Effects of Bacterial Products on Human Blood Leukocytes

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Effects of Bacterial Products on Human Blood Leukocytes.

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DEDICATION

I would like to dedicate this to two groups of people without whom I may not have made it thus far. First, I would like to thank my family. I want to recognize my paternal grandmother who suffered from sepsis and was never the same after. She was a wonderful person who taught me many things. I would like to acknowledge my parents and sister, who have given me support and understanding throughout my life. I appreciate all the opportunities you have given me. You have done so much for me over the years that I cannot begin to list them nor express my gratitude. I know I could not have always been easy going. The second group consists of my greatest friends, Jen and Carrie. If it wasn't for a stupid game late one night I may not be here. Thank you for setting me straight.
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INTRODUCTION

In 2002 and 2003 septicemia was ranked as the 10th leading cause of death for the overall U.S. population and 9th for children, aged 1-19 y (Hoyert, Mathews et al. 2006). Over a 22 year period, Martin et al identified 10,319,418 cases of sepsis with an 8.7% increase in the incidence of sepsis (Martin, Mannino et al. 2003). It is also reported that the long term quality of life of sepsis survivors is significantly lower than that of the general U.S. population (Heyland, Hopman et al. 2000) and the annual cost of treatment of severe sepsis is approximately $16.7 billion (Angus, Linde-Zwirble et al. 2001).

Sepsis is the systemic response to infection characterized by an increase in temperature, heart rate, and a change in white cell counts. It can present with organ dysfunction and lead to severe sepsis and septic shock (Bone, Balk et al. 1992). Tumor necrosis factor $\alpha$ (TNF-$\alpha$) is one of the first cytokines detected in models of sepsis where Escherichia coli (E. coli) are infused. TNF-$\alpha$ is closely followed by an increase in Interluekin-1 (IL-1) and is characterized by nausea, fever, and headaches (van Deventer, Buller et al. 1990; Taylor, Chang et al. 1991). Infusion of TNF-$\alpha$ can mimic the symptoms seen after infusion of endotoxin (Michie, Spriggs et al. 1988). It can induce a shock-like state leading to pulmonary inflammation, organ failure, and death (Tracey, Beutler et al. 1986; Tracey, Lowry et al. 1987; Johnson, Meyrick et al. 1989). Use of a monoclonal antibody to TNF-$\alpha$ protects against shock (Tracey, Fong et al. 1987), prevents the appearance of Interleukin-1$\beta$ (IL-1$\beta$) and Interleukin-6 (IL-6) (Fong, Tracey et
al. 1989), but can not inhibit coagulation and actually decreases fibrinolysis (van der Poll, Levi et al. 1994).

For these reasons it is important to understand the mechanisms leading to the release of TNF-α. Release from leukocytes and the subsequent systemic response is due to the shedding of bacterial products from an infection site into blood and lymph. LPS (lipopolysaccharide) and fMLP (N-Formyl-Methionine-Leucine-Phenylalanine) are, for example, two powerful bacterial products that can induce the release of cytokines. The signaling cascade, via Toll-like-receptor 4 (TLR4), is initiated upon LPS binding and culminates in activation of nuclear-factor κ B (NF-κB) (Opal and Huber 2002). fMLP can cause chemotaxis by signaling through the formylpeptide receptor (FPR) (Schiffmann, Corcoran et al. 1975) which is a seven transmembrane, G-protein coupled receptor (Boulay, Tardif et al. 1990; Boulay, Tardif et al. 1990).

Through the use of mutants and wild type FPR, and the wild type TLR4, we have shown that fMLP and LPS in combination can increase TNF-α mRNA in a synergistic manner. Mutations of the FPR can increase or decrease the transcription of TNF-α compared to the wild type receptor. The synergistic effect seen in TNF-α mRNA, however, is not seen in the synthesis and secretion of the protein. We have also determined a possible role for G_i-protein in TNF-α induction by LPS and calcium mobilization.
LITERATURE REVIEW

Sepsis

In 1991, The American College of Chest Physicians/Society of Critical Care Medicine met with a specific goal in mind. It was their opinion that a set of definitions needed to be agreed upon that could be applied to patients with sepsis and its symptoms. Up until then the term “sepsis” was used in a broad sense to describe a systemic inflammatory response to infection. Bone et al describes the historical reasoning behind a lack of established definitions; until the mid-1960’s patients with sepsis and its associated symptoms were rarely seen, sepsis was considered to be primarily due to Gram-negative bacteremia, molecular and cellular events in sepsis have only recently been elucidated, and a lack of epidemiological studies (Bone, Sibbald et al. 1992). The conferences first recommendation was to introduce a new phrase, “Systemic inflammatory response syndrome (SIRS)”. SIRS is used to describe the systemic inflammatory process regardless of its cause. A diagnosis of SIRS indicates a patient that has more then one of the following symptoms; a body temperature greater than 38°C or less than 36°C, a heart rate greater than 90 beats per minute, increased respiration, or changes in the white blood cell count. It can manifest from several causes including infection, multiple trauma, ischemia, or immune-mediated organ injury. A diagnosis of sepsis can only be used when a confirmed infection is present with the above symptoms (Bone, Balk et al. 1992).
The conference further defined sepsis into clinically distinct stages. If sepsis is present with organ dysfunction, sepsis-induced hypotension, or hypoperfusion abnormality, such as lactic acidosis or an alteration of mental status, it was termed severe sepsis. The diagnosis of septic shock is used when a patient has severe sepsis persisting in the presence of adequate fluid resuscitation. Also accompanying these conditions is hypoperfusion abnormalities and organ dysfunction (Bone, Balk et al. 1992). A prospective study found that SIRS and its related conditions, sepsis, severe sepsis, and septic shock, “represent a hierarchical continuum of increased inflammatory response to infection” (Rangel-Frausto, Pittet et al. 1995).

Sepsis is gradually becoming a significant agent of death, making it important to study the causes, symptoms, and molecular sequences of sepsis. In 1991, the CDC listed septicemia, the presence of microorganisms or their toxins in the blood, as the 13th leading cause of death in the United States (NCHS 1993). Less then 20 years later septicemia is ranked as the 10th leading cause of death for the overall U.S. population and 9th for children aged 1-19 years (Hoyert, Mathews et al. 2006). The annual total costs for hospitalization of patients with severe sepsis in 1995 was $16.7 billion (Angus, Linde-Zwirble et al. 2001) and could range from $38,000 to $50,000 for individuals with Gram-negative sepsis (Chalfin, Holbein et al. 1993). A study of hospital data over a 22 year period revealed that the overall mortality rate for patients with sepsis was decreasing, however, the incidence of sepsis and number of sepsis related deaths increased.
The same study found that the average age of patients with sepsis had steadily increased over time, from 57.4 years to 60.8. The causative agents of sepsis also seem to be changing over time also. Between the years 1979 and 1987, Gram-negative bacteria were the predominant cause of sepsis, however, cases of Gram-positive sepsis have steadily increased and in 2000 accounted for >50% of all cases (Martin, Mannino et al. 2003).

Several approaches are made to help a patient survive sepsis. The initial resuscitation of a patient includes increasing central venous oxygen and blood pressure. After bacterial cultures have been taken, the patient should undergo appropriate antibiotic therapy. If sepsis is due to a localized infection, removal of the device, debridement, or drainage should be utilized to help control the infection. When appropriate, measures should be taken to treat the secondary symptoms of sepsis and shock. This includes fluid therapy, vasopressors, and steroids (Dellinger, Carlet et al. 2004).

Antibiotics are administered to almost all patients with a suspected infection, even if the results of cultures have not come back. This leads to problems in the prescribing of appropriate antibiotics. Patients receiving appropriate antibiotics were found to have a shorter length of hospitalization and increased survival rate (Leibovici, Shraga et al. 1998; Kollef, Sherman et al. 1999; Ibrahim, Sherman et al. 2000). The use of antibiotics for the treatment of sepsis has come under scrutiny, though. A study of rabbits with *E. coli* sepsis showed that treatment with gentamicin rapidly increased the levels of endotoxin.
as bacteremia decreased, and those treated with a placebo had levels of endotoxin proportional to the level of bacteremia. The study also found that the rabbits treated with antibiotics displayed clinical shock with no bacteremia, but had significantly high levels of free endotoxin. The take-home message being that a significant amount of endotoxin is released following administration of antibiotics (Shenep and Mogan 1984).

There are some radical treatments for sepsis. The use of glucocorticoids, known to be anti-inflammatory and immunosuppressive (personnel communication with Carrie Owen, RPh), has undergone a second look. Several studies have found that low doses of hydrocortisone in the early to late stages of septic shock can lead to improvement of organ function, reduce time to shock reversal, and improve a patients outcome both mentally and physically (Bollaert, Charpentier et al. 1998; Brieger, Forst et al. 1999; Schelling, Stoll et al. 1999; Keh, Boehnke et al. 2003). Ibuprofen, an inhibitor of cyclooxygenase and blood clotting (personnel communication with Carrie Owen, RPh), was used in a trial looking at overall outcome and organ failure. In sepsis, organs can fail for a number of reasons including blood clots. A study found that ibuprofen reduced fever, tachycardia, and lactic acid levels in septic patients but had no significant effect on mortality or organ failure (Bernard, Wheeler et al. 1997). A second study pretreated healthy volunteers with ibuprofen prior to intravenous endotoxin administration. These volunteers were asymptomatic as compared to volunteers without ibuprofen. They did not experience the fever, rapid heart beat nor
elevation of stress hormones as was seen in the volunteers receiving no ibuprofen; however, they did experience the same rise in TNF-α levels (Michie, Manogue et al. 1988).

The new treatment with the most promise is recombinant human activated protein C (rHPC). Inflammatory cytokines, such as TNF-α, activate coagulation and inhibit fibrinolysis leading to the formation of blood clots (Janeway, Travers et al. 2005). Activated protein C promotes fibrinolysis and inhibits blood clotting, but has reduced activation in sepsis. Infusion of activated protein C with a lethal dose of E. coli prevented the coagulopathic and lethal effects seen in baboons without protein C treatment (Taylor, Chang et al. 1987). A trial of septic patients found a significant reduction in the relative risk of death (19.4%) between the group receiving rHPC and the placebo group (Bernard, Vincent et al. 2001).

Based on first hand experience and epidemiological studies, patients lucky enough to survive sepsis have a reduced quality of life. Patients with Adult Respiratory Distress Syndrome (ARDS) were broken down into two groups, those where trauma was the risk factor and those where sepsis was the risk factor. Patients in whom sepsis was the risk factor had worse health related quality of life (HRQL) for all subjects on a questionnaire compared to those where trauma was the risk factor. The questionnaire, short form 36 (SF-36), consisted of rating physical functioning, bodily pain, vitality, and social functioning. The same study compared septic ARDS patients to a control group
and found that there was a significant reduction in 6 of the 8 health related measures (Davidson, Caldwell et al. 1999). Others using SF-36 concluded that survivors of sepsis scored significantly lower on the physical functioning, role physical, general health, vitality, and social functioning domains when compared to established U.S. general population norms. Survivors of sepsis had similar HRQL to medical patients with chronic disease (Heyland, Hopman et al. 2000). A comparison of bacteremia survivors and a control group found that the median survival was 16.2 months for the study group and more than 75 months for the control (Leibovici, Samra et al. 1995). Taken together, septic survivors have a high short term mortality and a lower quality of life.

**The Septic Response**

The immune response taking place during sepsis is vast, complicated, and poorly understood. A baboon model of sepsis, in which a lethal dose of *E. coli* is infused intravenously, can be broken down into four stages. The inflammatory response is characterized by a decrease in white cells, and a rise in elastase, TNF-α, IL-1β, IL-6, tissue plasminogen activator, and endotoxin. Stage one completes in two hours and is followed by the coagulopathic stage. A decrease in platelet and fibrinogen levels accompanies this stage. Baboons also incurred a fall in blood pressure, increased cardiac output, higher temperatures, and transient vasodilatation. Looking at the baboon kidney, it was found that leukocytes had become adherent and that there were small deposits of fibrin. Stage three comprises a period of edema and cell injury. A steady decline in
blood pressure followed by death represents stage four (Creasey, Stevens et al. 1991; Taylor, Chang et al. 1991).

In baboon models, lethal doses of *E. coli* was followed by a rapid increase in TNF-α, reaching peak levels at 120 min. IL-1β levels paralleled the rise of TNF-α but was offset by one hour. A slow but steady increase in the acute phase protein IL-6 begins one hour after TNF-α levels begin to rise and peaks at 360 min. What is interesting to note is that baboons with a sublethal bacteremia, 10-fold fewer organisms, had minimal increases in all the above cytokines and recovered from the infection (Creasey, Stevens et al. 1991). This raises the possibility that in baboons, at least, lethality is caused by the host’s inability to respond to the excessive levels of these cytokines.

Another study compared acute non-life threatening sepsis in healthy human males to life threatening septic shock in baboons. In both the human and primate models, TNF-α levels were again the first to increase followed by IL-1, peaking at 90 min and 2.5 hours, respectively, after endotoxin or *E. coli* were administered. The levels of cytokines between the two models were drastically different. TNF-α levels were almost 60 times greater in the primate model than in the human and IL-1 levels were 6 times greater. Primate models also showed an increase in interferon-γ (IFN-γ) starting at 4 hours and peaking at 8 (Hesse, Tracey et al. 1988). Patients with meningococcal septic shock had detectable amounts of IL-6 and IL-1 after a rise in TNF-α. High levels of IL-6 and the
presence of IL-1 were associated with fatal outcome (Waage, Brandtzaeg et al. 1989).

In a human model of non-life threatening sepsis, TNF-α levels were seen to rise significantly as compared to saline infusion in the same volunteers. The rise in TNF-α was associated with the onset of symptoms such as chills headache, fever, and an increase in stress hormones. In contrast, no IFN-γ was detected and no significant rise in IL-1β was observed (Michie, Manogue et al. 1988). Another study also found no significant release of IL-1β. Van Deventer et al focused on the coagulopathic stage of sepsis and found that after a rise in TNF-α and IL-6 the coagulation pathway was activated (van Deventer, Buller et al. 1990).

Cachectin/Tumor Necrosis Factor-α (TNF-α)

In 1975, Carswell et al discovered a substance in mouse serum that could induce tumor necrosis (Carswell, Old et al. 1975). It had long been known that endotoxin could produce hemorrhagic necrosis of tumors (Parr, Wheeler et al. 1973; Green, Dobrjansky et al. 1982). The study by Carswell found that the endotoxin, previously believed to mediate the killing of the tumor, was acting indirectly. Serum from bacillus Calmette-Guerin infected mice treated with endotoxin contained a substance termed Tumor Necrosis Factor which was not endotoxin, but could kill tumors and tumor cells (Carswell, Old et al. 1975). This provided evidence that endotoxin was causing the release of a substance that mediated the necrosis of tumors.
Another team of scientists studied the wasting effects of bacterial endotoxin on mice and rabbits. Lipoprotein lipase (LPL), an enzyme involved in the metabolism of triglycerides, was found to be decreased in mice (CeH/HeN) sensitive to the effects of LPS. Mice resistant to endotoxin (CeH/HeJ) had relatively normal levels of LPL. LPL suppression, could be induced if serum from the sensitive mice was injected into the resistant strain. This suggested that endotoxin mediated the release of a substance that causes the inhibition or suppression of LPL (Kawakami and Cerami 1981). It was later determined by Beutler et al that a macrophage factor, cachectin, was the suppressing agent. Cachectin, named after its wasting effect, was a 17 Kd protein with an isoelectric point of 4.7 and was found in vivo as a dimer (Beutler, Mahoney et al. 1985). Cachectin and TNF-α are now known to be the same protein, having an effect on both normal and neoplastic cells (Beutler, Greenwald et al. 1985).

Tumor necrosis factor characterization has been a hot topic since the substances discovery with conflicting data. Rabbit TNF-α has an estimated M.W. of 68,000 by sodium dodecyl sulfate-polyacrylimide electrophoresis (SDS-PAGE). Studies have confirmed its protein nature, as digestion with pronase renders it inactive (Ruff and Gifford 1980). Many studies have used mouse models. A study using mice both resistant and sensitive to endotoxin found that the resistant mice could only produce the cytotoxic effects after transfer of bone marrow from the sensitive strain. This serum factor had a molecular weight of 60 Kd and an isoelectric point of 4.8 (Mannel, Meltzer et al. 1980). Others found that
the substance responsible for tumor necrosis fractionated into a high and a low molecular weight of 225 and 50 Kd. The high molecular weight form migrated with \( \alpha \)-globulin and the low form with albumin (Kull and Cuatrecasas 1981). Another study found that the tumor necrosis factor is a glycoprotein with a molecular weight of about 150 Kd. This factor was made up of at least four subunits with different molecular weights (Green, Dobrjansky et al. 1976). These discrepancies may be explained by species variability, contamination or an impure preparation. Lymphotoxin, later termed Tumor Necrosis Factor-\( \beta \) (TNF-\( \beta \)), can be seen in some preparations of tumor necrosis serum.

In 1985, human TNF-\( \alpha \) protein and its messenger RNA was isolated from an adherent monocytic cell line. The isolated TNF-\( \alpha \) could produce cytotoxic effects on mouse fibroblast cell line used to determine presence of TNF-\( \alpha \). This activity was not due to TNF-\( \beta \) contamination, since anti-TNF-\( \beta \) antibodies failed to neutralize the effects of the cytokine. This indicated a difference between TNF-\( \beta \), which can produce some of the same effects, and TNF-\( \alpha \). Complete neutralization of TNF-\( \alpha \) activity was only possible in the presence of antiserum raised against TNF-\( \alpha \). A cDNA clone was engineered using isolated mRNA. The cloned cDNA could be expressed in *E. coli* and was biologically active. In this experiment, it was found that 1,300 bp coded for human TNF-\( \alpha \). The calculated molecular weight was about 17 Kd and was in agreement with natural TNF-\( \alpha \). Based on the deduced amino acid sequence, TNF-\( \alpha \) is not a glycoprotein but
could have two possible cysteine residues enabling it to form dimers and trimers (Pennica, Nedwin et al. 1984). This protein sequence has an 80% homology to mouse TNF-α (Marmenout, Fransen et al. 1985). Another study found that human TNF-α cDNA is 1585 bp with the protein beginning at residue 77. This cDNA encoded a protein containing 233 amino acids (Wang, Creasey et al. 1985).

TNF-α can be produced from lymphoblastoid cell lines (Rubin, Anderson et al. 1985), monocytes (Aggarwal, Kohr et al. 1985; Marmenout, Fransen et al. 1985), macrophages (Urban, Shepard et al. 1986) and peripheral blood leukocytes (Kelker, Oppenheim et al. 1985). As stated above TNF-α is a 17 Kd protein when determined by SDS-PAGE. However, gel permeation chromatography shows TNF-α to have an approximate molecular weight of 45,000. This leads one to believe that TNF-α in vivo is either a dimer or trimer (Aggarwal, Kohr et al. 1985; Kelker, Oppenheim et al. 1985).

Two cytokines that produce similar effects can be produced in cultures of unseparated human mononuclear cells. One cytokine is TNF-β and is neutralized by antibody to only TNF-β. The second cytokine is TNF-α and is only neutralized in the presence of anti-TNF-α serum (Kelker, Oppenheim et al. 1985). TNF-α can be closely aligned with TNF-β both structurally and functionally. TNF-α and TNF-β sequences are 36% identical and when conservative substitutions are used have 51% homology (Aggarwal, Kohr et al. 1985). In fact, TNF-α and TNF-
β are found to have a tandem arrangement in the human genome (Nedospasov, Shakhov et al. 1986). Both TNF-α and TNF-β contain 3 introns with the last exon, which codes for more than 80% of the secreted protein, having a 56% homology. The tandemly arranged genes of TNF-α and TNF-β can be mapped to chromosome 6 in close proximity to the HLA gene cluster (Nedwin, Naylor et al. 1985). This and the similar structure suggest a duplication of an ancestor gene.

Radio iodinated murine TNF-α was utilized to determine the fate of TNF-α in vivo. The iodinated TNF-α had a half life of 6-7 minutes and could be found in various tissues including liver, kidneys, and gastrointestinal tract and was rapidly degraded (Beutler, Milsark et al. 1985). But the question became then, how was the protein acting on the target cells? High affinity binding sites were located on 2 tumorigenic mouse cell lines, two transplantable mouse tumors and human cervical carcinoma line, all of which were sensitive to the cytotoxic action of TNF-α. Cells that were resistant to TNF-α were found to be unable to bind the radioligand (Kull, Jacobs et al. 1985; Rubin, Anderson et al. 1985). Iodinated TNF-α was used to covalently cross-link to its receptors on sensitive cells. Two bands of receptor-ligand complex were determined by SDS-PAGE. A major band was located at 95 Kd and a fainter band was found at 75 Kd. It was believed by the authors that the 95 Kd band was the authentic receptor-ligand complex and that the 75 Kd band represented a precursor or a degradation product (Kull, Jacobs et al. 1985). What is interesting to note is that binding of labeled TNF-α
to its receptors could be inhibited by TNF-β, suggesting a shared receptor (Aggarwal, Eessalu et al. 1985).

TNF-α can have various effects on target cells beyond tumor necrosis. Recombinant human TNF-α can enhance adherence of human peripheral blood neutrophils to human umbilical vein endothelial cells (HUVE) through its activities on both cell types (Gamble, Harlan et al. 1985). It was found that ICAM-1 (intercellular adhesion molecule 1) and endothelial cell specific activation antigen had increased expression on HUVE cells after exposure to TNF-α (Pober, Gimbrone et al. 1986). Clotting of small vessels leading to organ failure is a hallmark of sepsis. TNF-α is involved in the procoagulant shift found in most septic patients. Experimental endotoxemia in humans induces the release of TNF-α and IL-6 followed by activation of the common pathway of blood coagulation (van Deventer, Buller et al. 1990). TNF-α was found to induce the fibrinolytic system for the first hour, but inhibited it thereafter. Subjects receiving an intravenous bolus of TNF-α showed sustained coagulation for 6-12 hours after infusion (van der Poll, Buller et al. 1990; van der Poll, Levi et al. 1991). TNF-α could also shift the coagulant-fibrinolytic balance by inducing endothelial tissue factor, a procoagulant, and suppressing endothelial cell dependent protein C activation (Nawroth and Stern 1986). TNF-α can also act as a pyrogen, causing a rapid monophasic fever (Dinarello, Cannon et al. 1986) and as an inducer of chemotaxis, leading to the recruitment of phagocytic cells from the vascular
system to the site of infection (Ming, Bersani et al. 1987). TNF-α has broad effects and target cells.

TNF-α can play a major role in sepsis and septic shock. Levels of plasma TNF-α were elevated in septic patients and correlated with severity of illness (Cannon, Friedberg et al. 1992). It appears to be the first cytokine released after injection of endotoxin or a lethal dose of *E. coli* in humans or baboons. The release of TNF-α is followed shortly by a rise in IL-6, then IL-1 (Hesse, Tracey et al. 1988; van Deventer, Buller et al. 1990; Taylor, Chang et al. 1991). In studying patients with sepsis, Pinsky *et al.* found no relation between peak TNF-α levels and outcome. It was observed, however, that patients with multiple system organ failure (MSOF) had elevated TNF-α and IL-6 levels. The levels of these cytokines in patients with MSOF did not decrease over time, as they did in patients that did not develop MSOF. It was concluded that persistence of TNF-α and IL-6 rather then their magnitude predicted poor outcome (Pinsky, Vincent et al. 1993). A mutation of the TNF-α promoter, leading to an increased rate of TNF-α transcription, is associated with an increased risk of severe sepsis after burn trauma (Barber, Aragaki et al. 2004). TNF-α is believed to be the principle mediator in endotoxic shock.

Based on this belief, several investigators have used TNF-α to induce a shock like state. In two studies in which rats and dogs were given intravenous injection of TNF-α induced hypotension, metabolic acidosis, and death from
respiratory arrest ensued. Autopsy of both found acute pulmonary inflammation, and adrenal and renal necrosis. Autopsy of the rats found hemorrhagic lesions of the gastrointestinal tract and intravascular thrombosis in dogs (Tracey, Beutler et al. 1986; Tracey, Lowry et al. 1987). Recombinant human TNF-α can induce hypoxia, leukopenia, and increased lung lymph flow characteristic of increased pulmonary microvascular permeability in sheep. Autopsy of the lung tissue indicated congestion, neutrophil sequestration, and pulmonary edema (Johnson, Meyrick et al. 1989). Michie et al studied the effects of increasing amounts of recombinant human TNF-α on tumor bearing humans. Low doses of rTNF-α had a minimal effect, whereas, higher doses gave similar metabolic responses as subjects given endotoxin intravenously (Michie, Spriggs et al. 1988). It was therefore concluded that TNF-α can mediate the pathogenic effects of endotoxin.

Due to the appearance of TNF-α in relation with sepsis and septic shock, it was postulated that treatment with anti-TNF-α antibodies may increase survival. Pretreatment of rats with monoclonal anti-TNF-α antibodies fully protected these animals from lethal doses of TNF-α (Michie, Spriggs et al. 1988). Gram-negative bacteremia induces a rapid increase in both IL-1 and IL-6 levels in a baboon model, and passive immunization against TNF-α before a lethal dose of E. coli inhibits the appearance of these cytokines (Fong, Tracey et al. 1989). In a baboon study, those given TNF-α-specific antibody 30 min after receiving lethal doses of E. coli survived greater then 7 days with an increased quality of life. Baboons that had not received the antibody survived for 19 hours.
or less. Postmortem histopathologic comparison of the two groups revealed that
the changes in the control group were not present in those treated with antibody.
These changes included edema, fibrin-thrombin deposition, and vascular
congestion. The absence of the above changes indicated protection of critical
organs by the anti-TNF-α antibody (Tracey, Fong et al. 1987; Hinshaw, Tekamp-
Olson et al. 1990). Anti-TNF-α antibodies given in low grade endotoxemia of
chimpanzees reduced levels of IL-6 and IL-8, but did not effect the activation of
the coagulation system and instead inhibited fibrinolysis leading to microvascular
thrombosis (van der Poll, Levi et al. 1994). A study of septic patients with
increased levels of IL-6 were treated with monoclonal antibodies to TNF-α and
saw no significant difference in survival rate. Those who were treated did have a
trend toward earlier resolution of organ failure and a decrease in IL-6 levels
relative to the untreated (Reinhart, Menges et al. 2001). It is possible that
treatment of sepsis by anti-TNF-α antibodies could work, but must be carried out
before TNF-α has had a chance to induce its full effects. This would indicate
treatment at a time when patients are not seeking medical attention.

**Lipopolysaccharide and TLR4**

Lipopolysaccharide, or LPS/endotoxin, is the major component of the
outer membrane of Gram-negative bacteria (Nowotny 1969). LPS can be shed
into the host fluids by bacteria or it can be released by dying bacteria in response
to antibiotics or the host’s immune system (Shenep and Mogan 1984). LPS can
have profound effects on the host cells, immune system, and physical well being.
These effects include induction of tissue factor, TNF-\(\alpha\), a decline of CD4+ T cells, and monocytopenia (Richardson, Rhyne et al. 1989; Guha, O’Connell et al. 2001). These effects are due to LPS binding to its receptor, TLR4, on host cells, mainly monocytes and macrophages, and eliciting a signaling cascade (Figure 1) culminating in the activation of NF-\(\kappa\)B and transcription of effector genes (Opal and Huber 2002).

The family of Toll-like receptors currently consists of eleven receptors, TLR1-11. Each receptor recognizes molecules derived from invading organisms ranging from Gram-negative and positive bacteria to the genetic makeup of viruses (Pasare and Medzhitov 2004). Toll-like receptors were first discovered by comparing the drosophila Toll protein morphology to that of human proteins. Five human Toll-like receptors (TLR-1-5) were closely tied to that of drosophila Toll (Rock, Hardiman et al. 1998). In drosophila, Toll is used to sense Gram-negative bacteria and fungi. Activation of this receptor causes the nuclear translocation of the fly NF-\(\kappa\)B homologue. It also serves a purpose in fly development (Wang and Ligoxygakis 2006).

Several lines of mice have been characterized as unresponsive to LPS, most notably C3H/HeJ. The LPS\(^d\) allele of these mice have a missense mutation in the third exon of the TLR4 gene (Poltorak, He et al. 1998). The Toll-like receptor 4 gene has been mapped to chromosome 4 in mice (Qureshi, Lariviere et al. 1999) and chromosome 9 in humans (Rock, Hardiman et al. 1998). Mice that have been engineered to be deficient in TLR4 had macrophages that did
Figure 1. TLR4 signaling pathway

The intracellular signaling pathways of the Toll-like receptor 4 (TLR4) complex. IKB, inhibitor of NF-κB; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; Mal, myeloid differentiation factor MyD88-like; MyD88, myeloid differentiation factor; NF-κB, nuclear factor-κB; NIK, nuclear factor-κB inducing kinase; PKR, RNA binding protein kinase; TIR, Toll IL-1 receptor; TRAF6, tumor necrosis factor receptor-associated factor 6.

(Opal and Huber 2002)
not respond to LPS, similar to the hyporesponsiveness of C3H/HeJ mice (Hoshino, Takeuchi et al. 1999). As NF-κB is activated in response to LPS, its activation was looked at to determine if TLR4 was the receptor for LPS. When TLR4 was transiently expressed in cells that normally did not express that receptor, it was able to subsequently mediate NF-κB activation (Chow, Young et al. 1999). TLR4 from LPS resistant mice failed to activate a NF-κB reporter whereas, TLR4 from LPS sensitive mice could (Hoshino, Takeuchi et al. 1999). It had been proposed that TLR2 may respond to LPS also (Kirschning, Wesche et al. 1998), however, it was found that cells and mice unable to express TLR2 could respond normally to LPS but lacked a response to Gram-positive stimulus (Heine, Kirschning et al. 1999; Takeuchi, Hoshino et al. 1999). Taken together, TLR4 is responsible for LPS signaling.

Humans with mutations in TLR4 have also been found to express a hyporesponsiveness to LPS. Two missense mutations (Asp299Gly and Thr399Ile) have been associated with a reduced response to inhaled LPS. These missense mutations effect the extracellular domain of the receptor (Arbour, Lorenz et al. 2000). Carriers of the Asp299Gly allele are associated with a greater risk of severe sepsis in low risk burn patients (Barber, Aragaki et al. 2004).

**Formyl Peptide Receptor, FPR**

A number of prokaryotic and eukaryotic cells, including leukocytes, can undergo chemotaxis. This is the ability to migrate along a chemical concentration
gradient. Chemotaxis can take place in response to a number of compounds including sugars, amino acids and bacterial products. One such bacterial product is the formyl peptide fMLP, N-formylmethionyl-luecyl-phenylalanine. In a study on neutrophils and macrophages it was shown that N-acylation of the peptides was required for the cells to undergo chemotaxis. Neither nonacylated peptides nor free methionine had an effect on chemotaxis of either leukocyte. It was also concluded that the cells preferred formylation rather then acylation. Several formylamino acids were tested and only formylmethionine was found to be active. The formylamino acids were also more active if they contained at least two residues, especially if they were hydrophobic (Schiffmann, Corcoran et al. 1975). Using 24 synthetic peptides, some differing in only the order of amino acids, Showell et al found that a relationship existed between structure and activity. F-Met-Leu-Phe and F-Met-Phe-Leu differ only in the order of the placement of phenylalanine and leucine, but F-Met-Leu-Phe was 500 times more active then F-Met-Phe-Leu. The authors reported that any neutral nonpolar amino acid will work well in the second position but that a phenylalanine in the third and final position had the greatest effect on leukocytes. This suggested that the receptor was stereospecific (Showell, Freer et al. 1976).

Until 1984, formylated peptides as bacterially derived chemoattractants was hypothetical, as there was no direct evidence. All formylpeptides tested had been synthetic. Heat-stable peptides of low molecular weight (150-1500 daltons) that could elicit chemotaxis had been found in E. coli culture filtrates (Schiffmann,
Corcoran et al. 1975). Nine years later, a group using HPLC technology was able to determine that fMLP is the major chemotactic component of bacterial filtrates. These formylpeptides are believed to be from bacterial protein degradation (Marasco, Phan et al. 1984), but they can also be found on mitochondrial proteins (Carp 1982).

The first step in the chemotactic response was studied by using titrated N-fMLP. Studying the interaction of fMLP and the leukocyte, Williams et al was able to directly identify a binding site and determine that binding is rapid and reversible. It was also found that in polymorphonuclear leukocytes (PMNL) only 5-10% of the receptors needs to be activated to cause a chemotactic response (Williams, Snyderman et al. 1977).

Covalent affinity labeling of the formyl peptide receptor opened many doors. First, the molecular weight of the receptor from neutrophils was determined to be between 55 and 70 Kd on SDS-PAGE, suggesting more than one receptor (Niedel, Davis et al. 1980). It was later determined using PMNLs that two major entities existed. One had a molecular weight of 50,000 and an isoelectric point of 6.0, whereas, the other had a molecular weight of 60,000 and an isoelectric point of 6.5 (Schmitt, Painter et al. 1983). Secondly, it made it possible to determine the formyl peptide receptor backbone. Using three different cell types, it was elucidated that the formyl peptide consisted of two N-linked oligosaccharide side chains located on the 1-3 Kd portion cleaved by papain and are not required for binding (Malech, Gardner et al. 1985). Receptor-mediated
internalization was demonstrated using a rhodamine-labeled formyl peptide. Fixing human monocytes at various time points after incubation with the labeled peptide showed that, within 1 min, the peptide was diffusely distributed over the cell membrane. Aggregation of the fluorescent peptide began at 3 min and small endocytic vesicles could be seen at 15 min. These events were receptor mediated, as co-incubation with non-labeled peptide prevented them. This study also suggested that for every peptide internalized, one receptor became unavailable for binding. The authors determined that the receptors were being replenished on the cell membrane, as the amount of internalized peptide exceeded the amount of receptors (10,000-18,000). It was evident that de novo synthesis was not occurring since cycloheximide treatment did not alter replenishment (Weinberg, Muscato et al. 1981).

In 1990, a group led by P.M. Murphy used a Xenopus oocyte protein expression system to determine if functional FPR is a single peptide or made of multiple subunits. Using poly(A) RNA from differentiated HL60 cells, a single mRNA of 2 Kb was able to confer functions specific to fMLP (Murphy, Gallin et al. 1990). A second group using a COS-7 cell expression system found two cDNA clones that could confer a functional fMLP receptor, fMLP-R26 and fMLP-R98. Both cDNAs had a predicted product of 350 amino acids and a molecular weight of 38,420 daltons. The cDNAs had two potential N-linked glycosylation sites in the amino-terminal region and a possible third site. Small differences between the two cDNAs, including two non-silent base substitutions, led the researchers
to believe that the two were allelic variations rather than two separate genes. However, given enzyme restriction hybridization patterns of genomic DNA, it was suggested that either FPR has two isoforms encoded in two genes or was a single gene with introns in the coding sequence. Based on the predicted amino acid sequence and the Kyte and Doolittle method, a hydropathy profile shows the FPR to have seven hydrophobic regions. Based on comparison to other similar receptors, the FPR is predicted by the authors to have seven transmembrane \( \alpha \)-helices, with the N-terminal region extracellular and the C-terminus region intracellular (Boulay, Tardif et al. 1990; Boulay, Tardif et al. 1990). A schematic of the FPR is shown in Figure 2.

It has been found that three human genes, FPR1, FPRL1 and FPRL2 (for FPR like) are located on chromosome 19 (Bao, Gerard et al. 1992). FPR1 and FPRL1 are expressed mainly on neutrophils, monocytes, some epithelial cells, liver hepatocytes and kupffer cells, and various endocrine cells. FPRL2 is only found on monocytes (Durstin, Gao et al. 1994; Becker, Forouhar et al. 1998). FPRL1 and FPRL2 can not, however, confer binding of fMLP in transfected cells. Analysis of the sequences shows that FPRL1 and FPRL2 are 56% and 66%, respectively, identical to FPR, with the regions of similarity being in the predicted transmembrane domains and the cytoplasmic segments (Bao, Gerard et al. 1992). Using cross hybridization methods six mouse genes have been cloned and designated \( fpr1 \) and \( fpr-rs1 \) through \( fpr-rs7 \) (Gao, Chen et al. 1998; Wang and Ye 2002). The orthologue of \( FPR1 \) is predicted to be \( fpr1 \), which encodes
Figure 2. Secondary structure model of FPR

(Gripentrog and Miettinen 2005)
a functional mouse fMLP receptor. Based on amino acid sequence fpr-rs1 and fpr-rs2 are similar to FPRL1, whereas fpr-rs3, 4, and 5 lacks any similarity to human FPR genes (Gao, Chen et al. 1998). Mice which lack a functional FPR show an increased bacterial burden in the liver and spleen, and an accelerated mortality when exposed to Listeria monocytogenes compared to mice with normal functioning FPR. In vivo and in vitro chemotaxis was also hindered in FPR−/− mice. The authors felt that this suggested a role for the FPR in innate immunity (Gao, Lee et al. 1999).

Several studies have looked into the binding pocket of the FPR using different approaches. Quehenberger formed chimeras from FPR, having high affinity binding, and FPR2, having low affinity binding. The binding affinity of FPR to fMLP was decreased by replacing the first and third extracellular loops of FPR with those of FPR2. The opposite was seen when FRP2’s first and third extracellular domains were replaced by those of FPR. Looking into the amino acid sequences of the two extracellular loops, it was found that FPR2 had 6 non-conserved amino acid substitutions, four of which were on the third extracellular loop. Two aromatic residues in close proximity to an amino acid with a large side chain could possibly change the shape of the binding pocket in FPR2. It has two amino acids with non-polar side chains (Ser84 and Met85) replacing the two charged amino acids in FPR (Arg84 and Lys85) on the first extracellular loop (Quehenberger, Prossnitz et al. 1993). Replacement of Ser84 and Met85 on FPR2 with Arg84 and Lys85 allowed the FPR2 receptor to have a binding affinity for
fMLP close to that of FPR. Two other clusters of amino acids (Gly\textsuperscript{89}/His\textsuperscript{90} and Phe\textsuperscript{102}/Thr\textsuperscript{103}) in the first extracellular loop also conferred a higher affinity for fMLP (Quehenberger, Pan et al. 1997). Using 28 site directed mutations of transmembrane domains, Miettinen \textit{et al} located the ligand binding pocket to be within the membrane bilayer 3 to 7 Angstroms from the extracellular surface (Miettinen, Mills et al. 1997). Mills \textit{et al} postulated that the likely position of fMLP in the binding pocket of FPR is parallel to the 5\textsuperscript{th} transmembrane helix. The formide group of fMLP hydrogen bonds to both Asp\textsuperscript{106} and Arg\textsuperscript{201} and its COOH terminal carboxy group is ion-paired with Arg\textsuperscript{205} (Mills, Miettinen et al. 2000).

The sequence comparison of FPR to other possible receptors shows that it belongs to the G protein coupled receptor (GPCR) superfamily (Boulay, Tardif \textit{et al}. 1990). It had long been known prior to this that non-hydrolyzable GTP converted high affinity FPR receptors to low affinity receptors. It was also known that guanine nucleotides could increase the rate of dissociation of fMLP from its receptor and that the affinity state was regulated by guanine nucleotides and GTPase, as GTP hydrolysis was activated in the presence of fMLP (Koo, Lefkowitz \textit{et al}. 1983; Hyslop, Oades \textit{et al}. 1984; Snyderman, Pike \textit{et al}. 1984). G-proteins are composed of invariant β- and γ-subunits and a variable α-subunit. The G-proteins are named according to the α-subunit (α\textsubscript{s}, α\textsubscript{i}, α\textsubscript{o}) (Probst, Snyder \textit{et al}. 1992). By 1984, a number of studies provided insight into which G-protein was coupled to FPR. Neutrophils incubated with fMLP generated superoxide anions, increased release of arachidonic acid, and mobilized calcium, and these
effects could be suppressed using pertussis toxin. The substrate of pertussis toxin is a subunit of the guanine nucleotide regulatory protein involved in adenylate cyclase inhibition (Okajima and Ui 1984) and is involved in ADP ribosylation of G\textsubscript{i} (Smith, Lane et al. 1985). Purified G\textsubscript{ai} subunits mixed with bovine G\textsubscript{\beta\gamma} subunits were able to reconstitute with solubilized FPR receptors. G\textsubscript{ai3} had the greatest affinity for the receptor followed by G\textsubscript{ai2} and the interactions could be inhibited by monoclonal antibodies to the various G\textsubscript{ai} subunits (Bennett, Key et al. 2001).

Mutants of the FPR were used to further elucidate the interactions of G-protein and the receptor for formyl peptides. The third intracellular loop has been shown to be important in a majority of seven transmembrane G-protein coupled receptors (Probst, Snyder et al. 1992). The third intracellular loop of FRP is one of the smallest loops of all GPCRs, consisting of only 15 amino acids. To determine if this loop is necessary in the FRP for G-protein coupling, several point and deletion mutants were made in this region. The mutants were able to bind fMLP normally and stimulated Ca\textsuperscript{2+} mobilization as seen in the wild type receptors (Prossnitz, Quehenberger et al. 1993). To further determine which sites of the FPR interacted with G-protein, Schreiber et al synthesized peptide segments contained in the intracellular loops and the cytoplasmic tail for inhibition studies. It was found that the peptides from the second intracellular loops and the cytoplasmic tail could inhibit binding of anti-G\textsubscript{ai} antibody and pertussis toxin catalyzation of ADP-ribosylation of G\textsubscript{ai}. In agreement with data
from Prossnitz et al, peptides from the third intracellular loop had no such effects (Schreiber, Prossnitz et al. 1994).

Two mutants, D71A and R123G, were studied for their inability to interact with G-protein. D71A has a mutation in the 2\textsuperscript{nd} transmembrane domain, which is not accessible to G-protein, but could possibly stabilize the low affinity state of the FPR (Prossnitz, Schreiber et al. 1995; Miettinen, Gripentrog et al. 1999). This aspartate is conserved in almost all GPCRs and is necessary for G-protein activation (Probst, Snyder et al. 1992). This mutant also had no significant receptor phosphorylation and was not internalized. The mutation, R123G, is located on the 2\textsuperscript{nd} transmembrane loop and is part of the “DRY” domain conserved in all GPCR. R123G was able to be phosphorylated and was subsequently internalized. Both of these receptors failed to activate G-protein, indicating that internalization of the receptor can take place in the absence of G-protein activation. Ligand binding to R123G confers the active conformation but is not recognized by G-protein (Prossnitz, Gilbert et al. 1999). A synopsis and location of the FPR mutants D71A, R123G, and Δst can be seen in Figure 3.

\textbf{Synergy}

Many substances can have a synergistic effect on cell activation, cytokine production, and other cellular processes. A response is synergistic when it is greater than the additive. This means that if a sample is activated with either of two stimulants, when combined the effect is greater than if you were to add the response of the two separately. There are several examples of this phenomenon
Figure 3. Synopsis of FPR mutants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ΔST</th>
<th>D71A</th>
<th>R123G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>50%</td>
</tr>
<tr>
<td>Desensitization</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Internalization</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>70%</td>
</tr>
<tr>
<td>Binds G-protein</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calcium Mobilization</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Binds Arrestin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mutation</td>
<td></td>
<td></td>
<td>aspartate 71 to alanine</td>
<td>arginine 123 to glycine</td>
</tr>
</tbody>
</table>

(Prossnitz, Schreiber et al. 1995; Hsu, Chiang et al. 1997; Prossnitz 1997; Bennett, Maestas et al. 2000; Vines, Xue et al. 2002)
in the literature. In one study, it was found that LPS and IFN-γ can act together to synergistically induce TLR2 expression in vascular endothelial cells. Semiquantitative PCR was used to analyze TLR2 expression in cells stimulated with 1 ng/ml LPS, 1 ng/ml IFN-γ or a combination of both. It was found that LPS or IFN-γ alone had little effect on TLR2 expression over the control. However, when used in combination they induced significant up-regulation of TLR2 expression (Faure, Thomas et al. 2001).

TLR2 was studied in another paper in which IL-12 mRNA levels were used to determine synergistic activity. Depleted zymosan particles are specifically recognized by the receptor dectin-1, a receptor in the lectin family for β-glucans, but not TLR-2. The lipopeptide, PAM₃CSK₄, does not activate dectin-1, but can activate TLR-2. IL-12 p40 mRNA was assessed by quantitative real-time PCR. It was found that depleted zymosan or PAM₃CSK₄ alone did not produce much of an effect on IL-12. However, when the two were used together they had a strong synergistic effect. The data led the authors to suspect that signaling by dectin-1 and TLRs cross talk in response to β-glucan-containing particles (Gantner, Simmons et al. 2003).

Muramyl peptides (MDP), the minimum active fragment of peptidoglycan, can act synergistically with endotoxins on a variety of cells. Flak et al found that the muramyl peptide tracheal cytotoxin (TCT), from Bordetella pertussis, can affect IL-1α, type II nitric oxide, and nitric oxide production when complemented with endotoxins (B. pertussis lipooligosaccharide and E. coli lipopolysaccharide)
in hamster tracheal epithelial cells. The authors also found that TCT in conjugation with lipooligosaccharide (LOS) or LPS can synergistically inhibit DNA synthesis. It is believed that the TCT, LOS and LPS activates a unique H7-sensitive kinase (Flak, Heiss et al. 2000). Another study focused on interleukin-8 (IL-8) production in differentiated THP-1 cells. They found that MDP with either LPS or lipoteichoic acid could produce IL-8 protein greater than the additive effect (Yang, Tamai et al. 2001).

Wolfert et al used Mono Mac 6 cells to study the synergistic effects of MDP, peptidoglycan (PGN) and LPS. Incubation with MDP alone could increase the amount of TNF-α mRNA, but had little effect on TNF-α protein levels. When LPS or PGN was used with MDP, the effect was additive on mRNA levels but synergistic at the protein level. Based on various experiments, the authors concluded that while MDP induced TNF-α mRNA, “a block” prevented it from being translated. This block could be removed in the presence of either LPS or PGN in a synergistic manner (Wolfert, Murray et al. 2002).
Materials and Methods

Cells
The human monocytic THP1 cells (ATTC TIB-202) were maintained in RPMI (Invitrogen, Carlsbad, CA), and supplemented with 10% Fetal Bovine Serum (FBS) (Omega Scientific, Tarzana, CA), and 5% penicillin-streptomycin (Invitrogen). The human lung epithelial A549 cells (ATCC CCL-185) were maintained in Ham’s F12 (Cellgrow, Mediatech Inc, Herndon, VA), supplemented with 11.8% FBS, 2mM L-Glutamine, 1.2% penicillin-streptomycin (Invitrogen), and 2.4% Sodium bicarbonate solution (Invitrogen). THP1_D71A and THP1-Δst were made previously in that lab by Dr. Astrid Doerner. A retroviral packaging cell line, 293FT, was maintained in DMEM (Invitrogen) with 10% FBS and 5% penicillin-streptomycin. All cells were grown at 37°C at 5% CO₂.

Stable transfection of THP1 cells
Using Clontech’s (Palo Alto, CA) qMSCV Retroviral Expression system (K1062-1), the gene of interest was cloned into the pMSCVpuro retroviral vector previously by Dr. Astrid Doerner. The packaging cell line, 293FT, was placed in a T75 tissue culture flask with 15 ml media but without penicillin-streptomycin and grown to confluency. When confluent, the cells were transfected with 5 μg PCL10, 5 μg VSVG and 10 μg pMSCVpuro containing the gene of interest with 35 μl Lipofectamine 2000 (Invitrogen) in 1.8 ml Optimem (Invitrogen). The packaging cells were incubated overnight. The transfection media was replaced 24 h later with complete media. After a 24 h incubation, 12 ml of virus laden
media was removed. To remove any cells, the media was centrifuged for 5 min at 400 g. Three milliliters of viral media was removed and 6 μl of Lipofectamine 2000 (Invitrogen) was added. The remaining virus was frozen at -80°C in 1 ml aliquots. THP1 cells were plated at a density of 1x10⁶ cells per well in three wells of a six-well plate. To each well, 1 ml of the virus-lipofectamine media was added and the plate was wrapped in parafilm. The plate was then centrifuged for 2 h at 833 g. The cells were resuspended with 1 ml media and incubated overnight. After 12-14 hours all three wells were placed together, the media was removed, and the cells were resuspended in 10 ml and placed in two T25 tissue culture flasks for 48 h. The cells were then selected with 0.5 μg/ml puromycin (BD biosciences, San Jose, CA), and cell death was monitored. This was done with four genes of interest; FPRwt, FPR-R123G, EGFP (Enhanced Green Flourescent Protein), and an empty vector. The EGFP transfected cells were monitored for greenness in a fluorescent microscope. When 90-100% of THP1 _EGFP cells were positive, analysis via flow cytometery was run on all cells to determine if the receptor was located on the cell surface.

**Stable transfection of A549 cells**
A549 cells were plated in three wells of a six-well plate at a density of 5x10⁵ cells per well in media without penicillin-streptomycin. Twenty-four hours later, 1 ml of previously frozen retrovirus and 2 μl Lipofectamine 2000 (Invitrogen) was added to each well. The plate was wrapped in parafilm and centrifuged for 2 h at 833 g after which 1 ml of complete media was added and the cells were
allowed to incubate overnight. The media was removed and cells from all three wells were mixed together. The cells were then plated in two T25 tissue culture flasks and placed in the incubator for two days. The media was replaced and cells were selected with 0.5 \( \mu \text{g/ml} \) of puromycin (BD Biosciences). This was done with three genes of interest; FPRwt, EGFP, and an empty vector. The EGFP transfected cells were monitored for greenness in a fluorescent microscope. When 90-100% of A549_EGFP cells were positive, analysis via flow cytometry was run on all cells to determine if the receptor was located on the cell surface.

**Flow Cytometric Analysis for the Formyl Peptide Receptor**

Flow cytometry was performed on THP1wt, THP1_FPRwt, THP1_FPRD71A, THP1_FPR\_Δst, and THP1_FRPR123G cells. A cell density of \( 1 \times 10^6 \) cells per sample was used. The cells were washed once in cold DPBS (Delbeco’s Phosphate Buffered Saline) (Invitrogen) and once with FACS buffer (DPBS +0.5% Bovine Serum Albumin (BSA) (Sigma, Saint Louis, MO)). Each sample was resuspended in 2 ml of FACS buffer and placed in FACS tubes. The tubes were centrifuged for six minutes at 50 g and 4°C. The buffer was decanted and the samples were resuspended in 1 ml buffer. One microliter formyl-Nle-Lue-Phe-Nle-Tyr-Lys fluorescein derivative (Molecular Probes, Invitrogen, Carlsbad, CA) was diluted in 19 \( \mu \text{l} \) buffer per sample, and added to the appropriate samples. The samples were then gently vortexed and placed on ice for 15 minutes. Each sample was measured in an EPICS Elite flow cytometer.
(Beckman Coulter, Fullerton, CA). THP1wt cells that were not incubated with the ligand were used for background readings.

**Flow Cytometeric Analysis for the Toll Like 4 Receptor**

Flow cytometry was performed on THP1wt and A549wt. A cell density of 1x10^6 cells per sample was used. The cells were washed once in cold DPBS (Invitrogen) and once with FACS buffer. Each sample was resuspended in 2 ml FACS buffer and placed in FACS tubes. The tubes were centrifuged for 6 min at 50 g at 4°C. The buffer was decanted, and 3 μg FitC conjugated mouse anti-human TLR4 (Imgenex, San Diego, CA) monoclonal antibody was added to the appropriate samples. The samples were then gently vortexed and placed on ice. After one hour the cells were washed twice with buffer and resuspended in 1 ml buffer. Each sample was then measured in an EPICS Elite flow cytometer. A sample of THP1wt and A549wt cells was not incubated with the antibody to allow for background readings.

**Stimulation and RNA Extraction of THP1 cells**

THP1-FPRwt, THP1-FPRR123G, THP1-FPRD71A or THP1-FPRΔst cells were plated at a density of 2x10^6 cells per well of a 24 well plate in 1 ml of complete media. After a 1 h rest the cells were stimulated for 2 h with stimulant. The THP1 cells were placed in a 1.5 ml eppendorf tube and the media was removed by vacuum after centrifugation for 10 min at 13.4 x1000 g at 4°C. The cells were lysed in 1 ml Trizol (Invitrogen) and RNA was extract with 200 μl chloroform (Fisher, Pittsburg, Pennsylvania). The aqueous phase was removed.
after centrifugation. RNA was precipitated with 0.5 ml isopropanol (Sigma) and placed overnight in a -20°C freezer. RNA was pelleted by centrifugation at 4°C and 13.4 x 1000 g for 10 min. The supernatant was decanted and the RNA was washed with 0.5 ml 75% ethanol. The pellet was allowed to dry and then dissolved in 25 μl nuclease free water (Ambion, Austin, TX).

cDNA Synthesis
Possible contamination of the RNA by genomic DNA was resolved with a DNAase treatment (DNA-free, Ambion). For each sample of RNA, 1 μl of DNA-free and 3 μl of buffer was added and then incubated for 30 min at 37°C. The DNAse was inactivated by a 2 min incubation with inactivating reagent (provided). The inactivating reagent was removed by centrifugation for 2 min at 13.4 x 1000 g. The supernatant was removed to a new tube and RNA concentration was found using BioRad’s spectrometer (Hercules, CA). cDNA was synthesized using 0.5-1 μg RNA. After heating the samples for 5 min at 65°C, cDNA Master Mix was added until the total equaled 20 μl. After a 1 h incubation, the samples were boiled for 5 min at 95°C and then placed on ice, and diluted with 80 μl nuclease free water (Ambion).
cDNA Master Mix
For each sample the following amounts were used;

2.0 μl 10x RT buffer

2.0 μl dNTP’s (5 mM)

1.0 μl RT

0.25 μl RNasin (Ambion)

0.5 μl random hexamer primers (4 μg/μl) (Amersham, GE Healthcare, Piscataway, NJ)

Nuclease free water to equal 20 μl

The RT buffer, dNTP’s and RT were purchased in Qiagen’s Omniscript kit (Valencia, CA).

Real time PCR
Quantitative real time PCR was performed using the Taqman method. The primers and probes used for real time PCR were the following oligonucleotides:

TNF-α, forward 5’-CTCCACCACCATGTGCTCCTCA-3’, reverse 5’-CTCTGGCAGGGGCTCTTGAT-3’ and probe 5’-FAMd(CACCATCAGCCGCATCGCCGTCTC)BHQ-1 3’ (Biosearch Technologies, Novato, CA); and human GAPDH, forward 5’-GGGAGGTGAAGGTGTCGGAGT-3’, reverse 5’-TCCACTTTACCAGAGTTAAAAGCAG-3’ and probe 5’-FAM-ACCAGGCAGCAATACGACCAA-BHQ1 3’ (Biosearch Technologies). Real time PCR was carried out using 2.5 μl of cDNA and 22.5 μl of Master Mix.

Measurements were done in triplicate using the Applied Biosystems 7500 Real
Time PCR system (Foster City, CA). The protocol for amplification was the following; Stage 1, one repetition, 50°C 5 sec, Stage 2, one repetition 95°C 2 min, Stage 3, 40 repetitions: step 1 95°C 15 sec, step 2 60°C 1 min. Data was collected at stage 3, step 2. To determine the absolute level of transcription, results were compared to standard curves (2-.00002 atamoles) obtained from known amounts of TNF-α or GAPDH standard plasmids, made previously in our lab. TNF-α transcript levels were then normalized using the GAPDH amounts.

**Real Time PCR master mix**

For each well the following amounts were used:

- 2.5 μl 10x Buffer (Invitrogen)
- 1.5 μl (GAPDH) or 2μl (TNF-α) MgCl₂ (50 mM) (Invitrogen)
- 0.5 μl dNTPs (10 mM) (Ambion)
- 1.0 μl forward and reverse primer mix (0.1 ug/ul)
- 0.5 μl ROX (Invitrogen)
- 0.125 μl Platinum Taq Polymerase (Invitrogen)
- 0.15 μl (GAPDH) or 0.25 μl (TNFα) probe (10 pmol/μl)

Nuclease free water (Ambion) to equal 22.5 μl.

**ELISA**

THP1_FPRwt, THP1_FPRR123G, THP1_FPRΔst and THP1_FPRD71A cells were each plated in eight wells of a 24 well plate at a density of 2x10⁶ cells per well. For each cell type, two wells were unstimulated, two wells received 100
nM fMLP, two wells were stimulated with 100 ng LPS and two wells were stimulated with both 100 nM fMLP and 100 ng LPS. The cells were stimulated for 2 h and then the media and cells were separated by centrifugation at 13.4 x 1000 g for 10 min at 4°C. The media was then used to determine protein amounts of TNF-α by ELISA.

A TNF-α ELISA kit by eBiosciences (San Diego, CA), with a sensitivity of 4 pg/ml, was used for all ELISA assays. Standards were diluted as stated in provided literature and 100 μl per well of each standard was used in duplicate. Each sample was run in duplicate using 100 μl per well of collected media. The plate was sealed and incubated for 2 h at room temperature. The samples were then aspirated and wells were washed five times with wash buffer (DPBS (Invitrogen) +.5% Tween 20(Fisher)). The plate was then incubated with detection antibody (100 μl/well) against human TNF-α. After 1 h the plate was washed as before and Avidin-HRP (100 μl/well) was placed on the plate and incubated for 30 min, after which the plate was washed as before. For 15 min the provided substrate solution (100 μl/well) was allowed to rest on the plate. The reaction was then stopped with Stop solution (50 μl/well). The plate was read using a Versamax™ microplate reader (Molecular Devises, Sunnyvale, CA) and analyzed using Softmax PRO software.

**Measurement of [Ca^{2+}]_{i}**

Cells were harvested, 1x10^6 cells per sample, by centrifugation, washed once with PBS and once with FLUX buffer (HBSS (Hank’s Buffered Salt Solution)}
(Invitrogen) + 0.25% BSA (Sigma) + 0.25 mM sulfinpyrazone (Sigma)). Cells were resuspended at 5x10^6 cells/ml in FLUX buffer. The cells were incubated with 5 μM indo-1-AM (Molecular Probes) for 30 min at 37°C with gentle shaking. The cells were washed once and resuspended at 5x10^5 cells/ml with FLUX buffer. The cells were incubated for 30 min at 37°C with gentle shaking. The increase in intracellular calcium by various stimulants was monitored using a spectrofluorometer (Photon Technology Inc, Birmingham, NJ) detecting at 405 and 485 nm. Values for R_{max} and R_{min} were determined using 200 μl 10% Triton 100 (Sigma) and 20 μl 0.5M EGTA (Ambion) respectively. The concentration of intracellular calcium was calculated using the formula [Ca^{2+}]_{i} = K_d[(R-R_{min})/(R_{max}-R)](S_f2/S_b2) where K_d equals 250 and R is the ratio of 405 to 485 nm. S_{f2}/S_{b2} is the ratio of R_{min} to R_{max} at 485 nm.

For studies in which cells were primed, intracellular calcium was measured as above with the exception of pretreatment and the FLUX buffer. Cells were pretreated for two hours with 100 nM fMLP, 100 ng/ml LPS, or a combination of both. The FLUX buffer used for measuring calcium in primed cells was 0.25% BSA(Sigma), .25% sulfinpyrazone(Sigma) and 1% FBS in HBSS (Invitrogen).
RESULTS

Cell Surface Receptors

Synergy has been seen in IL-12 (Gantner, Simmons et al. 2003), TLR2 (Faure, Thomas et al. 2001), and IL-8 (Yang, Tamai et al. 2001) expression. One study found that TNF-α expression could be synergistically activated in response to MDP in combination with either PGN or LPS (Wolfert, Murray et al. 2002). Our preliminary results indicated that multiple bacterial products, namely fMLP and LPS, could have a synergistic effect on the induction of TNF-α. To test this, the human monocytic cell line THP1 was used, as monocytes/macrophages are considered the first line of defense in the human immune system and are part of the innate immune response. THP1 wild type cells were exposed to media, 100 nM fMLP, 100 ng LPS or 100nM fMLP plus 100 ng LPS for 2 hours. The media was removed, RNA was extracted from the cells and cDNA was made. Quantitative Real Time PCR (Q-RT-PCR) was used to determine the fold induction of TNF-α expression. Results are the average of three independent experiments. The wild type cells were unable to respond to fMLP, but did respond to LPS, with TNF-α mRNA increased 50-fold compared to control cells exposed to media alone. THP1 wild type cells exposed to both LPS and fMLP had a response similar to that of LPS alone (Figure 4).
Figure 4. TNF-α mRNA levels in THP1 wild type cells in response to bacterial products

THP1 wild type cells were incubated with 100nm fMLP, 100ng LPS or a combination of both for two hours. The RNA was extracted and real time PCR data was obtained as outlined in methods. Data from cells exposed to media alone was set to one and all data from others were compared to it. Data presented are the average of three independent experiments done in duplicate + standard error.
Figure 4. TNF-α mRNA levels in THP1 wild type cells in response to bacterial products
THP1 wild type cells can respond adequately to LPS due to the expression of TLR4 on the cell surface, as determined by flow cytometry (Figure 5). A549 cells which have a low level of TLR4 expression do not respond to LPS (Data not shown). THP1 cells showed a mean shift of 10^{-2} in comparison to A549 cells.

Expression levels of FPR on THP1 cells were below the detection threshold of the flow cytometer and no induction of TNF-α was observed after stimulation with fMLP. THP1 cells were therefore stably transfected using a retroviral plasmid containing either wild type FPR, or one of three mutants, D71A, Δst, or R123G. The three mutants were chosen for their different abilities to activate G_i-protein, mobilize calcium, become phosphorylated and be internalized (Please refer to Figure 3 for a synopsis and references) Cell surface expression was determined by flow cytometry. The wild type FPR and the three mutants were expressed on the cell surface of THP1 cells with variable expression levels, which are 39.8% Δst, 16.5% R123G and 64.7% D71A (Figure 6, a and b). Expression levels were determined by setting mean fluorescence of THP1 cells transfected with FPRwt as 100%, dividing the mean fluorescence of the mutants by that of the FPRwt, and multiplying by 100.

**fMLP and LPS Dose Response**

To determine an adequate dose of LPS and fMLP for further study, a dose response was conducted on THP1-FPRwt cells. Cells were stimulated for 2 hours with LPS (0-1000 ng/ml) or fMLP (0-1000 nM). A curve was established for
Figure 5. Expression of TLR4 on THP1 and A549 cells

Expression of TLR4 was evaluated for THP1 (Black) and A549 (Red) wild type cells as described in the methods. Data are representative of three independent experiments.
Figure 5. Expression of TLR4 on THP1 and A549 cells
Figure 6. Expression of FRPwt and Mutants on THP1 cell surface

Expression was evaluated for THP1 (red) cells stably transfected with FPR wild type (black) and each mutant form (Δst, purple; D71A, blue; R123G, green) by flow cytometry as described in methods. A) Graph shows cell number plotted against fluorescent intensity. Graph is representative of four experiments.

B) Percent expression of FPR on cell surface where THP1-FPRwt is set at 100%. Data are from one flow cytometry experiment and representative of four.
Figure 6. Expression of FRPwt and Mutants on THP1 cell surface

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% expression</th>
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<tr>
<td>THP1-wt</td>
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<tr>
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<td>THP1-FPRΔst</td>
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<tr>
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<tr>
<td>THP1-FPRD71A</td>
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both LPS and fMLP by Q-RT-PCR of TNF-α mRNA (Figure 7, a and b). The minimum response for cells exposed to LPS was 4.6 fold and was induced by 0.1 ng/ml. The maximum response was found to be induced by 1000 ng/ml and had an induction level of 46.9 fold (Figure 7a). fMLP induced a minimum and maximum fold induction of 5.2 (1000 nM) and 18.5 (10 nm), respectively. A dose dependent response to fMLP was also determined by calcium mobilization (Figure 8). Doses below 10 nM had very little effect on calcium levels and those above 100 nM either did not change compared to 100 nM or even decreased. THP1-FPRwt cells respond to LPS and fMLP in a dose dependent manner. Based on this data and data previously collected in the lab, 100 ng/ml of LPS and 100 nM fMLP was used for further studies.

Figure 9 shows the synergistic effect of LPS and fMLP doses on TNF-α RNA using Q-RT-PCR. When used alone, fMLP and LPS act in a dose dependent manner on TNF-α RNA induction. When in combination, fMLP and LPS act in a synergistic manner. For example, at 100 nM fMLP, TNF-α induction is 20-fold over the negative control and 10 ug/ml of LPS alone induces TNF-α induction about 110-fold. The additive effect of these agonists would be an expected increase of 130-fold. However, the added effects caused an increase of 225-fold, suggesting a synergistic effect.
Figure 7. TNF-α dose response to LPS and fMLP

THP1-FPRwt cells were incubated with various doses of LPS or fMLP for 2 hours. RNA was extracted and quantitative real time PCR was performed as stated in Methods. Data from cells exposed to media alone was set to one and all data from others were compared to it. The results are expressed as means ± standard error and are representative of three (LPS) or four (fMLP) different experiments done in duplicate.
Figure 7. TNF-α dose response to LPS and fMLP

A. LPS

B. fMLP
Figure 8. Calcium mobilization in response to various doses of fMLP

THP1-FPRwt cells were analyzed for fMLP-stimulated elevation of intracellular calcium at various doses as described in Methods. Results are representative of 3 experiments performed in duplicate.
Figure 8. Calcium mobilization in response to various doses of fMLP
Figure 9. Synergistic dose response of LPS and fMLP on TNF-α induction

THP1-FPR wt cells were incubated with increasing doses of fMLP (0-10000nM) and LPS (0-10000ng) in a grid like fashion. mRNA was extracted and real time PCR was performed on cDNA as described in methods. Graph is representative of two independent experiments.
Figure 9. Synergistic dose response of LPS and fMLP on TNF-α induction
FPR mutations and the synergistic response

To determine if mutations in the FPR have any effect on TNF-α induction, quantitative real time analysis was performed on wt and mutant forms of the FPR (Figure 10). THP1 cells expressing the wild type FPR or three mutant forms were exposed for 2 hours to media, 100 nM fMLP, 100 ng/ml LPS or fMLP plus LPS. THP1 cells expressing wild type FPR induced TNF-α mRNA production in response to both fMLP (10-fold) and LPS (17-fold). When the stimulants are combined, the expected fold increase would be 27 fold, but it actually increased 45-fold. The FPR receptors with point mutations, D71A and R123G, did not respond to fMLP, however, they respond normally to LPS. When fMLP and LPS were used in combination, the THP1 cells expressing these FPR receptors induced TNF-α mRNA to the same levels as LPS alone. The Δst mutant form of FPR induced TNF-α in response to fMLP to a greater extent than wild type, 32 compared to 10. The THP1 cells expressing the Δst form of the FPR also had a synergistic response to fMLP and LPS when used in combination. It would be expected that the fold induction would be 47, when in fact it is 62. In summary, fMLP and LPS can act in a synergistic manner on TNF-α RNA in THP1 cells expressing wild type and Δst FPR.
Figure 10. Quantitative Real time PCR analysis of TNF-α induction

Stably transfected THP1 cells expressing the wt or mutant forms of the FPR were stimulated for two hours with 100nM fMLP, 100ng LPS or a combination of both. mRNA was extracted and quantitative real time PCR analysis was performed as described in Methods. Data from cells exposed to media alone was set to one and all data from others were compared to it. Data expressed as mean ± standard error and representative of 5 experiments done in duplicate.
Figure 10. Quantitative Real time PCR analysis of TNF-α induction
To determine if the synergistic effect found in TNF-α mRNA levels could also be seen in the secreted TNF-α protein, an ELISA analysis was performed (Figure 11). This figure represents the average protein concentration of three experiments ± standard error. The average of the three experiments shows no synergistic effect from co-stimulation of fMLP and LPS. The FPR mutants R123G and D71A do not induce TNF-α protein, as would be expected since neither were shown to induce TNF-α transcription. All show a similar protein concentration in response to LPS. In the fMLP plus LPS group there is no synergistic response for any FPR receptor; In fact, the FPR wild type actually decreases.

Pertussis toxin can inhibit FPR signaling and the synergistic response normally seen in cells stimulated with fMLP and LPS. Pertussis toxin catalyzes the ADP-rybosilation of the α subunit of the guanine regulatory protein G_αi. This prevents the G-protein from interacting with GPCR, leaving the receptors unable to activate a signaling cascade (Product literature from Sigma for pertussis toxin from *Bordetella pertussis*). Cells were incubated for 4 hours in the presence or absence of pertussis toxin prior to being stimulated for two hours with fMLP, LPS or both (Figure 12). In the absence of pertussis toxin, as seen previously, fMLP and LPS together can increase TNF-α mRNA to a greater extent than the additive. When pertussis toxin is added, fMLP fails to induce TNF-α mRNA as a result of the uncoupling of the FPR and the G_αi-protein. Pertussis toxin also seems to inhibit TLR-4 signaling by 50%. This indicates that LPS at least partly signals through a G-protein mediated signaling pathway.
Figure 11. TNF-α protein concentration in THP1 cells expressing FPR wild type and mutant forms

Stably transfected THP1 cells expressing the wt or mutant forms of the FPR were stimulated for two hours with 100nM fMLP, 100ng LPS or a combination of both. Cell media was separated from cells and used for ELISA analysis which was performed as described in methods. Data represents the average of three experiments ± standard error.
Figure 11. TNF-α protein concentration in THP1 cells expressing FPR wild type and mutant forms
Figure 12. Pertussis toxin inhibits FPR signaling, but not TLR4

THP1-FPRwt cells were either pretreated for 4 hours with 200ng/ml Pertussis toxin or with media. They were then stimulated with 100nM fMLP, 100ng LPS, or a combination of both. RNA was extracted and quantitative real time PCR was carried out with cDNA as described in methods. Control cells exposed to media alone were set to 1 fold and all data from stimulated cells were compared. Data are representative of 3 independent experiments ± standard error.
Figure 12. Pertussis toxin inhibits FPR signaling, but not TLR4
Effects of bacterial products on intracellular calcium

Calcium mobilization is an important second messenger in the signaling cascade of FPR. To determine what effects the mutations would have on calcium release in response to fMLP, calcium mobilization was determined for all three mutations and wild type FPR. Figure 13 shows representative data of one of three independent experiments done in duplicate. One-hundred nM fMLP was administered to each cell line after a base line of intracellular calcium was taken for 1 minute. Wild type FPR showed the greatest increase in calcium release over the base line. The mutants R123G and D71A have a minimum release of intracellular calcium when stimulated with fMLP. The Δst mutation increased calcium in response to fMLP to relatively the same levels as wild type. It is apparent that Δst base line readings are considerably higher then wild type FPR. This was seen in all calcium mobilization experiments where THP1 cells expressing Δst FPR were used.

Figure 14 represents a calcium mobilization in which the THP1 cells expressing either the FPR wild type or mutant Δst were stimulated at 60 and 300 sec with 100 nM fMLP. Wild type FPR is desensitized, as a second stimulation of fMLP causes a calcium release of approximately one quarter of the original. The FPR mutant Δst, however, releases calcium in response to a second stimulation of fMLP about equal to the first response. This indicates that, while wild type FPR is desensitized and unable to respond a second time, the mutant Δst is not, due
Figure 13. Calcium mobilization by wild type and mutant forms of FPR

THP1 cells expressing wild type and mutant forms of the FPR were analyzed for elevation of intracellular calcium in the presence of 100nM fMLP. Data are representative of three experiments done in duplicate.
Figure 13. Calcium mobilization by wild type and mutant forms of FPR
Figure 14. Desensitization of wild type and mutant Δst forms of FPR

THP1 cells expressing wild type and the Δst mutant form of FPR were analyzed for elevation of intracellular calcium in the presence of 100nM fMLP at two time points. Data are representative of three experiments done in duplicate.
Figure 14. Desensitization of wild type and mutant Δst forms of FPR
to its loss of phosphorylation sites, and can respond quickly to a second stimulus
of fMLP.

As LPS is a major endotoxin contributing to the release of TNF-α it was
used in an experiment in which THP1 cells expressing the wild type FPR were
primed for two hours with either media, 100 nM fMLP, 100 ng/ml LPS or a
combination of both. In Figure 15, intracellular calcium mobilization was
determined in response to 100 nM fMLP, 100 ng/ml LPS, or both for each set of
primed cells. For all primed cells (Figure 15a, b, c, and d), stimulation with LPS
had minimal effect on intracellular calcium release. In cells primed with media
(Figure 15 a), a combination of fMLP and LPS gave no increase in calcium
mobilization over that of fMLP alone. However, priming the cells with LPS for two
hours (Figure 15 b) causes a decrease of less then half in intracellular calcium
mobilization in response to fMLP, and fMLP and LPS together. Priming with
either fMLP or fMLP plus LPS (Figure 15 c and d, respectively), causes an even
greater decrease. These primed cells stimulated with either fMLP or fMLP plus
LPS mobilized calcium to a degree less then a quarter that of cells primed with
media alone.
Figure 15. Effects of priming on intracellular Calcium mobilization

THP1-FPRwt cells were pretreated for two hours with media (A), 100nM fMLP (B), 100ng LPS (C), or 100nM fMLP+ 100ng LPS (D). Each set of cells were stimulated with 100nM fMLP, 100ng LPS or a combination at 60 sec and intracellular calcium mobilization was analyzed as described in Methods. The data are representative of three separate experiments.
Figure 15. Effects of priming on intracellular Calcium mobilization

A. Media primed

B. LPS primed
C. fMLP primed

D. fMLP + LPS primed
DISCUSSION

Sepsis is fast becoming a leading source of death in the United States. Within 13 years it has moved up from the 13th to the 10th leading cause (NCHS 1993; Hoyert, Mathews et al. 2006). The treatment of sepsis is a multi-billion dollar endeavor with extended hospital stays, and in some cases, a need for long term care of survivors (Angus, Linde-Zwirble et al. 2001). The long term quality of life for the survivors is diminished and comparable to that of patients with chronic diseases (Davidson, Caldwell et al. 1999; Heyland, Hopman et al. 2000). For these reasons it is important to understand the mechanisms behind the disease.

A major player in the etiology of sepsis and septic shock is TNF-α. It is one of the first cytokines released in response to endotoxin (Hesse, Tracey et al. 1988; Creasey, Stevens et al. 1991; Taylor, Chang et al. 1991) and when administered can mimic the symptoms of sepsis (Tracey, Beutler et al. 1986; Tracey, Lowry et al. 1987; Michie, Spriggs et al. 1988). Understanding the causes behind the massive production of TNF-α in the septic response is the first step in elucidating the mechanism involved in sepsis and septic shock. As of yet, the Gram-negative endotoxin, LPS, has been the major focus in studies of sepsis and its symptoms. However, other bacterial products, such as formylpeptides, could contribute to the induction of TNF-α.

Our study focused on two bacterial products, the endotoxin LPS and the formylpeptide fMLP. Since monocytes are the progenitor of the phagocytic macrophage, the human monocytic cell line THP1 was chosen for this study. The
wild type THP1 cells produce TNF-α RNA in response to LPS (Figure 4). When stimulated with fMLP, however, the cells failed to induce TNF-α RNA. As the cells are a pro-monocytic myeloid cell line and are not yet completely differentiated into monocytes, they may express little to no fMLP receptor. Flow cytometry revealed little cell surface expression of FPR (data not shown). The THP1 cells do express TLR4 on the cell surface and respond readily to LPS (Figures 4 and 5). In order to study the effects fMLP and LPS have on TNF-α levels, THP1 cells were stably transfected with the wild type FPR and the three mutants R123G, D71A and Δst (Figure 3). All receptors were expressed on the cell surface but at different levels. This may be due to variability in the transfection rate, expression rate, or presentation on the cell surface. THP1 cells were stably transfected with the R123G mutant several times in our lab and in all instances it had an extremely low expression level (Data not shown). This suggests the low expression on the cell surface is caused by problems with receptor processing, transport or miss-folding.

LPS and fMLP can induce the production of TNF-α mRNA (Figure 7) and activate calcium mobilization (Figure 8) in a dose dependent manner. Maximum intracellular calcium is released in response to 100 nM fMLP. Above this dose calcium mobilization is prolonged and actually decreases at 10 mM. This prolongation of calcium could be due to the attainment of a steady state of calcium release.
Some chemicals and bacterial products can cause a synergistic effect on receptors (Faure, Thomas et al. 2001) and cytokines (Yang, Tamai et al. 2001; Gantner, Simmons et al. 2003). One study found that MDP with either LPS or PGN had a synergistic effect on secreted TNF-α protein levels (Wolfert, Murray et al. 2002). We wanted to determine if this could also be true for fMLP and LPS. THP1-FPRwt cells were incubated with fMLP and LPS at varying doses and TNF-α mRNA was determined by quantitative real time PCR (Figure 9). It can be seen that fMLP and LPS have a synergistic effect in a dose dependent manner. This could be due to LPS’s ability to increase the length of the poly(A) tail of TNF-α mRNA, leading to a more stable mRNA (Crawford, Ensor et al. 1997). This synergistic increase in TNF-α mRNA could also be influenced by a prolonged half-life. In one study of bacterial DNA and LPS, it was found that the half-life of TNF-α mRNA was longer in dual treated cells than that of cells treated with either bacterial DNA or LPS (Gao, Xue et al. 2001).

By comparing wild type FPR and the three mutants, we determined that the mutants D71A and R123G are unable to respond to fMLP and inhibited the synergistic effect seen in the wild type FPR in response to fMLP and LPS (Figure 10). Stimulation with fMLP had no effect on D71A and R123G mutants. This is due to an inability of both to bind G-protein. D71A, in contrast to R123G, is also unable to be phosphorylated and internalized. R123G, which has a mutation in the highly conserved “DRY” motif is phosphorylated to a small degree and is internalized 70% that of WT (Prossnitz, Schreiber et al. 1995; Bennett, Maestas
et al. 2000). As there is no difference in the induction of TNF-α RNA in cells expressing R123G or D71A, it can be inferred that phosphorylation and internalization of the receptor plays no role in TNF-α transcription. Cells expressing the Δst mutation can bind G-protein, but it is not phosphorylated nor is it internalized (Hsu, Chiang et al. 1997; Prossnitz 1997; Vines, Xue et al. 2002). Cells expressing the Δst mutation respond readily to fMLP and to a greater extent than wild type receptor. As Δst can bind G-protein, but is not phosphorylated and can signal for TNF-α transcription, G-protein activation is involved in the signal for TNF-α transcription. This can also be seen in cells expressing the wild type FPR that are incubated with pertussis toxin, which inhibits G protein-protein from interacting with a receptor, in which induction of TNF-α RNA is reduced.

LPS signaling in all four cell lines was relatively the same. When stimulated with both fMLP and LPS, D71A and R123G had TNF-α RNA levels almost equal to that of LPS stimulation alone, showing that only TLR4 is activated and the FPR mutants D71A and R123G are unable to activate the FPR signaling cascade. Wild type FPR and Δst were seen to have a synergistic response upon stimulation with both fMLP and LPS. In both cases, the response is greater than if one was to add the responses of fMLP and LPS alone. Δst has a greater induction of TNF-α RNA in response to fMLP than does the wild type, even though Δst had a lower cell surface expression level. The expression level
for Δst on THP1 cells is 60% less than that of wild type expression, yet Δst induction of TNF-α nearly triples. This may be explained by Δst’s inability to be phosphorylated and consequently internalized (Hsu, Chiang et al. 1997; Vines, Xue et al. 2002). If the Δst receptor is unable to be internalized and the FPR signal stopped then perhaps it is constantly activated in the presence of fMLP.

It is important to see if the synergistic effects observed in TNF-α mRNA levels are also found in the secreted TNF-α protein. It was determined by ELISA that D71A and R123G were unable to induce TNF-α protein in response to fMLP (Figure 11). This was to be expected, as no RNA was determined for these mutants. Again, Δst had greater induction of protein when compared to the wild type receptor. The synergistic effect on TNF-α mRNA levels in cells expressing wild type and Δst FPR is not observed at the protein level. This loss may be caused by a threshold level of translation of TNF-α mRNA or the determination of protein levels at the incorrect time point.

The FPR is a seven transmembrane G-protein receptor that is sensitive to pertussis toxin. It was determined that pertussis toxin could inhibit the signaling of the FPR and the synergistic effect seen in stimulation with both LPS and fMLP (Figure 12). Pertussis toxin also had an effect on LPS signaling. Treatment with the G-protein inhibitor decreased TNF-α induction in response to LPS by half. This could not be due to cell death, as equal amounts of RNA were extracted in treated and non-treated cells. Cholera toxin, which increases cAMP levels,
reduces LPS stimulated induction of TNF-α RNA (Data not shown). The increased cAMP acts on a cAMP responsive element in the promoter region of TNF-α, leading to a decrease in TNF-α transcription (Hajishengallis, Nawar et al. 2004). The reduction in LPS-stimulated TNF-α mRNA in the presence of pertussis toxin is not mediated by cAMP, as pertussis toxin does not effect unstimulated or stimulated levels of cAMP (Becker, Kermode et al. 1985). A study of murine B cell proliferation found that pertussis toxin significantly reduced LPS induced proliferation, NF-κB activation, and inhibited phosphorylation of ERK (Kim, Yang et al. 2006). It is perhaps this reduced activation of NF-κB, a downstream factor in LPS signaling, that leads to the decrease in TNF-α transcription seen in pertussis toxin treated cells. The above data is in direct opposition to other studies. In mouse peritoneal macrophages, treatment with pertussis toxin increased TNF-α production when induced by LPS (Zhang and Morrison 1993; Altavilla, Squadrito et al. 1996). Either data suggests a role for Gαi-proteins in TNF-α induction via LPS signaling.

An important second messenger in FPR and other GPCRs signaling is calcium, which is released in response to an increase in IP3 (Kiselyov, Shin et al. 2003). Elevated levels of calcium can activate a cyclic AMP response element binding protein (CREB). Activation of CaMKIV, which phosphorylates and activates a CREB-protein, mediates TNF-α transcription (Lobo, Zanjani et al. 1999). We determined that cells expressing the wild type FPR and the Δst mutation are able to mobilize calcium and possibly activate downstream signaling
events, such as induction of TNF-α mRNA synthesis (Figure 13). The FPR-Δst can mobilize calcium to the same degree as wild type. Cells expressing FPR with the R123G or the D71A mutations were unable to mobilize calcium in response to fMLP. This is expected, as calcium release is initiated by activation of G-protein (Le, Murphy et al. 2002).

It is interesting to note that FPR-Δst has a higher base line level of calcium than that of wild type. This higher base line and the fact that Δst has increased TNF-α mRNA could be due Δst’s inability to be desensitized (Prossnitz 1997). Cells expressing wild type or Δst FPR were stimulated with fMLP at 1 min and again at 5 min (Figure 14). Wild type FPR had a second calcium response that was a fraction of the first. Δst on the other hand could produce a calcium response equal to the first. This indicates that the wild type FPR is desensitized or internalized, whereas FPR-Δst is not.

We next wanted to determine if LPS can effect the mobilization of calcium. Calcium as a second messenger has been linked to LPS signaling. Human keratinocytes expressing CD14 and TLR4 had a rapid intracellular increase in calcium when induced with LPS (Song, Park et al. 2002). Pugin et al found that stable transfection of THP1 cells with CD14 could enhance the cellular response to LPS and induced intracellular calcium release, whereas untransfected THP1 cells did not (Pugin, Kravchenko et al. 1998). THP1 cells expressing the wild type FPR were primed for two hours with media, LPS, fMLP or both fMLP and LPS (Figure 15). Each set of cells was then stimulated with fMLP, LPS or both. In all
cases, stimulation with LPS did not increase intracellular calcium amounts to a significant level. This indicated that, in this system, LPS does not signal in a calcium-dependent manner. CD14 was transfected into cells used for studies in which LPS was able to mobilize calcium. It is possible that CD14 has an important role in calcium mobilization in response to LPS. In this study, CD14 levels on the cell surface were not determined. It is possible that the levels were insufficient to propagate a calcium response. In all cases, there was little difference between cells stimulated with fMLP and those stimulated with both fMLP and LPS. There was a change in calcium mobilization when cells primed with either fMLP, LPS, or both are compared with media treated cells. Priming the cells with LPS caused a decrease of about half in intracellular calcium mobilization in response to fMLP and fMLP plus LPS. This decrease in calcium release in cells primed with LPS may be due to a role for G-protein in LPS signaling. It was determined that pertussis toxin could increase LPS induced induction of TNF-α via a pertussis toxin sensitive G-protein (Zhang and Morrison 1993; Altavilla, Squadrito et al. 1996). G-protein is also linked to LPS proliferation of murine B cells. It was found that LPS induced proliferation of B cells is mediated by a G-protein associated ERK pathway (Kim, Yang et al. 2006). Perhaps priming the cells with LPS caused the cells to down-regulate receptors or G-protein. Intracellular calcium mobilization was about 33% that of media primed cells when cells were primed with fMLP or fMLP and LPS. This is due to the wild type FPR being desensitized and internalized when it first encounters
fMLP in the priming stage. A study conducted by Wilde et al found that basal and peptide stimulated PMN membranes had a decrease in receptors, efficiency of signal transduction, and GTPase activity when pre-incubated with the ligand (Wilde, Carlson et al. 1989).

In the future, it would be interesting to find the link between the fMLP and LPS induced synergistic effect seen in TNF-α RNA. Perhaps looking further into the published reports of calcium’s involvement in LPS signaling would lead to an answer. This effect could potentially be mediated through activation of two transcription activators, NF-κB and CREB-protein, which are known to be involved in similar pathways.
CONCLUSION

1) TNF-α RNA induction and intracellular calcium mobilization respond in a dose dependent manner to fMLP and LPS.

2) LPS and fMLP can synergistically induce TNF-α RNA transcription in wild type and Δst FPR, but can not activate transcription in a synergistic manner.

3) Wild type and Δst can release intracellular calcium in response to fMLP whereas, R123G and D71A can not. In contrast to wild type, Δst can rapidly respond to a second fMLP signal. This suggests that it is not desensitized.

4) Priming of THP1-FPRwt cells with LPS or fMLP plus LPS reduces the intracellular calcium response to fMLP.

5) Pertussis toxin inhibits the synergistic response in TNF-α RNA and reduces the activity of LPS transcription. This suggests a role for Gt protein in LPS induction of TNF-α
REFERENCES


ABSTRACT

Tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) is a protein that can be produced in large amounts by tissue macrophages and monocytes in response to bacterial products. Systemic release of TNF-\( \alpha \) has been shown to mediate severe septic shock. Fever, hypotension, cardiovascular changes, disseminated intravascular coagulation (DIC), multiple organ failure and death are characteristics of this disease state.

Our study has indicated that the bacterial products, lipopolysaccharide (LPS) and formylated peptides (fMLP), each induce activation of cytokine gene transcription in leukocytes. Interestingly, fMLP induces some intracellular signaling events that differ from those elicited by LPS. Both stimuli, however, activate the signaling pathways that converge to produce TNF-\( \alpha \). We have found that the bacterial products, fMLP and LPS, can act synergistically in the induction of TNF-\( \alpha \) mRNA in monocytes, but is not seen in TNF-\( \alpha \) protein levels. Our results demonstrate that multiple bacterial products may regulate the production of pro-inflammatory cytokines in a synergistic manner. Control of this inflammation is best understood at the level of synergistic regulation of intracellular signaling. Our results have also exposed a novel and potentially important pathogenic mechanism occurring during bacterial infection. Thus, our overall hypotheses are that leukocyte activation is regulated by multiple bacterial products that operate synergistically by activating multiple signaling pathways,
and that this synergy is likely to play a significant role in the induction of host defense to bacterial infections and in the pathogenesis of inflammatory disorders.