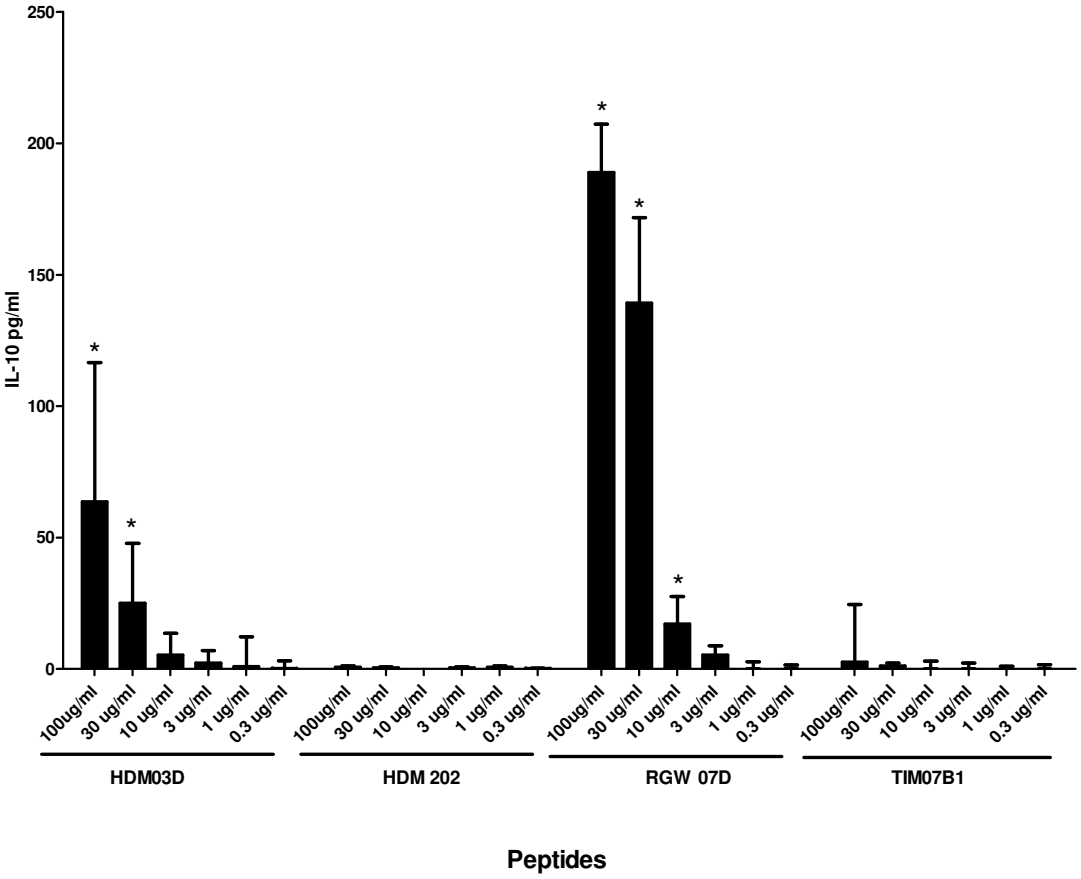

Figure 2. IL-10 production is induced by allergen peptide stimulation. Fresh PBMCs from non-allergic patients were isolated, diluted to a concentration of 2.5 million cells per mL in medium and a 100µlwere added to peptide solutions at 100µg/ml, 30µg/ml, 10µg/ml, 3µg/ml, 1µg/ml, or 0.3µg/ml concentrations. The supernatants were collected after 3 days of stimulation and were analyzed by ELISA for the presence of IL-10. '*' indicates $p \leq 0.05$. P values were determined using Duncans post-hoc test. Data are presented as mean +/- SEM (n=6).

Peptide	Purpose	100µg/ml	30µg/ml	1µg/ml
HDM03D	Positive Control	+	(+)	-
HDM202	Negative Control	-	-	-
TIM07B1	Negative Control	-	-	-
RGW07D	Experiment	-	-	-

Table 1. *Limulus* amebocyte lysate (LAL) assay. Three dilutions of each peptide was prepared; 100µg/ml, 30µg/ml or 100µg/ml and incubated with amebocyte lysates according to the manufacturer's instructions. Coagulation of the lysate occur when LPS is present in the peptide solution. The sensitivity of the assay was 0.03 EU/ml. The "+" indicates positive for endotoxin. A "-" indicates a negative test. The use of a parenthesis indicates a possible positive test since the measurement is qualitative, being subject to visual interpretation. Results are from one experiment and each point in figure are duplicated.

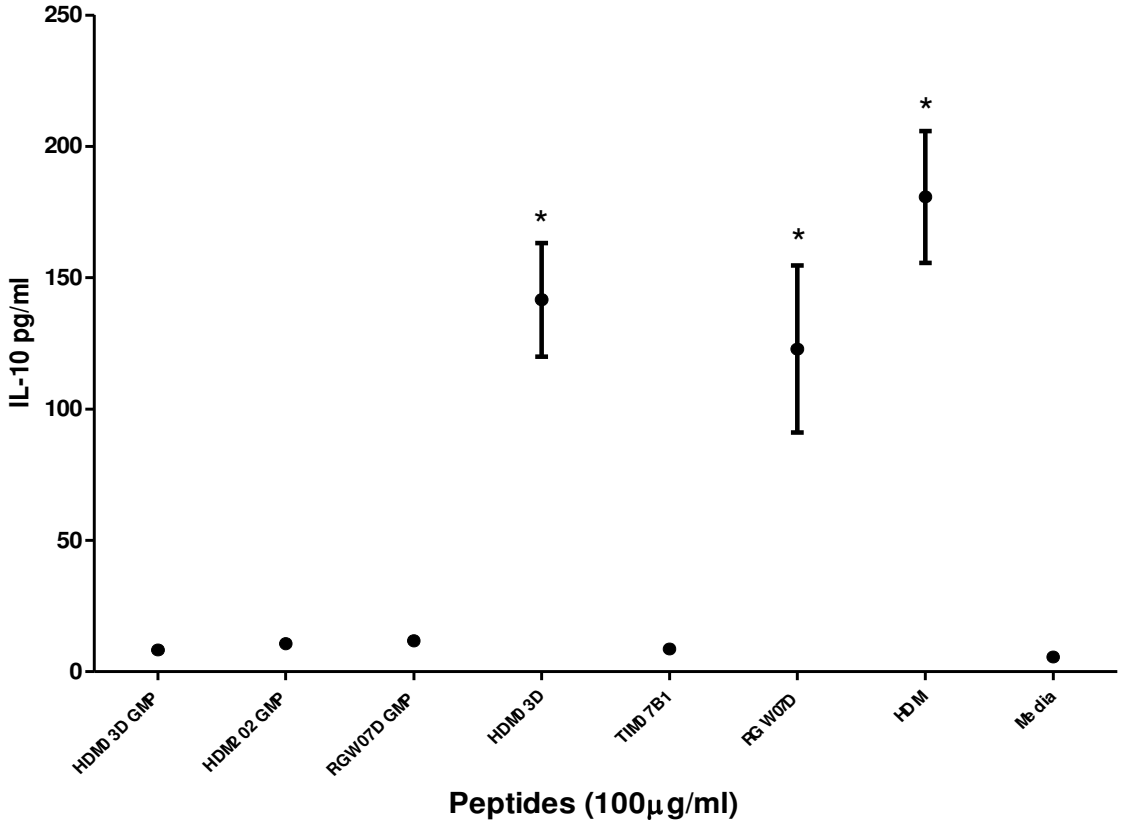

Figure 3. Highly purified peptides (generated in a Good Manufacturing Practice (GMP) compliant facility) did not elicit IL-10 compared to non-GMP purified peptides. Fresh human PBMCs from 5 patients were isolated, diluted to a concentration of 2.5 million cells per mL in cellular medium and 100 µl were added to peptide solutions at 100µg/ml. The cells were incubated for 3 days. Supernatants were then collected and analyzed by ELISA for the presence of IL-10. '*' indicates $p \leq 0.05$. P values were determined using Duncans post-hoc test. Data indicate the mean +/- SEM. Medium and TIM07B1 were used as negative controls.

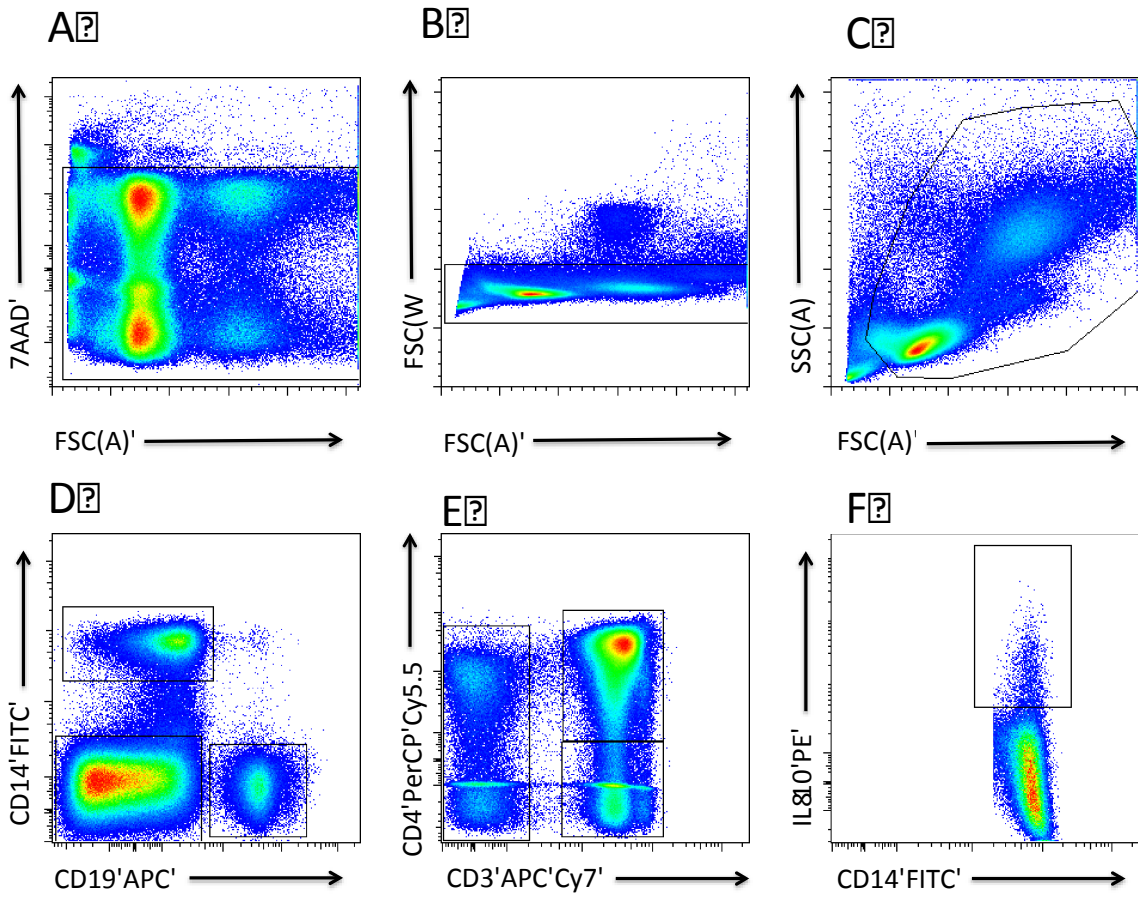

Figure 4. Flow cytometric analysis and gating strategy for PBMC peptide stimulation. Sequential gating is shown from (A) to (D). (A) Live cells included by 7AAD binding and front scatter analysis (FSC) which judges cell size (B) Single cell gating based on FSC (A) and FSC (W). (C) Light scatter profile based of FSC-A, related to cell size and side scatter (SSC-A) related to granularity. (D) Cells were stained for CD 14 versus CD 19. (E) Cells were stained for CD 3 versus CD 4. (F) Gating strategy for IL-10 versus CD 14.

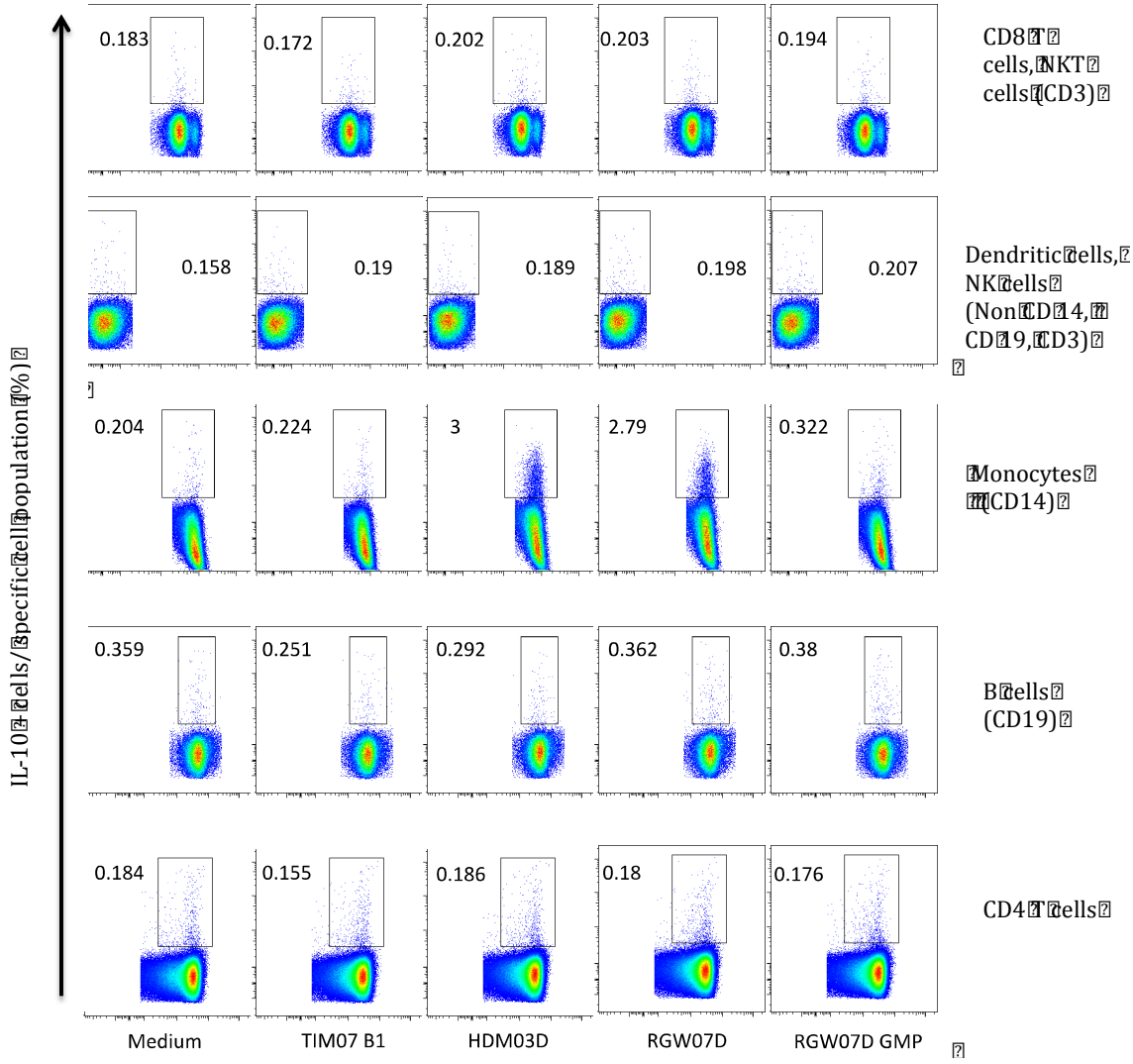

Figure 5. RGW07D peptide elicits IL-10 production from monocytes. 5 million cells per sample of PBMCs were stimulated for 4 hours with the peptide RGW07D, RGW07D GMP, HDM03D, TIM07 B1 and medium. After 4 hours, PBMCs were stained with CD14 (monocytes), CD4 (T cell) and CD19 (B cell), CD 3 (CD8 and NKT cell) and IL-10 antibodies. 500,000 events were analyzed and the number values indicate percentage of cells. The experiment was duplicated.

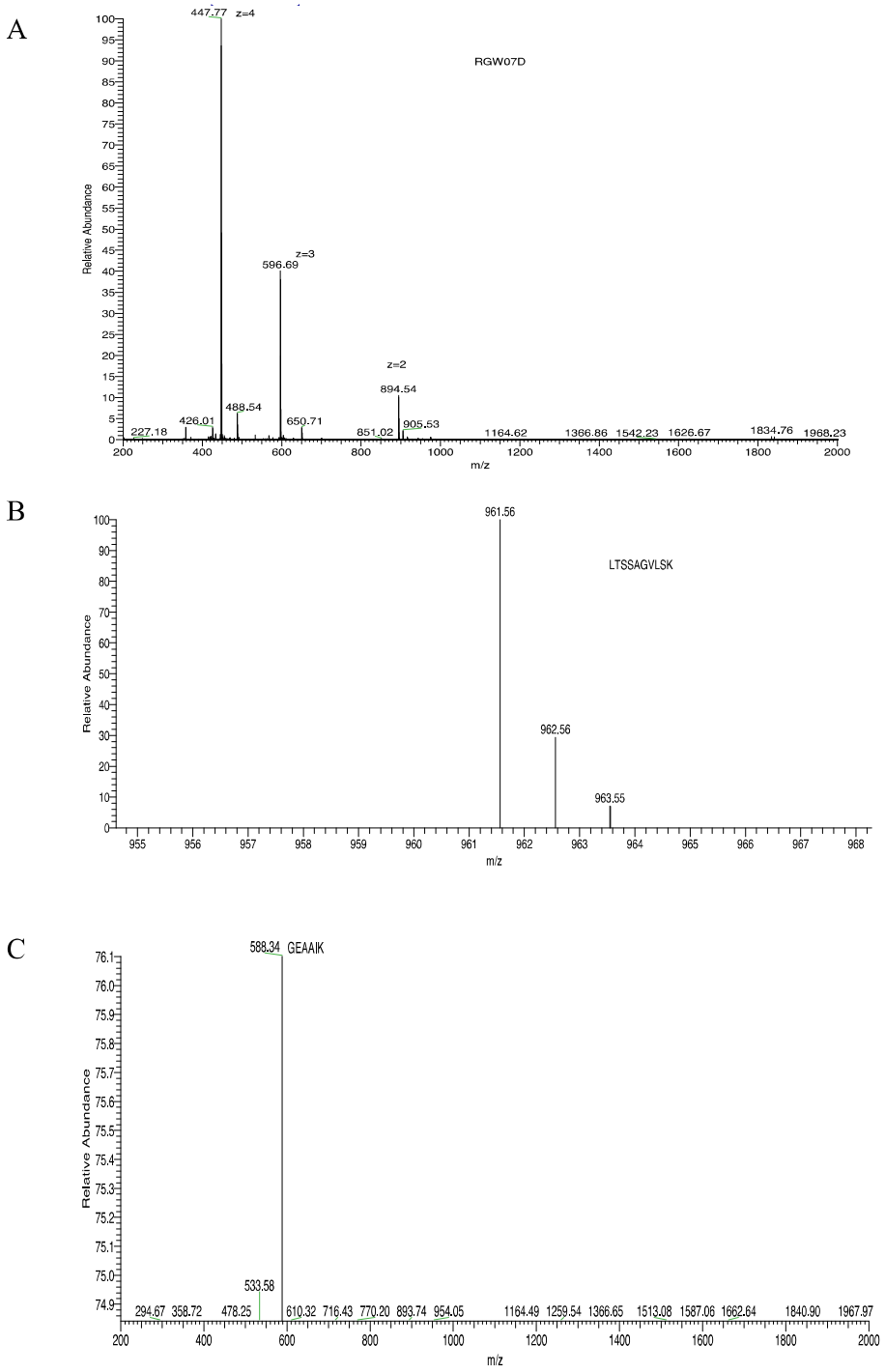

Figure 6. Mass spectrometry of RGW07D peptide and successful trypsin homologue digests. A micro-flow liquid chromatography electrospray tandem mass spectrometry was used to analyze the RGW07D peptides and digests (A) Mass spectrometry trace of undigested RGW07D peptide. (B) Mass spectrometry trace of longer fragment of digested RGW07D peptide. (C) Mass spectrometry trace of the smaller fragment of digested RGW07D peptide.

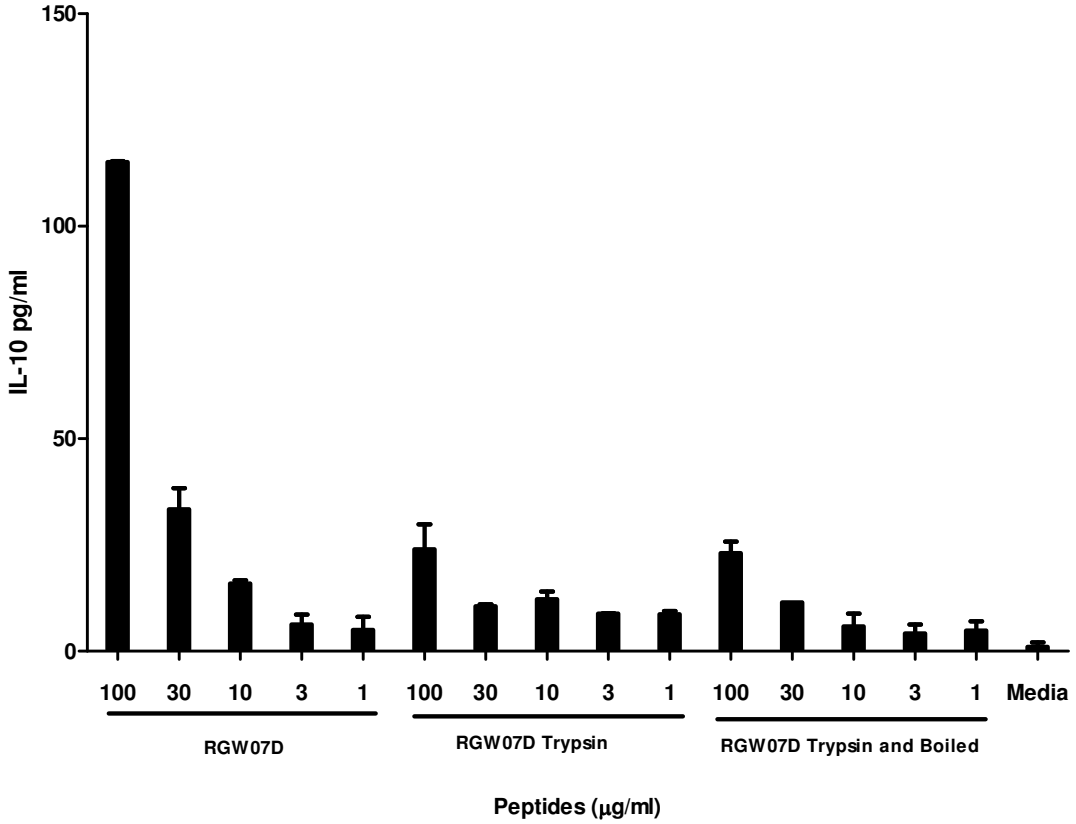

Figure 7. IL-10 production is induced by native RGW07D or digested/heated RGW07D. Fresh human PBMCs were isolated, diluted to a concentration of 2.5 million cells per mL in medium and a 100 µl were added to peptide and enzyme digests. Intact RGW07D peptide was diluted to concentrations of 100µg/ml, 30µg/ml, 100µg/ml, 3µg/ml, and 1µg/ml. The digest concentrations were derived from pre-digest peptide concentration. The digests were diluted to a concentration of 100µg/ml, 30µg/ml, 100µg/ml, 3µg/ml, and 1µg/ml. The supernatants were collected after 3 days of stimulation and were analyzed by ELISA for the presence of IL-10. Data are presented as mean +/- SEM (n=2).

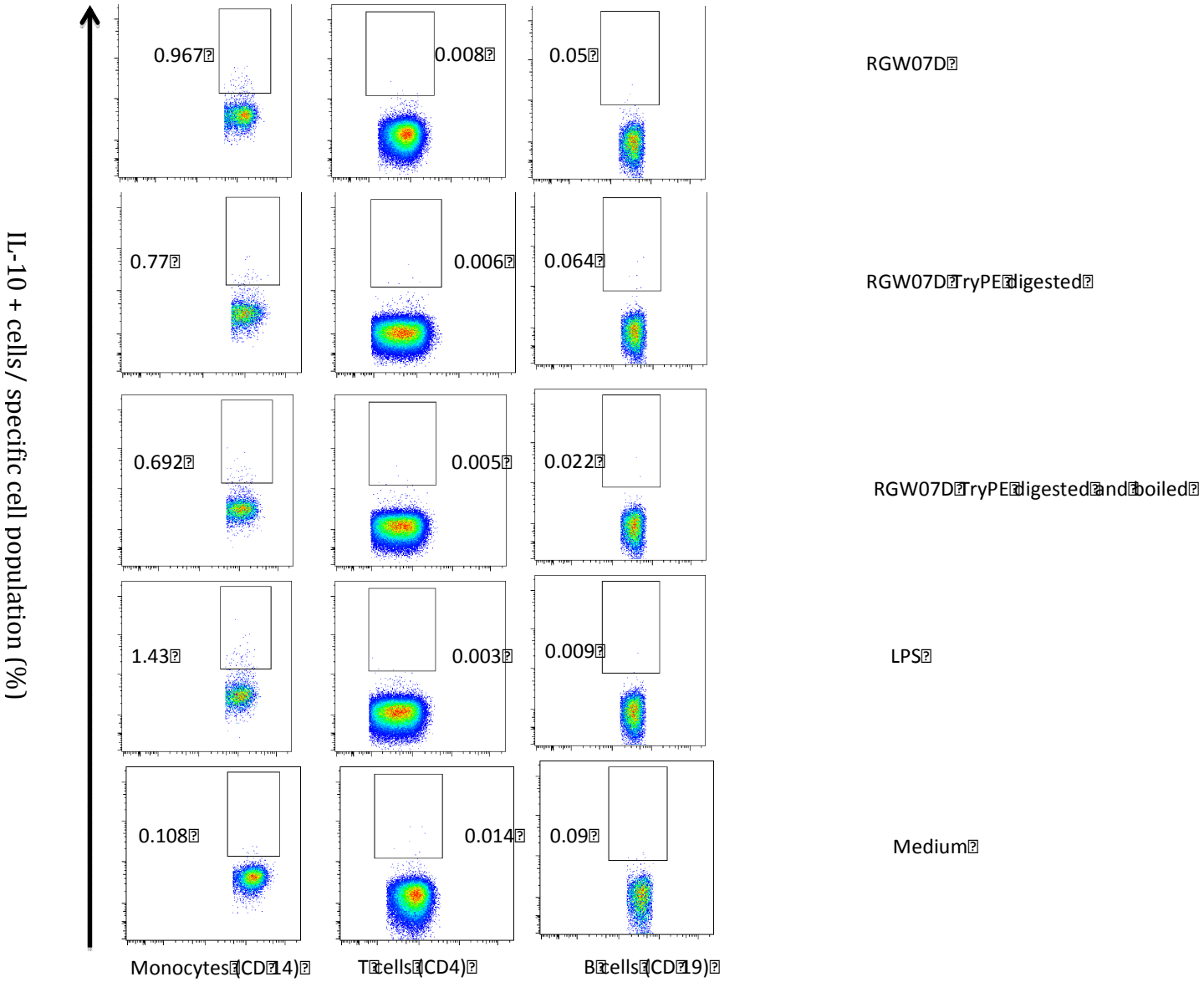

Figure 8. RGW07D peptide and RGW07D digests elicit IL-10 production by monocytes. 5 million cells per sample of PBMCs were stimulated for 4 hours with the peptide RGW07D, RGW07D boiled, RGW07D trypsin homologue digest, RGW07D boiled and trypsin digest. After 4 hours, PBMCs were stained with CD14 (monocytes), CD4 (T cell) and CD19 (B cell) antibodies. The cells were permeabilized with a saponin solution and intra-cellularly stained with anti-IL-10. LPS stimulation was used as a positive control. 500,000 events were analyzed and the number values indicate percentage of cells. The experiment was established in duplicate.

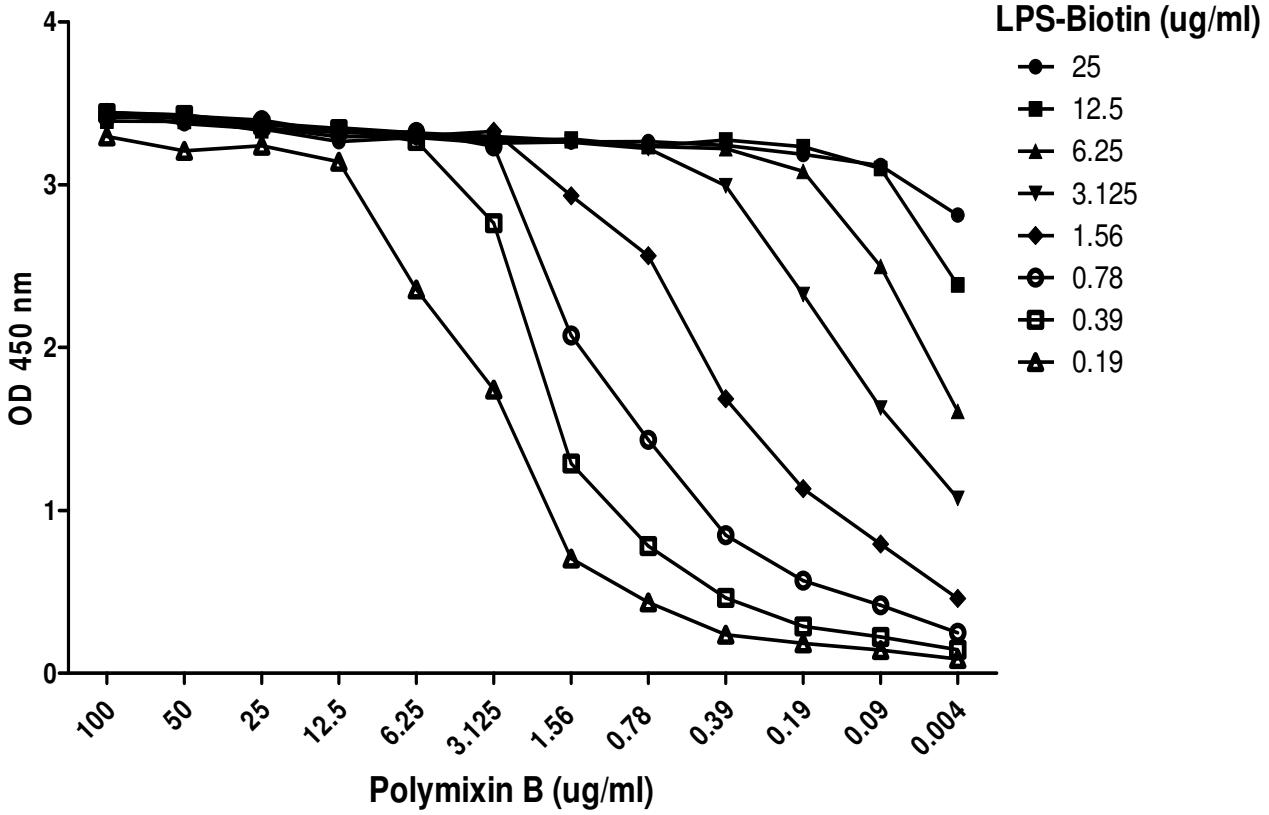

Figure 9. Determination of optimal polymixin B and biotin LPS concentration in the LPS peptide binding assay. Polymixin B was immobilized a 96-well plate beginning at a concentration of 100 µg/ml and diluted two-fold to 0.004µg/ml. Each polymixin B concentration was incubated with a concentration of LPS-biotin beginning at a concentration at 25µg/ml and diluted by two-fold to 0.19µg/ml. Optical density (OD) (450nm) was measured from the TMB substrate being cleaved by streptavidin-conjugated horseradish peroxidase (HRP) when bound to biotinylated LPS. Data are derived from a single experiment.

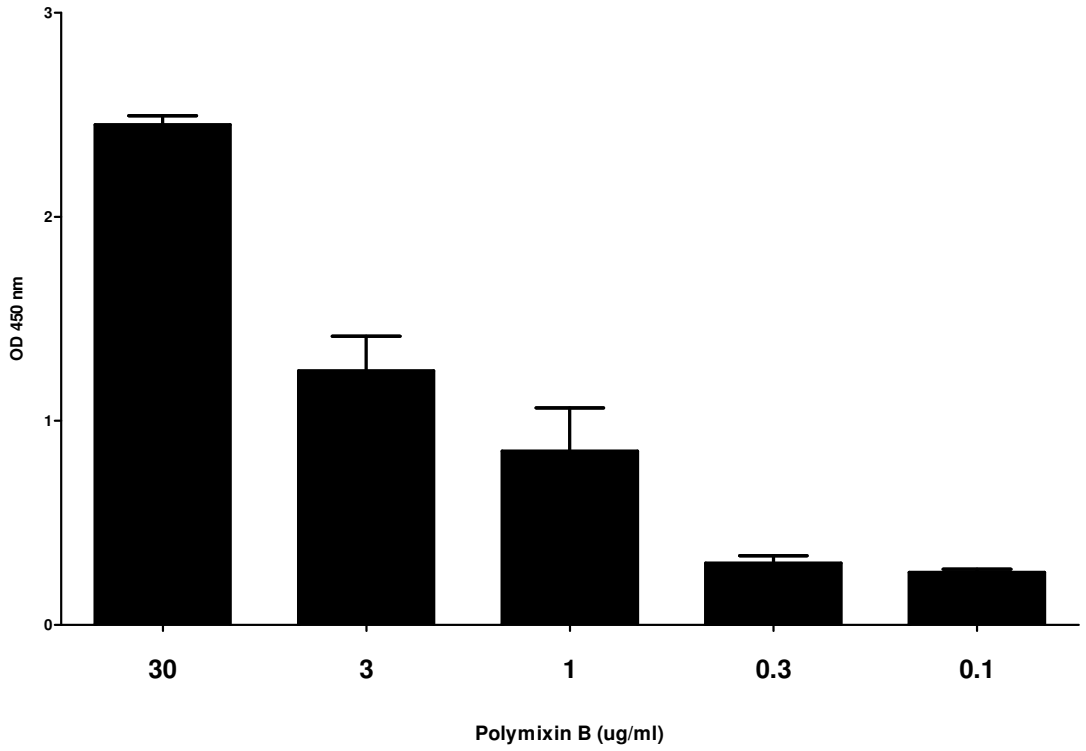

Figure 10. Biotinylated *E. coli* LPS binds to immobilized polymixin B. Polymixin B was immobilized onto a 96 well plate at concentrations: 30µg/ml, 3µg/ml, 1µg/ml, 0.3µg/ml, 0.1µg/ml. Biotinylated LPS (1.56µg/ml) was incubated with the immobilized polymixin B. OD (450nm) was measured from the TMB substrate being cleaved by streptavidin conjugated-horseradish peroxidase (HRP) when bound to biotinylated LPS. Data was background corrected and are presented as mean (n=3, in duplicates) +/- SEM.

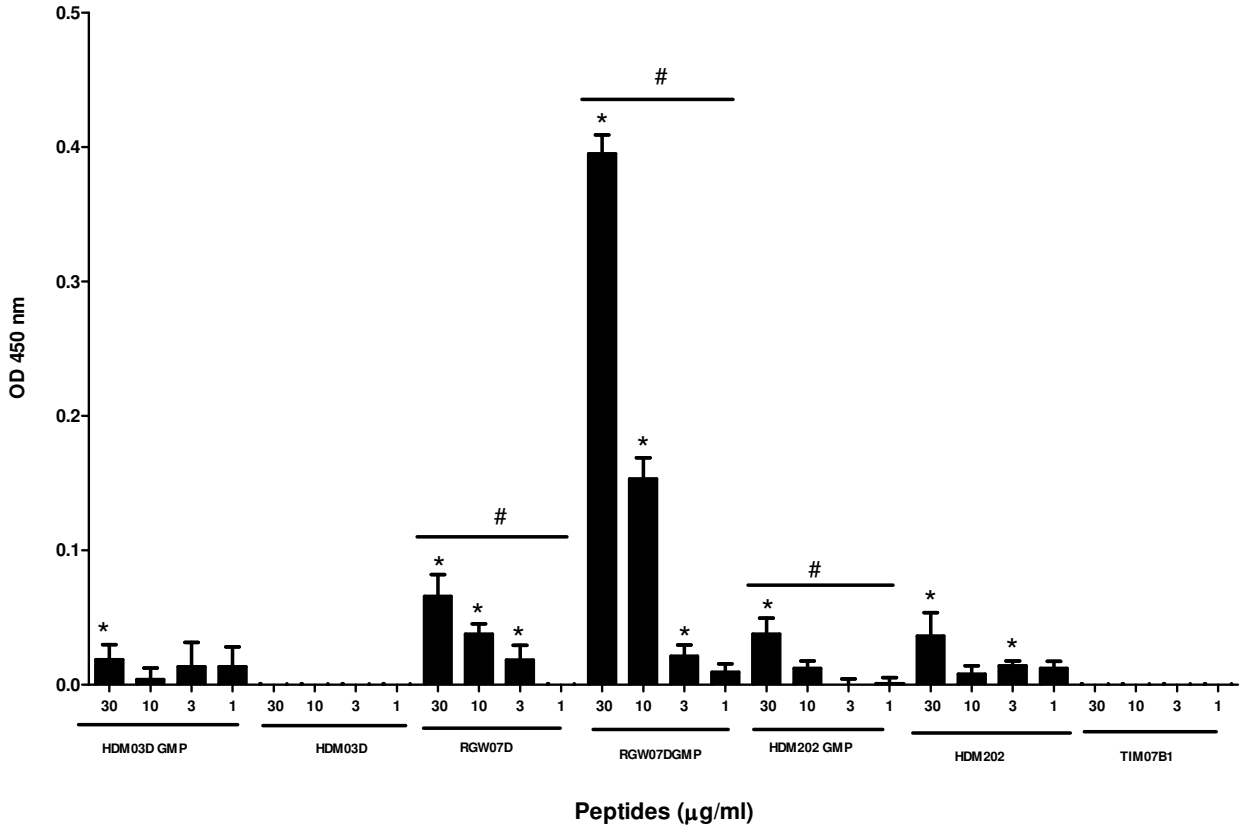

Figure 11. *E.coli* biotinylated LPS binds to allergen peptides. Allergen peptides were immobilized onto a 96 well plate at concentrations: 30μg/ml, 100μg/ml, 3μg/ml, 1μg/ml. Biotinylated LPS (1.56ug/ml) was incubated with the immobilized allergen peptides. OD (450nm) was measured from the TMB substrate being cleaved by streptavidin-conjugated horseradish peroxidase (HRP) when bound to biotinylated LPS. '*' indicates $p \leq 0.05$. '#' indicates $p \leq 0.05$ within an group. P values were determined using Duncans post-hoc test. P values for dose response were calculated by a Students test of the Pearson Correlation. Data were background corrected and are presented as mean (n=5, in duplicates) +/- SEM

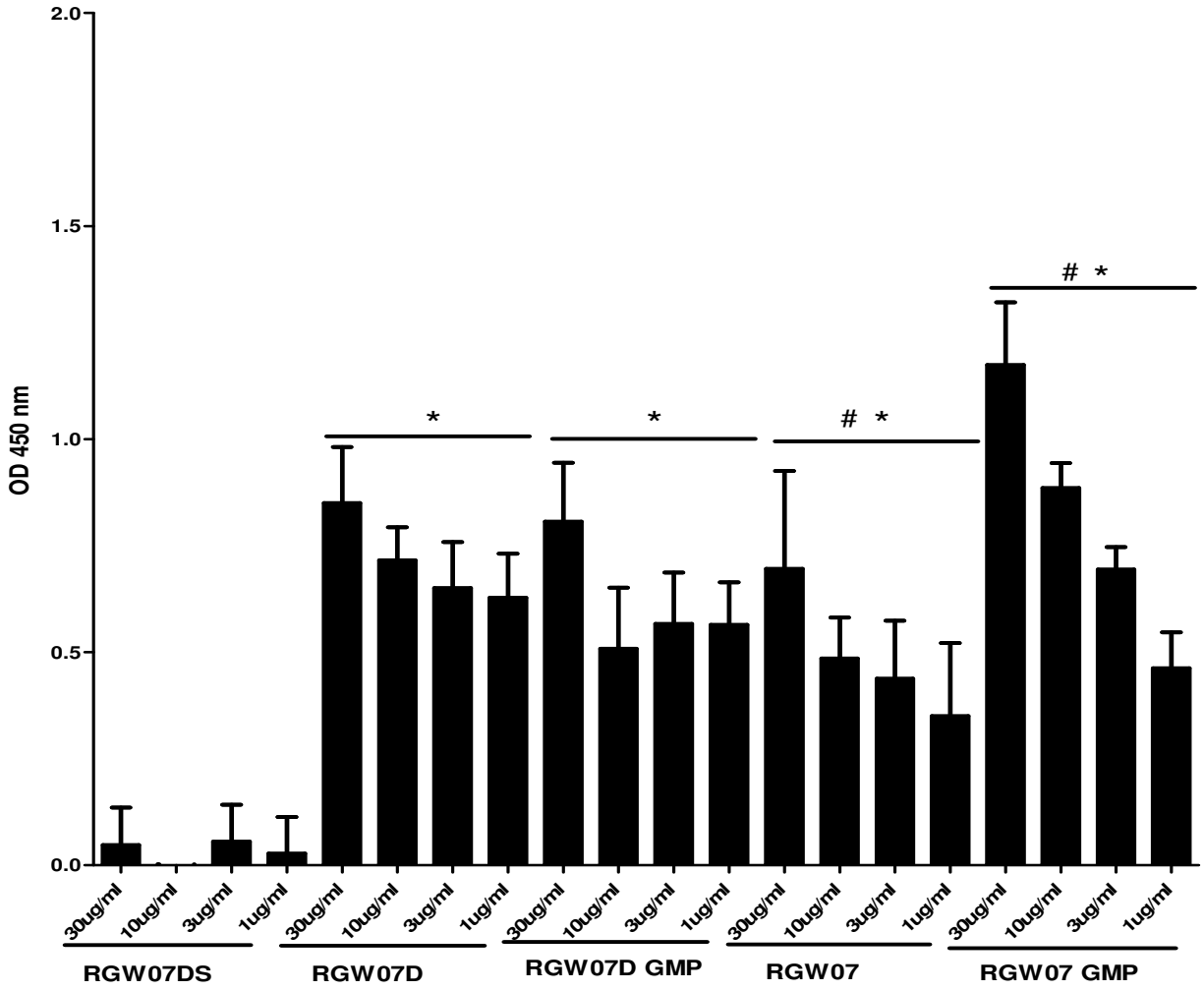

Figure 12. RGW07D peptide and its variants bind to biotinylated *E.coli* LPS. RGW07D peptides and variants were immobilized on to a 96-well plate at concentrations; 30µg/ml, 100µg/ml, 3µg/ml, 1µg/ml. Biotinylated LPS (1.56ug/ml) was incubated with the immobilized allergen peptides to allow interaction. Biotinylated LPS was incubated with the immobilized peptides. Absorbance (450nm) was measured from the TMB substrate being cleaved by streptavidin conjugated-horseradish peroxidase (HRP) bound to biotinylated LPS. A negative control scramble peptide, RGW07DS, was used to account for non-specific binding.'*' indicates $p \leq 0.05$. '#' indicates $p \leq 0.05$ within an group. P values were determined for means using Duncans post-hoc test. P values for dose response were calculated by a Students test of the Pearson Correlation. Data were background corrected and are presented as mean (n=5, in duplicates) +/- SEM

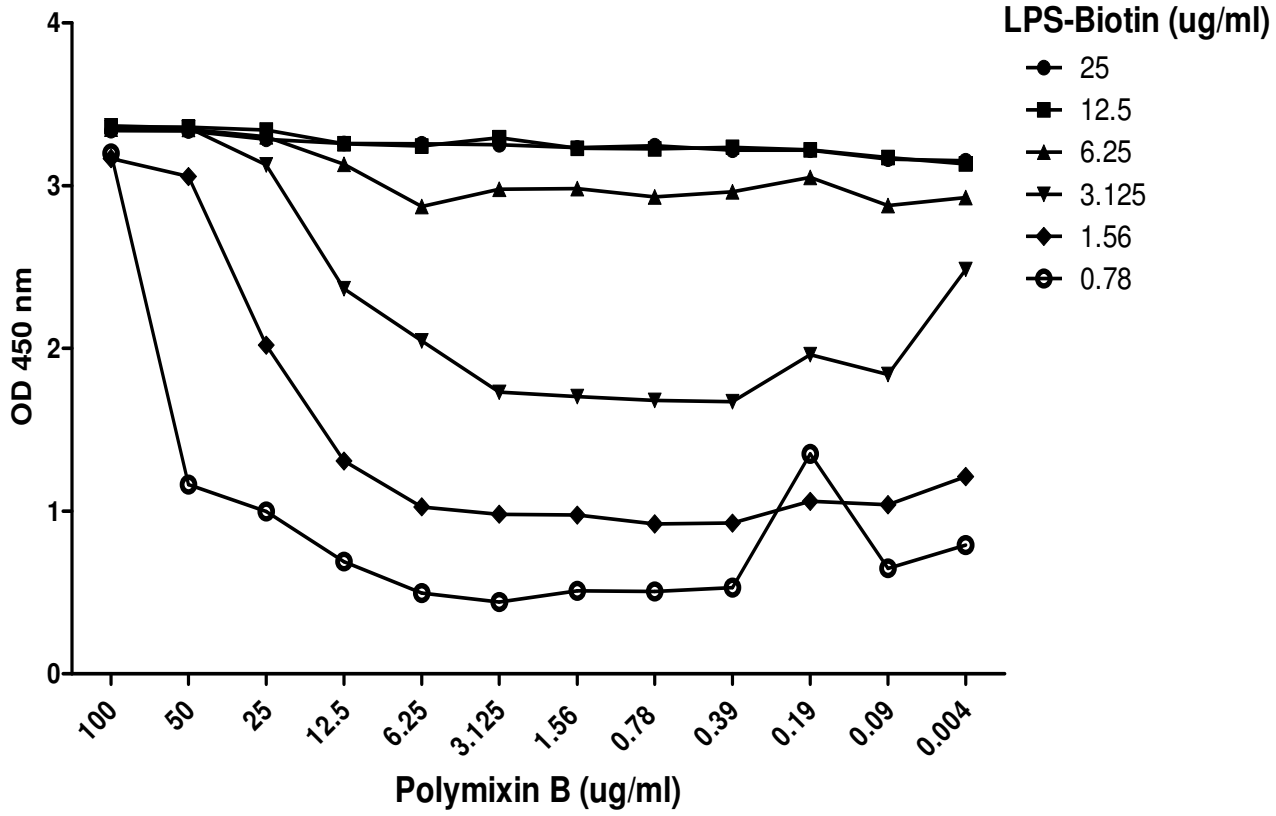

Figure 13. Determination of optimal biotin *P. aeruginosa* LPS concentration in the LPS peptide binding assay. Polymixin B was immobilized a 96-well plate beginning at a concentration of 100 µg/ml and diluted two-fold to 0.004µg/ml. Each polymixin B concentration was incubated with a concentration of LPS-biotin beginning at a concentration at 25µg/ml and diluted by two-fold to 0.78µg/ml. Optical density (OD) (450nm) was measured from the TMB substrate being cleaved by streptavidin-conjugated horseradish peroxidase (HRP) when bound to biotinylated LPS. Data are derived from a single experiment.

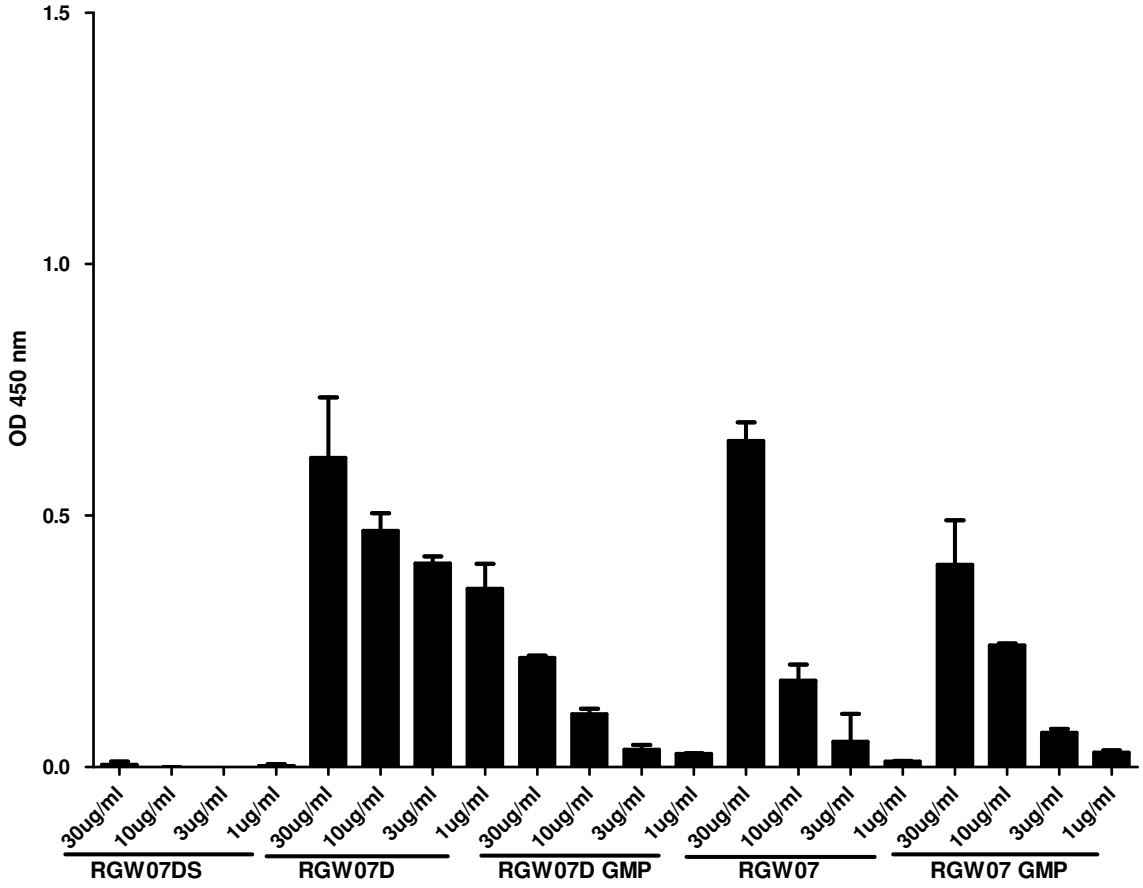

Figure 14 *P. aeruginosa* biotinylated LPS binds to RGW07 variant peptides. RGW07 peptides were immobilized to a 96-well plate at concentrations: 30µg/ml, 100µg/ml, 3µg/ml, 1µg/ml. Biotinylated LPS (3.75ug/ml) was incubated with immobilized allergen peptides to allow interaction. OD (450nm) was measured from the TMB substrate being cleaved by streptavidin- conjugated horseradish peroxidase (HRP) bound to biotinylated LPS. A negative control scramble peptide, RGW07DS, was used to account for non-specific binding. Data were background corrected and are presented as mean (n=2, in duplicates) +/- SEM

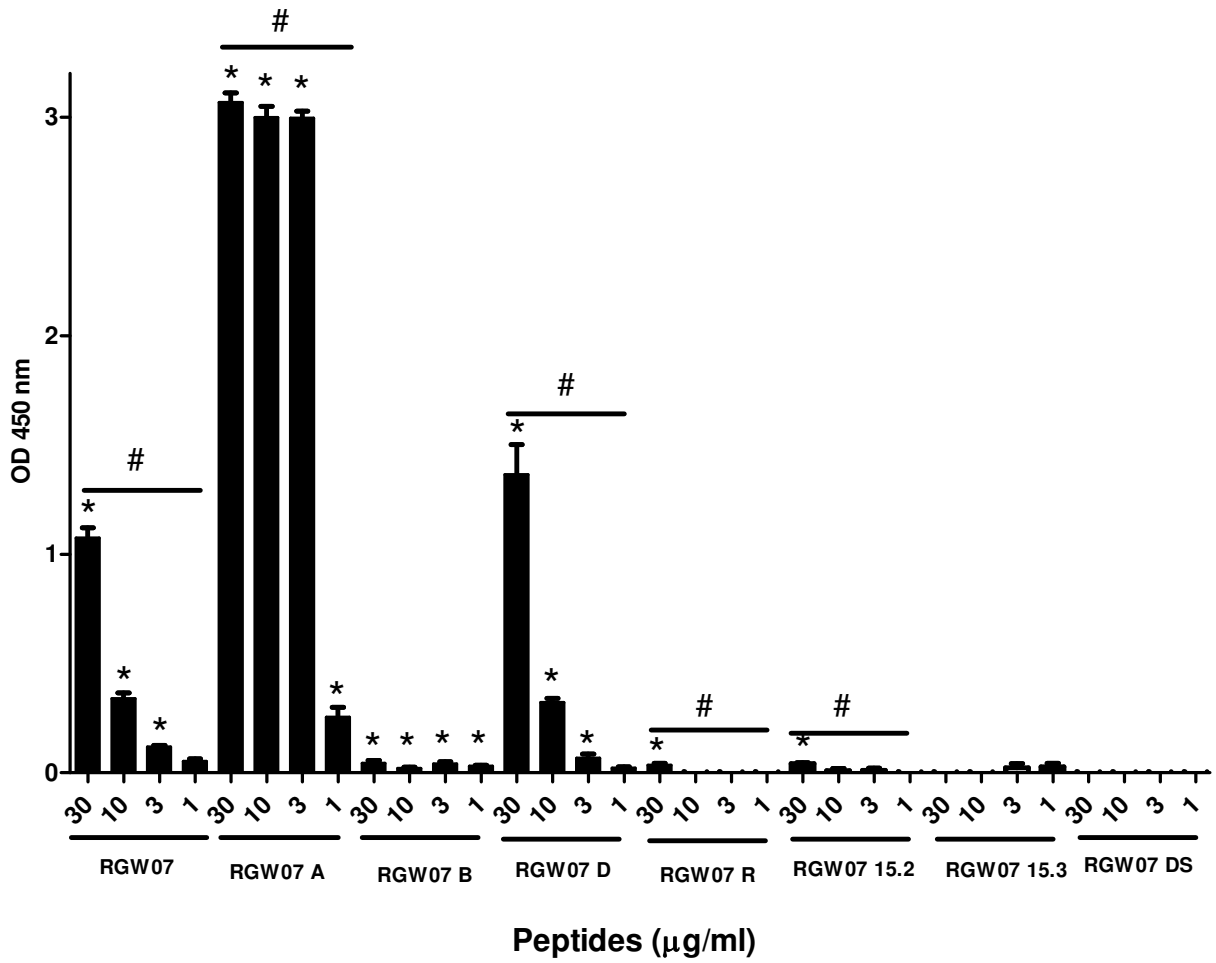

Figure 15. RGW07 and its isoforms bind to biotinylated *E. coli* LPS. RGW07D peptides and variants were immobilized on to a 96-well plate at concentrations; 30μg/ml, 100μg/ml, 3μg/ml, 1μg/ml. Biotinylated LPS (1.56ug/ml) was incubated with the immobilized allergen peptides to allow interaction. Biotinylated LPS was incubated with the immobilized peptides. Absorbance (450nm) was measured from the TMB substrate being cleaved by streptavidin conjugated-horseradish peroxidase (HRP) bound to biotinylated LPS. A negative control scramble peptide, RGW07DS, was used to account for non-specific binding. P values were determined for means using Duncans post-hoc test. .'*' indicates $p \leq 0.05$. '#' indicates $p \leq 0.05$ within an group. P values for dose response were calculated by a Students test of the Pearson Correlation. Data were background corrected and are presented as mean (n=5, in duplicates) +/- SEM

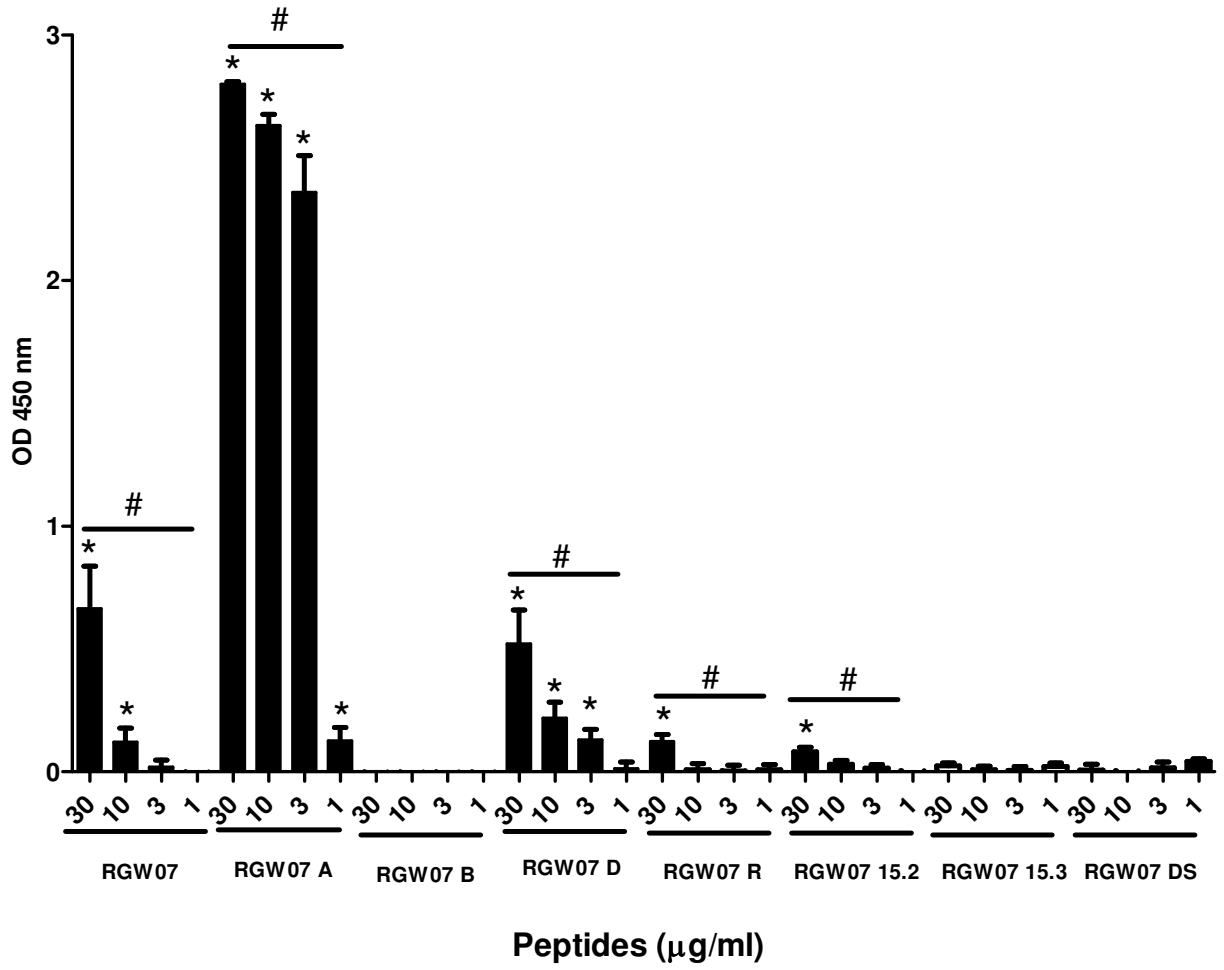

Figure 16. *P. aeruginosa* biotinylated LPS binds to RGW07 isoform peptides. Peptides were immobilized to a 96-well plate at concentrations: 30μg/ml, 100μg/ml, 3μg/ml, 1μg/ml. Biotinylated LPS (3.75 ug/ml) was incubated with immobilized allergen peptides to allow interaction. OD (450nm) was measured from the TMB substrate being cleaved by streptavidin- conjugated horseradish peroxidase (HRP) bound to biotinylated LPS. A negative control scramble peptide, RGW07DS, was used to account for non-specific binding. '*' indicates $p \leq 0.05$. '#' indicates $p \leq 0.05$ within an group. . P values were determined for means using Duncans post-hoc test. P values for dose response were calculated by a Students test of the Pearson Correlation. Data were background corrected and are presented as mean (n=6 to 8, in duplicates) +/- SEM

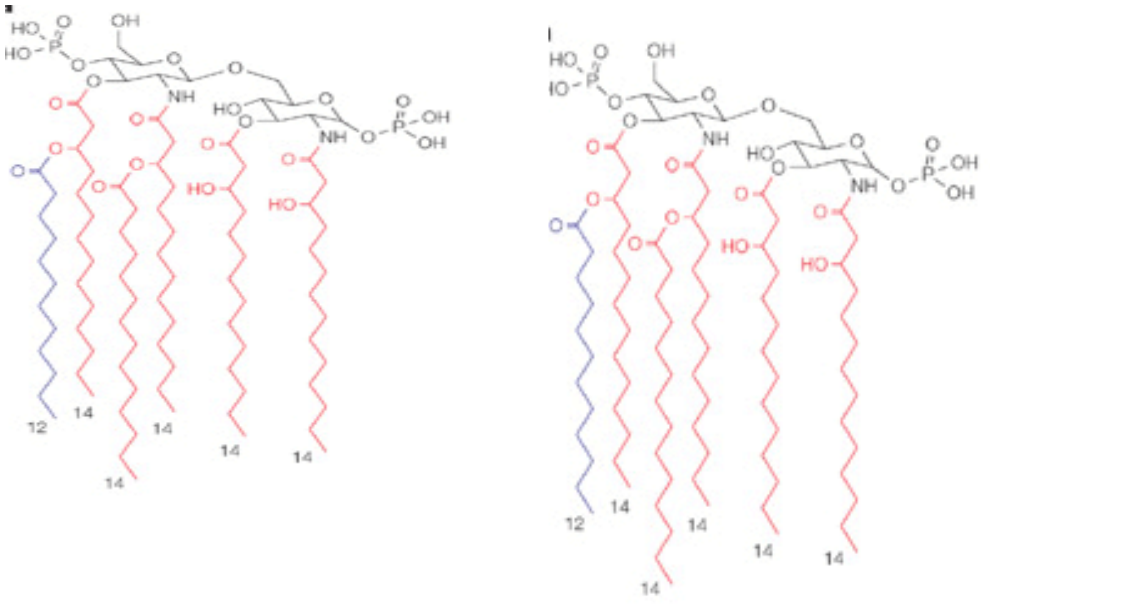

Figure 17 Comparison of E. *coli* and P. *aeruginosa* lipid A moiety. The structural difference can be noted in the length of the acyl chains (carbon length is presented) and the number of acyl chains. Figure was adapted from [29]

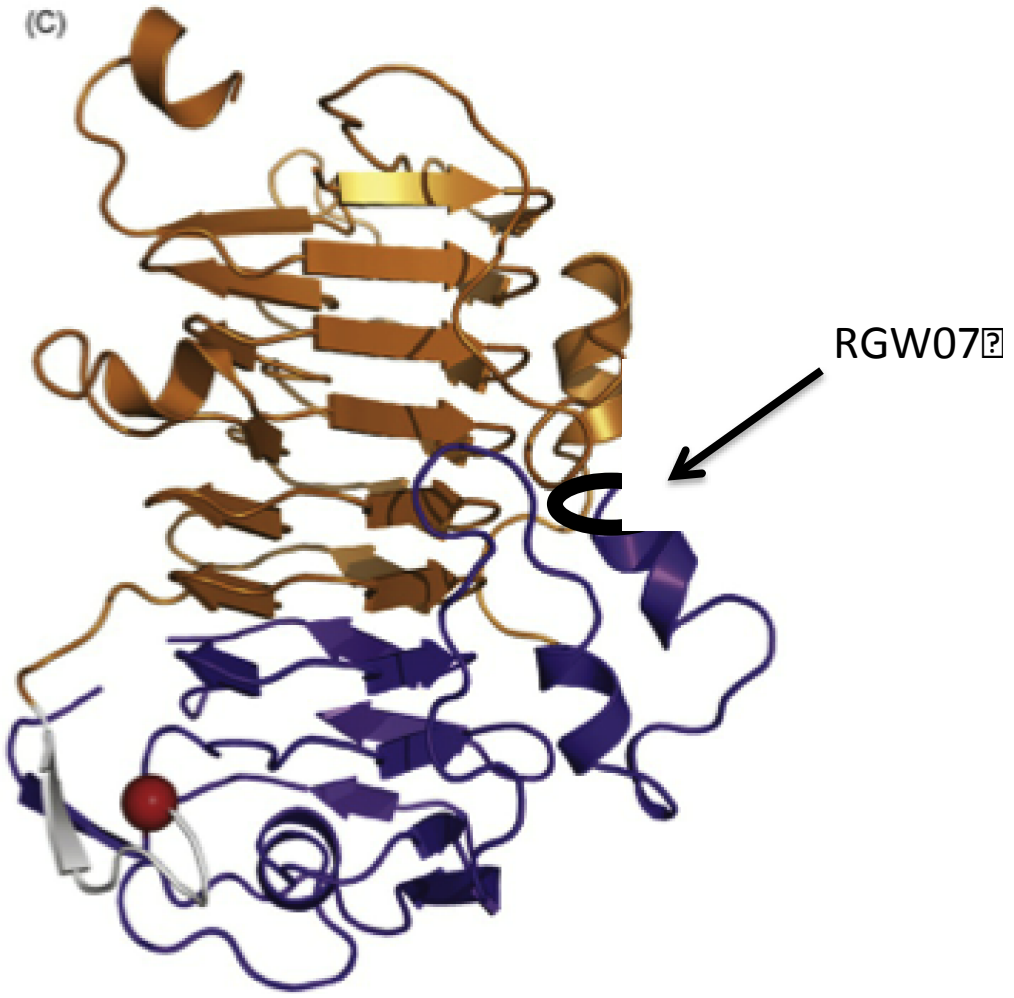

Figure 18 Ribbon structure of Amb a 1. The RGW07 sequence is contained in the β-chain of the Amb a 1 protein. Figure was adapted from [38]

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9.0 APPENDECES

9.1 Appendix I: FMO IL-10 staining

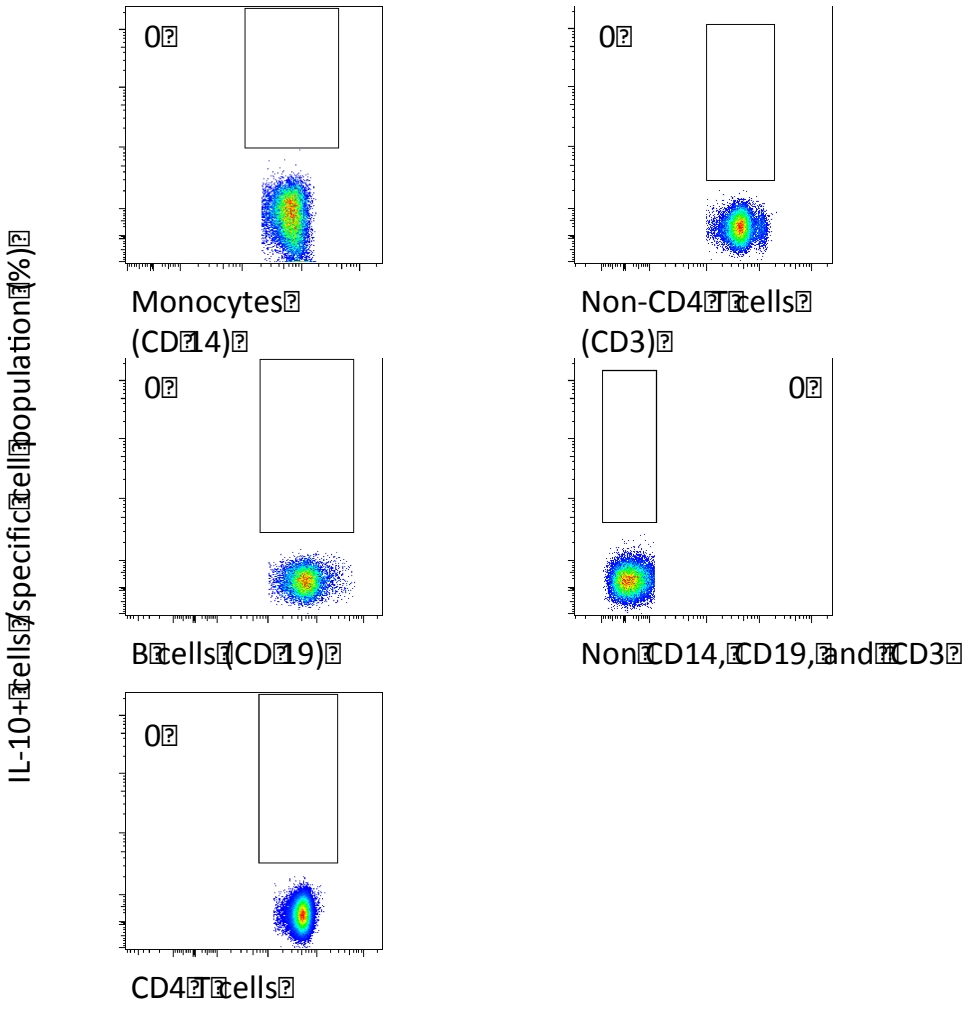

Figure 1. IL-10 FMO gating for extracellular staining

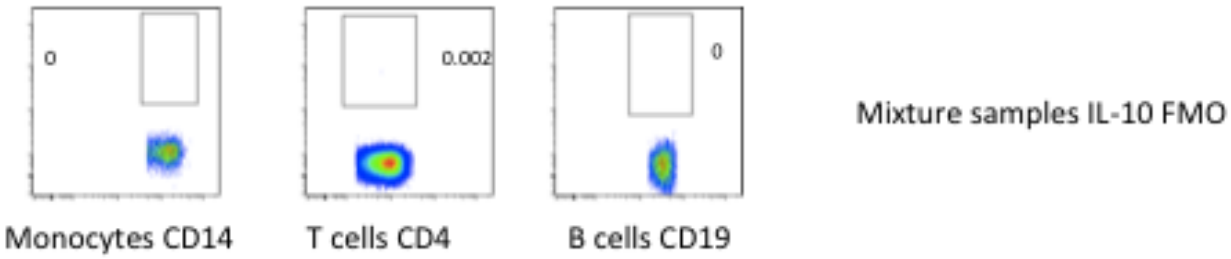

Figure 2. IL-10 FMO gating for intracellular staining

9.2 Appendix II: SPR Experiment

Surface Plasmon Resonance (SPR) instrument was attempted to quantify the interaction between peptides and E. coli *LPS*

An SPR instrument was utilized as an alternative form of analysis of the LPS peptide binding capacity of RGW07D. ProteOn XPR36 Protein Interaction Array System (SPR) determines macromolecule-binding capacities occurring between ligands bound on a sensor chip surface and an analyte in solution that flows over top the ligand-bound surface. Polarized light is projected into a prism and refracted by the sensor chip on to an optical detection unit. Binding capacity is quantified by changes in refractive index. When no ligand-analyte association is present, there are no refractive changes at the sensor chip. However, a ligand-analyte association causes a change in the refractive index. These quantitative changes are captured by the optical detection unit and graphed in Refractive Units (RU). Higher RU indicated a greater amount of association.

Immobilization of biotinylated E. *coli* LPS to the avidin sensor chip was attempted; however, LPS failed to bind to the sensor chip. Immobilization of LPS to the avidin sensor chip was required before the peptide binding capacity could be measured. The immobilization step was performed in the horizontal orientation for 3 channels of the chip. The ProteOn XPR36 system used a running buffer of 1xPBS .05% Tween (PBST). The chip was air initialized as *per* instructions from the manufacturer. A regenerator step was performed alternating 1M NaCl and 50 mM NaOH at a flow rate of 30ul/min for 60

seconds. This was repeated 4 times. Biotin-conjugated E.*coli* LPS O111:B4 was diluted in PBST to 0.01 mg/mL, 0.02 mg/mL and 0.03mg/mL to identify an ideal concentration to saturate the avidin chip. The flow rate for the immobilization step of biotinylated LPS was set to 25ul/min for 180 seconds. In **Figure 1**, the signal lines at negative time (s) indicated the background before injection of LPS, which was approximately zero (0) RU. At time 0, the LPS was injected into the channels and there was a spike in the RU signal. Once the injection of LPS was completed, at 180 seconds, there was a decline in the RU signal to background. This indicated that the LPS did not bind. If the signal was elevated above zero, then this would have indicated binding. It was recommended by the manufacturer to increase the range of the concentrations of LPS. Therefore, the LPS was diluted in PBST to 0.005mg/mL, 0.01mg/mL, 0.02mg/mL, 0.03mg/mL, and 0.04mg/mL. The same protocol was followed as mentioned in the previous experiment. **Figure 2**, displays similar results to the previous experiment (**Figure 1**). There was no binding of the biotin-LPS to the avidin chip. In SPR experiments, the immobilization of ligands is ideal when the ligand's molecular weight is low, compared to the analyte. Since LPS aggregates and its molecular weight is variable (10kDa to 1000kDa), it was hypothesized that the intrinsic ability of LPS to aggregate was the reason for its inability to bind to avidin. It was required to break up aggregation of LPS. Many groups [44, 45] have used sonication and therefore, sonication of LPS was attempted to assist in facilitating biotin-avidin binding.

A sonication water bath was used to sonicate LPS prior to the SPR experiment and LPS bound to the avidin chip. LPS was diluted in PBST to 0.005mg/mL,

0.01mg/mL, 0.02mg/mL, 0.03mg/mL, and 0.04mg/mL. The diluted LPS was sonicated for 10 minutes in a sonication water bath. The chip was air initialized as *per* instructions from the manufacturer. A regenerator step was performed alternating 1M NaCl and 50 mM NaOH at a flow rate of 30ul/min for 60 seconds, which was repeated 4 times. The flow rate for the immobilization step of biotinylated LPS was set to 25ul/min for 180 seconds. In **Figure 3**, the background RU was equivalent to zero before the injection of the sonicated LPS. From t=0 to 180s, the LPS was flowed over the avidin chip. After t=180s, the RU signal for all five LPS concentrations was at approximately 20 RU. Therefore, this indicated that the LPS bound to the avidin on the sensor chip. However, the ideal RU for ligand binding is approximately 100 RU, as suggested by the manufacturer. Therefore, a more vigorous sonication was needed to disassociate LPS to improve biotin-avidin binding, so a sonication probe was used.

A sonication probe was used to sonicate LPS prior to the SPR experiment; however, LPS did not bind to the avidin chip. LPS was diluted in PBST to 0.005mg/mL, 0.01mg/mL, 0.02mg/mL, 0.03mg/mL, and 0.04mg/mL. The diluted LPS was sonicated with a probe for 3 minutes. Since probe sonication was much more vigorous, it was decided to decrease the time from 10 min to 3 min. The chip was air initialized as *per* instruction from the manufacturer. A regenerator step was performed alternating 1M NaCl and 50 mM NaOH at a flow rate of 30ul/min for 60 seconds, which was repeated 4 times. The flow rate for the immobilization step of biotinylated LPS was set to 25ul/min for 180 seconds. In **Figure 4**, the background RU was equivalent to zero before the injection of the sonicated LPS. From t=0 to 180s, the LPS was flowed over the avidin

chip. However, the RU signal returned to background levels. Therefore, it appeared that sonication with the probe did not provide better immobilization of LPS to avidin. Since immobilization of LPS to avidin failed, peptide binding capacity was not measured and no further studies were undertaken.

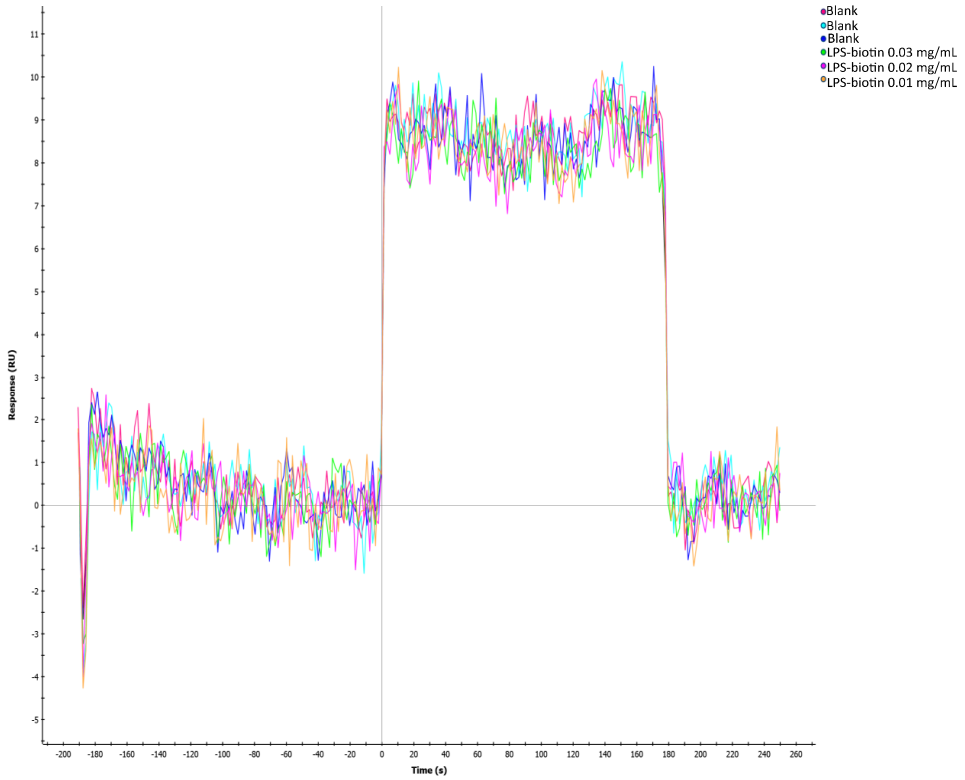

Figure 1. Biotinylated E.*coli* LPS failed to bind to the avidin SPR sensor chip. The immobilization step was performed in the horizontal orientation for 6 channels. The ProteOn XPR36 system used a running buffer of 1xPBS 0.05% Tween (PBST). The chip was air initialized. Biotin-conjugated E.*coli* LPS O111:B4 was diluted in PBST to 0.01 mg/mL, 0.02 mg/mL and 0.03mg/mL. The flow rate for immobilization of biotinylated LPS was set to 25ul/min for 180 seconds. Measurement of immobilization occurred in refractive units (RU). Data presented were normalized and one of three separate experiments. Display is one vertical channel.

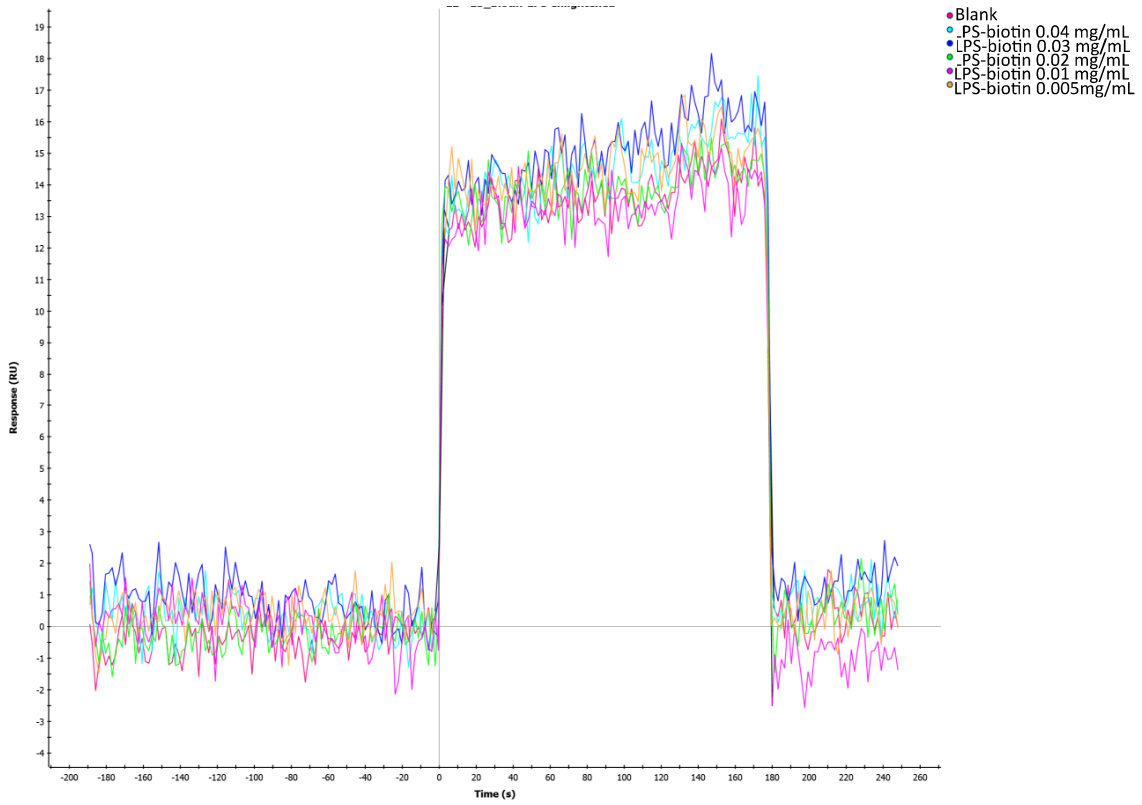

Figure 2. Biotinylated E.*coli* LPS failed to bind to the avidin SPR sensor chip. The immobilization step was performed in the horizontal orientation for 6 channels. The ProteOn XPR36 system used a running buffer of 1xPBS 0.05% Tween (PBST). The chip was air initialized. Biotin-conjugated E.*coli* LPS O111:B4 was diluted in PBST to 0.005mg/mL, 0.01 mg/mL, 0.02 mg/mL, 0.03mg/mL and 0.04mg/mL. The flow rate for immobilization of biotinylated LPS was set to 25ul/min for 180 seconds. Measurement of immobilization occurred in refractive units (RU). Data presented were normalized and one of three separate experiments. Display is one vertical channel.

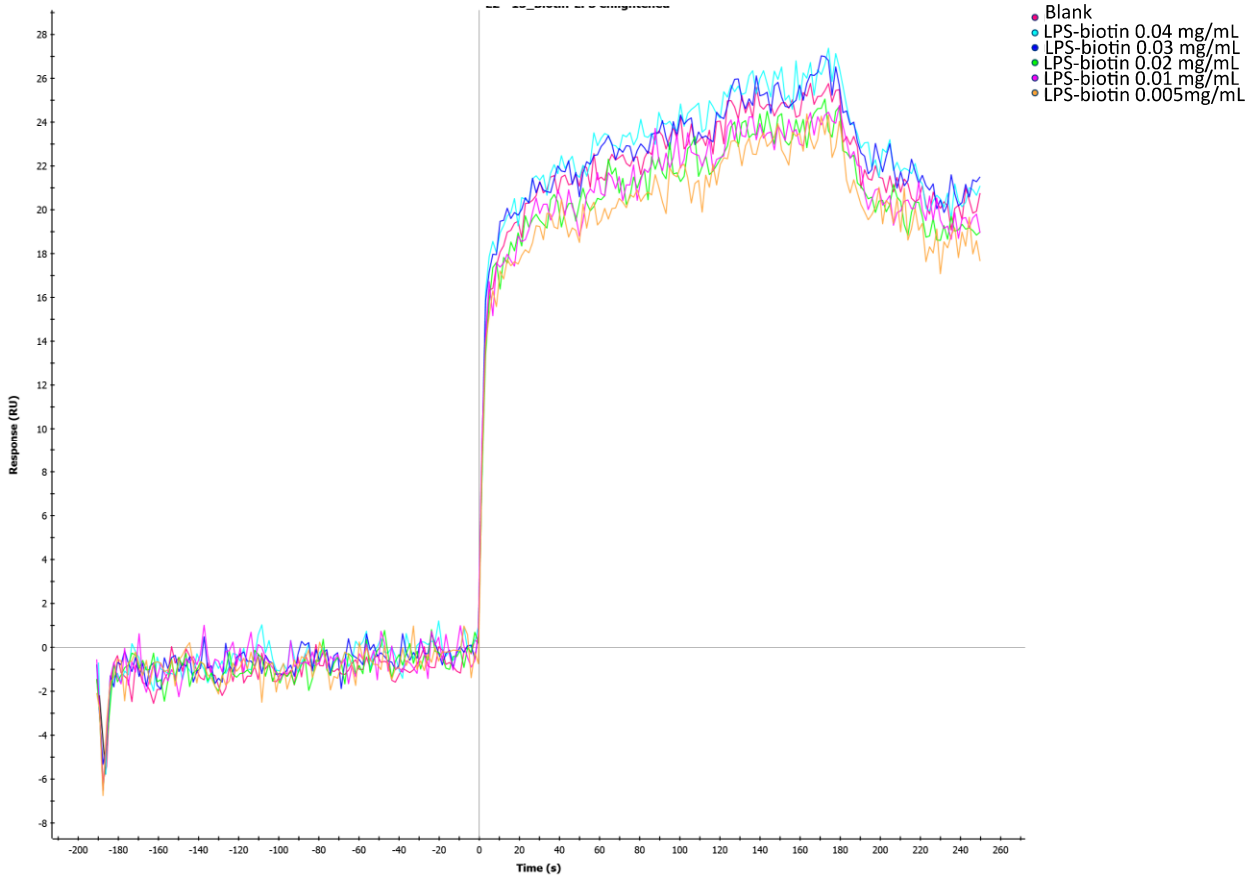

Figure 3. Biotinylated E.*coli* LPS that was sonicated in a sonication water bath and immobilized to the avidin SPR sensor chip. The immobilization step was performed in the horizontal orientation for 6 channels of the chip. The ProteOn XPR36 system used a running buffer of 1xPBS 0.05% Tween (PBST). The chip was air initialized. Biotin-conjugated E.*coli* LPS O111:B4 was diluted in PBST to 0.005 mg/mL, 0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL and 0.04 mg/mL. The diluted LPS was sonicated for 10 mins in sonication bather prior to immobilization. The flow rate for immobilization of biotinylated LPS was set to 25ul/min for 180 seconds. Measurement of immobilization occurred in refractive units (RU). Data given was normalized and one of two separate experiments. Display is one vertical channel.

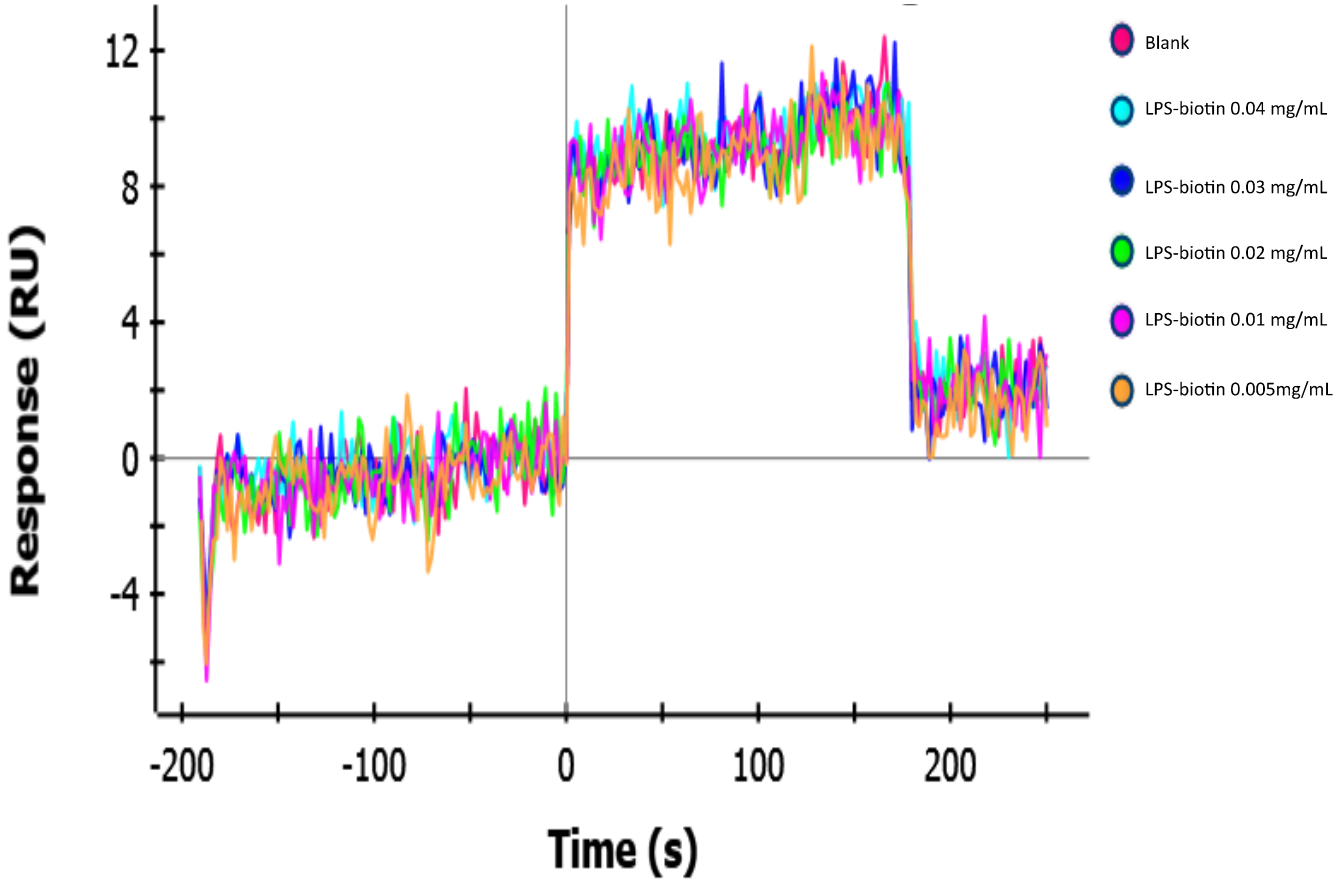

Figure 4. Biotinylated E.*coli* LPS that was sonicated by sonication probe and immobilized to the avidin SPR sensor chip. The immobilization step was performed in the horizontal orientation for 6 channels of the chip. The ProteOn XPR36 system used a running buffer of 1xPBS 0.005% Tween (PBST). The chip was air initialized. Biotin-conjugated E.*coli* LPS O111:B4 was diluted in PBST to to 0.005 mg/mL, 0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL and 0.04 mg/mL. The diluted LPS was sonicated for 10 mins with sonication probe prior to immobilization. The flow rate for immobilization of biotinylated LPS was set to 25ul/min for 180 seconds. Measurement of immobilization occurred in refractive units (RU). Data presented were normalized and one of three separate experiments. Graph displayed is one vertical channel.