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Activation of toll-like receptor 2 through TLR2-LRR binding synthetic peptides

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ACTIVATION OF TOLL-LIKE RECEPTOR 2 THROUGH TLR2-LRR BINDING SYNTHETIC PEPTIDES

BY

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ABSTRACT

ACTIVATION OF TOLL-LIKE RECEPTOR 2 (TLR2) THROUGH TLR2-LRR BINDING SYNTHETIC PEPTIDES

By

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University of New Hampshire, December 2006

Bacterial sepsis and systemic inflammatory immune responses continue to be major causes of illness and death despite extensive research into the development of antimicrobial agents. There is a need for novel therapeutic reagents designed to modulate these responses. Toll-like receptor 2 plays a key role in the development of innate and adaptive immune responses to microbial products that interact with the receptor's extracellular leucine-rich repeat ligand-binding domain. In a preliminary study, five peptides were synthesized that bound to the leucine-rich repeat region of Toll-like receptor 2 (TLR2). We tested the hypothesis that the novel TLR 2 leucine rich repeat binding peptides affects TLR2-mediated immune function by examining the ability of the five peptides to induce the maturation of bone marrow derived-dendritic cells in vitro. The dendritic cells were cultured in the presence of the peptides and maturation was determined through flow cytometry and cytokine analysis. We discovered that dendritic cells produced interleukin-6 to the JT1 leucine-rich repeat binding peptide. Some activation of the MHC class II dendritic cell maturation marker was observed in response to peptide JT1. Our results indicate that the JT1 synthetic toll-like receptor 2 leucine rich repeat binding peptide induces maturation of bone marrow derived dendritic cells in vitro.

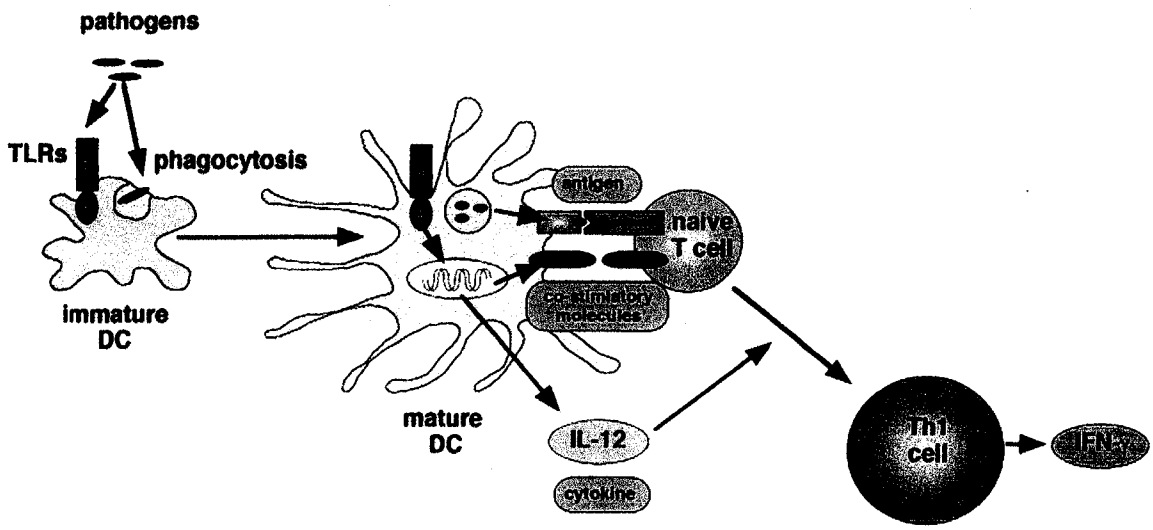
CHAPTER I

INTRODUCTION

All healthy human beings possess both innate and adaptive immunity. The innate immune system is the first defense against invading microbial pathogens and elicits a generalized response through recognition of specific molecular patterns by effector cells. The innate response is invariant however frequently exposure to the microbe occurs. Innate immunity is essential for timely detection and containment of an infection (Aderem and Smith, 2004).

Adaptive immunity prevents pathogen re-infection and often includes antibody production. This response usually occurs no later than 96 hours after the start of infection (Janeway 2005). Though the adaptive response is more complex than the innate response, the two are strongly interrelated. Adaptive immunity involves clonal selection of a repertoire of T and B-lymphocytes from an extremely diverse pool of cells bearing antigen-specific receptors that mediate pathogen binding. In response to activation, T-cells bearing receptors that bind to the cognate antigen undergo clonal selection; they also up-regulate cell surface molecules that are co-stimulatory to B cells, including B7 and CD40c. Antigen-specific lymphocytes proliferate and differentiate into effector cells leading to pathogen destruction via a variety of mechanisms that include direct killing by cytotoxic T-cells and phagocytosis (Janeway 2005, Paul, 1993). Clonal selection also results in the production of differentiated memory lymphocytes, allowing a more rapid and specific response in recurrent infections.

Figure 1. Toll-like Receptor Activation of Adaptive Immunity



(Figure adapted from Takeda and Akira, 2001)

Dendritic cell maturation occurs upon TLR-mediated pathogen recognition and phagoctyosis. TLR activation leads to the production of co stimulatory molecules as well as inflammatory cytokines that assist in antigen presentation to naïve T cells and with T cell maturation to Th1 cells.

The Role of Innate Immunity

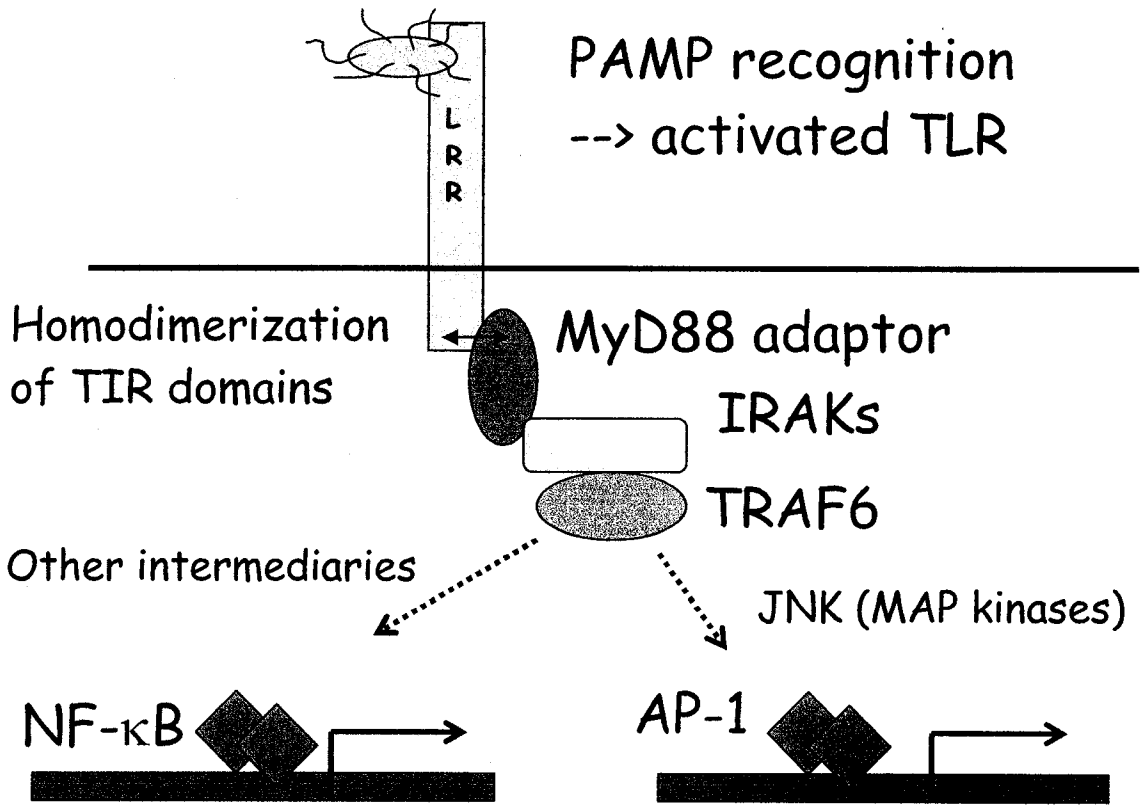
Antigen-presenting cells (APCs), such as dendritic cells and macrophages, are key effector cells of the innate immune response. These cells recognize pathogens via a family of extracellular receptors known as Toll-like receptors (TLRs) (Aderem and Smith, 2004, Satthaporn and Eremin, 2001). Toll-like receptor activation causes the phagocytosis and subsequent degradation of a pathogen via proteolytic cleavage. Intracellular processing results in the presentation of antigenic peptides on the cellular surface within the context of the class I major histocompatiblity complex proteins (MHC). Dendritic cells (DCs) possess the ability to stimulate naïve T cells (Satthaporn and Eremin, 2001), upregulating the production of inflammatory cytokines such as interleukins 6, 8, 10 (IL 6, 8, 10) and interferon gamma (IFN-γ). The inflammatory response results in blood vessel dilation and phagocyte migration, localizing the pathogen and inhibiting the further spread of infection.

The inflammatory response can result in serious negative consequences such as the release of reactive oxidizing compounds and lysosomal contents by effector cells resulting in tissue damage and necrosis. Systemic bacterial infections result in a widespread inflammatory response called sepsis. The consequences are high fever, hypotension, severe tissue destruction, respiratory distress, shock, organ failure, and eventual death (Janeway, 2005). The innate immune response has a critical role in the maintenance of human health. Therefore the ability to modulate or control this response could save thousands of lives (Martin, 2003), or significantly improve the quality of life for millions.

Toll-Like Receptors

An inflammatory response is the result of phagocyte activation through recognition of pathogen-associated molecular patterns (PAMPs), which are microbe-associated lipoproteins, or of other cell wall components such as lipopolysaccharides and peptidoglycan. Recognition occurs through a family of receptors on the surface of leukocytes known as TLRs. TLRs are transmembrane proteins containing an extracellular region consisting of a leucine-rich repeat domain (LRR) that mediates binding to the PAMP. There are eleven human TLRs currently known. Each is thought to recognize a specific PAMP associated with a wide variety of pathogens. Toll-like receptor 4 (TLR4) produces an immune response when activated by lypopolysaccharide (LPS), a major constituent of gram-negative bacteria cell walls. Toll-like receptor 5 (TLR5) recognizes flagellin protein; CpG DNA activates Toll-like receptor 9 (TLR9) and peptidoglycan activates TLR2 (reviewed in Takeda and Akira, 2001). A single pathogen can activate several different TLRs via its associated PAMPs. The activation of each TLR brings about both an individual specific response, such as the production of tumor necrosis factor α (TNF-α) by TLR2 and more generally the activation of NF-κB. Toll-like receptors have an intracellular carboxy-terminus that contains a highly conserved region called the Toll/interleukin-1 receptor (TIR) homology domain. Toll-like receptor activation and subsequent pathway stem from heterodimerization of the TIR domain and the MyD88 adaptor protein's domain (figure 2). This results in the recruitment and phosphorylation of the interleukin –1 (IL-1) receptor associated kinase (IRAK) a serine/threonine kinase whose death domain interacts with the death domain of MyD88. IRAK associates with the TNF receptor-associated factor 6 (TRAF6) adaptor protein,

Figure 2. The Toll-like Receptor MyD88-dependent Signaling Pathway



activating the TGF activated kinase and mitogen-activated protein (MAP) kinase kinase (MKK6) which activates NF-κB, c-Jun N-terminal kinase (JNK) and AP-1 and the p38 MAP kinase signaling pathways (reviewed in Armant and Fenton, 2002). This results in the production of pro-inflammatory cytokine production such as interleukins, TNF-alpha and of inducible nitrogen oxide synthesis (NOS-2) (Hallman et al., 2001).

The recognition of microbial PAMPs by TLRs may occur via interactions of a monomeric TLR with its ligand or via the homodimerization of the TLRs, as is seen with TLR2. Ligand recognition may also occur through cooperative binding between different TLRs forming heterodimers. This cooperative binding allows a greater repertoire of PAMPs to be recognized (Ozinsky, 2000). Both TLR2 and TLR6 have been shown to work cooperatively to bring about phagocyte activation through exposure to peptidoglycan, phenol soluble modulin (PSM), Outer surface A lipoprotein (Osp-L), and Soluble Tuberculosis Factor (STF) (Bulut, 2001).

The PAMPs recognized by TLR2 may explain how a variety of pathogens can interact with a single receptor, but does not explain the complex repertoire of TLR2 responses to pathogen-derived molecules, such as the activation of transcription factors, pro-inflammatory responses, MyD88-dependent and independent pathways, as well as modulation of Th1 and Th2 responses. How TLR-mediated immune responses are specifically tailored to particular pathogens is a key question that needs to be answered. One explanation is that TLRs can discriminate between complex molecular signatures that characterize specific pathogen subsets, and that the specificity of this subset is translated into an appropriate immune response. We have begun to explore this model by

asking what specifically at the molecular level, is the repertoire of residues or epitopes recognized by the TLR2-LRR.

Leucine Rich Repeat Proteins

TLRs are members of a growing super family of Leucine Rich Repeat (LRR) proteins that participate in reversible high-affinity interactions with other peptides. Leucine Rich Repeat proteins have been implicated in complex cellular processes such as hormone binding and cell adhesion, as well as signal transduction pathways (Kajava, 1998). The LRR domains of these proteins are highly conserved and a typical motif is characterized by 5-17 tandem arrays of a leucine-rich consensus sequence containing 20-29 leucine residues. A single repeat is typically characterized by the sequence LxxLxxLxLxxNxLxxLpxxoFxx, where x is any residue, p is any polar residue, and o is any nonpolar residue. This sequence represents an N-terminal beta-strand, an asparagine turn-region, and a C-terminal alpha helix (Kobe and Deisenhofer, 1994; Kajava, 1998).

The porcine ribonuclease inhibitor is a protein (Figure 3) consisting entirely of LRRs. The crystal structure of this protein indicates that each LRR is a modular unit, made up of a beta strand, an asparagine turn region, and an alpha helix. Tandem repeats are parallely aligned on a mutual axis (Kobe and Deisnhofer, 1994; Kajava 1998) resulting in a horseshoe shaped molecule consisting of parallel beta sheets along the inner portion of the domain, with the helices flanking the outer surface (Kobe and Deisenhofer, 1994). Ligand binding to the LRR is mediated by the interstitial non-consensus residues, occurring as areas on either surface of the LRR. The expansive solvent-exposed inner and outer surfaces may accommodate the simultaneous binding of multiple ligands. The

Figure 3. X-ray Crystallographic Structure of Porcine Ribonuclease Inhibitor Protein.

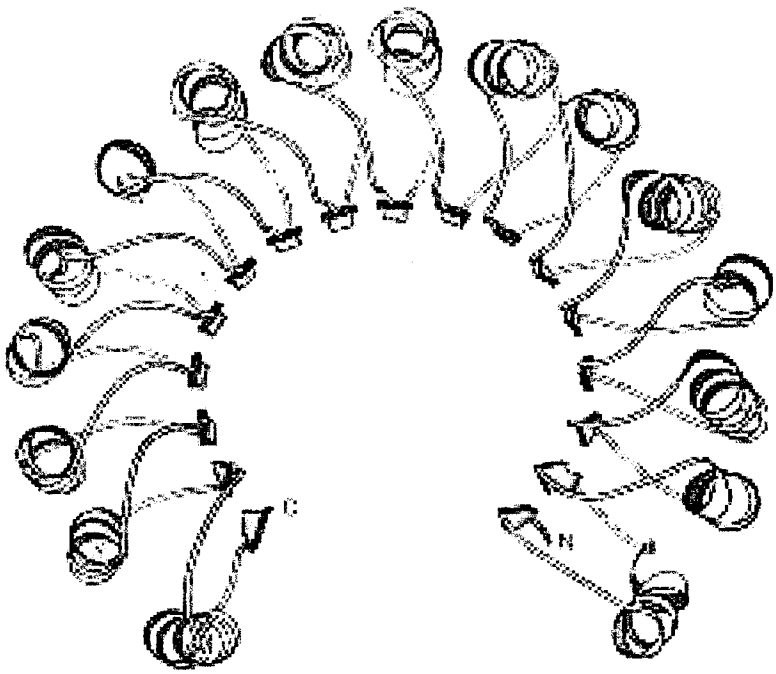


Figure adapted from Chen and Shapiro, 1997

conserved leucines within the LRRs are structural and not involved in ligand recognition. (Kajava, 1998, Clark et al.).

The yeast CCR4 deadenylase protein contains one partial and five complete LRRs (Malvar et al., 1992). A study determined that the LRR of CCR4 binds to its own deadenylase domain and deletion of this LRR resulted in the loss of CCR4's enzymatic function. Mutations in the beta-sheet surface of the CCR4-LRR resulted in a significant reduction in the proteins enzymatic activity (Clark et al., 2004, Meng et al. 2003). A small number of residues modulate ligand binding to CCR4-LRR, with multiple ligands able to bind simultaneously to the surface of a single LRR (Clark et al., 2004).

Toll-Like Receptor 2

TLR2 is expressed on monocytes, peripheral blood lymphocytes, polymorphonuclear leukocytes, and dendritic cells (reviewed in Hallman et al., 2001) and recognizes ligands from a wide variety of bacterial, fungal, and viral components. The repertoire of known TLR2 ligands is expanding, but it is not known if these ligands contain a common motif. Toll-like receptor 2 contains approximately 19 LRRs within its extracellular domain (Kirschning and Schumann, 2002) that are responsible for ligand recognition (Iwaki et al., Meng et al., 2004). The TLR2-LRR domain interacts with *Staphylococcus aureus* peptidoglycan, as well as various lipoproteins (Fujita et al., 2003; Mitsuzawa et al., 2001). It is not known if the TLR2-LRR alone is responsible for all interactions between TLR2 and its respective ligands.

The utility of developing new TLR2 directed agonist/antagonists is that human autoimmune diseases and antibiotic-resistant bacterial infections are on the rise, and a critical need exists to characterize receptor-ligand interactions that play key roles in

human health and develop novel therapeutic reagents designed to modulate these interactions. Expanded knowledge of specific peptide sequences recognized by the TLR2-LRR could reveal the existence of a common binding motif among PAMPs. Such motifs could represent novel targets for development of therapeutic molecules.

Phenol soluble modulin (PSM) is a known TLR2 ligand and is produced by *Staphylococcus epidermidis* (Hajjar, 2001) and other staphylococci species which have been implicated in catheter related sepsis. The development of a TLR2 antagonist could prevent PSM from initiating an inflammatory response, lessening the likelihood of sepsis.

Mycobacterium tuberculosis is a TLR2 agonist and the most common of the tuberculosis-causing pathogens. Tuberculosis affects more than 60 million individuals world wide, and kills approximately 10 million annually (Janeway, 2005). *M. tuberculosis* produces soluble tuberculosis factor (STF), a known TLR2 antagonist (Bulut, 2001). This bacterium is an intracellular pathogen that evades innate immune responses (Paul, 1993). Modulation of TLR2 via competitive inhibition by small therapeutic peptides could allow individuals to mount a more effective immune response against this pathogen. While this may not be a complete cure for the disease, peptide-based therapeutics could expand treatment options.

Atherosclerosis is another human disease in which TLR2 involvement contributes to pathology. Peptidoglycan (PG), a known TLR2 agonist, has been discovered in atherosclerotic plaques. One study found that arterial TLR2 when activated by the peptidoglycan found in the plaques contributed to plaque formation (Schoneveld et al., 2005). Therapeutically regulating TLR2 activation by PG could serve to prevent

atherosclerosis.

Preliminary Studies

In a preliminary study, a yeast two-hybrid system was used to identify the specific ligand residues that mediate binding to the CCR4-LRR. Twenty-one unique peptides from a random peptide aptamer library of $2x10^9$ possible clones were isolated based on their ability to bind to the CCR4-LRR. Through sequencing, a highly conserved consensus sequence consisting of [G/P]-(3)[L/V/I/F] was found in the majority of the peptides (Lisa Clark, personal communication). The findings suggested that a motif consisting of four residues could play a key role in the interactions of the peptide aptamers with the CCR4-LRR peptide-binding surface.

Based upon these results, a study was undertaken to determine whether a similar conserved motif could be found among TLR2-LRR-peptide ligands. A FliTrx ™ random peptide display library (Figure 4) was used as a tool to discover peptides that physically bind to the TR2-LRR. The FliTrx™ system contains approximately $1x10^8$ unique clones and functions by displaying random 12-mer peptides on the surface of *E.coli* flagella. The peptides are inserted into the active loop of the thioredoxin scaffold protein. The thioredoxin A peptide fusions are contained within the dispensable region of the major bacterial flagellar gene (FliTrx™) (Figure 4). Working with this peptide library led to the discovery of 17 peptides that bound to the TLR2-LRR. Sequence analysis showed that 11 of the 17 peptides contained a common motif of [Gxx(V/L/I)] (Tierney, 2005 Master's thesis). Five of these eleven peptides were designated JT1-JT5, synthesized, and used in cellular activation assays to determine whether they were biologically active (Table 1).

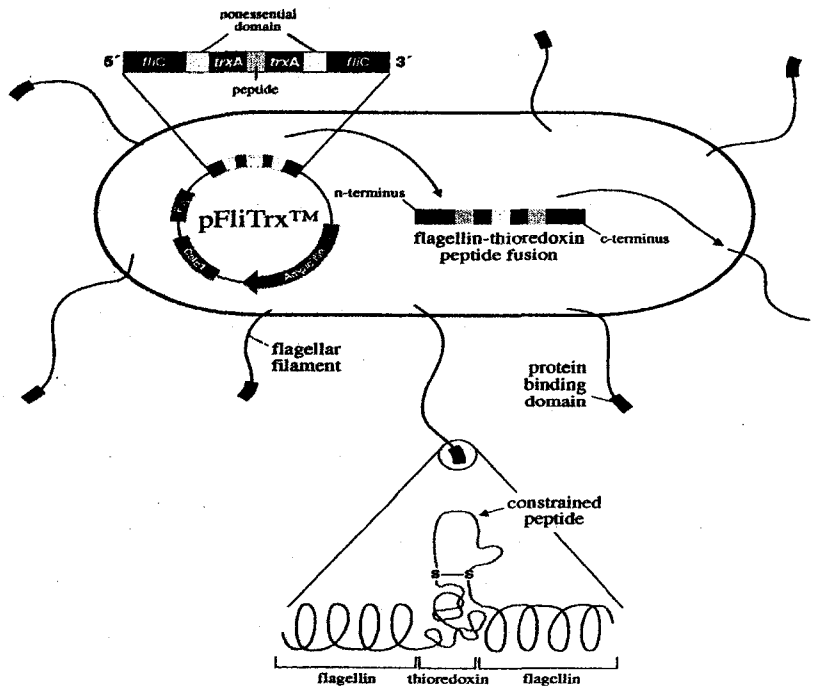
Peptide Therapeutics

The development of novel peptide-based therapeutics has been under intensive investigation in recent years. Peptide based drugs contain fewer than 100 amino acids (Wieland, 1995) and are easily degraded and rapidly removed from the circulation by the kidneys. The short life of these peptides can be advantageous, such as in peptide-based cytotoxic cancer therapy (Boerman, et al., 2000). The half-life of small peptides can also be extended using a variety of techniques, like glycosylation (Haubner et al., 2001) and serum albumin association (Koehler et al., 2002). Small molecule mimics of peptide ligands have been shown to compete and interfere with the binding of natural ligands of receptor-mediated binding interactions of therapeutic interest; for instance, in receptor-mediated binding that takes place in both the gastrointestinal and neuro-cranial systems (Mizejewski, 2001). Synthetic peptides can also provide the basis for the development of novel pharmaceuticals, such as drugs that are non-peptide in nature that mimic the actions of the peptide ligand. The low molecular-weight TIR peptide mimic of the TIR domain [(F/Y)-(V/L/I)-(P/G)] of the MyD88 adaptor protein involved in TLR signaling, has been shown to have a significant effect upon EL4 thyoma cells and murine lymphocytes. The TIR peptide mimic inhibited MAP kinase p38 phosphorylation by IL-1beta, and also led to a reduction in IL-1beta-induced fever responses in mice (Bartfai et al., 2003). The ability of a three-residue peptide mimic to have a significant biological effect indicates that it is possible to develop small peptide mimics from TLR ligands that have a significant therapeutic effect.

Specific Aims

The aim of this thesis project was to determine whether the five TLR2-LRR-binding

Figure 4. The FliTrx™ Random Peptide Display System.

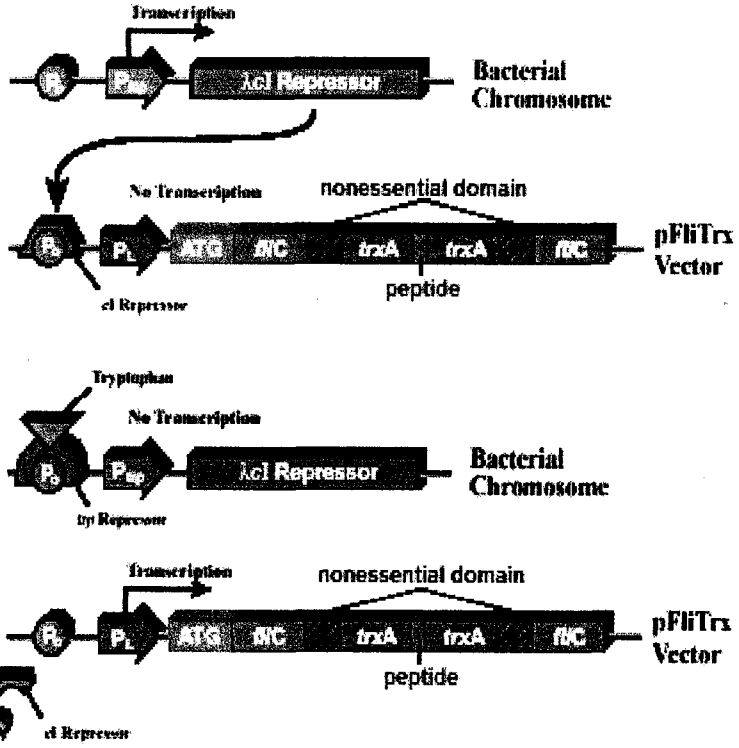


1. In the absence of tryptophan, expression of the cI repressor is driven by the *trp* promoter.

2. The cI repressor protein binds to the operator region upstream of the P_L promoter and prevents transcription of the FLITRX peptide fusion.

3. Tryptophan is added to the medium and a tryptophan-*trp* repressor complex is formed. This complex binds tightly to the *trp* operator blocking expression of the cI repressor

4. The cI repressor dissociates from the P_L operator, allowing transcription of the flagellin/ thioredoxin peptide fusion.



peptides synthesized based on the previous FliTrx™ study can have a significant biological effect on TLR2 activation in vitro. The biological effect of these peptides was tested, by examining their ability to induce BMDC maturation and macrophage activation in a series of *in vitro* assays.

The effect of the synthetic peptides on in vitro maturation of BMDCs was evaluated by flow cytometry and cytokine analysis. In this study, we determined that one synthetic peptide, JT1, significantly activated the BMDCs in vitro, as measured by increased IL-6 production and upregulation of the MHC class II marker. In contrast, we found that none of the peptides tested modulated the binding of *Salmonella typhimurium* to human macrophages. Finally, a TLR screening assay performed by an outside consultant (Invivogen Corp.) found that no significant upregulation of NF-κB occurred by TLR2 in response to any of the synthetic peptides.

Table 1. Synthetic Peptides and Their Sequences

Peptide	Sequence
JT1	C-WRFGAQIKGE-C
JT2	C-PRLPIVLAGA-C
JT3	C-MEGRGRVAE-C
JT4	C-LGVVSGRSYR-C
JT5	C-SRRIGKLGGLV-C

Table represents the identities and specific sequences of the 5 peptides used in this study. These peptides were chosen based on a preliminary study in which they were shown to physically bind to the LRR of TLR2.

CHAPTER II

MATERIALS AND METHODS

Bone Marrow-Derived Dendritic Cells

Femurs removed from two adult C 57 Black 6 micewerewashed by briefly soaking 15-ml of Roswell Park Memorial Institute (RPMI) 1640 medium. They were then transferred transferred to a sterile petri dish and soaked in 70% ethanol for two to three minutes, followed by two additional washes with RPMI 1640 medium. The femurs were transferred to a sterile petri dish and placed in a laminar flow cell culture hood and flushed with cold RPMI 1640 using a sterile 20-gauge needle attached to a 3cc syringe and the bone marrow was strained through a 40-μm cell strainer. The cells were pelleted at 1,000 x g for 10 minutes and resuspended in 10 mL of RPMI 1640 containing 5% granulocyte macrophage colony-stimulating factor (GMCSF) as a conditioned supernatant (GMCSF was a generous gift of the Brent Berwin laboratory. Dartmouth Medical School), 1% penicillin streptomycin, and 10% fetal bovine serum (FBS). The cells were again pelleted at 1,000 x gravity for ten minutes and resuspended in 5 mL of supplemented RPMI 1640. We maintained Primary BMDC cultures were for a total of eight days. On day zero the cells were seeded at approximately 0.5-1.0 x 10^6 cells/mL in a 24-well plate. The cells were washed and new medium was added every two days, with

stimulations occurring on day six.

U937 Culture

The U937 cells used in this study were purchased from the American Type Culture Collection (ATCC, #CRL-1593.2)) and were grown in 5% CO_2 at 37^0C. The supplemented media used was RPMI 1640 with 1% Penicillin and Streptomycin and 5% FBS. The cells were passaged every 4 to 5 days. The cells were fed every two days by addition of 15 mL of media to each flask.

TLR2-LRR-Binding Peptides

Synthetic peptides JT1-JT5 (Table 1) were synthesized and HPLC purified by EZ Biolab, Inc (Westfield, IN). Peptides were greater than 95% pure as determined by mass spectrometry analysis. Peptide stock solutions for this study were made by dilutions with endotoxin free water. All peptide dilutions were tested for endotoxin contamination using a Cambrex Limulus amebocyte lysate (LAL) (Pyrogent Plus^{TM}) gelation assay kit. Maximum levels of endotoxin contamination for peptides diluted to 100μg/mL were less than 60 EU/mL. Because peptide JT1 was determined to activate BMDCs in our studies, a follow up endotoxin test of JT1was conducted by the Associates of Cape Cod commercial laboratory, using kinetic chromogenic characterization. JT1 endotoxin levels as determined by the Associates of Cape Cod was .04-.06 EU/μg or 4-6 pg/mL. The lowest reported stimulation level for antigen presenting cells is 15 pg/mL (Reed, Berwin, Baker and Nicchitta, 2003).

Stimulation of Cells

The BMDCs were stimulated on the sixth day after explantation, using several different stimuli that included the non-biotinylated synthetic peptides JT1-JT5,

biotinylated versions of peptides JT1-JT5 with or without streptavidin cross-linking and LPS as a positive control. Initial experiments were performed using a variety of cell densities and peptide concentrations in an effort to determine optimal conditions for stimulation.

Thereafter, stimulations with the non-biotinylated peptides consisted of using each peptide at a concentration of approximately 50 μg/mL. The individual peptides were added to wells on the 24-well plate on day 6 after washing and feeding had been performed. The cells were allowed to incubate with the peptides for 36-48 hours. After incubation, the supernatants from each well were saved and the cells were harvested for analysis via flow cytometry. Cell harvesting consisted of repeated pipetting of the medium to loosen cells, then transferring the suspended cells to flow cytometry tubes. The tubes were centrifuged at 250x gravity for 10 minutes to allow the cells to pellet and the supernatant was collected and saved for analysis, and the cells were resuspended in 1x PBS to be stained for flow cytometry.

Biotinylated Peptides

The five synthetic peptides were biotinylated using the Pierce EZ-link ™ Sulfo NHS-LC Biotin kit according to the manufacturer's directions. The biotinylated peptides were separated from the biotinylating reagents using a 1-ml gel filtration column (sephadex G-15 resin, Sigma Product #G-15-120).

Cross-Linked Peptides

The biotinylated peptides were cross-linked using Neutravidin™-coated beads (Pierce) as follows. A packed volume of 250 μL of beads was placed in a sterile microcentrifuge tube together with approximately 1 mL of the biotinylated peptides at a

concentration of about 1mg/mL. Each protein-bead slurry was rocked at room temperature for 30 minutes; the beads were then washed with sterile 1 x PBS and resuspended in a 1:1 solution with cell culture medium. 250 µL from the final slurry suspension was added to individual wells of a 24-well culture plate. The cells were incubated for 36 to 48 hours and the supernatants were removed for cytokine analysis.

Invivogen NF-κB Study

The NF-κB activation study was performed by the Invivogen Corporation using a secreted alkaline phosphatase reporter gene under the control of a promoter induced by the activated NF-κB transcription factor. Details of the assay are available on the Invivogen Corporation website (www.invivogen.com). The stimulation of TLRs was assessed by measuring the NF-κB activation in HEK293 cells in response to each of the JT peptides. A panel of HEK293 cells transfected with individual TLRs 2,3,4,5,7,8 and9 was used and involved testing each TLR against all five peptides. A 96 well plate was seeded with 2.5-5.0 x 10^4 cells/ well with 20 µl (50µg/ml) of experimental peptide added for a total volume of 200 µl. The cells were incubated for 16-20 hours at 37^0C and OD_{650} was determined using a Beckman Coulter AD 340C Absorbance Detector. The assay for each peptide was performed in duplicate and data were compared against a panel of positive control reagents specific for a panel of TLRs.

***Salmonella* Binding Assay**

The U937 macrophage cells were activated by adding 1 x 10^{-9} M phorbol myristic acid (PMA) to the culture. Cultures were then incubated for 24 hours, and the cells were washed 3x with unsupplemented RPMI to remove the PMA. The cells were resuspended to a final concentration of 3x 10^5 cells/ml and 300 µl of the solution was seeded into each

well of an 8-chambered Lab-Tek ^{tm} glass slide. One of the experimental peptides was then added to each well at a concentration of approximately 100 μg/mL, with the exception of peptide JT1, which was added at ~33 μg/mL due to limited quantities of peptide. The slides incubated at 37^0C for 2 hours to allow the U937 cells to attach. The culture of *Salmonella typhimurium* was prepared by growing the cells aerobically for 8 hours, then inoculating 20 mL of media with 1 mL of the initial culture and incubating it anaerobically for ~24 hours. The bacterial cells were washed 3x in 1x HBSS and resuspended in 10 ml of 1x Hanks Buffered Salt Solution (HBSS), giving a concentration ~$1x10^9$ bacterial cells/ml. The U937 cells were incubated for two hours and the Salmonella were added at a concentration of ~$3x10^7$ cells/ml in seven of the eight wells. A chamber without bacteria was the negative control. The slides were incubated for 1 hour and stained differentially by the hemostat staining technique and viewed using a light microscope at 1000x oil immersion.

Toxicity Test

Toxicity testing of the five synthetic JT peptides was done using the U937 cell line. Approximately 2 ml of a $2.0x10^5$ cells/ml solution was seeded into three sets of six culture tubes (18 tubes total). We added each peptide to three tubes, at a concentration of 100ug/ml. We incubated the cells and determined viability at three intervals using one set of six tubes, (5 experimental and 1 control with no peptide) as one time point. Counts were done at 24, 48 and 65 hours using trypan blue exclusion dye.

Cytokine Analysis

All cytokine analysis was performed by the Dartmouth Hitchcock Medical Centers Immune Monitoring facility. Supernatant from stimulated BMDCs were loaded

onto a 96 well plate and were analyzed for cytokine content through multiplex enzyme-linked immunosorbant assays (ELISA) and quantified using a Luminex array reader at OD_{650}.

CHAPTER III

RESULTS

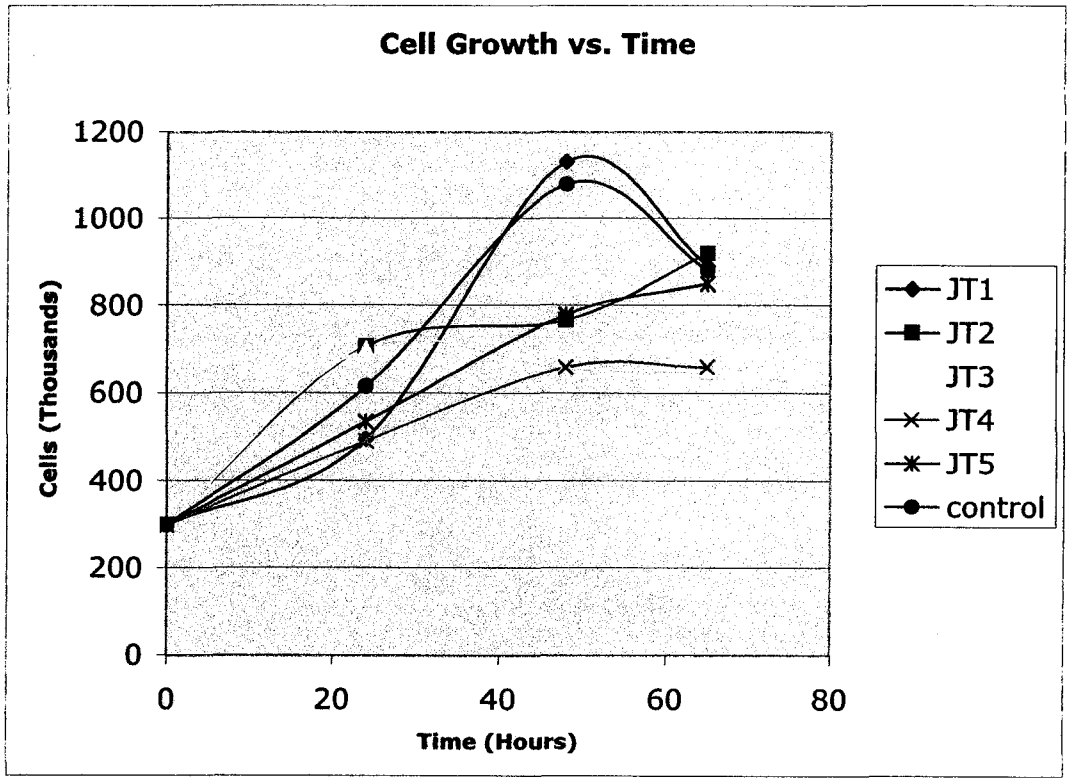
Toxicity Testing of TLR2-LRR binding peptides.

In vitro toxicity testing of the TLR2-LRR binding peptides was performed via growth curve analysis using the U937 cell line. We did not observe a significant decline in cell numbers in response to addition of the synthetic peptides at 100 μg/mL. The viability of the cells growing in the presence of the peptides JT3 and JT4 was approximately 30% lower than that of the other experimental groups after 48 hours. However, since no significant decline in cell viability was observed after the 24 hour incubation period relative to our experiments (Figure 5), we concluded that the peptides were not toxic and that stimulation experiments could proceed.

Flow Cytometry Analysis

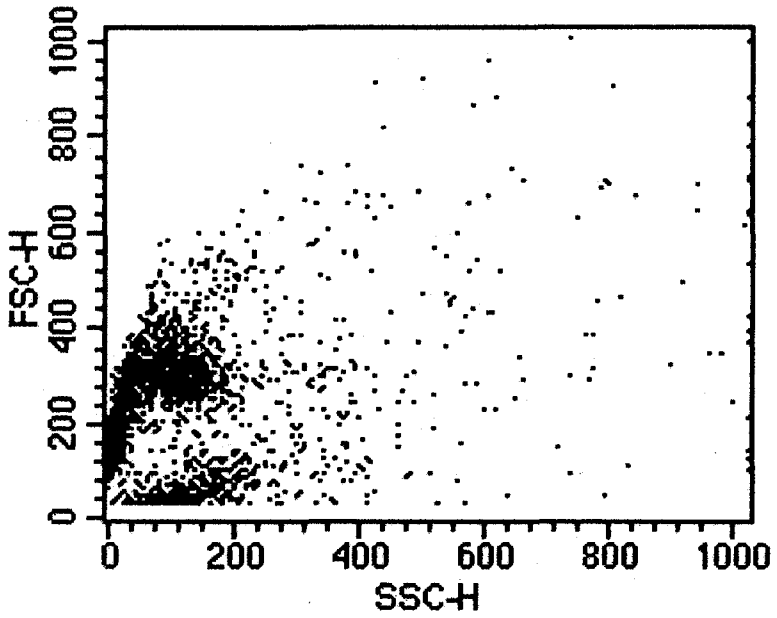
Flow cytometry analysis revealed that newly explanted bone marrow cells (figure 6) were significantly smaller than the same cells after 6 days incubation with GMCSF, and had lower percentage of BMDCs based on staining for the CD11c dendritic cell marker. The day six cells were more consistent in their forward and side-scatter profiles, which is typical of expansion BMDC's in culture with GMCSF (Brent.Berwin, personal communication). Low-level activation of the CD11c positive cells in response to peptide JT1 was observed by flow cytometry staining for the MHC class II maturation marker in several replicate experiments. No significant stimulation, as measured by increased

Figure 5. Growth of U937 Cells in the Absence or Presence of TLR2-LRR Binding Peptides

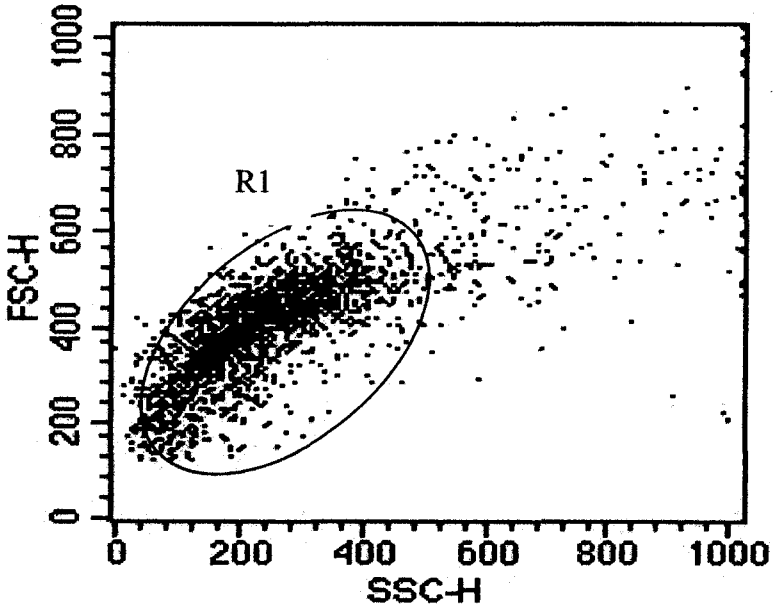


Graphical representation of the growth of U937 cells (thousands) *in vitro* with respect to time (hours) in the presence or absence of the 5 experimental JT peptides and a control (unstimulated) group. This graph represents a single trial of this experiment.

Figure 6.Forward Scatter Versus Side Scatter Profiles of Day 0 and Day 6 BMDCs.



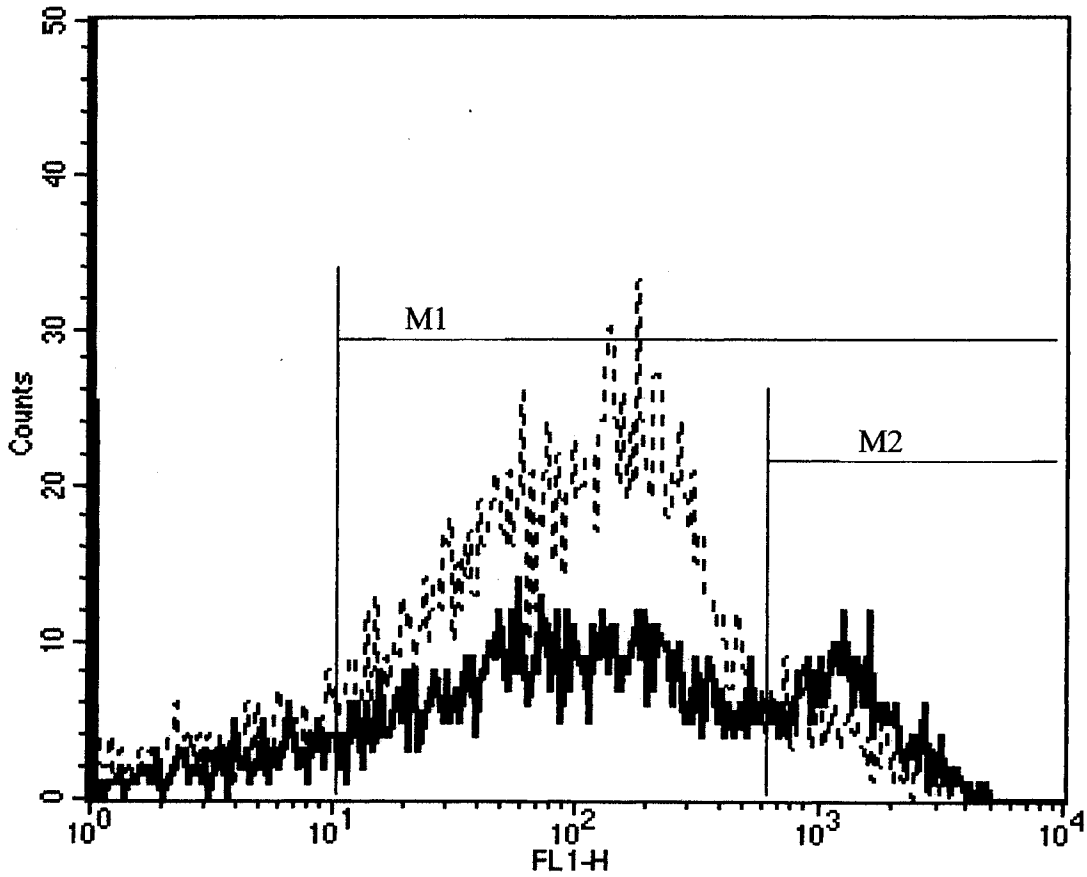
BMDC Day 0



BMDC Day 6

This figure displays the forward versus side scatter flow cytometry profiles performed on freshly explanted (day 0) or after 6 days in culture with GMCSF. Day 6 cells were larger in size and exhibited increased granularity and were more positive for the CD11c BMDC marker. The R1 region is the region containing the cell population of interest.

Figure 7.MHC-Class II Upregulation of Day 6 BMDCs in Response to stimulation with Peptide JT1.



A comparison between stimulated (solid line) and unstimulated (broken line) BMDC when stained with FITC conjugated MHC class II antibody. In this experiment, day 6 BMDCs were cultured for 36 hours in the presence or absence of peptide JT1 @ 50 μg/ml. Flow cytometry profiles are for cells on region (R1, figure 7) positive for the CD11c BMDC marker. In this experiment only 11.8 % of the unstimulated cells fell into the MHC class II very bright population (indicated by marker M2), relative to 22.9 % of the stimulated population. The median brightness of unstimulated cells positive for the MHC class II BMDC maturation marker (as indicated by marker M1) was 172; compared with the median brightness of stimulated cells which was 319.

Table 2.Compilation of Flow Cytometry Data

	JT1		JT2		JT3		JT4		JT5		No stim		LPS	
Cell Density	M2%+	M1	M2%+	M1	M2%+	M1	M2%+	M1	M2%+	M1	M2%+	M1	M2%+	M1
5.00 E5 / well	7.4	116.0							11.0	173.0	7.1	131.0	12.0	179.0
50ug/ml											7.6	141.0		
1.00 E6 / well	10.6	154.0							14.8	207.0	13.0	178.0	7.3	146.0
50ug/ml	7.4	122.0							17.4	228.0	7.5	126.0	17.6	225.0
	15.9	219.0									12.6	173.0		
1.50 E6 / well	22.9	319.0	3.4	45.7	5.0	50.0	2.8	41.0	13.4	188.0	11.8	172.0	14.5	220.0
50ug/ml	15.2	183.0	2.9	43.0	8.0	52.3			7.5	54.0	11.3	165.0	14.0	107.0
	15.1	80.6							2.0	43.5	7.0	53.2	13.6	113.0
	10.2	64.4									7.4	55.2		
											1.4	36.2		
2.00 E6 / well	5.6	73.0	7.0	101.0	6.4	82.0	12.2	143.0	4.8	84.3	5.7	88.2	13.4	142.0
50ug/ml	25.3	161.0	8.6	99.0	10.3	115.0	10.3	107.5	7.0	96.5	11.0	121.9	19.9	184.0
2.00 E6 / well	5.5	81.3	7.0	97.3	5.7	84.3	10.0	119.7	4.5	82.0	5.7	88.2	13.4	142.0
100ug/ml														
1.00 E6 / well	6.5	45.3	5.4	51.9	6.7	50.7	8.0	61.5	8.4	47.0	6.1	52.8	23.5	191.0
10ug/ml														

This chart represents a compilation of single peptide flow cytometry data. The data in the far left column represents the approximate BMDC density used in each well of a 24-well culture plate as well as the concentrations of the peptides used in the experiments. Each row represents the data collected for that particular density and peptide concentration. The data in each peptide column represent the percentage of gated cells that fell into the M2 shoulder population (left), and the median brightness levels in the gated population (right) for peptides JT1-5, unstimulated cells, and cells stimulated with LPS at 50 μg/mL. Blank cells indicated no data.

upregulation of MHC class II relative to non-stimulated controls was observed for the other peptides tested, JT2,3 and 4. peptides and negative control group. Figure 6 shows an increase in median brightness (FLI) of cells stimulated with peptide JT1 relative to unstimulated controls. The unstimulated control group showed a greater percentage of cells at a peak median brightness of 10^2 units (FLI) than the JT1 stimulated cells 10^3-10^4 units (FLI).

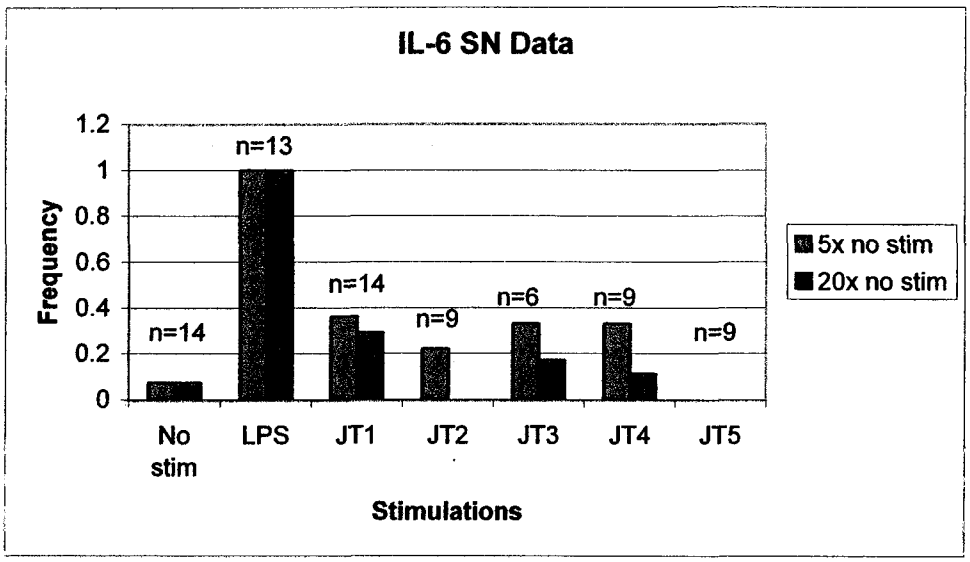
The stimulation data collected in multiple flow cytometry experiments exhibited a great deal of variability largely correlated with BMDC density (Table 2). Our data suggest that $1.5x10^6$ cells/mL was the most appropriate cell density for these experiments based upon observed greater sensitivity to LPS. Cells plated at this density showed the most consistent activation in the presence of peptide JT1 (Table 2).

During 12 flow cytometry experiments, peptides JT2-5 showed no MHC class II activation, having data similar to that of the negative control. The flow cytometry experiments staining for the upregulation of the B7 co-stimulatory marker were negative for all peptides, including JT1 as well as the LPS positive control.

Cytokine Analysis

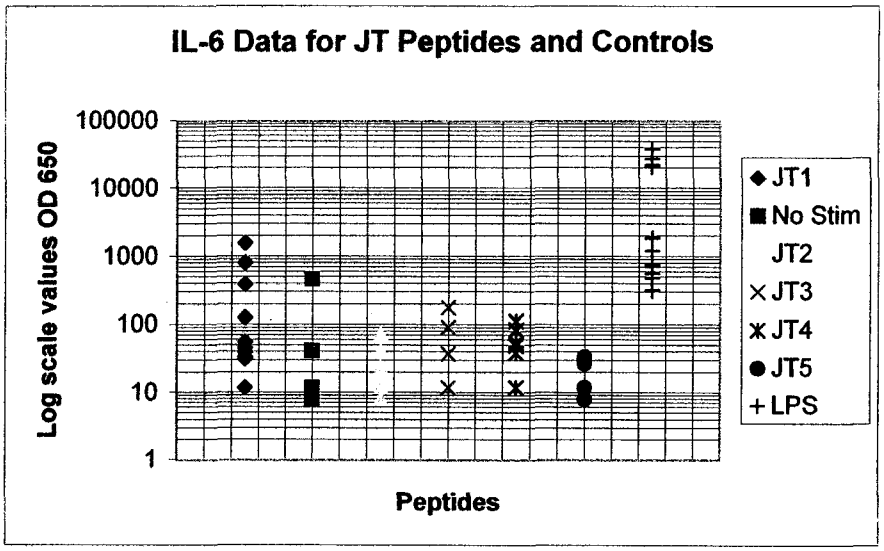
The supernatants from the BMDCs stimulated *in vitro* with TLR2-LRR-binding peptides were analyzed for levels of IL-6, IL-8, IL-1β, IL-12 and TNF-α. The cytokine analysis of the supernatants of cells stimulated with JT1 indicated showed significant levels of IL-6 in comparison to the unstimulated control (Figures 8 and 9). Statistical analysis of these data using a student's t-test with 13 degrees of freedom resulted in a t value of 1.42, suggesting that the difference between the JT1 values and the unstimulated

Figure 8. Frequency of IL-6 Secretion in Response to Peptides JT1-JT5



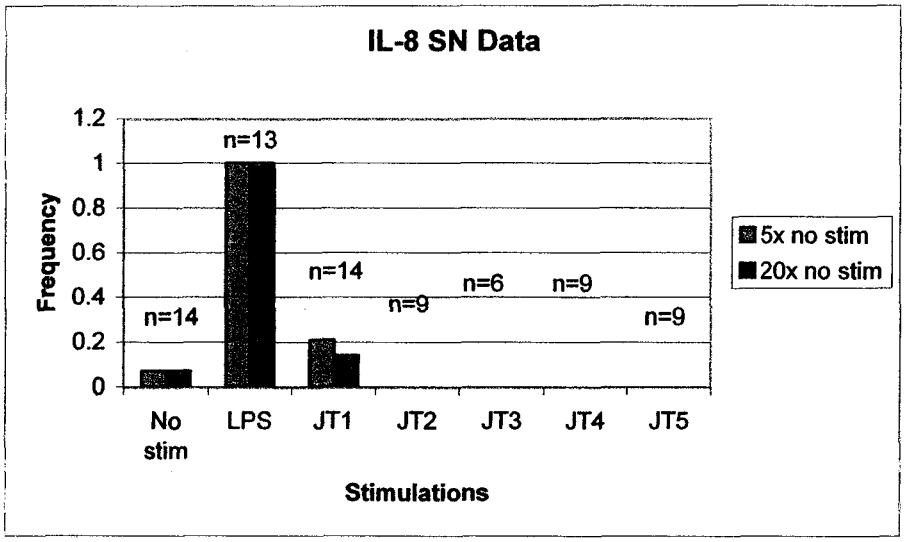
This is a graphical representation of the supernatant data collected for IL-6. Supernatants derived from BMDCs cultured with LPS, no peptides, or peptides JT1-JT5 were analyzed for IL6 as described. Bars represent the frequency of wells in each experimental group for which the IL-6 level detected (pg/mL) was greater than 5x (blue) or greater than 20x (red) the baseline detection level of 5 pg/mL. Number of replicate wells tested for each experimental group is indicated above the bars. All peptides stimulated a low level increase in IL-6 production, with the exception of JT5.

Figure 9. IL-6 Secretion in Response to Peptides JT1-JT5.



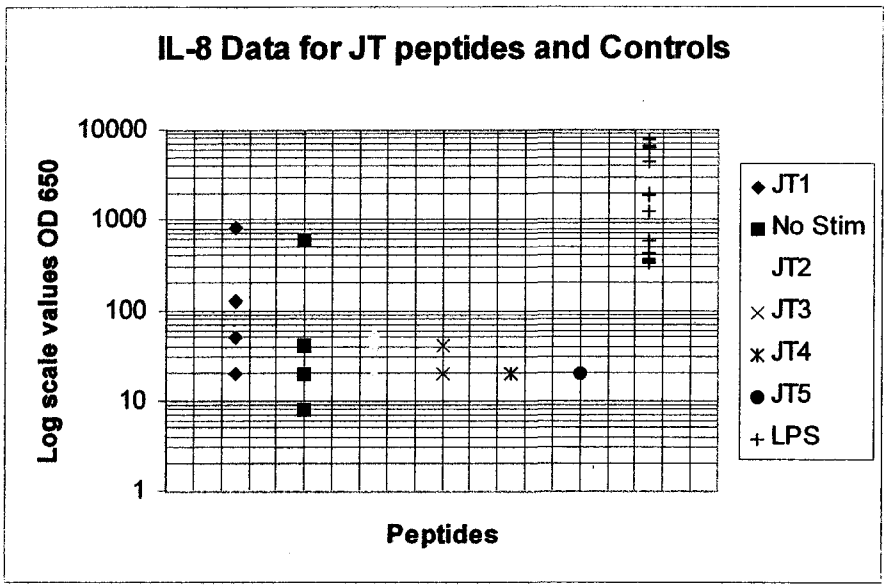
This graph is a log scale representation of the supernatant data collected for each of the 5 JT peptides as well as the two control groups in reference to IL-6 production. Values are based on multiplex spectrophotometric assay at OD_{650}. Based on these results, peptide JT1 was determined to be stimulatory for IL-6 production in several replicate experiments.

Figure 10. Frequency of IL-8 Secretion in Response to Peptides JT1-JT5



This is a graphical representation of the supernatant data collected for IL-8. Supernatants derived from BMDCs cultured with LPS, no peptides, or peptides JT1-JT5 were analyzed for IL-8 as described. Bars represent the frequency of wells in each experimental group for which the IL-8 level detected (pg/mL) was greater than 5x (blue) or greater than 20x (red) the baseline detection level of 5 pg/mL. Number of replicate wells tested for each experimental group is indicated above the bars. Secretion of IL-8 did not occur in response to peptides JT2, 3, 4 and 5.

Figure 11. IL-8 Secretion in Response to Peptides JT1-JT5.



This graph is a log scale representation of the supernatant data collected for each of the 5 JT peptides as well as the two control groups in reference to IL-8 production. Values are based on multiplex spectrophotometric assay at OD_{650}.

values are significantly different at a .179 confidence interval. The levels of IL-6 produced in response to JT1 were 5x and 20x times the lowest detection level limit at frequencies of 0.36 and 0.29 respectively (n=14 experimental wells), in contrast to the observed frequencies for the unstimulated control group at 5x and 20x which were .07 and .07 respectively (n=14), and 100% of all wells stimulated with LPS exhibited IL-6 levels greater than 20x the background detection level (n=13). Some increased IL-6 activation was also seen with peptides JT2, 3, and 4, but not with JT5 (Figure 8).

The levels of IL-8 produced in response to JT1 had 5x and 20x the baseline detection limit at frequencies of 0.21 and 0.14. In contrast no increased IL-8 production was observed in response to JT2,3,4 and 5 (figure 10).

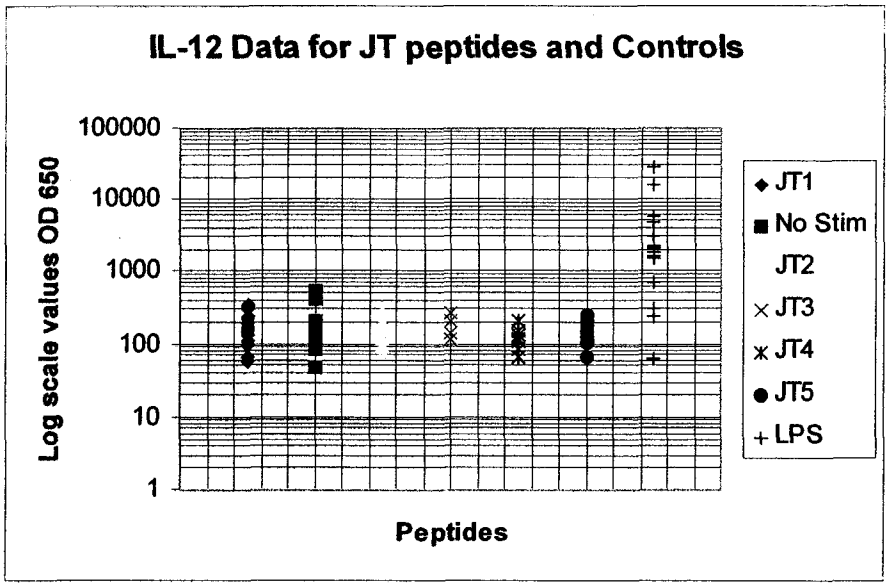
No increased production of IL-12 or IL-1β for BMDCs cultured with any of the JT peptides. Increased production of IL-12 (Figure 12) occurred only in response to LPS. Production of IL-1β (Figure 13) was not detectable in response to any of the stimuli.

Cytokine results for TNF-α (figure 14) were positive in response to JT1 and JT2. We found frequency stimulation values 5 and 20 times the baseline detection limit at frequencies .21 and .21 for JT1, and .22 and .11 for JT2. The activation in response to these peptides was not statistically significant.

Invivogen NF-κb Study

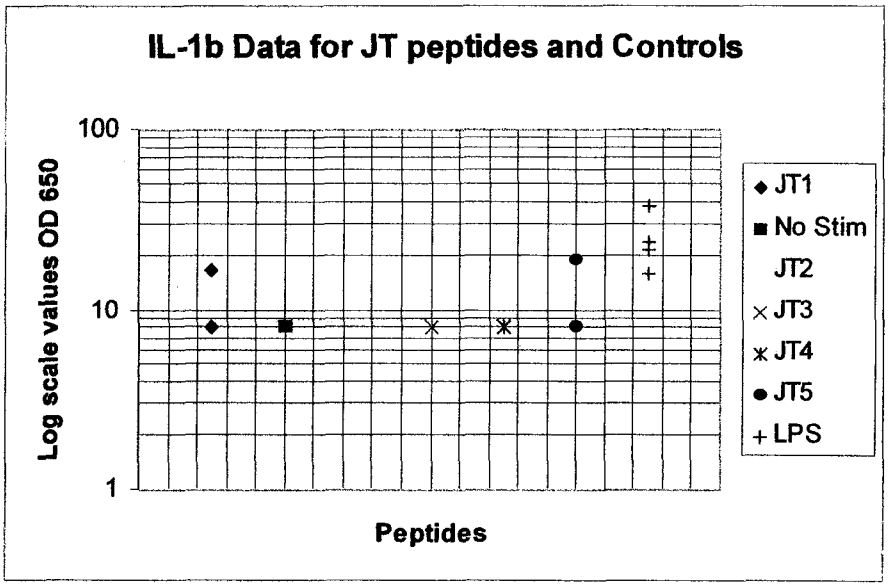
The Invivogen Corporation determined NF-κB activation did not occur at a significant level in response to the TLR2-LRR-binding peptides. The assay used individual HEK293 cell lines expressing seven different TLRs: TLR2,3,4,5,7,8 and 9 (Table 3; Figure 15). NF-κB Activity was monitored by measuring the OD_{650} of a secreted alkaline phosphatase reporter, which was induced by NF-κB. This assay was

Figure 12. IL-12 Secretion in Response to Peptides JT1-JT5.



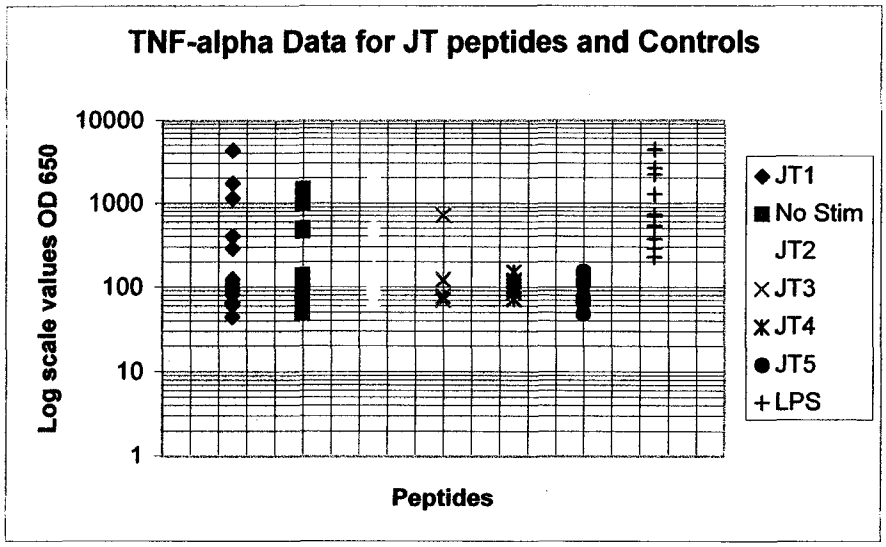
This graph is a log scale representation of the supernatant data collected for each of the 5 JT peptides as well as the two control groups in reference to IL-12 production. Values are based on multiplex spectrophotometric assay at OD_{650}.

Figure 13. IL-1β Secretion in Response to Peptides JT1-JT5.



This graph is a log scale representation of the supernatant data collected for each of the 5 JT peptides as well as the two control groups in reference to IL-1B production. Values are based on multiplex spectrophotometric assay at OD_{650}.

Figure 14. TNF-α Secretion in Response to Peptides JT1-JT5.



This graph is a log scale representation of the supernatant data collected for each of the 5 JT peptides as well as the two control groups in reference to TNF-α production. Values are based on multiplex spectrophotometric assay at OD_{650}.

Table 3. Results of Invivogen NF-κB Study

Results

Results are provided as OD (650nm) values

Screening #1

293/TLR Cell Line	No Ligand	JT1	JT2	JT3	JT4	JT5	Control +
hTLR2	0.091	0.144	0.109	0.141	0.093	0.097	2.654
hTLR3	0.161	0.154	0.163	0.142	0.150	0.130	2.454
hTLR4(MD2-CD14)	0.090	0.093	0.081	0.079	0.080	0.076	2.187
hTLR5	0.113	0.133	0.143	0.163	0.115	0.120	2.826
hTLR7	0.075	0.074	0.070	0.063	0.071	0.065	2.356
hTLR8	0.160	0.139	0.144	0.146	0.145	0.136	2.165
hTLR9	0.159	0.123	0.116	0.109	0.116	0.118	2.612
Parent cells	0.080	0.078	0.080	0.077	0.075	0.074	2.316

Screening #2

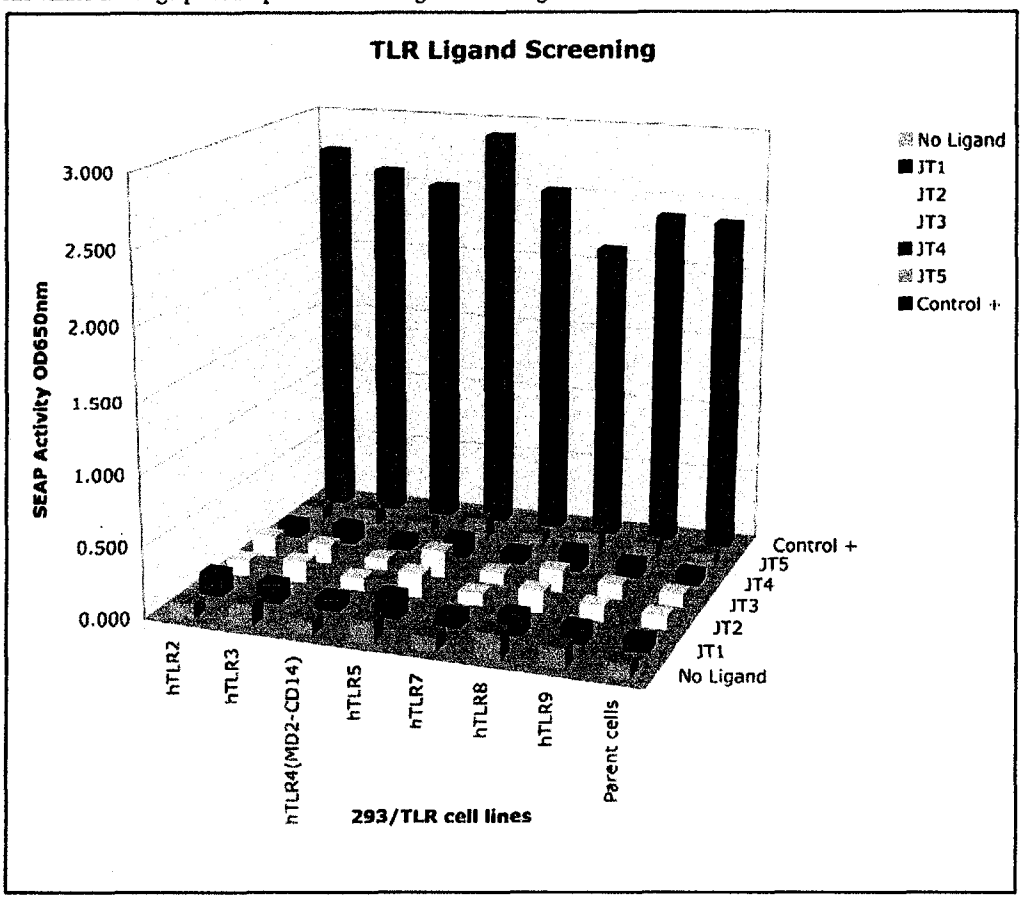
293/TLR Cell Line	No Ligand	JT1	JT2	JT3	JT4	JT5	Control +
hTLR2	0.106	0.174	0.119	0.155	0.111	0.105	2.813
hTLR3	0.137	0.115	0.144	0.128	0.119	0.119	2.749
hTLR4(MD2-CD14)	0.095	0.090	0.099	0.081	0.081	0.075	2.837
hTLR5	0.183	0.192	0.202	0.218	0.166	0.190	2.973
hTLR7	0.116	0.097	0.100	0.104	0.096	0.094	2.698
hTLR8	0.196	0.146	0.167	0.158	0.148	0.151	2.093
hTLR9	0.124	0.101	0.106	0.103	0.106	0.099	2.172
Parent cells	0.109	0.098	0.103	0.101	0.095	0.098	2.438

Final concentration of samples is 50μg/mL

Tables represent two identical screenings measuring NF-kB activation at OD_{650} as described in materials and methods. The activation of peptides JT1 through 5 and their effect on hTLRs: 2, 3, 4, 5, 7, 8, and 9.

Figure 15. Graphical Results of NF-κB Study

The values on the graph corresponds to an average of screening 1 and 2



Final concentration of samples is 50μg/mL

Control ligands

hTLR2: HKLM (heat-killed *Listeria monocytogenes*) at 10^8 cells/ml
hTLR3: Poly(I:C) at 1 μg/ml
hTLR4: *E. coli* K12 LPS at 100 ng/ml
hTLR5: *S. typhimurium* flagellin at 1 μg/ml
hTLR7: Loxoribine at 1 mM
hTLR8: ssRNA40 at 5 μg/mL
hTLR9: CpG ODN 2006 at 1 μg/ml
Parent cells: PMA 10μg/mL (parent cells have no TLR and serve as control)

marginally positive in response to JT1, 2 and 3 through TLR2, however this effect was not statistically significant. The response to peptides JT4 and JT5 was negative, with values at or below the unstimulated control cells. Of the three peptides that did show some low level activity in duplicate screenings, the most activity was observed in response to JT1. There was no activation seen through TLRs 3,4,5,7,8, and 9 in response to any of the peptides.

***Salmonella* Binding Assay**

The *Salmonella* binding assay indicated that the JT peptides are not stimulatory to the U937 cells, and that the JT peptides do not block the binding of a natural TLR2 ligand (Table 5).

Table 4. Summary of *Salmonella* Binding Assay

Experiment 1

Experimental	Phagocytic Activity	Phagocytic Index
JT1 33μg/mL	26%	3
JT2 100μg/mL	14%	2.7
JT3 100μg/mL	10%	2
JT4 100μg/mL	8%	2
JT5 100μg/mL	8%	2
No Peptide	4%	2
No Peptide	16%	2
No Bacteria	0%	0

Experiment 2

Experimental	Phagocytic Activity	Phagocytic Index
JT1 100 μg/mL	38%	4.4
JT1 67μg/mL	56%	4.2
JT1 33μg/mL	44%	3.1
JT1 17μg/mL	50%	4.
JT1 1mg/mL	54%	4.4
No Peptide	42%	3
No Peptide	42%	3
No Bacteria	12%	2.3

Experiment 1 summarizes data gathered from the *Salmonella* binding to U937 cells in response to the five JT peptides. Experiment 2 summarizes the data gathered when U937 cells were stimulated with varying concentrations of peptide JT1. Percentages represent the number of U937 cells positive for 2 or more bound *Salmonella*. The phagocytic index represents the average number of bound *Salmonella* to positive U937 cells.

CHAPTER IV

DISCUSSION

The objective of this thesis was to evaluate the biological effect of five synthetic TLR2-LRR binding peptides through flow cytometry and cytokine profiling. We hypothesized that the peptides may be able to either elicit, or down-regulate an innate immune response, as has been seen with a small peptide TIR mimic (Bartfai et al 2003). The flow cytometry results showed variable BMDC activation in response to JT1 and the cytokine analysis indicated that JT1 induces IL6 production. The assay performed by the Invovogen Corporation determined that no significant NF-κB upregulation occurred in response to the peptides.

Flow Cytometry

Although the results of the BMDC stimulation experiments were variable and dependent on cellular density, peptide JT1 was found to induce BMDC maturation in multiple experiments. At cell densities of $1.5x10^6$ cells/well and peptide concentrations of 50 μg/mL peptide JT1 was as effective as LPS in upregulation of MHC class II. The MHC class II very bright population (indicated by M2 in Figure 6) averaged 15.8% (Table2, n=4) for JT1, and 14.0% (n=3) for LPS, versus 7.7% (n=5) for unstimulated controls. In contrast, BMDCs stimulated with peptides JT2-5 were not significantly different from unstimulated controls in selected experiments. Some stimulation of BMDCs plated at $1.00x10^6$ and $2.00x10^6$ cells/wellwas also observed.

We observed an inconsistent stimulatory effect in response to peptide JT1at a concentration of 50μg/mL. Cell counting was performed before seeding the cells, but some variation in cell densities occurred, causing variability in BMDC activation experiments. Variability of BMDC cultures has been observed in the Berwin lab (personal communication) because of "over-handling" of cells, such as feeding and washing, causing the BMDCs in culture to activate. The peptides may have formed multimeric complexes, varying activation results. This would be difficult to control as several independent dilutions of the peptides were used. In order for consistent activation to be seen, it may be necessary to cross-link the peptides.

We performed most of our stimulations with peptide JT1, due to early success in its activation of BMDCs. More assays with the other JT peptides may have shown eventual BMDC activation. Future work would include further experimentations with the other synthetic peptides and a repeated attempt to cross-link.

Stimulated BMDCs were also stained for flow cytometry using the B7 maturation marker, but no activation was observed in response to the peptides or LPS. Upregulation of the B7 co-stimulatory marker may require more optimal conditions, or may occur through an independent pathway than through TLR2. Due to time constraints, we used the MHC class II marker for BMDC maturation, as we had already seen activation of this marker in response to the peptides and LPS control.

We designed several lab experiments to examine the effects of cross-linking of the peptides on BMDC activation. One experiment was designed based on streptavidin's four biotin binding sites. Biotinylated peptides were added to BMDCs being cultured in streptavidin coated wells. Flow cytometry data indicated that no activation had occurred

in response to any of the peptides. This required using a 96 -well plate system instead of 24-well plates. This added variable changed the conditions of the cells being cultured, and was not optimized due to time constraints. Any future efforts with these peptides would involve more focus on cross-linking.

Attachment of the peptides to streptavidin-agarose beads was performed to cross-link the peptides, such that the beads would represent a type of psuedo-bacterium. The large size of the beads was inappropriate for flow cytometry analysis because of bead auto-fluorescence (Alice Givan, personal communicatio). Cytokine analysis of these supernatants yielded no positive results. The results do not necessarily indicate that cross-linking of the peptides cannot activate the cells; it is more likely that the preliminary cross-linking performed in lab were not optimal. Another variable is the degree of biotinylation of the peptides. If these experiments were to be repeated, the peptides would be commercially synthesized and biotinylated for consistency. Another possibility would be to use smaller beads more appropriate for flow cytometry analysis.

Cytokine Analysis

The supernatants for all stimulation experiments underwent cytokine analysis at the DHMC Immune Monitoring Facility. All supernatants were examined for a series of cytokines, which included IL-6, IL-8, IL-1B, IL-12 and Tumor Necrosis Factor Alpha (TNF-α). In these assays, IL-6 was consistently and significantly upregulated when cells were cultured in the presence of JT1. We evaluated this using a Students T-test and JT1 treatment proved to be statistically significant compared to the unstimulated control (t-value of 1.42, n=13); the confidence interval was 0.179, indicating less than a 20% chance that the observed difference was due to random sample error. These data

represent two different screenings for IL-6 with 14 individual test wells where JT1 was used to activate the BMDCs. We interpret these results as positively indicating that JT1 does play a biological role in the upregulation of IL-6 by BMDCs.

Some low level IL-6 production was observed in response to peptides JT2, 3, and 4. This indicates that activation of IL-6 was a specific effect of JT1 and not just due to the presence of a foreign peptide in culture. More data points could have strengthened the statistical analysis as several experiments of poor activation data in regards to the positive control were included in the T-test.

The IL-8 cytokine analysis indicated marginal activation with JT1, but not with any of the other peptides, however this effect was not found to be statistically significant. Slight TNF-α production was also seen in response to JT1 and JT2. The analysis for IL-12 and IL-1b was negative for all the peptides. The ability of some peptides to upgregulate certain cytokines and not others indicates the specificity of the cytokine profiles produced in response to particular PAMPs.

Invivogen Studies

Because the stimulation of all TLRs is known to result in upregulation of the NF-κB transcription factor, making it was of interest to determine whether the JT peptides could also activate NF-κB. In assays performed for this study by the Invivogen Corporation, translocation of NF-κB was observed in cells expressing only TLR2, in response to co-culture with peptides JT1, 2 and 3. The effect was only slight, with the most activation observed in response to peptide JT1. When compared to the positive control groups the degree of activation in response to these peptides was statistically insignificant. However, in duplicate assays, the response was clearly greater than that

seen with the negative control, therefore JT1 may have some minimal biological effect on TLR2 activation. In future studies, it would be of great interest to determine whether activation of NF-κB would be enhanced by biotinylated peptides if optimally cross-linked. It is possible that the NF-κB results observed reflect lack of optimization of the assay.

***Salmonella* Binding Assay**

The *Salmonella* binding assay was performed to examine the ability of the peptides to effect the binding of the human macrophage U937 cell line to *Salmonella typhimurium* by using all five synthetic peptides, as well as a negative control containing no peptide, and a control that contained only U937 cells. The only peptide that increased increase both the phagocytic activity as well as the phagocytic index of the macrophages was JT1. This peptide was added at a lower concentration (33μg/mL) due to low amounts of stock JT1. An experiment with varying concentrations of JTI, did not indicate an increase in binding of bacteria at a significant level. If this were repeated, another attempt would be made to optimize the amount of peptide required to effect U937 binding. The peptides do not block the binding of a natural ligand and there was no difference between the positive control cells and peptides JT2, 3, 4 and 5 in regards to phagocytic activity or index.

More careful testing is needed, as the negative control cells that contained no bacteria were positive for some binding. I was careful to maintain proper aseptic technique but some contamination of the wells did occur. This was likely due to the close proximity of the wells containing the bacteria with the negative control well and that the same reagents were used for all wells.

Future Directions

Based on the results of this study, we anticipate that cross-linking the peptides is likely to be the most effective way to continue studying the biological role of the JT peptides in BMDC activation. Experiments involving different functional endpoints, such as cell division, or the up-regulation of other cell surface markers would also be appropriate, as would flow cytometry experiments which focus on staining for the B7 co-stimulatory markers.

Since only JT1 was observed to have a biological function and not JT2,3,4,or 5, the conclusion can be drawn that the motif (GxxV/L/I) was not relevant. All of the peptides had the same number of residues and only differed in the location of the motif and the residues that surrounded it. If the motif allowed the peptides to interact with TLR-2, all of the peptides should have caused BMDC maturation and cytokine secretion. Future work with these peptides could determine why JT1 has a biological effect, while the other peptides do not. This could be done by substitutions and deletions in the JT1 peptide sequence.

The IL-6 cytokine results and other data collected from stimulations with peptide JT1 a significant biological role. Other published TLR studies have only determined the ligands responsible for TLR activation at the macromolecular level (Bulut, 2001, Hajjar, 2001, reviewed in Takeda and Akira, 2001) and not the specific peptide sequences involved. It is known that activation of the TLRs upregulates NF-κB production (Hallman et al., 2001), what is not known is how a ligand is able to shape cytokine profiles.

We have shown that IL-6 production occurs in response to peptide JT1, and not the other peptides. Of the several cytokines we looked at, IL-6 was the only one produced in response to JT1 at a significant level. IL-6 was initially thought to be only be an inflammatory cytokine, however it has been shown to inhibit TNF-α production, as well as T-lymphocyte and macrophage function. The presence of IL-6 has also been shown to markedly reduce NF-κB activity and augment immunoglobulins (Wheeler et. al, 1999, Hegde et. al, 2004). Inhibition of inflammatory cytokines such as TNF-a has been shown to improve both organ function and survival in animal sepsis models (Wheeler et. al, 1999). The role of TLR2 activation in an immune response could be to downregulate an inflammatory immune response, while promoting an adaptive response to occur.

This study has increased the current understanding of ligand recognition by TLR2, as well as the ability of this receptor to shape an immune response through production of IL-6. This knowledge will be beneficial in future studies with TLRs, as well as in the development of novel peptide based therapeutics.

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APPENDIX

UNIVERSITY of NEW HAMPSHIRE

January 11, 2006

Clark, Lisa
Microbiology, Rudman Hall
Durham, NH 03824

IACUC #: 051203
Approval Date: 12/22/2005
Review Level: B

Project: Dendritic Cell Maturation by Novel TLR2-LRR-Binding Peptides

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building, 51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564